UNIVERSITY OF
LIVERPOOL

Mechanisms of Haem Acquisition by the Black-Pigmenting Anaerobes, *Prevotella intermedia* and *Porphyromonas gingivalis*

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor of Philosophy

by

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JULY 2011

AKNOWLEDGEMENTS

The work described in this thesis was completed in- and funded by the School of Dentistry between October 2007 and July 2010.

First and foremost I would like to express my sincerest gratitude to my supervisor, Dr John Smalley, for his guidance and support throughout my studies. I have hugely benefited from his research experience and knowledge, and could not have hoped for a better mentor. Many thanks are also due to my secondary supervisor, Dr Ed Yates, whose supervision, advice, and financial assistance made this research project possible.

I am also indebted to my EU supervisor, Professor Jan Potempa, for giving me the opportunity to work in the laboratory at the Jagiellonian University, Poland, and for his overwhelming generosity. In this respect I also gratefully acknowledge all of the staff and students who helped me during my visits to Jagiellonian University, with special thanks extended in particular to Aneta Sroka, Borys Szmigielski, and Anna Jaworska

I would also like to express my appreciation to Professor Teresa Olczak and Dr Halina Wojtowicz of the University of Wroclaw for kindly donating generous quantities of the HmuY haemophore and for performing the circular dichroism analysis of Hb-LPS interactions. Special thanks must also go to Dr Svetlana Antonyuk and Dr Richard Strange for carrying out the X-ray diffraction experiments and for tutoring me in crystallographic data processing and refinement.

Finally I would also like to thank everyone from the new (and old) Edwards building for aiding me throughout my studies, and Lee Cooper for his keen eye for details and for being a dab hand at all things Microsoft. Many thanks, in particular, go to the finest technician to ever adorn a white lab coat, Mr Andrew Birss, for all of his help, advice and friendship over the past four years. On a personal note, I would also like to thank my parents and family for everything they have done for me and Rachel for her patience and support.

ABSTRACT

Periodontitis describes a set of related inflammatory diseases that damage the periodontal tissues that support the teeth. It is caused by bacterial infections that, if left untreated, can lead to progressive loss of alveolar bone and subsequent loosening and loss of teeth. Periodontitis is the primary cause of tooth loss in adults and treatment of more severe forms of the disease can be both painful and costly.

Black-pigmenting bacterial species, such as *Porphyromonas gingivalis* and *Prevotella intermedia*, are among the major periodontal pathogens associated with severe periodontitis. These bacteria form black pigments consisting of iron protoporphyrin IX (haem; Fe(III)PPIX) when grown on blood-containing media. Development of the haem-containing black pigments by these organisms serves an important defensive role as the intrinsic catalase activity of ferrihaem destroys H_2O_2 (Smalley *et al.*, 2000). Furthermore, formation of the pigment by strictly anaerobic *P. gingivalis* is a process which consumes oxygen and promotes anaerobiosis (Smalley *et al.*, 1998; 2002).

This study sought to further characterise some of the facets of the mechanisms of haem acquisition by these two bacteria, with particular emphasis on the role of specific virulence factors (InpA of *P. intermedia*, HmuY and the gingipains of *P. gingivalis*, and LPS from both species). This resulted in identification of Interpain A (InpA), a 90 kDa cysteine protease, and homologue of *Streptococcus pyogenes* streptopain (SpeB), which is expressed by *P. intermedia* as a potential major virulence factor in haem acquisition through its ability to degrade haemoglobin, haemalbumin and haem-haemopexin. InpA-mediated proteolysis of these haemoproteins was highly dependent on experimental factors such as pH and redox potential, which profoundly affected the protease sensitivity of the haem-containing protein substrates. This suggested that InpA activity would be hugely influenced by the changing conditions of the periodontal pocket during progressive periodontitis.

A unique syntrophic relationship of haem acquisition from haemoglobin was also identified between the HmuY haemophore and the K- and R- gingipains of *P. gingivalis*. Furthermore, InpA facilitated greater haem extraction by HmuY by promoting haemoglobin oxidation to the methaemoglobin form. This suggested a

degree of mutualism and possible cross-reactivity between the haem acquisition systems of these two bacteria which has possibly arisen on account of their close proximity within the periodontal pocket biofilm. This study also identified a possible central role for LPS in binding and storage of haem at the cell surface of these black-pigmenting bacteria and presents evidence suggesting that the lipophilic nature of this molecule, in addition to the respective pericellular pHs of *P. intermedia* and *P. gingivalis* cells, may affect the species of haem present in the pigments.

PREFACE

Chapter 1 introduces the thesis with an overview of periodontal disease, the mechanisms of haem acquisition used by bacteria and the virulence factors of *P. intermedia* and *P.gingivalis*.

Chapter 2 provides a detailed account of the experimental protocols undertaken in the study, including bacterial protein purification procedures.

Chapter 3 describes the studies undertaken to characterise the interaction of *P. intermedia* cysteine protease, Interpain A (InpA), with oxyhaemoglobin (oxyHb).

Chapter 4 discusses experiments to further characterise the interaction of InpA with oxyHb using an inactivated mutant of InpA, InpA C154A.

Chapter 5 examines potential synergy between InpA and the Arg- and Lys-specific gingipain proteases of *P. gingivalis* in regards to proteolytic haem release from oxyHb.

Chapter 6 investigates the ability of InpA and R- and K-gingipains to facilitate haem extraction from oxyHb by the HmuY haemophore of *P. gingivalis*.

Chapter 7 further characterises the role of InpA in haem acquisition be examining the susceptibility of haem containing albumin and haemopexin to the protease.

Chapter 8 presents evidence suggesting that LPS of *P. gingivalis* and *P. intermedia* may play several important roles in haem acquisition, including providing a site for haem binding at the surface of the bacterium.

Chapter 9 provides and overall discussion of the findings of the thesis and suggestions for further experimental work.

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ABBREVIATIONS

A	Absorbance
AC-LYS	N-a-benzoylcarbonyl-l-lysine-p-nitroanilide
Ala/A	Alanine
Ap	Ampicillin
A-PS	Anionic polysaccharide
Arg/ R	Arginine
Asn/N	Asparagine
AzometHb	Azide-ligated haemoglobin
L-BAPNA	Bz-L-Arg- pNA
BPG	2,3-bisphosphoglycerate
BPA	Black pigmenting anaerobes
BSA	Bovine serum albumin
CD	Circular dichroism
Cm	Chloramphenicol
CP	Cysteine proteases
CPA	Carboxypeptidase A
Cys/C	Cysteine
DBBF	bis(3,5-dibromosalicyl)fumarate
DeoxyHb	Deoxyhaemoglobin
DMSO	Dimethylsulphoxide
DTT	Dithiothreitol
° C	Degrees Celsius
ε	Extinction coefficient
E_h	Redox potential
EDTA	Ethylenediaminetetraacetic acid
eTSB	Enriched Trypticase Soy Broth
FA titration	Fluorescence Anisotropy Titration
Fe(III)PPIX	Iron(III) protoporphyrin IX
Fe(III)PPIX.OH	Monomeric iron(III) protoporphyrin IX
$[Fe(III)PPIX]_2O$	μ-oxo iron(III) protopotphyrin dimer
FPLC	Fast Protein Liquid Chromatography
FTIR	Fourier Transform Infrared Spectroscopy
Fur	Ferric Uptake Regulator
g	Acceleration due to gravity
GCF	Gingival crevicular fluid
h	Hour
H_2O_2	Hydrogen peroxide
HA	Haemagglutinin/adhesin domain
Hb	Haemoglobin
Hib	*Haemophilus influenzae* type b
His	Histidine

HRgpA	Non-covalent complex of RgpA catalytic domain HA domain
HRgpA-metHb	Methaemoglobin produced by pre-treatment with HRgpA
HSA	Human serum albumin
IHP	*myo*-inositol hexakisphosphate
IL	Interleukin
InpA	Interpain
InpA C154A	Interpain Cys-Ala mutant
InpA-metHb	Methaemoglobin produced by pre-treatment with InpA
IPTG	Isopropyl β-D-thioglucopyranoside
ITC	Isothermal Titration Calorimetry
K_{av}	Fraction of the stationary gel volume which is available for diffusion of a given solute species
kDa	Kilodalton
Kdo	2-keto-3-deoxyoctonate
Kgp	Lysine-specific gingipain
Km	Kanamycin
l	litre
LAL	Limulus amoebocyte lysate
LB	Luria Bernani
LBP	Lipopolysaccharide binding protein
Leu/L	Leucine
LPS	Lipopolysaccharide
LSB	Laemmli Sample Buffer
Lys/K	Lysine
λ nm	Wavelength
λ_{max}	Maximum wavelength
λ_{ex}	Excitation wavelength
λ_{em}	Emission wavelength
M	Molar
MALDI-TOF MS	Matrix-Assisted Laser-Desorption Ionization-Time-Of-Flight-Mass Spectometry
2-ME	2-mercaptoethanol
metHb	Methaemoglobin
mg	Milligram
min	Minute
mM	Millimolar
mt-RgpA(cat)	Membrane type RgpA catalytic domain
mt-RgpB	Membrane type RgpB
Mv	Millivolt
µg	Microgram
µl	Microlitre
N_3^-	Azide
nm	Nanomolar

Na2S2O4	Sodium dithionite
OD	Optical density
OH-	Hydroxide
OPG	Osteoprotegerin
oxyHb	Oxyhaemoglobin
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
pH	$-\log_{10}$ of hydrogen ion concentration
Phe/F	Phenylalanine
pK_a	acid dissociation constant
PMNL	Polymorphonuclear leucocytes
RANK-L	Receptor activator of nuclear factor-κβ ligand
RgpA and RgpB	Arginine specific gingipain
RgpA(cat)	Rgp catalytic domain
rmsd	Root-mean-square deviation
S.O.C	Super Optimal Broth
SDS-PAGE	Sodium dodecyl sulfate polyacrylamide gel electrophoresis
SpeB	Streptopain B
TLR	Toll-like receptor
TMB	3,3' 5,5' tetramethylbenzidine
TNF	Tumor necrosis factor
Trp/W	Tryptophan
Tyr/Y	Tyrosine
V_0	Void volume
V_t	Total volume
VSC	Volatile sulphur compound
v/v	Volume per unit volume
wt	Wild-type
w/v	Weight per unit volume

CHAPTER 1

GENERAL INTRODUCTION

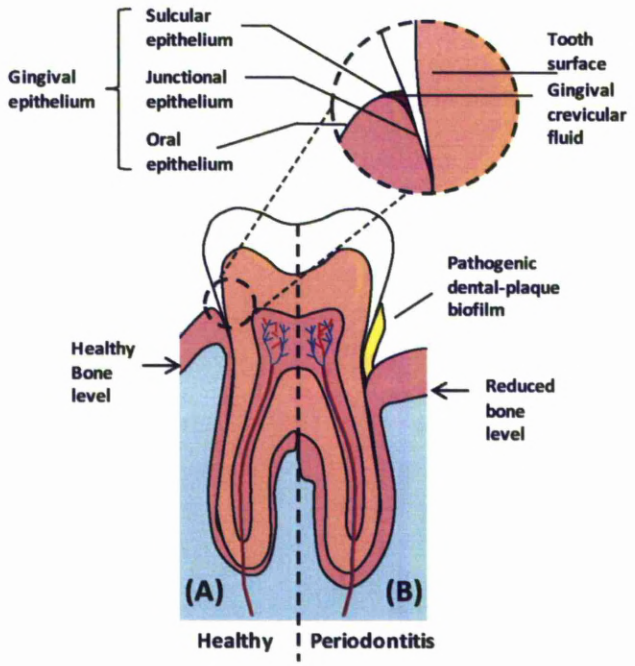
Prevotella intermedia and *Porphyromonas gingivalis* belong to a unique class of bacteria that accumulate a cell surface black pigment consisting of ferrihaems when grown on blood-containing solid media. Development of the black pigments by these organisms serves an important defensive role as the intrinsic catalase activity of ferrihaem destroys H_2O_2 (Smalley *et al.*, 2000). Furthermore, under certain conditions, formation of the pigment by *P. gingivalis* (which will be discussed in greater detail later) consumes oxygen and promotes anaerobiosis (Smalley *et al.*, 1998; 2002).This unique pigmentation phenomenon is the main focus of this thesis, which seeks to further elucidate the mechanisms of haem acquisition by these two oral bacteria with particular emphasis on the methods of haem liberation from the host and haem binding to bacterial cell surface. Firstly, however it is important to understand the relevance of these bacteria, and hence their pigments, in relation to human health. Both *P. gingivalis* and *P. intermedia* belong to a group of Gram-negative oral anaerobes associated with periodontal diseases in humans and animals (Holt & Esserbole, 2005). The following section will discuss the microbiology and disease processes of periodontal diseases in greater detail.

1.1. PERIODONTAL DISEASE.

1.1.1. An introduction to periodontitis.

The term 'periodontitis' is used to describe a family of related diseases that share common pathways of tissue destruction, alveolar bone resorption and loss of teeth, but which differ in aetiology, disease progression and response to therapy (Fig 1.1 [Darveau, 2010]). Fossils of our early ancestors reveal evidence of alveolar bone loss around tooth root surfaces, which suggest that periodontitis, which is the most common cause of tooth loss in the world today, was also prevalent in ancient populations. It is caused by bacterial infection and affects the majority of the population mildly and 5 -15 % of the population more severely, worldwide (Research, Science and Therapy Committee of the American Academy of Periodontology, 2006). However, more severe periodontal disease is more prevalent for certain high-risk groups (e.g. individuals with Down's syndrome, Types I and II diabetes, AIDS, leukaemia, neutropenia and Crohn's disease). Disease progression and tissue destruction in inflamed periodontal tissue is a direct result of excessive

host cytokine production (e.g. interleukins [IL-6, IL-11 etc] and Tumour Necrosis Factor-α [TNF-α]) in response to bacteria and their products (Darveau, 2010).



(A) Connective tissue and alveolar bone support the tooth root in healthy periodontal tissue. Oral epithelium covers the supporting tissue; Junctional epithelium connects the tissue to the tooth, the gingival sulcus (filled with gingival crevicular fluid) is the space between the epithelial surface and the tooth. (B) In periodontis, destruction of periodontal connective tissue and alveolar bone is caused by a dental-plaque biofilm which accumulates on the surface of the tooth.

Fig 1.1. The effects of periodontitis. Adapted from Darveau, (2010)

Genetic, environmental and acquired risk factors also influence the pathology of the disease, and may differ in relevance from one stage and form of disease to another (Page *et al.*, 1997). Intrinsic factors, such as an inherited predisposition to susceptibility, and induced factors, which include the pollutants associated with smoking tobacco, can shape the immune response and thus alter the likelihood of developing periodontal disease (Page & Kornman, 2000; Taubman *et al.*, 2007).Thus, the variable clinical manifestations of periodontal diseases result from the complex interplay between microbial challenges and host risk factors (Darveau, 2010).

One of the characteristic features of periodontal ligament destruction in periodontitis is the formation of a distinctive crevice known as the periodontal pocket, which serves as a reservoir for bacteria and can lead to further systemic complications (Moore *et al.*, 1982). For example, epidemiological data strongly suggests that periodontal disease may be an important risk factor for coronary heart

disease (Spahr *et al.*, 2006). Treatment of more severe periodontitis includes regular mechanical cleansing of the afflicted area (which is costly, time consuming and can be painful) and antibiotic therapy which could ultimately result in spread of antibiotic resistance. Furthering our understanding of the microbiology and disease processes of periodontitis is thus an important issue.

1.1.2 Microbiology of periodontal disease.

Defining the microbiology of destructible periodontal diseases has long been problematic. Koch's postulates define the causal relationship between infectious agents and a disease as; 1) the agent must be recoverable from every case of the disease, 2) it must not be recovered from other forms of the disease and 3) once the microorganism is recovered and grown in pure cell culture it must induce disease experimentally in animals (Carter, 1987). These presumptions however, are not useful for establishing the aetiological agents of destructive periodontitis. For example, the large numbers of microbial species in both healthy and diseased gingival sites complicates postulates 1 and 2, and the difficulty of establishing an infection of an isolated, suspected pathogen over competing resident microbiota in gingival sites of animals compounds postulate 3. The criteria for defining pathogens in destructive periodontitis have therefore been modified and expanded upon to accommodate these considerations.

The association of *Aggregatibacter actinomycetemcomitans* with juvenile periodontitis and refractory adult periodontitis has been known for many years. Thanks largely to advancements in molecular techniques, it is now established that a small group of predominantly Gram-negative, anaerobic or microaerophilic subgingival microbiota are responsible for initiating and perpetuating human periodontitis. Bacteria associated with most cases of periodontitis include *P. gingivalis, Tannerella forsythensis,* and *A. actinomycetemcomitans* (Consensus Report for Periodontal Disease, 1996) and are likely spread by vertical or horizontal transmission. However, the story is not quite as simple because the presence of a confirmed periodontal pathogen does not necessarily guarantee disease, and, paradoxically, periodontal pathogens can also be isolated from the mouths of healthy individuals as well as from healthy sites from disease sufferers (Haffajee & Socransky, 1994).

In order to produce periodontal disease, a periodontal pathogen must first attach to a periodontal site (epithelial cells, tooth surface) or co-aggregate with existing microorganisms, compete with the established microbiota, survive the host's immune defence and facilitate host tissue destruction. Bacterial virulence is dependent on gene expression for multiple products that carry out these functions, and for organisms to be considered virulent they must have, and express, all of these necessary genetic elements. For example, strains of *P. gingivalis* produce a number of proteolytic enzymes, which can be considered virulence factors as they degrade collagen and several other constituents of the extracellular matrix (Eley & Cox, 2000). Tissue destruction is thus a product of pathogen virulence factors that directly destroy host tissue (such as toxins and enzyme [Fives-Taylor *et al.*, 1999; Holt *et al.*, 1999]) and those that work indirectly through the induction of immunopathological reactions, such as lipopolysaccharide (LPS) of Gram-negative bacteria (Page & Kornman, 2000). Patients suffering from periodontitis possess high levels of systemic and topical antibodies against known periodontal pathogens (Kinane *et al.*, 1999), but effective clearance of the infection is not achieved unaided. Host cytokine responses aim to eradicate infections, but if a pathogen possesses virulence factors enabling survival *via* immune-modulation, then acute or even chronic inflammation may ensue.

Clinically-relevant host tissue destruction is uncommon despite continual colonization by a variety of pathogenic and non-pathogenic bacteria. The appearance of pathogenic organisms in healthy sites suggests clonal varieties of bacteria lacking important virulence factors. This was confirmed for *P. gingivalis* which displays varying levels of virulence between different strains (Haffajee & Socransky, 1994). Pathogens colonising healthy periodontal sites lacking key genetic elements (from bacteriophages, plasmids or transposons) do not contain a full library of virulence factors needed to induce tissue destruction. In other cases, expression of virulence factors by a periodontal pathogen in a healthy site may not be triggered unless specific environmental criteria are satisfied (Socransky & Haffajee, 1991). Furthermore, threshold numbers of virulent pathogens at specific susceptible locations are required to initiate disease. Changes in the local environment, which alter the composition of bacterial species and expression of virulence factors, thus also play an important role in the pathogenesis of periodontal disease.

1.1.3 Bacterial co-aggregation and the maturation of periodontal biofilm.

The majority of resident microflora must be compatible with the host and may even serve a beneficial role. Interactions between species regulate establishment, spread and propensity to cause disease of pathogenic species at specific sites. Beneficial species could, for example, compete with pathogens for occupancy of sites and even destroy virulence factors (Socransky & Haffajee, 1991). *Streptococcus sanguis*, *Streptococcus uberis* and *Actinomyces viscosus* produce hydrogen peroxide which is refractory to growth of *A. actinomycetemcomitans* (Hillman & Socransky, 1987), which conversely produces bacteriocins inhibitory only to growth of the aforementioned species (Stevens *et al.*, 1987). Environmental fluctuations which reduce numbers of resident microbial species (e.g. by antibiotic treatment), remove antagonism exerted on *A. actinomycetemcomitans*. Inversely, bacterial interactions could also be harmful by allowing pathogenic bacteria to colonise through surface co-aggregation or by metabolic alterations of the local environment. Certain species combinations are highlighted as instigators of periodontal disease, and include, *Fusobacterium nucleatum*, *T. forsythensis*, and *Campylobacter rectus, Streptococcus intermedius*, *P. gingivalis*, and *Micromonas micros*, and *S. intermedius* and *F. nucleatum* both with and without *P. gingivalis* (Haffajee *et al.* 1988, Socransky *et al.*, 1988a).

Oral microbes form supra- and subgingival plaque biofilms. The early dental plaque predominantly consists of Gram-positive bacteria and is gradually replaced by a more complex and predominantly Gram-negative flora if left undisturbed. Colour designations have been assigned to bacterial complexes within the periodontal pocket based on their association with periodontal infection (Socransky & Haffajee, 2005). The early colonisers are designated as the Blue, Yellow, Green and Purple complexes, while the colours Red and Orange were assigned to bacterial complexes representing the mature subgingival flora. These complexes are associated with health (e.g. early colonisers) and disease, with the red complex most likely to be associated with periodontitis (Socransky & Haffajee, 2005).

Naked enamel tooth surfaces become immediately coated with an acquired pellicle, making the surface receptive to colonization by specific early predominantly Gram-positive bacteria. As adherent bacteria grow and secrete extracellular polysaccharides that form the biofilm matrix, additional bacterial

species adhere to these early colonisers through the process of co-aggregation (Gibbons, 1984; Whittaker *et al.*, 1996), which is defined as the recognition between surface molecules of two bacterial cell types, so as to allow a mixed-cell aggregate to form. As the biofilm matures and proliferates, bacterial communities provide a broader habitat for growth, nutrition, waste elimination, new colonization, environmental safety and protection against antimicrobial drug therapy and the host's immune system which enables new types of bacteria to join (Thomas & Nakaishi, 2006; Marsh, 2004). The plaque eventually becomes thick enough to form anaerobic zones due to the activities of oxygen-consuming aerobes, permitting anaerobes of the mature plaque to colonise (Marsh, 2004; Bowden & Hamilton, 1998). Of particular note is the anaerobe *F. nucleatum*, which co-aggregates with many species of oral bacteria (Whittaker *et al.*, 1996) and acts as a co-aggregation bridge between aerobes and anaerobes (Bradshaw *et al.*, 1998). Association with aerobic bacteria can lengthen the in-air survival time of strict anaerobes such as *F. nucleatum*, which has a mean aerobic culture survival time of less than 5 min in the absence of aerobes (Bradshaw *et al.*, 1997). Co-aggregation also facilitates metabolic communication between species with distinct metabolic capabilities. Through enzymic cleavage of host glycoprotein glycan chains, strains of streptococci produce fermentable carbohydrates which are used by a broad range of other bacteria (Byers *et al.*, 1999). The biofilm also provides vitamin K for *P. gingivalis*, N-acetylmuramic acid for *T. forsythensis* and volatile fatty acids for *Treponema denticola* (Nishihara & Koseki, 2004). Additionally, non- or weakly-proteolytic *F. nucleatum* binds plasminogen which is converted to plasmin by *P. gingivalis* proteases and streptokinase during co-aggregation (Darenfed *et al.*, 1999) which, through its serine protease activity, may contribute to periodontal-tissue destruction by enabling host defence evasion and tissue invasion by *F. nucleatum*. This represents a unique form of synergy essential for *F. nucleatum* lesion formation in murine models (Feuille *et al.*, 1996). Furthermore, close proximity of bacteria in biofilm co-aggregates facilitate genetic transfer leading to spread of antibiotic resistance and virulence factors.

As the dental plaque expands, adjacent gingival epithelial cells become inflamed and secrete gingival crevicular fluid (GCF). Whereas bacteria inhabiting the supra-gingival plaque make use of salivary components (e.g. mucins and host-

diet carbohydrates) as a major nutrient source (De Jong & Van der Hoeven, 1987; Van der Hoeven *et al.*, 1990) subgingival microbiota make use of GCF (ter Steeg *et al.*, 1987) which contains a vast array of host proteins (e.g. transferrin, albumin and haemoglobin) but is relatively devoid of carbohydrate. Unsurprisingly, growth of subgingival plaque microbiota relies on proteolytic breakdown of proteins, and microbial infections associated with periodontal disease and dental abscesses often require an established community of bacterial species capable of synergistically breaking down host proteins and glycoproteins (Homer & Beighton, 1992; Bradshaw *et al.*, 1994; Wickstrom *et al* 2009; Ter Steeg & Van der Hoeven, 1989).

The ecological niche within the dental plaque gradually becomes protein-rich and strictly or near anaerobic, encouraging proliferation of proteolytic organisms such as *P. gingivalis*, *T. forsythensis* and *T. denticola*, which only appear in dental plaques once an anaerobic environment, with modest levels of local inflammation and a supply of essential growth factors made available through the biofilm food-web, has been established (Marsh & Devine, 2011). Increased proteolytic metabolic activity consequently alters the local environment by increasing pH and lowering redox potential (Kenny & Ash, 1969) which further competitively favours the growth of the above putative pathogens over species associated with gingival health.

Changes in the local subgingival environment such as these also affects expression of bacterial virulence factors (Socransky & Haffajee, 1991). As is often the case, a global regulon affected by specific environmental factors, such as temperature, pH, osmotic pressure or concentrations of iron, magnesium or calcium, up- or down-regulates production of multiple virulence factors. Increased haem availability as a result of inflammation and increased GCF flow can dramatically alter the phenotype of certain bacteria. For example, *P. gingivalis* has increased protease activity and virulence (McKee *et al.*, 1986), altered LPS structure (Jain & Darveau, 2010) and differential expression of outer-membrane proteins (Barua *et al.*, 1990; Bramanti & Holt, 1990; Smalley *et al.*, 1993) when grown under haem excess conditions. A species of bacterium could theoretically inhabit the same site for many years until changes in the environment encourage the expression of host-damaging virulence factors.

Periodontal infections are highly complex, having no clearly defined individual cause or course of treatment to control the infection. Chemotherapy therefore needs to be highly case-specific and determined by the nature of the infectious microbiota. A great deal of additional research is needed to define the contribution of key bacterial species and the pathogenic mechanisms they deploy, and explore the syntrophic relationships that have developed between them.

1.1.4 Consequences of periodontal inflammation.

Enrichment of anaerobic and proteolytic bacteria and their immuno-modulatory virulence factors through this process of biofilm maturation exacerbates the inflammatory immune response leading to subsequent gingival tissue damage (O'Brien-Simpson *et al.*, 2003; Ryder, 2010). As well as the aforementioned direct tissue destruction by bacterial secretion of proteases, oral bacteria can also indirectly induce tissue destruction by activating matrix metalloproteinases (Sorsa *et al.*, 1992) and by activating the plasmin cascade system (Uitto *et al.*, 1989). Cytokine release also modulates the chemotactic recruitment of polymorphonuclear leucocytes (PMNL) to infected sites, where they degranulate and release enzymes such as elastase, collagenase, and cathepsin G resulting in local tissue destruction (Van Dyke *et al.*, 1993). *P. gingivalis* and *P. intermedia* can also degrade the serum proteinase-inhibitors α_1-antitrypsin and α_2-macroglobulin (Carlsson *et al.*, 1984b) which modify PMNL proteolytic activities.

The initial response to bacterial infection is activation of the innate immune system resulting in cytokine release, thus propagating inflammation throughout the gingival tissue (Darveau, 2010). If the inflammatory front is not contained, it can expand to sites adjacent to alveolar bone and instigate the destruction of connective tissue and alveolar bone (Graves & Cochran, 2003). The importance of bacterially induced inflammatory immune response for bone loss was demonstrated in a primate model (Assuma *et al.*, 1998). When bone homeostasis is disrupted in certain inflammatory bone conditions, excessive bone resorption can be encouraged (osteoporosis or periodontitis [Lerner, 2006]). Bacterially induced inflammation results in pro-inflammatory cytokine release (eg IL-1β, IL-6, IL-11, IL-17, and TNF-α) which disrupt bone homeostasis by increasing and decreasing expression of

receptor activator of nuclear factor-κβ ligand (RANKL) and soluble decoy receptor osteoprotegerin (OPG), respectively (Nakashima *et al.*, 2000; Lerner, 2006; Boyle *et al.*, 2003). Pathological bone resorption thus occurs upon an increase in the RANKL:OPG ratio (Cochran, 2008).

For the purpose of this study, the most important consequence of gingival inflammation is the increase of haem availability in the periodontal pocket. Inflamed gingival tissue leads to vascular disruption and bleeding (Abrams *et al.*, 1984) and thus increased gingiva haemoglobin concentrations (Hanioka *et al.*, 1990), which is likely to play an important role in pigment formation by *P. gingivalis* and *P. intermedia* (Smalley *et al.*, 2007; 2008; Guan *et al.*, 2006). Free haemoglobin in inflamed gingivae may also act synergistically with bacterial LPS to promote further inflammation and progression of periodontitis (Bodet *et al.*, 2007). As discussed earlier, inflamed gingival epithelial cells also secrete GCF which is rich in host proteins (in particular albumin) and provides nutrients for bacterial growth (Curtis *et al.*, 1990; Makela *et al.*, 1991).

1.2. HAEM, IRON AND BACTERIA.

1.2.1. Iron requirements of pathogenic bacteria.

For any pathogen to successfully establish an infection it must colonise and proliferate in a niche within a host, and be able to acquire nutrients, such as iron, from tissues to survive. Iron is essential for cell maintenance, growth and pathogenicity of many organisms, and for cellular processes where it serves as the catalytic centre for redox reactions of certain enzymes involved in electron transport, peroxide reduction, and nucleotide biosynthesis (Heinemann *et al.*, 2008). Although there is abundant iron within the human host, its availability to pathogenic organisms is limited. At physiological pH, Fe^{3+} is insoluble in aqueous solution and therefore unavailable for microorganisms. Additionally, the majority of host iron is intracellular, sequestered in haemoglobin (76 %) or ferritin (23%) (Weinberg, 1984, 1993). Free extracellular iron is rapidly sequestered in serum by transferrin, and by lactoferrin on mucosal surfaces. As such, the amount of free iron is approximately 10^{-18} M, below levels that permit growth of microorganisms (Bullen *et al.*, 1978).

In environments with limited supplies of nutrients and growth factors, bacteria expressing the most effective machinery for their acquisition have an advantage over competing organisms. Bacteria colonising a human host must therefore deploy sophisticated iron scavenging mechanisms, including siderophores to chelate iron (Raymond *et al.*, 2003), reduction of the Fe^{3+} to the more soluble Fe^{2+} form (Coulanges *et al.*, 1997), acid-dependent utilisation of Fe^{3+} (Imbert & Blondeau, 1998), and iron capture from transferrin, lactoferrin or ferritin *via* specific outer-membrane receptors (Brown & Holden, 2002). Haem also serves as both a source of iron and porphyrin for certain pathogens. However, such is the affinity of mammalian haem for iron, that siderophores are ineffective in its removal (Weinberg, 1993). Many Gram-negative pathogenic bacteria have thus developed specific outer- membrane receptors that function to utilise haem or haem-containing proteins (Cescau *et al.*, 2007).

1.2.2. Iron protoporphyrin IX (haem; Fe(III)PPIX).

As discussed earlier, haem is essential for growth of *P. gingivalis* and *P. intermedia.* Haem is a prosthetic group of many proteins that comprises an iron metal ion, chelated in the centre of an organic heterocyclic porphyrin ring. The structure of haem is shown in Fig 1.2. Its most important feature is that the iron can undergo reversible oxidation to the Fe^{3+} state (referred to as haemin), which makes the molecule highly biologically active. A variety of haemoproteins containing this prosthetic group, utilise this intrinsic reversible oxidation to perform numerous diverse biological functions (Rajagopal *et al.*, 2008), such as oxygen transport (haemoglobin) and storage (myoglobin), respiration and electron-transfer (cytochromes b and c), signal transduction and gas sensing (coenzyme A, nitric oxide synthase, and soluble guanylate cyclase [Poulos, 2007]), control of gene expression and microRNA processing (Paoli *et al.*, 2002). It can also behave as a catalase and peroxidase (Heinermann *et al.*, 2008).

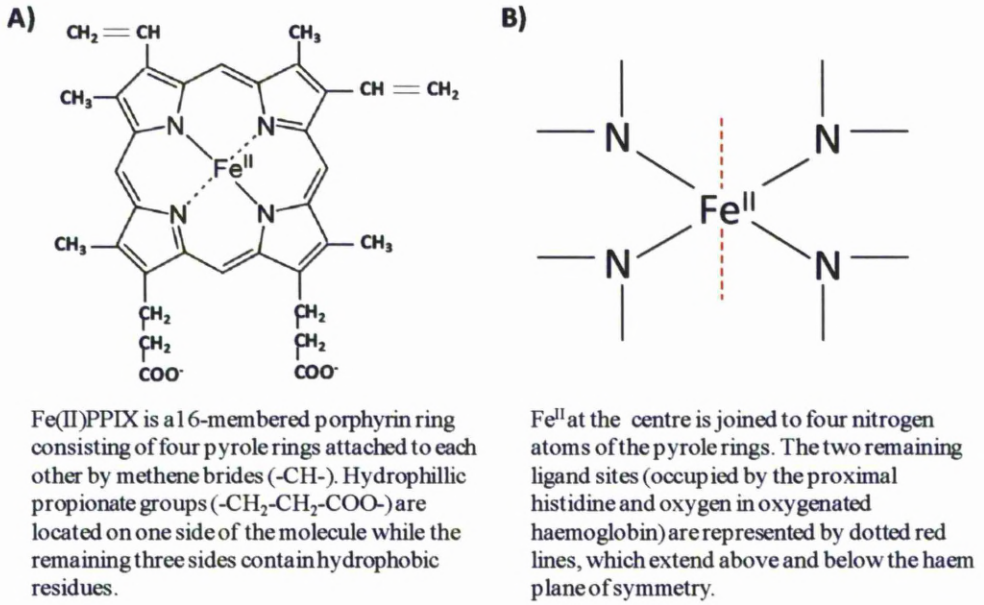


Fig 1.2. Structure of Fe(II)PPIX as found in haemoglobin.

To understand the mechanisms of haem binding and uptake by bacteria (Ascenzi *et al.*, 2005) it is important to first appreciate the complex behaviour of haem in solution. The equilibrium between haem species present is highly dependent on pH, haem and electrolyte concentration, and the type of solvent present in mixed aqueous solution (Asher *et al.*, 2009). The current theory is that in pure aqueous solution two five-coordinated haems will spontaneously associate via π-π stacking interactions of the unligated faces to form an amphipathic dimer (π-π dimer) with outward directed H_2O/HO axial ligands (de Villiers *et al.*, 2007). However, until recently it was thought that haem primarily existed as a μ-oxo bridged dimer (Shack & Clarke, 1947) which is now known to only form in basic-aprotic solvent mixtures (de Villiers *et al.*, 2007; Asher *et al.*, 2009). In pure aqueous solutions the axial H_2O/HO ligands of the π-π dimer are expected to strongly solvate with water molecules (de Villiers *et al.*, 2007; Asher *et al.*, 2009). Under basic conditions, if water is replaced by a miscible aprotic solvent, such as dimethylsulphoxide (DMSO), acetone, or pyridine (10 % v/v in 0.1 M NaOH), the axial solvation sphere will be disrupted, and the equilibrium will shift to favour the μ-oxo dimer (de Villiers *et al.*, 2007; Asher *et al.*, 2009). Strong salt concentrations have a similar effect by competing with axial ligands for hydration (Asher *et al.*, 2009). Under such

polar conditions π-stacking of the unligated surfaces, leading to large aggregates of μ-oxo dimer, would occur. The formation of either dimeric species is thus dependent on the solvent and is completely reversible (Asher *et al.*, 2009).

The equilibrium between haem monomers and dimers is also dependent on the concentration of haem in solution. In water, even at relatively low concentrations of haem, the π-π dimer and not the monomer is overwhelmingly dominant regardless of pH (Asher *et al.*, 2009). However, in an aprotic solvent (which disfavours the π-π dimer) low pH results in protonation and disruption of the μ-oxo bridge to form Fe(III)PPIX.OH monomers (Asher *et al.*, 2009; Casabianca *et al.*, 2009). Monomers are also the prevailing species in hydrophobic solvents such as ethanol or 80 % methanol (Asher *et al.*, 2009). A graphical representation of Fe(III)PPIX species is shown in Figure 1.3.

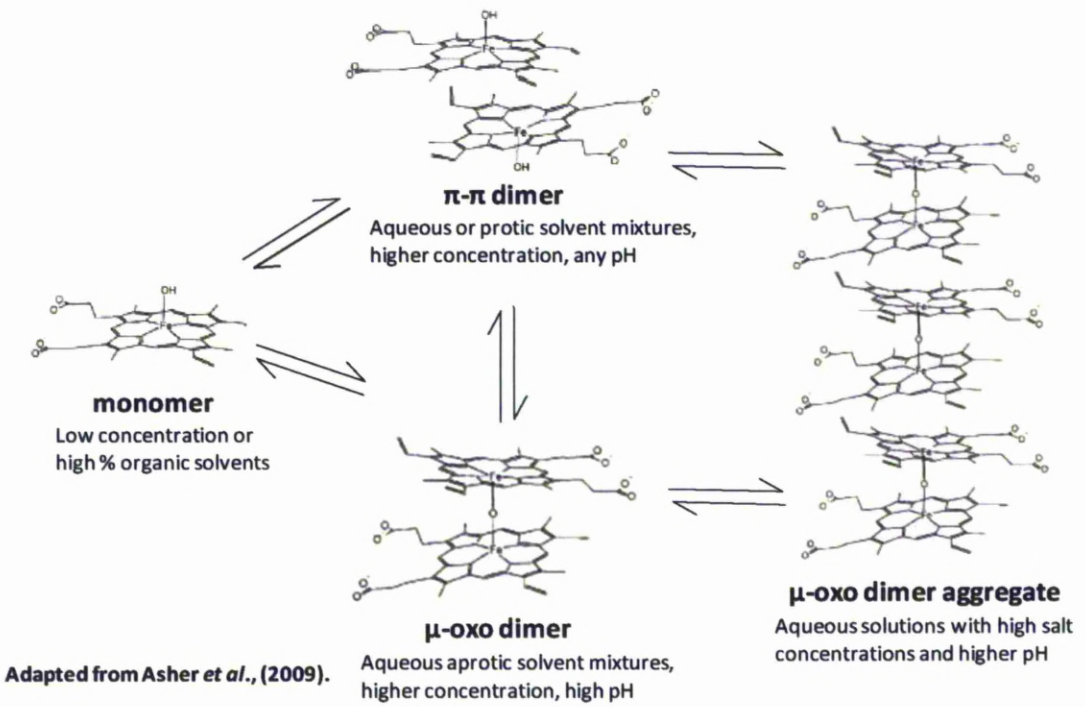


Fig 1.3. Representation of Fe(III)PPIX species in solution.

Haem species in solution can be distinguished by their UV-visible spectra, being characterised by distinctive intense absorbance peaks in the blue region, known as Soret bands, and by weaker absorptions at longer wavelengths (450 – 700

nm), known as Q bands. For example, the μ-oxo dimer is characterised by 394 nm Soret peak, a shoulder close to 365 nm and another peak and shoulder at 575 nm and 600 nm respectively. The spectrum of the π-π dimer is pH-dependent with a Soret λ_{max} at ~ 387 nm, a prominent shoulder at 365 nm and a Q band at ~605 nm with OH axial ligands at alkaline pH, and Soret λ_{max} at 364 nm, a shoulder at 400 nm and a Q band at 634 nm for acid pH H_2O liganded species (de Villiers *et al.*, 2007; Asher *et al.*, 2009). The pH-dependent changes in spectra of π-π dimers are attributed to protonation of the two outwardly directed axial ligands. Monomeric haem can also be identified with UV-visible spectroscopy. Alkaline pH monomeric haem, with OH axial ligand, can be distinguished by a Soret peak and prominent shoulder at 395 nm and 365nm respectively and a visible maximum at 597 nm. H_2O axial-liganded monomeric haem has a sharp 396 nm Soret band and an additional band at ~620 nm. Examples of spectra from different haem species are shown below (Fig. 1.4)

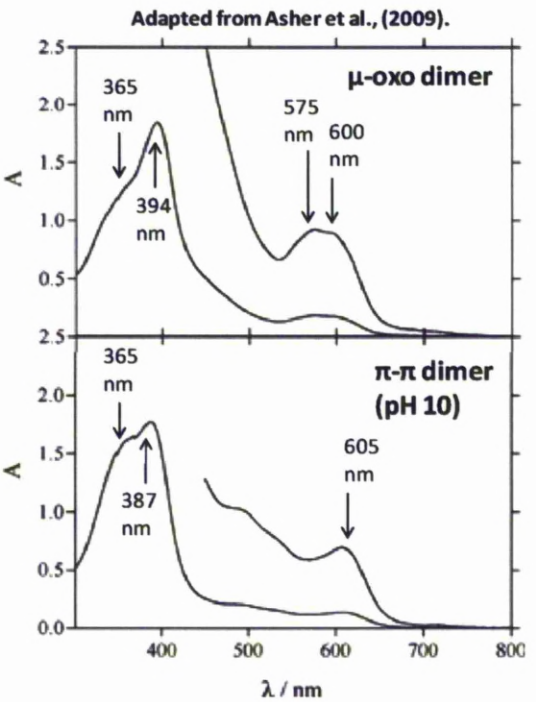


Fig 1.4. UV-visible spectra of Fe(III)PPIX species.

Free haem is poorly tolerated by cells. As a low molecular weight (616 Da) and lipophilic molecule, haem will readily intercalate into cell membranes, thus impairing lipid bi-layers and certain organelles (e.g. mitochondria) and also

destabilising the cytoskeleton (Ryter & Tyrell, 2000). Free haem also catalyzes the formation of reactive oxygen species which can lead to cellular damage, oxidative stress and tissue damage (Jeney *et al.*, 2002). Furthermore, free haem plays an important role in numerous acute and chronic pathological conditions including atherogenesis (Jeney *et al.*, 2002), intestinal carcinogenesis (Larsson *et al.*, 2005), and haemolysis and inflammation through induction of leukocyte chemotaxis and neutrophil responses (Kumar & Bandyopadhyay, 2005). Due to the enormous biological reactivity of free haems, they are almost universally sequestered within haemoproteins such as haemopexin (Hrkal *et al.*, 1974) and albumin (Beaven *et al.*, 1974) which also renders them unavailable to pathogenic organisms. In addition, any haem in the form of haemoglobin released from lysed erythrocytes will be rapidly bound to haptoglobin and transported to the liver.

1.2.3. Haemoglobin and myoglobin.

Approximately 67 and 5 % of total body intracellular iron (~4 g) consists of haem bound to haemoglobin and myoglobin, respectively (Bridges *et al.*, 1995). The remainder is located in enzymes (2 %), as insoluble ferric iron stored in ferritin (25 %), and in the poorly defined labile iron pool (~1.75 %; Bridges *et al.*, 1995).

Haemoglobin is a tetrameric protein composed of two α- and two β-globin chain subunits (see Fig 1.5) and is the principal component of oxygen transport in the blood. Each haemoglobin subunit contains an individual haem group wedged into the haem pocket by a phenylalanine residue (Phe CD1; Perutz, 1990). The haem iron atom is coordinated by four equatorial pyrrole nitrogen atoms of the porphyrin and forms a covalent bond with the globin proximal histidine imidazole group (His F8, Fig 1.5,C). The tetrapyrrole ring of the porphyrin also forms additional contacts with other residues of the haem pocket globin chain (Griko *et al.*, 1988). Three edges of the porphyrin (which interact with sites of the B, C and E globin helices) become buried in the protein interior and are thus protected from the solvent. The haem-6- and -7-propionates, on the fourth side of the porphyrin, are exposed to, and are able to interact with, the solvent and several other globin surface residues (Griko *et al.*, 1988).

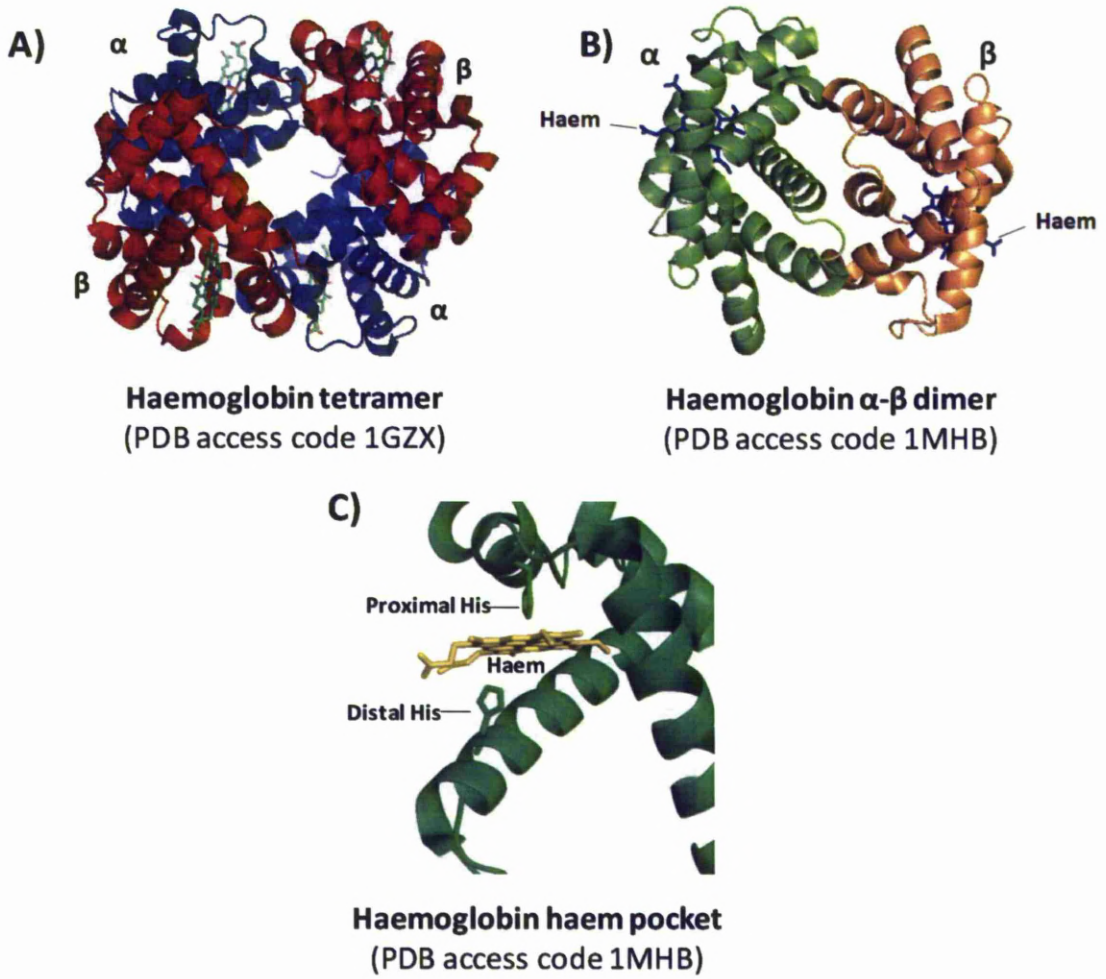


Fig 1.5. Structure of haemoglobin. Diagram created using Pymol.

Plots of equilibrium O_2 uptake for tetrameric haemoglobin reveal a sigmoidal curve reflecting the cooperative nature of oxygen binding. This is explained by binding of oxygen by one subunit which makes oxygen binding by the adjacent subunit more favourable. Haemoglobin subunits exist in two states that differ in relative tertiary structure and orientation within the tetramer. These are the low O_2 affinity structure (T or tense state) and the high O_2 affinity structure (R or relaxed state) (Perutz *et al.*, 1987). When oxygen binds to the five-coordinate haem iron of the first subunit in the T-state, the haem converts from a high to a low spin state which simultaneously causes the iron atom to move towards the porphyrin ring plane and also triggers several tertiary structural changes which impose a strain on the subunit (Perutz *et al.*, 1998). This strain eventually becomes great enough to alter

nonpolar, electrostatic, and hydrogen bond interactions between subunits, and results in similar modifications in the tertiary structures of the remaining subunits (Baldwin & Clothia, 1979; Brzozowski *et al.*, 1984). The successive binding of O_2 relieves tension of adjacent subunits which adopt relaxed (R) states and bind oxygen more easily (Perutz, 1970). The binding affinity is thus increased with successive oxygen binding. Because of these changes, native deoxyhaemoglobin (deoxyHb) and oxygen liganded oxyhaemoglobin (oxyHb) are seen to differ in structure (Perutz, 1970).

1.2.3.1. Haemoglobin oxidation.

OxyHb (containing an Fe^{2+} haem iron) spontaneously oxidizes (auto-oxidation, with the production of a superoxide anion, O_2^-) into the physiologically inactive ferric form known as methaemoglobin (metHb), which is incapable of oxygen binding, (Wever *et al.*, 1973; Tsuruga & Shikama, 1997; Shikama, 1998).The Fe-O_2 bond is relatively stable and auto-oxidation *via* spontaneous O_2^- dissociation is thermodynamically unfavourable, due to O_2 being a relatively poor one-electron acceptor (Shikama, 1985). The mechanisms for auto-oxidation *in vivo* instead involve entry of solvent OH^- or H_2O molecules into the haem pocket, inducing nucleophilic displacement of O_2^- (Shikama, 1990; Tsuruga & Shikama, 1997). These nucleophiles remain bound to the now ferric iron at the sixth coordinate position, forming aquo-metHb (for bound H_2O) or hydroxyl-metHb (for bound OH^-). As such, pH hugely influences auto-oxidation due to increases in OH^- or H_2O concentrations. Protection of the haem from nucleophiles is therefore important and is achieved by its containment within the globin moiety in haemoglobin and myoglobin (Sugawara *et al.* 1995). Encapsulation within erythrocytes which contain numerous methaemoglobin-reductases, further protects haemoglobin from oxidation (Rockwood *et al.*, 2003). Despite this however, approximately 3 % of circulating adult haemoglobin is oxidized on a daily basis (Eder *et al.*, 1949). It is also noteworthy that haemoglobin oxidation can be induced by a great number of oxidants such as sodium nitrite and potassium ferricyanide. Haemoglobin oxidation is also encouraged by dissociation into $\alpha1\beta1$ and $\alpha2\beta2$ dimers (Zhang *et al.*, 1991), which occurs as a result of haemoglobin dilution (Nagel & Gibson, 1971) and at low pH (Katz *et al.*, 1973; Myshkin, 1984).

Distal amino acid residues surrounding the haem control its immediate environment by inducing polar, hydrophobic and steric interactions which regulate affinities of bound ligands (such as O_2, H_2O, OH^-, CO, NO_2^-, and alkyl isocyanides), the type of which is largely dependent on the oxidation state of the molecule (Wever *et al.*, 1973; Dickerson & Geis, 1983). Haemoglobins of many species have a distal histidine residue (His E7; Fig 1.5,C) which protects against autoxidation by controlling distal pocket ligand access (Shikama, 1998). The imidazole N-H group of the distal histidine forms an H bond which stabilises bound dioxygen in the sixth coordinate position in haemoglobin and myoglobin, so much so that displacement of O_2^- by water molecules occurs at an exceedingly slow rate (Shaanan, 1982; Phillips & Schoenborn, 1981). Proton catalysis at acid pH can enormously enhance the reductive displacement of bound dioxygen by H_2O. The solvent catalytic proton transiently protonates the remote imidazole nitrogen (N^δ) of the distal histidine, initiating a proton relay mechanism that catalyses a full charge transfer from Fe^{2+} to O_2, resulting in the release of O_2^- in the form of HO_2 (see Fig 1.6) (Phillips & Schoenborn, 1981; Shikama, 1988). As such, proton-catalysis accounts for the majority of the auto-oxidation occurring under physiological conditions (Tsuruga *et al.*, 1998).

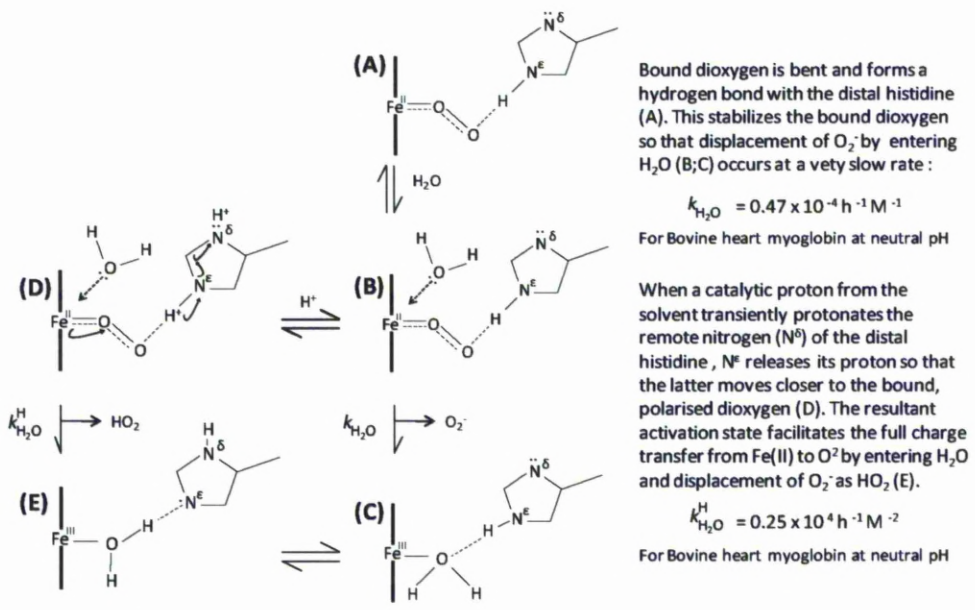


Fig 1.6. Schematic representation of the spontaneous autoxidation process in water (natural rate constant k_{H2O}) and the proton-catalyzed process ($k^H_{H_2O}$) in neutral pH range. Adapted from Shikama *et al.*(1988).

Human oxyHb exhibits biphasic auto-oxidation, with α chains oxidizing at a faster rate relative to β chains, in acid or neutral conditions (Mansouri & Winterhalter, 1973). However, at basic pH, human oxyHb oxidation is monophasic as a result of high concentrations of the powerful nucleophile OH^-, which oxidizes both chain variants at equal rates (Yasuda *et al*, 2002). Chain heterogeneity is observed for haemoglobin tetramers due to packing constraints manifest upon formation of the α1β1 and α2β2 contacts (Fig 1.7) which tilt the β chain distal histidine (His E7) away from dioxygen (Shaanan, 1982: Tsuruga *et al.*, 1998). This weakens the His-O_2 H-bond and prevents proton mediated catalyzed displacement of O_2^- by water molecules, delaying β chain's oxidation under acidic conditions (Tsuruga *et al.*, 1998).

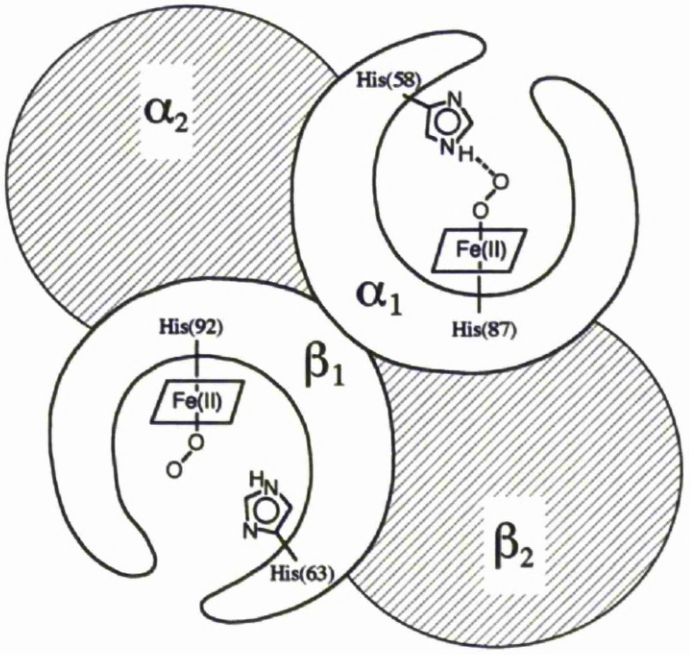


Fig. 1.7. Schematic representation of human oxyHb as seen in the $\alpha_1\beta_1$ contact to produce tilting of the distal histidine in the β chain. From Tsuruga *et al.* (1998).

1.2.3.2. Dissociation of haem from haemoglobin.

At neutral pH, apomyoglobin has an association equilibrium constant for haem binding of ~ 10^{14} M^{-1} which is determined, in addition to the proximal His(F8)-haem iron covalent bond, by a complicated set of hydrophobic and ionic

interactions (Hargrove *et al.*, 1996). The importance of the covalent bond is demonstrated by near 1000-fold increase in haem dissociation in metmyoglobin mutants incapable of forming this interaction. The location of the amphipathic porphyrin within the hydrophobic haem pocket (formed and maintained by proximal residues such as Leu89 and Ile99) and exclusion of solvent also limit haem dissociation (Hargrove *et al.*, 1996). This apolar environment strengthens the proximal histidine-iron bond by lowering the pK_a of its imidazole group and the side chains of proximal residues that hold it in a fixed orientation (Hargrove *et al.*, 1996). Mutations resulting in increased polarity around the proximal histidine destabilise this bond due to solvation and protonation of the imidazole group (Hargrove *et al.*, 1996). Many of the interactions with the tetrapyrrole ring are also affected by polar residues (Momenteau & Reed, 1994). Haem-globin stability is also reduced by oxidation of the haem iron and αβ dimer dissociation (Gattoni *et al.*, 1996), low pH (Hargrove *et al.*, 1994) and certain types of ligand binding to iron at the sixth coordinate position (e.g. hydroxide and phenoxide [Hargrove *et al.*, 1994]).

1.2.4. Haptoglobin.

Hb freed from erythrocytes will rapidly oxidise leading to haem dissociation. Extracellular Hb, liberated by intravascular haemolysis (also bacterially mediated), is consumed by the serum haptoglobin which has a binding affinity of $K_{as} \sim 10^{-12}$ M (Evans *et al.*, 1999). The irreversible non-covalent haptoglobin-Hb complex is subsequently absorbed by the liver and delivered to the reticuloendothelial system by CD163 receptor-mediated uptake or endocytosis (Kristiansen *et al.*, 2001). The haptoglobin tetramer is composed of two heavy (beta) and light (alpha) chains held together by a series of disulphide bonds (Kurosky *et al.*, 1980). Haptoglobin binds one molecule of tetrameric haemoglobin, with each haptoglobin heavy chain binding an alpha-beta heterodimer of haemoglobin (Valette *et al.*, 1981). The structure of either protein is unaltered (Waks *et al.*, 1971).

1.2.5. Non-haemoglobin haem sources.

The most important serum protein in haem catabolism is haemopexin (57 kDa; see Fig 1.8) (Seery *et al.*, 1972) which specifically binds one molecule of haem (Heide *et al.*, 1964) with a greater affinity than albumin ($K_d \sim 10^{-13}$ M [Muller-

Eberhard *et al.*, 1969; Drabkin, 1971]). Haemopexin, is found at serum concentrations of 0.3 to 1.5 mg ml^{-1} (Braun, 1971).

Serum albumin, which also contributes to transport of extracorpuscular haem to liver parenchymal cells, is the most predominant protein in GCF from both healthy and inflamed periodontal sites (Curtis *et al.*, 1990, Makela *et al.*, 1991). Moreover, because it is present in serum at concentrations between 80 and 40-fold greater than haemopexin, albumin binds more of any free haem (Muller-Eberhard & Morgan, 1975). This is also despite the fact that it has a 75-fold lower affinity for haem binding (K_d~10^{-8} M; Hrkal *et al.*, 1980) than haemopexin. Serum albumin (Fig 1.8) consists of a single polypeptide chain (66 kDa) arranged into nine loops and stabilized by 17 disulfide bridges but possesses a complex multidomain structure allowing for great flexibility and conformational adaptability (Peters, 1985). The three distinct domains (I, II, and III) are further subdivided into subdomains A and B that are connected by a random coil (He & Carter, 1992). The structure in solution is highly pH-dependent (Peters, 1985; Carter & Ho, 1994), and it possesses a high-affinity site ($K_a = 5 \times 10^7$ mol^{-1}), in addition to at least ten subsidiary lower-affinity sites for haem (Beaven *et al.*, 1974). Initial haem-albumin association is followed by internalisation within a narrow hydrophobic D-shaped cavity in subdomain IB stretching from Leu112 to Cys200 (Zunszain *et al.*, 2003) in a process that sequesters and reduces haem bioavailability (Adams & Berman, 1980). In normal plasma haemalbumin is present at a ratio of about 1 to 5000 molecules of apoalbumin (Walji *et al.*, 1993).

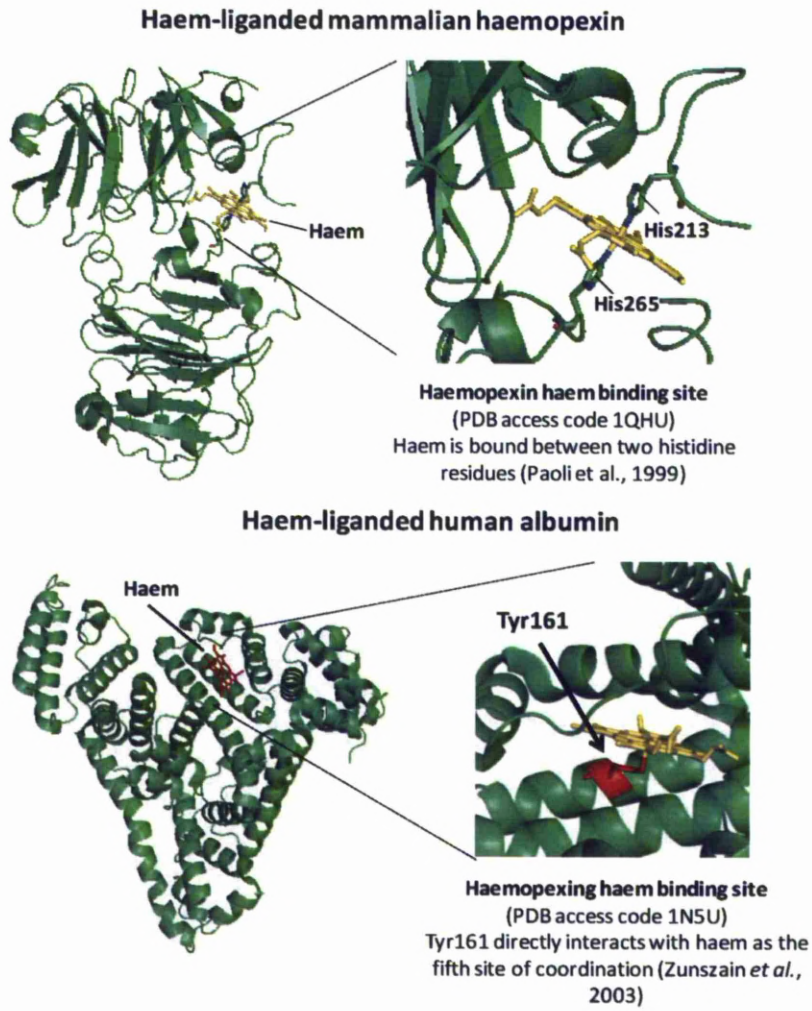


Fig 1.8. Structures of haemopexin and albumin. Diagrams created using Pymol.

1.2.6. Bacterial haem acquisition.

1.2.6.1. Intracellular haem sources.

To gain access to iron and haem, pathogenic bacteria such as *P. gingivalis* and *P. intermedia* must utilise host intracellular reserves. This can be achieved by several mechanisms such as the secretion of haemolysins (Picket *et al.*, 1992; Takada *et al.*, 2003; Hoshi *et al.*, 1993), proteases (Simpson & Oliver, 1993) and cytolysins (Lagergard *et al.*, 1993). Many bacteria produce transmembrane pore-forming toxins to lyse erythrocytes, such as the alpha haemolysin of strains of *Escherichia coli* (Bhakdi *et al.*, 1986), haemolysins of *Staphylococcus aureus* (Dinges *et al.*, 2000),

streptolysins of strains of streptococci (Sekiya *et al.*, 1993), *Aggregatibacter pleuropneumoniae* haemolysin (Lalonde *et al.*, 1989), and the adenylate cyclase toxin (AC toxin) of *Bordetella perussis* (Szabo *et al.*, 1994). Depending on the size, the pore produced by a particular toxin permits flux of ions or macromolecules (Hugo *et al.*, 1986). Erythrocyte membrane damage enables haemoglobin molecules to leak out or leads to total cell lysis. Some bacteria, such as *Haemophilus influenzae* type b (Hib), rely on immunological mechanisms to release Hb, in which anti-Hib capsular polysaccharide antibodies adsorb onto erythrocyte membranes and induce complement-mediated intravascular lysis (Shurin *et al.*, 1986).

Importantly, oral bacteria possessing haemolytic activity have also been identified. Pore-forming haemolysins (Deshpande & Khan, 1999; Karunakaran & Holt, 1993) and proteases of *P. gingivalis* (Chu *et al.*, 1991; Hoshi *et al.*, 1993) are thought to facilitate haemolysis. Potent haemolytic activity has been observed by *P. intermedia* (Okamoto *et al.*, 1999; Silva *et al.*, 2003). *T. denticola* (Chu *et al.*, 1995) and *A. actinomycetemcomitans* (Balashova *et al.*, 2006) also synthesize haemolysins, and are thought to mutually benefit other periodontal-pocket bacteria. Also, the stronger haemolytic activity of *P. intermedia* may benefit *P. gingivalis* growth by augmenting the levels of Hb (Okamoto *et al.*, 1999).

1.2.6.2. Haemoglobin as a source of haem for microorganisms.

Chronic periodontitis is characterised by vascular disruption and bleeding induced by inflammatory responses to pathogenic infectious agents. Bleeding is accompanied by increased availability of gingival crevicular fluid and haem-containing proteins. However, haem from freshly lysed erythrocytes must be liberated from Hb. Haemoglobin binding activity has been identified in *Neisseria meningitidis* (Lee & Hill 1992), *Vibrio cholerae* (Henderson & Payne, 1994) and *H. ducreyi* and *H. influenzae* (Elkins, 1995). Proteases are also used to affect this. For example, *P. gingivalis* R- and K-specific cysteine proteases (gingipains) sequentially breakdown Hb to liberate haem (Lewis *et al.*, 1999; Smalley *et al.*, 2007; 2008). The haem-binding protein (Hbp) secreted from pathogenic strains of *E. coli* also degrades Hb (Otto *et al.*, 1998). In addition, several species including Hib (Stull, 1987), *Vibrio vulnificus* (Helms *et al.*, 1984), *Campylobacter jejuni* (Picket *et al.*, 1992) and pathogenic *Neisseria* spp. (Dyer *et al.*, 1987) use haptoglobin-bound Hb as a haem

source. *P. gingivalis* also proteolyses the haemoglobin-haptoglobin complex (Tompkins *et al.*, 1997)

1.2.6.3. Haemopexin and albumin as sources of haem.

In order to survive, pathogenic bacteria must also circumvent the actions of haemopexin and albumin. Haemopexin-bound haem is used for growth *in vitro* by *C. jejuni* (Picket *et al.*, 1992) and by Hib (Cope *et al*, 1994), and *P. gingivalis* also proteolytically degrades haemopexin (Jansen *et al.*, 1994). Albumin also provides a non-haemoglobin haem, carbon and nitrogen supply for bacteria such as *P. gingivalis* and *P. intermedia* (Jansen at al., 1994; Milner *et al.*, 1996; Smalley & Birss, 1997; Ichiro, 2000).

1.2.7. Haem uptake systems of Gram-negative bacteria.

The outer membranes of Gram-negative bacteria are permeability barriers that block passive transfer of molecules larger than 600 Da into the cytoplasm (Braun & Killman, 1999), and so active transport across the cell membrane is required for haem internalisation. The best characterised haem acquisition systems involve direct haem or haemoprotein (haemopexin, haemoglobin, haptoglobin-haemoglobin) uptake by bacterial outer-membrane surface receptors (Tong & Guo, 2009). The archetypal system is that described for *P. aeruginosa* (see Fig 1.9 [Tong & Guo, 2007]). Over thirty other outer-membrane haem receptors, varying in the number of ligands they can bind, have been reported for several lineages of Gram-negative bacteria (Wandersman & Stojiljkovic, 2000). Haem receptors have been described for *Neisseria gonorrhoeae* (Lee, 1992), *N. meningitidis* (Lewis *et al.*, 1998), *H. influenzae* (Cope *et al.*, 1995), *V. cholerae* (Henderson & Payne, 1994), *Yersinisa enterocolitica* (Stojiljkovic & Hantke, 1994), and *S. dysenteriae* (Burkhard & Wilks, 2007). A haem utilisation receptor, HmuR, of *P. gingivalis* which is involved in haem and haemoglobin internalisation has also been characterised (Simpson *et al* 2000; Olczak *et al.*, 2008). Interestingly, several haem and haemoprotein receptors (including HmuR) share extensive sequence homology which may suggest shared ancestry (Simpson *et al.*, 2000).

Many of the described haem and/or protein-bound haem transport systems are dependent on TonB-like proteins. In 2010, Noinaj *et al.* published a comprehensive

review on TonB-dependent transporters. TonB, in association with ExbB and ExbD proteins, provides energy required for active haem transport (Postle, 2007). It is proposed that the TonB system utilises energy produced by the proton motive force to open channels in the outer membrane and allow haem passage (Postle, 2007). *P. gingivalis* genes, *hemR*, *ihtA* and *tlr*, exhibit TonB-dependent receptor homology (Dashper *et al.*, 2000; Karunakaran *et al.*, 1997; Slakeski *et al.*, 2000).

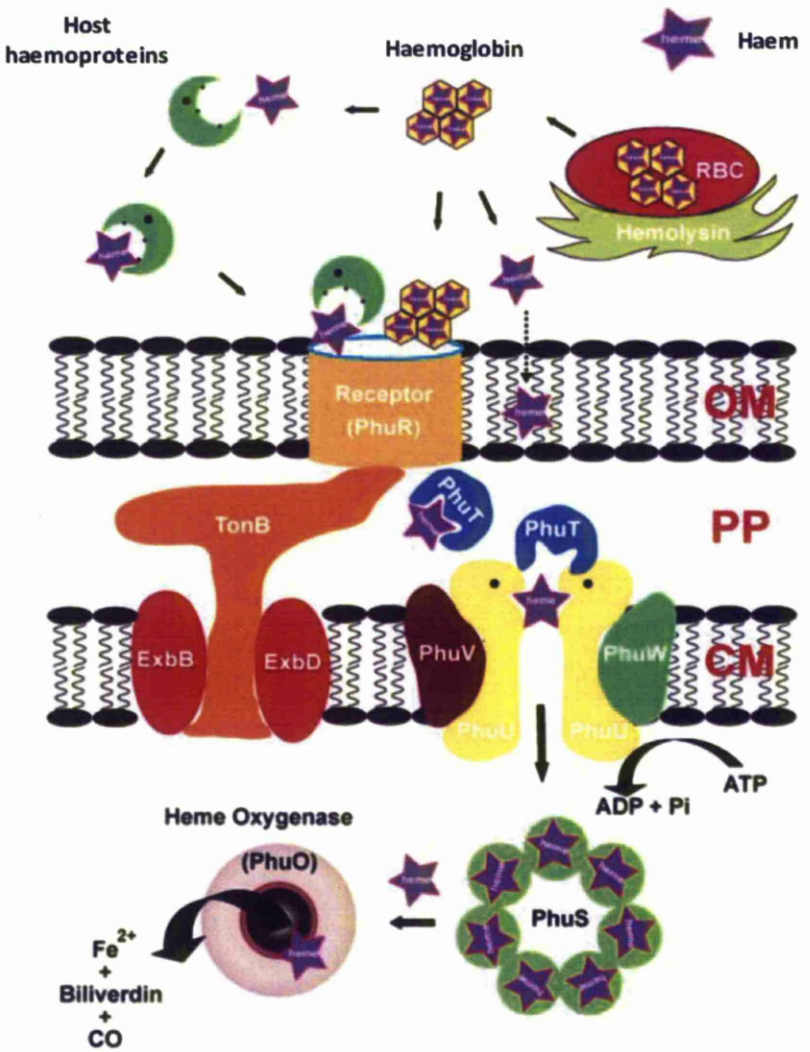


Fig 1.9. Schematic representation of haem uptake (purple star) by *P. aeruginosa*. Taken from Tong & Guo, 2009.

Several haem uptake system also utilise extracellular proteins known as haemophores to capture haem. HasA of *S. marcescens* is a 19 kDa monomeric

protein that binds haem at an equimolar ratio with high affinity (K_d~10^{-11} M) and was the first haemophore to be identified (Deniau *et al.*, 2003). HasA binds external free haem, or extracts it from haemoproteins, and shuttles it to a surface-located haemophore specific receptor (Lefevre *et al.*, 2008). Haemophore systems have been reported for *S. marcescens*, *P. aeruginosa*, *Pseudomonas fluorescens*, *Y. pestis*, *Y. enterocolitica*, and *H. influenzae* (Cescau *et al.*, 2007). Of particular interest for this study is HmuY of the periodontal pathogen *P. gingivalis* (Olczak *et al.*, 2008) which will be discussed in greater detail later.

In Gram-negative bacteria, once haem is transferred across the outer-membrane it is captured by a haem-specific periplasmic transport protein (HTP) which interacts with ABC transporters to facilitate transport across the cytoplasmic membrane (Stojiljkovic & Perkins-Balding, 2002). Periplasmic HemT, transmembrane HemU, and the ATPase HemV of *Y. enterocolitica* form the first characterised permease (Stojiljkovic & Perkins-Balding, 2002). To limit the toxic effect of free haem, once internalised, haem is sequestered by an analogue of PhuS before being delivered to haem oxygenase (HO) for catabolism (Stojiljkovic & Hantke, 1992).

1.2.8. Regulation of haem uptake.

Although iron is essential for pathogenic bacteria, it is toxic at high levels. Environmental iron levels frequently regulate expression of haem/iron uptake system components in many Gram-negative pathogens; the global determinant being Fur (Ferric Uptake Regulator). This is a homodimer of 17 kDa subunits (Hantke, 2001) and has been described in *V. cholerae* (Occhino *et al.*, 1998), *Y. pestis* (Thompson *et al.*, 1999), *Y. enterocolitica* (Stojiljkovic & Hantke, 1994), and *P. aeruginosa* (Ochsner *et al.*, 2001). Its N-terminal domain interacts with a 19 bp "Fur box" DNA sequence in response to divalent iron binding by the Histidine-rich C-terminal domain and dimerisation, which positively or negatively regulates outer-membrane receptor protein gene expression (Coy & Neilands, 1991; Vanderpool & Armstrong, 2004). Non-Fur regulatory mechanisms have also been identified for *S. marcescens* which uses haemophore dependent signal transduction to regulate transcription of the haemophore-specific outer membrane receptor, HasR (Rossi *et al.*, 2003). Iron

and haem also regulate virulence factor expression in *P. gingivalis* which profoundly impacts its pathogenicity and ability to acquire haem (McKee *et al.*, 1986).

Although haem is a major iron source and haem scavenging is ubiquitous amongst pathogenic bacteria, few organisms accumulate haem to the extent of black pigmenting anaerobes (BPAs). The main aim of this investigation will be to characterise and elucidate the mechanisms used by two BPAs (*P. gingivalis* and *P. intermedia*) to acquire host-derived haem, and these species are discussed in greater detail in the following sections.

1.3. *PORPHYROMONAS GINGIVALIS.*

1.3.1. *Porphyromonas gingivalis* and periodontal disease.

P. gingivalis is strongly associated with development and progression of periodontal disease, and as a potential risk factor for cardiovascular disease (Spahr *et al.*, 2006, Kuramitsu *et al.*, 2003) and pre-term delivery of low birth weight infants (Dasanayake *et al.*, 2001). It is often isolated from healthy individuals but found in far greater numbers within periodontal lesions (Choi *et al.* 1990). Increase in abundance of *P. gingivalis* (and other opportunistic pathogens) and a change in the overall composition of sub-gingival plaque microflora is correlated with the advancement of inflammatory periodontal disease (Dzink *et al.*, 1988). Removal of, or immunization against, *P. gingivalis* in experimental models significantly reduces progression of periodontitis, demonstrating the importance of the organism (Persson *et al.*, 1994). *P. gingivalis* produces energy through fermentation of amino acids and thus produces large quantities of proteolytic enzymes which are also critical for disruption of host-defence, penetration and destruction of connective tissues, and maintenance of inflammation in the periodontal pocket (Potempa *et al.*, 2000).

P. gingivalis exists predominantly in complex biofilm communities within the periodontal pocket, co-aggregating with oral bacteria such as *Actinomyces viscosus, Streptococcus mutans, S. gordonnii, F. nucleatum and P. intermedia* (Nonaka *et al.* 2001; Li *et al.*, 1991; Kamaguchi *et al.*, 2003; Kinder & Holt, 1989). These interactions provide growth substrates and reduce oxygen tension allowing *P.*

gingivalis growth. Furthermore, *P. gingivalis* adheres to numerous host surfaces such as epithelial cells (Cutler *et al.*, 1995).

1.3.2. Haem requirement of *P. gingivalis.*

P. gingivalis, which lacks siderophores and is deficient in protoporphyrin ring synthesis, utilises exogenous sources to satisfy its growth requirements for haem and iron (Roper *et al.*, 2000; Nelson *et al.*, 2003). Haemproteins, in addition to supplying peptides and amino acids, fulfil this need (Lewis *et al.*, 1999). Development of the cell surface haem pigment is a defining phenotype and is an important virulence factor (Smalley *et al.*, 1998, 2000). Formation of the pigment, predominantly consisting of μ-oxo dimers (bishaem), creates an anaerobic microenvironment around the bacterium by absorbing O_2 during its formation from Fe(II) haems of haemoglobin (Smalley *et al.*, 1998). Although *P. gingivalis* possesses superoxide dismutase (Nakayama, 1994), it lacks catalase and peroxidase enzymes to protect against neutrophil attack (Marquis, 1995). The pigment compensates for this inadequacy due to the inherent catalase activity of ferric protoporphyrin IX (Jones *et al.*, 1973) to degrade H_2O_2 (Smalley *et al.*, 2000). *P. gingivalis* cells carrying a surface layer of μ-oxo bishaem are predictably less susceptible to peroxidation (Smalley *et al.*, 2000).

Smalley *et al.* (2002) proposed two routes of μ-oxo bishaem generation by *P. gingivalis*. The first involves proteolytic degradation of haemproteins (e.g. oxy- and deoxyHb) to release Fe(II) haems which combine with and consume dioxygen according to equation (1)

$$4\text{Fe(II)PPIX} + O_2 \rightarrow 2[\text{Fe(III)PPIX}]_2O \quad (1)$$

The second generates μ-oxo bishaem via the bringing together of two OH^- bearing ferrihaems (Fe(III)PPIX.OH), derived from methaemoglobin, and enables pigmentation to occur in oxygenated environments (Smalley *et al.*, 2002).

$$2\text{Fe(III)PPIX.OH} \rightarrow [\text{Fe(III)PPIX}]_2O + H_2O \quad (2)$$

However, it has recently been revealed that spontaneous formation of μ-oxo bishaem is unlikely in aqueous solution (Villiers *et al.*, 2007). *P. gingivalis* must

therefore utilise some unique, and as of yet uncharacterised, mechanism to facilitate μ-oxo dimer formation.

Gene sequences encoding haem cofactor enzymes (e.g. cytochrome *d*, ubiquinol oxidase and cytochrome *c* nitrite reductase) have been identified in *P. gingivalis* (Roper *et al.*, 2000). Although putative *hemD* (uroporphyrinogen III synthase), *hemG* (protoporphyrinogen oxidase), *hemN* (coproporphyrinogen oxidase), and *hemH* (porphyrin ferrochelatase) genes encoding enzymes involved in the protoporphyrin IX (PPIX) biosynthetic pathway have been identified in the *P. gingivalis* genome, the entire repertoire of genes required for *de novo* biosynthesis is incomplete (Nelson *et al.*, 2003). Therefore, environmental haem capture is essential for energy metabolism (Roper *et al.*, 2000). However, expression of a ferrochelatase (HemH) by *P. gingivalis* may catalyse insertion of iron into exogenous iron-free PPIX to form haemin or extract iron from haem (Olczak *et al.*, 2005; Nelson *et al.*, 2003). Furthermore, HemN, D and G may modify related tetrapyrrole ring side chains to generate essential porphyrins (Olczak *et al.*, 2005). Thus, transport of haem into the bacterial cell is probably energetically more favourable than PPIX biosynthesis in a haem rich medium (Genco & Dixon, 2001; Olczak *et al.*, 2005). Transferrin, which is abundant in GCF of patients with destructive periodontitis (Makela *et al.*, 1991), is degraded by K- and R-gingipains to provide non-haem derived iron (Sroka *et al.*, 2001; Brochu *et al.*, 2001). Lactoferrin also serves as an iron source (Grenier *et al.*, 2001a).

1.3.2.1. Haem sources.

Chronic periodontitis is characterised by vascular disruption, bleeding, and increase in GCF containing haemproteins. *P. gingivalis* possesses five major haemagglutinins (HagA, B, C, D, and E; Lepine *et al.*, 1996) and six haemagglutinin-like proteins (Nelson *et al.*, 2003) which mediate adsorption and invasion of the bacterium into host cells and participate in iron and haem utilisation (Lantz *et al.*, 1991; Pike *et al.*, 1996; Scragg *et al.*, 1999). Haemolysins have also been identified in cells and extracellular vesicles of *P. gingivalis*, some of which are haem-regulated (Chu *et al.*, 1991; Hoshi *et al.*, 1993; Kay *et al.*, 1990). *P. gingivalis* has a preference for Hb-derived haem which is liberated by cysteine proteases (gingipains) (Shizukuishi *et al.*, 1995; Lewis *et al.*, 1999; Aduse-Opoku *et al.*, 2000;

Smalley *et al.*, 2004; 2007; 2008) and which are essential for pigmentation. Gingival crevice haemoproteins such as haem-haemopexin and serum haemalbumin also supplement Hb-derived haem (Lewis *et al.*, 1999; Sroka *et al.*, 2001). Albumin is degraded by *P. gingivalis* (Fishburn *et al.*, 1991; Smalley *et al.*, 1997; Grenier *et al.*, 2001c) and satisfies a requirement for carbon and nitrogen *in vitro* (Milner *et al.*, 1996), and haemalbumin is also degraded to provide haem for growth *in vitro* (Bramanti & Holt, 1991; Smalley & Birss, 1997). *P. gingivalis* can distinguish between apo- and haemalbumin (presumably by way of haem ligand conformational changes [Walji *et al.*, 1993]) with cells binding the latter more avidly (Smalley & Birss, 1999). The nature of the *P. gingivalis* apo- and haemalbumin binding component is unclear although a role for LPS has been suggested (Smalley *et al.*, 1999). Confusing matters further is contradictory evidence as to whether haem and albumin compete for the same binding site (Smalley *et al.*, 1999; Tompkins *et al.*, 1997). It is clear however, that growth on bovine serum albumin is dependent on the activity of both K- and R-gingipains (Shi *et al.*, 1999).

A putative high affinity binding site of *P. gingivalis* has a greater affinity for hemin than albumin but not haemopexin (Tompkins *et al.*, 1997). As such, auxiliary mechanisms are essential to satisfy its haem requirement in the presence of haemopexin. Unlike *E. coli, P. gingivalis* will grow on media containing haptoglobin or haemopexin as the only haem source (Bramanti & Holt, 1991), and haptoglobin and haemopexin are highly susceptible to proteolysis by *P. gingivalis* (Carlsson *et al.*, 1984a; Jansen *et al.*, 1994).

1.3.3. *P. gingivalis* proteases.

1.3.3.1. Gingipains.

P.gingivalis produces a wide variety of enzymes essential for pathogenesis such as aminopeptidases, carboxypeptidases, di- and tri- peptidyl peptidases, oligopeptidases and several endopeptidases (e.g. gingipains) (Travis *et al.*, 1997; Kumagai *et al.*, 2000; Banbula *et al.*, 1999; 2001; Grenier *et al.*, 2001b; Masuda *et al.*, 2002; Chen *et al.*, 2002).

P. gingivalis produces two types of gingipain differing in specificity for P1 Arg- (RgpA and RgpB) and P1 Lys- (Kgp). Together, they are responsible for ~ 85%

of extracellular proteolytic activity, 99% of so-called "trypsin-like" amidolytic activity (Smalley *et al.*, 2000) and the majority of 'haemoglobinase' activity (Dashper *et al.*, 2004). Gingipains provide nutrients and are thus vital for survival, growth and are major virulence factors (Lamont *et al.*, 1998; O'Brian-Simpson *et al.*, 2001). Gingipains bind haem and iron (Imamura, 2003) and degrade haemoglobin (Smalley *et al.*, 2008), haptoglobin and haemopexin (Sroka *et al.*, 2001), transferrin (Sroka *et al.*, 2001; Brochu *et al.*, 2001), lactoferrin (de Lillo *et al.*, 1996), and are essential for haem pigmentation (Shoemaker *et al.*, 1986; Chen *et al.*, 2000). Gingipains also have a wide range of other important roles besides providing nutrients for growth (See Table 1.1)

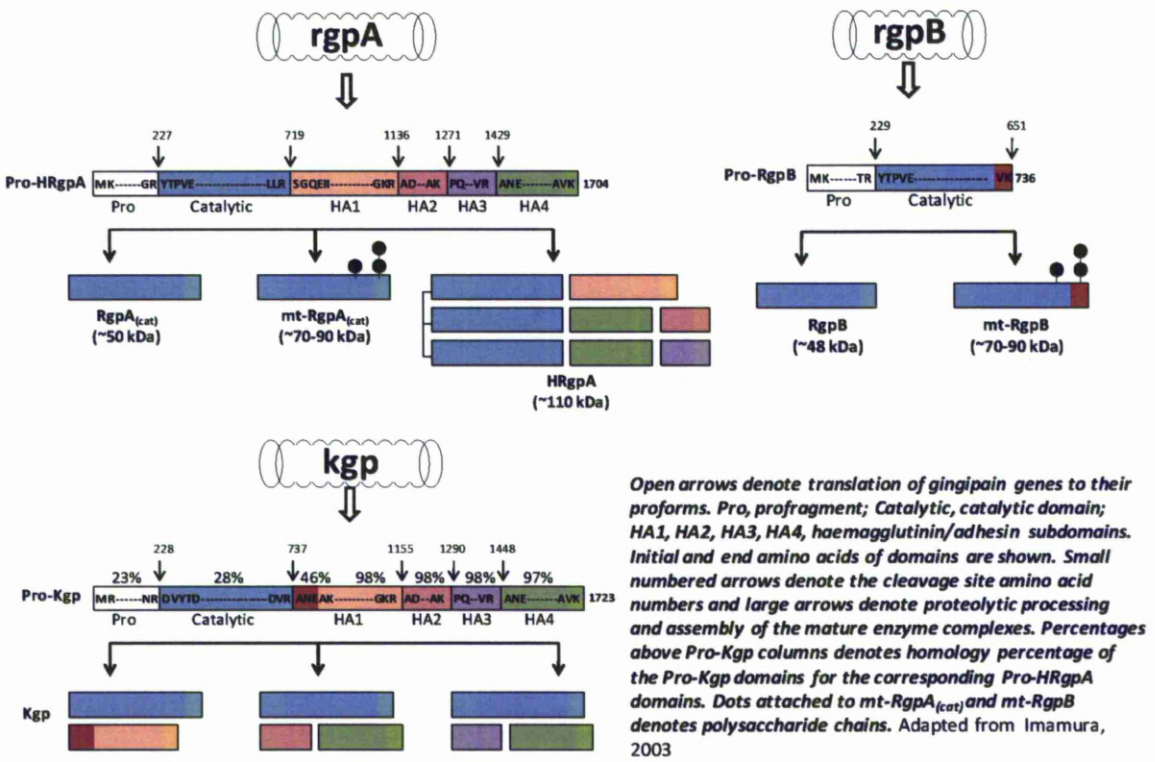
Table 1.1. The role of gingipains in the pathogensis of periodontal disease.

Physiological Role	Gingipain specificity	Ref
House Keeping Functions		
Processing of pro-Kgp	R	Potempa & Travis, 1996
Processes a 75 kDa outer membrane protein	R	Kadowaki et al., 1998
Disruption of immune response		
Degradation of complement factors (C3 and C5)	R	DiScriio et al., 1996
Antibody proteolysis (IgG, IgA, secretory IgA)	R & K	Kadowaki et al., 2000
Cleavage of C5a receptor	K	Jagels et al., 1996
Degradation of monocyte CD14	K	Sugawara et al., 2000
Cytokine Inactivation (TNF-α & IL-6)	R	Calkins et al., 1998; Kadowaki et al., 2000
Processing of ICAM-1 on oral epithelial cells	R & K	Tada et al., 2003
Direct destruction of host tissue		
Degradation of collagens type I and IV	R & K	Kadowaki et al., 2000
Degradation of fibronectin	R & K	Kadowaki et al., 2000
Degradation of laminin	R & K	Kadowaki et al., 2000
Contribution to tissue colonization		
Haemagglutinin domains adhere directly to extracellular matrix proteins	R & K	Lantz et al., 1991, Pike et al., 1996
Processing of fimbrillin	R	Nakayama et al., 1996, Onoe et al., 1996
Mediating cell detachment	R	Chen et al., 2001
Bleeding and vascular permeability		
Triggering bradykinin release	R	Imamura et al., 1994
Activation of coagulation factor IX & X	R	Imamura et al., 1997; 2001a
Activation of prothrombin	R	Imamura et al., 2001b
Degradation of fibrinogen and fibrin	R & K	Pike et al., 1996

Gingipains are the products of three genes, *rgpA*, *rgpB* and *kgp* (Potempa *et al.*, 1995; Curtis *et al.*, 1999). Once translated, RgpA consists of three separate domains, a pro-fragment with a signal sequence, a catalytic domain, and a

haemagglutinin/adhesin domain (HA) at the C-terminus (Potempa *et al.*, 1995; Curtis *et al.*, 1999). The original polyprotein undergoes proteolytic processing and post-translational modification to produce three alternative forms of the enzyme. The first type consists of the catalytic domain alone ($RgpA_{(cat)}$) produced either by catalytic processing of the initial protein or by interruption of the transcription process (Rangarajan *et al.*, 1997). The second version is membrane-associated ($mt\text{-}RgpA_{(cat)}$) consisting of a catalytic domain modified with lipopolysaccharide (LPS; Curtis *et al.*, 1999). HRgpA, the third version, is a non-covalent but highly stable complex of the catalytic domain and the HA domain (Pavloff & Potempa, 1995). RgpB consists of a catalytic domain only and lacks almost the entire section encoding HA domains bar a small carboxy-terminal segment (Slakeski *et al.*, 1998; Mikolajczyk-Pawlinska *et al.*, 1998). A membrane-associated form of RgpB with LPS attachments, known as mt-RgpB (mt standing for membrane-type) has also been described (Curtis *et al.*, 1999). Interestingly soluble RgpB does not contain LPS and is truncated at the carboxy terminus compared to the nascent gene product (Potempa *et al.*, 1998). RgpA and RgpB share 72%, 93% and 40 % pro-fragment, catalytic, and C-terminal extension domain homology respectively (Curtis et a., 1999). The *rgpA* and *rgpB* loci have been identified in all clinical and labratory isolates of *P. gingivalis* and their protein structures are highly conserved (Mikolajczyk-Pawlinska *et al.*, 1998; Aduse-Opoku *et al.*, 1995; Allaker *et al.*, 1997).

Kgp is a polyprotein containing a leader sequence, pro-fragment, and catalytic and HA domains (Pavloff *et al.*, 1997). The catalytic domains and pro-fragments of Kgp and RgpA share only 28% and 23 % amino acid sequence homology, respectively, yet the HA domains are highly homologous (Okamoto *et al.*, 1995, 1996). Nascent secreted Kgp and Rgp polyproteins, are non-covalent heteromultimeric complexes of a catalytic domain and discrete functional subunits comprising the HA domains (HA1, HA2, HA3 and HA4) (Pavloff *et al.*, 1995; 1997; Okamoto *et al.*, 1996; 1995; Pike *et al.*, 1994;Veith *et al.*, 2002). The HA2 (HbR) domains binds haemoglobin (K_d~ 10^{-9} M) and, *via* the porphyrin moiety, haem (K_d~ 10^{-8} M) (Paeamaesvaran *et al.*, 2003) and is essential for pigmentation (Nakayama *et al.*, 1998; Chen & Kuramitsu, 1999). A schematic representation of gingipain translation and processing can be found below (Fig 1.10).



Open arrows denote translation of gingipain genes to their proforms. Pro, profragment; Catalytic, catalytic domain; HA1, HA2, HA3, HA4, haemagglutinin/adhesin subdomains. Initial and end amino acids of domains are shown. Small numbered arrows denote the cleavage site amino acid numbers and large arrows denote proteolytic processing and assembly of the mature enzyme complexes. Percentages above Pro-Kgp columns denotes homology percentage of the Pro-Kgp domains for the corresponding Pro-HRgpA domains. Dots attached to mt-RgpA(cat) and mt-RgpB denotes polysaccharide chains. Adapted from Imamura, 2003

Fig 1.10. Isoform construction by proteolytic processing and assembly of the translated products of *rgpA*, *rgpB*, and *kgp* genes from *P. gingivalis* HG66.

1.3.4. Haem- and haemoglobin-binding proteins.

1.3.4.1. HmuY and HmuR.

HmuR is a major outer-membrane receptor involved in acquisition of free haem and that bound to haemoproteins in a TonB dependent manner (Simpson *et al.*, 2000; Olczak *et al.*, 2001, 2005). HmuR binds haem through His95 and His191 residues (Genco & Dixon, 2001; Lui *et al.*,2004) and has a β-barrel structure consisting of a β-barrel domain, arranged in loops and short turns on the extracellular and periplasmic sides, respectively, and a globular amino terminal domain that folds into the barrel pore (Liu *et al.*, 2006). The *hmuR* gene is located on an operon containing four, as yet uncharacterised genes, and the gene for the outer-membrane lipoprotein, HmuY (Olczak *et al.*, 2008). The importance of the *hmu* operon was demonstrated by the fact that its inactivation results in a decrease in haem binding and uptake by the cells (Lewis *et al.*, 2006). HmuY binds haem *in vitro*

and is constitutively expressed, but is produced at greater levels under low-haem conditions in biofilms (Olczak *et al.*, 2008; 2010; Dashper, *et al* 2009; Ang *et al.*, 2008). HmuY may also be involved in biofilm formation (Olczak *et al.*, 2010). HmuY and HmuR work together and play an important role in haem capture (Olczak *et al.*, 2008). HRgpA and Kgp may also function as haemophore like proteins to capture and shuttle haem to HmuR, and interact with the receptor through their HA domains (Olczak *et al.*, 2001; Simpson *et al.*, 2004).

1.3.4.2. Other haem-binding proteins.

IhtB and RagB are TonB-dependent outer-membrane lipoproteins potentially involved in haem binding (Kim *et al.*, 1996; Hanley *et al.*, 1999; Bonass *et al.*, 2000; Heinje, 1989). HBP35 is capable of binding haem (possibly via the iron) and is important for expression of virulence (Shibata *et al.*, 2003b; Hiratsuka *et al.*, 1999). Tla, a TonB-dependent haem-binding protein, has 98 % homology with Rgp at the C-terminus and may influence Rgp activity (Aduse-Opoku *et al.*, 1997). Tlr, Omp26 HemR may also function in haem acquisition (Slakeski *et al.*, 2000; Karunakaran *et al.*, 1997; Bramanti & Holt, 1993a). *P. gingivalis* also possesses a recently identified haemophore-like protein, HusA, which binds haem with a high binding constant and is expressed as both an outer membrane receptor and released from the organism (Gao *et al.*, 2010).

1.3.5. Expression of genes involved in iron/haem utilisation and virulence.

Environmental iron concentrations may influence expression of *P. gingivalis* proteins. A *P. gingivalis* homologue of Fur proteins, thought to regulate *hmuY* and *hmuR* genes, has been identified (Nelson *et al.*, 2003; Olczak *et al.*, 2005). Furthermore, a novel iron-dependent repressor (AAQ66163) has also been described but details of its function are unknown. Haem replete and deplete conditions also regulate expression of numerous virulence factors, some of which are known to be involved in haem acquisition (Bramanti & Holt, 1992; 1993b; Kesavalu *et al.*, 2003; Smalley *et al.*, 1991; 1993a; Marsh *et al.*, 1994). Unusually, gingipain expression may be regulated independently from haem and iron levels. Expression of *rgpA* and *kgp* is more likely to be regulated by growth phase (Liu & Genco, unpublished findings). Quorum sensing, using the LuxS protein (Chung *et al.*, 2001), may also control expression of genes involved in haem/iron acquisition including HmuR and

is likely to influence other components of the *hmu* operon such as HmuY (Chung *et al.*, 2001; James *et al.*, 2006).

1.4. *PREVOTELLA INTERMEDIA.*

1.4.1. *Prevotella intermedia and periodontal disease.*

P. intermedia is associated with acute necrotizing ulcerative gingivitis, adult periodontitis and pregnancy gingivitis (Alauzet *et al.*, 2010). Although primarily isolated from subgingival plaques in deep periodontal pockets, *P. intermedia* is also found on the oral mucosa, the tongue, and the tonsils (Van der Velden *et al.*, 1986; Van Winkelfoff *et al.*, 1986). It is also implicated in extra-oral infections, including bacterial tracheitis and noma in children (Brook, 1997; Falker *et al*, 1995). *P. intermedia* also plays an important role in the maturation of plaque by forming a complex with *Prevotella nigrescens* and *F. nucleatum* which bridges early colonizing Gram-positive with Gram-negative bacteria of the mature biofilm (Socransky *et al.*, 1998), and thus contributes indirectly to periodontal disease progression. As a biofilm member, and through exposure to various antibiotics, emergence of resistance often occurs (Walker, 1996). Elimination of *P. intermedia* is a matter of concern as it may represent a reservoir for antibiotic resistance genes.

P. intermedia carriage is prevalent throughout much of the population and is isolated from abnormally deep periodontal pockets in 15% of the adult Finnish population (Könönen *et al.*, 2007). *P. intermedia* is also isolated from sub-gingival plaque of periodontally-healthy subjects (Mättö *et al.*, 1996; Teanpaisan *et al.*, 1995) although in low numbers. There is variability of enzyme activity in *P. intermedia* strains isolated from healthy and active sites, with the greatest activity found in the latter (Maeda *et al.*, 1998; Tatami, 1992), where it is likely to increase in response to environmental stimuli and exacerbating infection (Socransky & Haffajee, 1992). Complicating matters further, five *P.intermedia* serotypes with variable levels of virulence have been identified (Gmür & Guggenheim, 1983), with serotype I being most commonly associated with deep periodontal pockets (Dahlén *et al.*, 1990). *P. nigrescens*, which is less virulent, has been identified as a separate species to *P.intermedia* (Shah and Gharbia, 1992), and although it is more commonly associated with periodontally healthy sites than *P. intermedia*, both species occur simultaneously in the oral cavity (Mättö *et al.*, 1996; Maeda *et al.*, 1998).

1.4.2. Haem requirements of *P. intermedia.*

Despite its clinical relevance, relatively little is known about the genetic and biochemical fundamentals of *P. intermedia* virulence. Like *P. gingivalis*, it lacks the ability to synthesize haem and has developed mechanisms to acquire exogenous sources which it accumulates as a cell-surface pigment. However, *P. intermedia* and *P. nigrescens* do not develop the same green-black pigment as *P. gingivalis*, but instead initially become dark orange-brown before blackening, indicating some other type of haem and mechanism of pigmentation exists (Smalley *et al* 2003a). Instead, the pigment is predominantly composed of monomeric haem, [Fe(III)PPIX.OH] (Smalley *et al* 2003a). Growth of *Prevotella* species is associated with a decrease in pH (as low as pH 5 [Smalley *et al.*, 2003a]) due to production of acidic fermentation products in the presence of fermentable carbohydrates (Shah & Williams, 1987; Takahashi & Yamada, 2000). Smalley *et al.*(2003a) propose that proteolytic liberation of Fe(III)PPIX would subsequently result in formation of the $[Fe(III)PPIX]_2O$ species which would, in an acidic environment, *de facto* be converted into [Fe(III)PPIX.OH] due to the pH-dependent equilibrium between these species (Silver & Lukas, 1983):

$$4Fe(II)PPIX + O_2 \rightarrow 2[Fe(III)PPIX]_2O \quad (3)$$

$$[Fe(III)PPIX]_2O + 2H^+ \leftrightarrow 2Fe(III)PPIX.OH \quad (4)$$

However, more recent findings suggest that in aqueous solutions a π-π dimer, and not the μ-oxo dimer, is overwhelmingly the predominant species (Villiers *et al.*, 2007). As such protonation may not result in the formation of a monomer. This suggests that formation of haem monomers in *P. intermedia* pigments may be a consequence of binding to the cell surface (a concept explored in Chapter 8). Low pH may however result in protonation of the haem carboxylates, decreasing its solubility and enhancing bacterial surface deposition (Smalley *et al* 2003a). In its monomeric form, Fe(III)PPIX.OH is 100-fold more potent in degrading hydrogen peroxide than $[Fe(III)PPIX]_2O$ (Jones *et al.*, 1973) which would enhance its defence against neutrophil attack.

Bacteria in the inflamed periodontal pocket will be exposed to erythrocytes from bleeding. *P. intermedia* has potent haemolytic activity (Okamoto *et al.*, 1999; Silva *et al.*, 2003) attributed to production of prevolysin O (Takada *et al.*, 2003) and

hydrogen sulphide (Yano *et al.*, 2009). In this regard, GCF from diseased sites is proven to contain Hb (Hanioka *et al.*, 2005) which is an effective haem source for *P. intermedia* growth (Leung *et al.*, 1998; Guan *et al.*, 2002). Importantly, Hb binding and digestion has been demonstrated for several species of prevotellae (Zwickel at el., 1992; Leung *et al.*, 1998; Guan *et al.*, 2002), with the culture supernatants degrading Hb over a wide pH range (Guan *et al.*, 2006). Although the precise mechanisms of Hb utilisation are yet to be elucidated, a 60 kDa cell surface Hb binding protein has been described (Guan *et al.*, 2004).

Both *P. nigrescens* and *P. intermedia* can also degrade transferrin, which is attributed to a 37 kDa cell-surface transferrin-binding protein (Duchesne *et al.*, 1999). However, although degradation has been characterised, transferrin and lactoferrin are not sufficient to support growth of *P. intermedia*, emphasising the importance of haem (Leung & Folk, 2002). Potential auxillary haem sources (albumin, haptoglobin and haemopexin) are degraded by *P. intermedia* (Jansen *et al.*, 1994; Carlsson *et al.*, 1984a), with albumin being of particular importance as *P. intermedia* exhibits intensified proteolytic activity and growth when supplemented with the protein (Ichiro, 2000).

Little is known of the mechanisms of haem transport across the bacterial cell surface. Haem binding occurs independently of, and is unaffected by, Hb binding (Leung *et al.*, 1998; Thompkins *et al.*, 1997). Haem-deplete conditions affect expression of 19 proteins thought to be involved in haem internalisation, including a predicted lipoprotein homologue of other bacterial haem receptors, and a homologue of TonB-dependent outer-membrane receptors of Gram-negative bacteria (Yu *et al.*, 2007). In addition, *P. intermedia* biochemistry and physiology is influenced by changes in environmental haemin levels (Stubbs *et al.*, 1999), with cell morphology changing from cocco-bacillary in haemin rich conditions to a predominantly bacillary form in haemin-deplete conditions. Cells grown in low haemin environments also produce greater numbers of extracellular vesicles and exhibit increased peptidolytic, haemagglutination and haemin binding activity (Stubbs *et al.*, 1999).

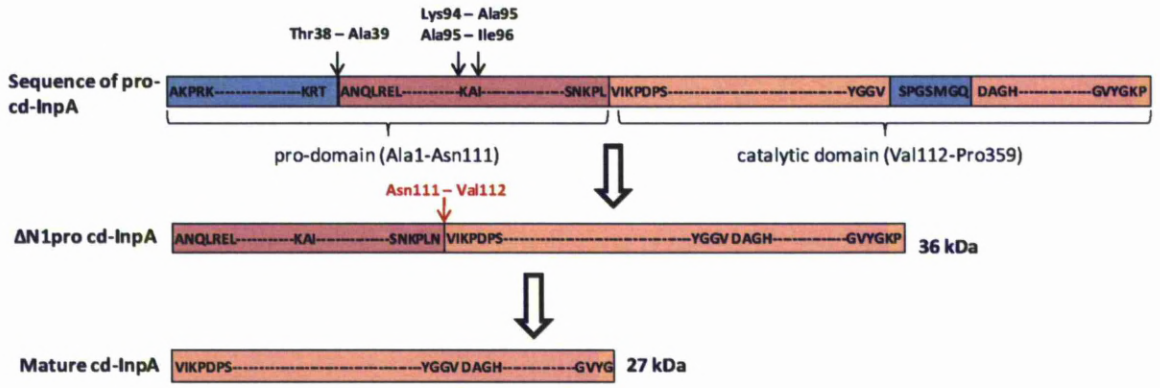
1.4.3. Virulence factors of *Prevotella intermedia.*

1.4.3.1. Interpain A.

Cysteine proteases (CPs) carry out various housekeeping functions or can serve as virulence factors in infectious bacteria, and include the clan-A papain-like CPs, such as staphopain A and B from *S. aureus* (Nickerson *et al.*, 2010), streptopain (streptococcal pyrogenic exotoxin B and SpeB) and IdeS endopeptidase from *Streptococcus pyogenes* (Wenig *et al.*, 2004), as well as the gingipains (see section 1.3.3.1). CPs display broad substrate specificity but are only found in single species or small groups of related species. Orthologues of *S. pyogenes* SpeB are found in several bacterial species but predominantly within *Bacteroidetes* (Sheldrick, 2002). The *P. intermedia 17* genome contains three open-reading-frames encoding cysteine proteases related to SpeB, including interpain A (InpA) of the cysteine-histidine-dyad class (Mallorquí-Fernández *et al.*, 2008; Potempa *et al.*, 2005).

SpeB and gingipains are well established virulence factors, with the gingipains being essential for pigmentation, working sequentially to first oxidise and finally degrade Hb to release haem (Smalley *et al.*, 2004; 2007; 2008). Due to the similarities between interpain and gingipain proteases, and considering the unique pigmentation profile that they both share, InpA may also play a role in this process. The *inpA* gene of *P. intermedia 17* encodes a 869 amino acid SpeB-orthologue, containing a 44-residue signal peptide, a pro-domain (Ala1-Asn111), a catalytic domain (Val112-Pro359) and a 465 residue C-terminal region arranged into distinct domains with putative regulatory and secretory functions (Nguyen *et al.*, 2007; Mallorquí-Fernández *et al.*, 2008). Zymogenic Interpain A (pro-cd-InpA) undergoes autolytic activation *in vitro* and possibly *in vivo* to remove the pro-domain, and the mature catalytic domain is a 27 kDa protein resistant to further degradation (Fig 1.11). The autocatalytic nature of InpA resembles that of pro-SpeB (in respect to the formation of intermediary species during maturation, ΔN1 pro-cd-InpA, and specific cleavage sites within the N-terminal pro-domain) and functions to enable latency maintenance independently of secreted- or host-proteases (Chen *et al.*, 2003; Mallorquí-Fernández *et al.*, 2008). Throughout the maturation process the catalytic cysteine, Cys154, is maintained at the same position

while the catalytic histidine, His305, undergoes major rearrangement to bring full function to the enzyme (Mallorquí-Fernández *et al.*, 2008).



Open arrow depicts direction of autocatalytic maturation of pro-cd-InpA. Amino acid residues not present in the respective three-dimensional structures are depicted in blue and red. The four autolytic cleavage points of pro-cd-InpA leading to wt cd-InpA formation are arrowed. The main cleavage point leading to the stable mature forms is characterized by a red arrow. Maturation of pro-cd-InpA occurs stepwise through a main 36 kDa intermediate (ΔN1pro-cd-InpA) which includes all the residues from Ala39 onwards except Ser295-Gln301. Mature cd-InpA comprises residues Val112 – Pro359.

Fig 1.11. Autocatalytic maturation of pro-cd-InpA. Adapted from Mallorquí-Fernández *et al.*, 2008.

P. intermedia and *P. nigresecens* are resistant to numerous host antimicrobial peptides, partly due to the production of several proteases (Devine *et al.*, 1999; Lee *et al.*, 2010). InpA contributes to virulence by degrading complement C3, thus inhibiting all three complement pathways and which may occur synergistically with gingipains (Potempa *et al.*, 2008). Much of this study will seek to elucidate the role of InpA with particular emphasis on its involvement in haem acquisition and the development of the haem pigment.

1.4.3.2. Other virulence factors.

P. intermedia produces a large array of virulence factors, including bacterial adhesins, fimbriae, haemagglutinins and various hydrolytic enzymes which contribute to infection and periodontal disease (Holt & Bramanti, 1991, Leung, *et al.*, 1999, Stubbs *et al.*, 1996). Several clinical isolates of *P. intermedia* can invade a variety of cell types including gingival epithelial cells, fibroblasts, and coronary

artery endothelial cells (Dogan *et al.*, 2000; Dorn *et al.*, 1999; Han *et al.*, 2000) in what is thought to be a fimbrial adhesin and an internalin-like protein (AdpC and AdpB) mediated process (Iyer *et al.*, 2010; Amano, 2010; Yu *et al.*, 2006). Proteases are secreted by *P. intermedia* for the purpose of degradation of host tissue and host tissue proteins, immunoglobulins and other host-derived molecules which therefore facilitate the progression of infection (Dorn *et al.*, 1999; Eley & Cox, 2003; Gazi *et al.*, 1997; Jansen *et al.*, 1995). *P.intermedia* and *P. melaninogenica* produce phospholipases which contribute to hydrolysis of membrane phospholipids (Bulkacz & Faull, 2009; Bulkacz *et al.*, 1981). Matrix metalloproteinases are also activated by proteases of *P. intermedia* and *P. nigresecens* which exacerbate extracellular matrix degradation (Bau *et al.*, 2008; Itoh *et al.*, 2009). *P. intermedia* strains also produce trypsin-like serine proteases, a dipeptidyl peptidase IV and cysteine proteases (Falkler *et al.*, 1995; Shibata *et al.*, 1993a; Deschner *et al.*, 2003), which are important for nutrient acquisition and evasion of the immune system (Collin & Olsen, 2001). *P. intermedia* also reduces host immunoglobulin-dependent responses through protease production and by binding human IgG via the Fc domain (Labbé & Grenier, 1995; Eley & Cox., 2003).

1.5. LIPOPOLYSACCHARIDES.

1.5.1. LPS structure.

The envelope of Gram-negative bacteria consists of an inner- and outer-membrane separated by the periplasmic space. The outer membrane is an asymmetric bilayer consisting of the lipid anchor domain, lipid A, of lipopolysaccharide (LPS) on the outer leaflet and phospholipid on the inner leaflet. LPS is a biologically active glycoconjugate of Gram-negative outer membranes and a major pathogenic factor, initiating inflammatory responses including pyrexia, intravenous coagulation, and shock (Jain & Darveau, 2010). LPS elicits potent immune responses via interaction with mammalian receptor protein complexes consisting of Toll-like receptor 4 (TLR-4), MD-2 and CD14 and accessory protein, and lipopolysaccharide binding protein (LBP) (Miyake, 2006). LPS is also crucial for maintaining the structural integrity of the bacterium and constitutes a permeability barrier to limit

passage of hydrophobic/toxic molecules (Nikaido, 2003). The archetypal LPS structure consists of three covalently linked domains: hydrophobic lipid A moiety which constitutes the outer leaflet of the outer membrane, core oligosaccharide (consisting of the inner and outer core) and O-antigen side chain consisting of several repeating units. LPS is extracted using the hot phenol and water method (Westphal & Jahn, 1965). Lipid A can also be separated by hydrating crude LPS fractions with acid (Westphal & Lüderitz, 1954).

Lipid A is of particular interest as it has been implicated with the majority of LPS biological activity and is also the most conserved of the structures (Loppnow *et al.*, 1990). Immune-stimulation by lipid A is so potent that bloodstream infections can result in severe clinical complications such as septic shock, which annually leads to ~200,000 deaths in the USA (Angus *et al.*, 2001). The prototypical lipid A structure, determined from *E.coli* (Fig 1.12) and conserved in most enterics, consists of a β-1,6-linked D-glucosamine disaccharide backbone phosphorylated at the C1 and C4' positions and also substituted with four fatty acyl side chains (Raetz & Whitfield, 2002). The four primary acyl groups are composed of 3-hydroxy myristic acid chains and are bonded via an amide- (at position C2 and C2') or an ester-linkage (at position C3 and C3') to the glucosamine dimer (Raetz & Whitfield, 2002). Two secondary acyl groups, lauric acid and myristic acid, are subsequently attached via ester links to the 3-hydroxy group of the C2' and C3' primary acyl chains respectively, resulting in a total of six acyl chains (Raetz & Whitfield, 2002). However, a wide variety of bacteria have been identified with lipid A that differ from the prototypical model and whose structures that can also be subject to environmental regulation (Raetz & Whitfield, 2002).

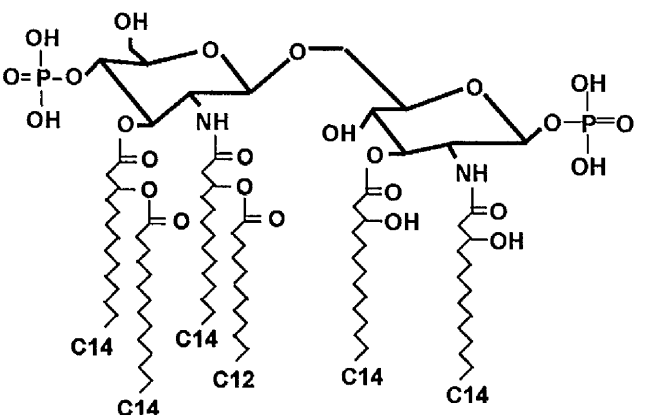


Fig 1.12. Schematic of the prototypical *E. coli* lipid A structure. Adapted from Raetz & Whitfield, 2002.

The remainder of the LPS molecule projects from the surface. The core oligosaccharide, which is covalently linked to lipid A, is relatively conserved in structure and usually contains an inner 2-keto-3-deoxyoctonate (Kdo) and L(D)-*glycero*-D-(L)-*manno*-heptose region and an outer hexose region (LuÈderitz *et al.*, 1982). The O-antigen is attached to the outer core and consists of a sequence of identical oligosaccharide repeats which are highly strain specific and determinative of the serological identity of a bacterium (Raetz & Whitfield, 2002). A considerable degree of chemical microheterogeneity is commonly found in the O-chain even within a single bacterial strain. Mutants lacking the O-antigen are referred to as so called 'rough mutants' (R-type LPS) while attachment of the O-antigen results in 'smooth' LPS (S-type LPS). A schematic representation of a typical Gram-negative bacterial inner and outer membrane can be found in Fig 1.13.

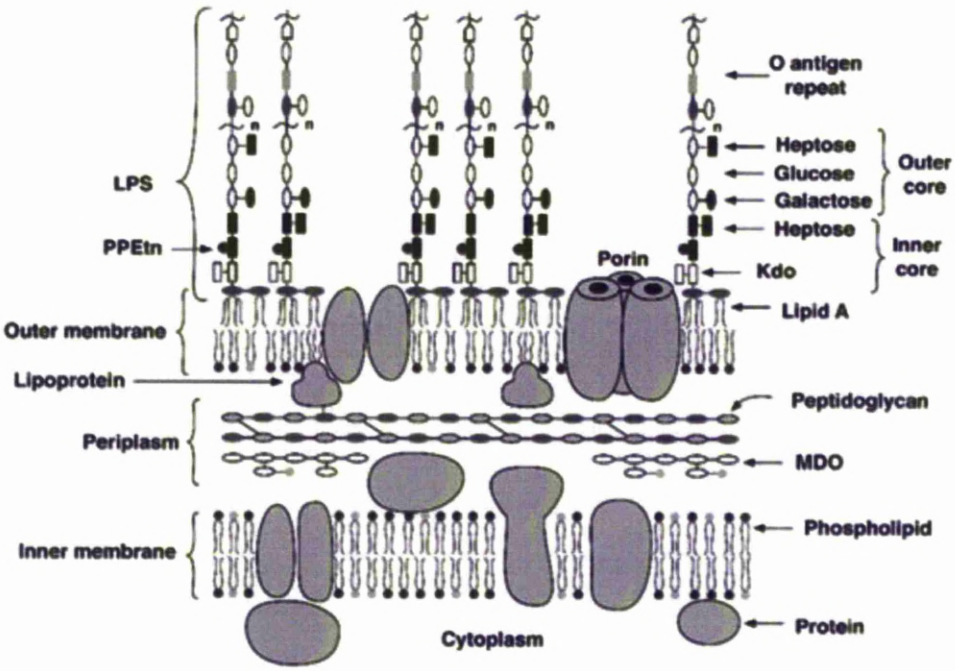


Fig 1.13. Schematic diagram of the inner and outer membranes of *E.coli* K-12 according to Raetz *et al*, 1991. Geometric form; ovals and rectangles represent sugar residues, as indicated, whereas circles represent polar head groups of various lipids. Abbreviations: PPEtn (ethanolamine phosphate); LPS (lipopolysaccharide); Kdo (2-keto-deoxyoctonic acid). Taken from Magkhães *et al*, (2007).

1.5.2. Lipopolysaccharide of *P. gingivalis.*

LPS is an important virulence factor of *P. gingivalis* (Lamont & Jenkinson, 1998). *P. gingivalis* synthesizes lipid A that exhibits remarkable structural heterogeneity and is a weaker stimulator of the immune response than the prototypical endotoxin of enteric bacteria (Jain & Darveau, 2010). Lipid A in *P. gingivalis*, of which there are four identified main subgroups (penta-acylated monophosphorylated; tetra-acetylated monophosphorylated; penta-acylated diphosphorylated; tetra-acylated nonphosphorylated), can either be stimulatory, antagonistic, or inert to TLR-4 (Jain & Darveau, 2010). All four distinct lipid A subgroups can be isolated from a single strain of *P. gingivalis* depending on the growth conditions (e.g. haem concentration [Al-Qutub *et al.*, 2006]) and extraction procedure (Darveau *et al.*, 2004) and each elicits a substantially different immune response via TLR-4 or TLR-2 dependent mechanisms (Darveau *et al.*, 2004; Ogawa *et al.*, 2007; Sawada *et al.*, 2007). Originally *P. gingivalis* W50 was only thought to synthesize a single LPS with a tetrasaccharide repeating unit of O-polysaccharide, [→6)-α-D-Glc*p*-(1→4)-α-L-Rha*p*-(1→3)-β-D-GalNAc-(1→3)-α-D-Gal*p*-(1→], modified by phosphoethanolamine (PEA) at position 2 of Rha in a nonstoichiometric manner (Paramonov *et al.*, 2001). However, a second LPS, A-LPS, with a phosphorylated mannan-containing anionic polysaccharide (A-PS) has also been identified (Rangarajan *et al.*, 2008).

1.5.3. Lipopolysaccharide of *Prevotella intermedia.*

Although information of *P. intermedia* LPS is limited, the lipid A structure, which is similar to that of *P. gingivalis*, consist of penta-acylated diglucosamine backbone with a phosphate at the 4-position of the non-reducing sugar (Hashimoto *et al.*, 2003). *P. intermedia* LPS has immune-modulatory functions by activating cells through a TLR4-independent pathway and triggers release of IL-1β, IL-6, IL-8 and TNF-α (Reddi *et al.*, 1995; Kim *et al.*, 2007). *P. intermedia* LPS has also been shown to enhance bone osteolysis by reducing alkaline phosphatase activity and mineralization, and inducing IL-6 and metalloproteinases in feral mouse osteoblasts (Chung *et al.*, 2006; Pelt *et al.*, 2002).

1.5.4. LPS interactions with haemoglobin.

LPS interactions with a variety of host target molecules are largely poorly characterised. Binding of certain proteins (e.g. lactoferrin and high-density lipoproteins) to free or membrane-located LPS reduces its biological activity

(Machnicki *et al.*, 1993; Yu & Wright, 1996). Of particular interest to this study is the interaction of LPS with Hb (Roth & Kaca, 1994). Hb is an important systemic high affinity LPS binding protein and the interaction (generally) leads to increased biological activity of rough and smooth LPS in LAL (Limulus amebocyte lysate) tests (Kaca *et al.*, 1994, Roth *et al.*, 1994), stimulation of endothelial tissue factor production (Kaca *et al.*, 1994), increased LPS binding to endothelial cells and enhanced lethal toxicity (Su *et al.*, 1997, Roth, 1996). Even modest intravascular haemolysis is associated with significantly increased mortality rates in patients with Gram-negative bacteraemia (Su *et al.*, 1997). LPS-oxyHb interactions also results in metHb and haemichrome formation (Kaca *et al.*, 1995).

Recent studies show that increased LPS-induced cytokine induction in the presence of Hb is due to dissagregation and reorientation of LPS aggregates into endotoxically active cubic conformations and an increase in the conical shape of the lipid A moiety (Jürgens *et al.*, 2001; Brandenburg *et al.*, 2003; Howe *et al.*, 2007). Binding and disaggregation of LPS by Hb may also augment its biological activity by increasing solubility (Kaca *et al.*, 1994a). Due to the contradictory nature of the evidence, the precise mechanisms through which LPS forms a complex with Hb and leads to the observed pathologies is unclear, although a role for lipid A is generally implicated (Currell & Levine, 2002; Belanger *et al.*, 1995; Kaca *et al.*, 1995a). Kaca *et al.* (1994a; 1995) have linked Hb binding to lipid A phosphate residues and ester-linked fatty acids but also proposed a role for the Kdo moiety. Although Jürgens *et al.* (2001) also postulate a role for lipid A it is their contention that this is a phosphate-independent process. An additional role for O chains in facilitating more effective binding has also been suggested, with smooth *E. coli* and *Salmonella minnesota* LPS variants inducing greater oxidation of oxyHb than their rough counterparts (Currell & Levin, 2002).

1.6. AIMS OF CURRENT STUDY.

The aim of the current study was to further characterise the mechanisms of haem capture and pigment formation by *P. gingivalis* and *P. intermedia* whilst considering the conditions of the periodontal pocket environment on these processes. For this purpose, virulence factors from both species were assessed for the ability to acquire haem from host haemoproteins or were judged as potential sites of haem binding and, thus pigment generation, at the surface of the bacterium. Specifically the aims were as follows:

1. Assessment of the role of a cysteine protease interpain A (InpA) in *P. intermedia* haem acquisition by investigation of haemoglobin breakdown.
2. Further characterisation of the interaction of InpA with haemoglobin using a catalytically intert InpA mutant InpA C154A to reveal more information about the catalytic and non-catalytic processes of the interaction with Hb.
3. To establish any possible synergistic interaction between InpA and the Arg- and Lys-specific gingipains (and hence between *P. gingivalis* and *P. intermedia* species) in haemoglobin breakdown.
4. Improve the understanding of haem uptake by the HmuY/HmuR system of *P. gingivalis* by assessing the involvement of gingipains in this process.
5. Examine the potential contribution of InpA to haem procurement by the HmuY/HmuR system.
6. To establish the role of InpA in haem acquisition from host haem-sequestering plasma proteins albumin and haemopexin.
7. To examine LPS from both BPAs as a haem binding molecule and determine its possible role in pigment formation.
8. Identify the role of LPS-Hb complex formation in haem acquisition for both BPAs.

CHAPTER 2

MATERIALS AND METHODS

2.1. BACTERIAL STRAINS AND GROWTH CONDITIONS.

2.1.1. Bacterial strains.

Table 2.1. Selected isolates of bacteria used throughout this study.

Species	Strain	Sources
P. gingivalis	A7436	Professor T Olczak, Laboratory of Biochemistry, Institute of Biochemistry and Molecular Biology, University of Wroclaw, Poland
	HG66	Professor J. Potempa, Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
	W50	Dr Smalley, J.W, University of Liverpool
	WPH35	Dr Smalley, J.W, University of Liverpool
P. intermedia	ATCC 25611	Professor J. Potempa, Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland
	NY653	Dr Smalley, J.W, University of Liverpool
E. coli	BL21(DE3)pLysS	Difco Laboratories, USA
	ER2566	New England Biolabs

2.1.2. Growth conditions.

Wild-type *P. gingivalis* and *P. intermedia* strains were grown anaerobically on horse blood agar plates (ABA; Biocorp), and in Schaedler broth (Biocorp; Oxoid) or Enriched Trypticase Soy Broth (eTSB; for strain HG66).

E. coli BL21(DE3)pLysS and ER2566 strains were maintained in 2 % Luria-Bertani (LB) medium/agar plates (Sigma) supplemented with 100 μg ml^{-1} ampicillin (Ap), 50μg ml^{-1} kanamycin sulphate (Km) or 50 μg ml^{-1} Km and 10 μg ml^{-1} chloramphenicol (Cm) where appropriate.

2.2. EXTRACTION OF BACTERIAL PROTEINS.

2.2.1. Interpain purification and isolation.

2.2.1.1. InpA mutant construction.

Wild type and mutant interpain A (InpA) plasmids were prepared by Jan Potempa's group at the Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland (Mallorquí-Fernández et al., 2007). In brief, *P. intermedia* ATCC 25611 genomic DNA was extracted, and the structural region of InpA, comprising the pro-domain and the catalytic domain (pro-cd-InpA) was amplified by PCR using forward primer 5'-ATGCCATGG**CA**AAGCCACGCACAAAGGAACAG-3' with an *Nco*I recognition site and reverse primer 5'-ATGCTCGAGTGGTTTTCCGTSSSCACCC-3' with an *Xho*I recognition site. As the *Nco*I site encompasses an ATG start codon, two bases (**CA**) were introduced into the forward primers after the *Nco*I site to allow in-frame target protein translation. As a result of this genetic manipulation a methionine was inserted before N-terminal alanine of InpA. Furthermore, additional codons, CTC and GAG, encoding for leucine and glutamate residues were introduced following InpA C-terminal proline by the reverse primer. The PCR (Polymerase chain reaction) products were then cloned into the *Nco*I/*Xho*I site of pET24d(+) expression vector (Novagen), which provided the coding sequence for C-terminal hexahistidine tag (6xHis). The wt construct was used to produce the $Cys^{154}Ala$ mutant InpA (pro-cd-InpA C154A) using overlap extension PCR (Aiyar et al., 1997). The correctness of the construct was verified by double-stranded DNA sequencing.

2.2.1.2. Transformation of E.coli BL21(DE3)pLysS.

Purification and isolation of InpA was performed by myself during several visits to the Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland. *E. coli* BL21(DE3)pLysS (90 µl) was transformed with the expression vector, pET24d(+) (1 µl), containing genes for the C-terminally truncated catalytic domain protease, interpain (InpA), of *P. intermedia* or pro-cd-InpA C154A. These cells were especially designed for expression of genes regulated by the T7 promoter. The cells were incubated on ice for 30 min, heat shocked for 1 min at 42 °C and immediately transferred to ice. The reaction was supplemented with 500 µl of S.O.C. (super optimal broth) medium (2 % (w/v) tryptone, 0.5 % (w/v) yeast extract, 10 mM NaCl, 2.5 mM KCl, 10 mM

$MgCl_2$, 10 mM $MgSO_4$, 20 mM glucose; Invitrogen) and incubated (shaking) at 37 °C for 1h. Aliquots of the transformation reaction mixture were plated on Km/Cm LB agar plates for selection of cells containing the vector, and incubated overnight at 37 °C.

2.2.1.3. Expression of InpA.

A single colony of freshly transformed *E. coli* BL21(DE3)pLysS was inoculated into 100 ml Km/Cm LB medium supplemented with 2 % glucose, and incubated with shaking overnight at 37 °C. Ten ml of the overnight culture was added to 1 L Km LB medium (2 % glucose) and incubated at 37 °C (with shaking) until the OD600 = 0.7-0.8. Target protein synthesis was then induced with 0.1 mM isopropyl β-D-thioglucopyranoside (IPTG; Sigma) at 26 ° C for 3 h. IPTG acts as an inducer by binding to lacI, the *lacO* and *lacUV5* repressor. Since *lacUV5* is the promoter for T7 RNA polymerase synthesis by *E. coli* BL21(DE3)pLysS, addition of IPTG inactivates lacI which dissociates from the *lacUV5* promoter, thus inducing expression of T7 RNA polymerase. IPTG also induces dissociation of lacI from the *lacO* operon on the plasmid, enabling binding of T7 RNA polymerase to *lacO* which initiates transcription of the T7 promoter. Cells were harvested by centrifugation (10,000 x g for 8 min).

2.2.1.4. Purification and activation of InpA.

InpA was purified by first suspending the cell paste in 40 ml binding buffer A (containing 20 mM Tris-HCl, 10 mM imidazole and 500 mM NaCl, pH 7.8) supplemented with 1.5 mM of the reversible cysteine protease inhibitor 4'-4'–dithidipyridine, followed by ultrasonication on ice for 5 min. The cell lysate was centrifuged at 26,000 x g for 1 h and the clear supernatant was filtered through a 0.45 μm membrane. The filtered His-tagged recombinant proteins were then purified by affinity chromatography on Fast Flow Ni-NTA Sepharose (Qiagen) as described previously (Mallorqui-Fernandez et al., 2008). In brief, the filtrate was resuspended with previously equilibrated Ni-NTA sepharose with binding buffer A overnight at 4 °C, and the slurry was then recollected in a column, and washed several times *in situ* with binding buffer A. The column was then eluted with 2 column volumes of elution buffer (binding buffer A with 500 mM imidazole, pH 7.8) and the flow through was collected. The eluant was then extensively dialysed in 20 mM Tris, pH 7.8 followed by further purification by anion exchange chromatography (MonoQ, GE Healthcare). The same protocol was employed for purification of pro-cd-InpA C154A but without the addition of 4'-

4'–dithidipyridine. InpA was activated before use by incubating 580 μM InpA with 2mM dithiothreitol for 15 min at 4 °C.

2.2.2. HmuY haemophore purification and isolation.

HmuY (NCBI accession number CAM 31898) was kindly donated by Professor Teresa Olczak (Laboratory of Biochemistry, Institute of Biochemistry and Molecular Biology, University of Wroclaw, Poland) and expressed by the department using a pHmuY11 plasmid and *E. coli* ER2566 cells (New England Biolabs) and purified from a soluble fraction of the *E. coli* lysate as previously described (Olczak *et al.*, 2008). In brief, the *hmuY* gene, lacking the first 25 residues encoding the predicted signal peptide (20 a.a), and five additional amino acids (CGKKK) was amplified on *P. gingivalis* A7436 DNA using forward primer 5'GGACG*CCATGG*ACGAGCCGAACCAACC 3' and reverse primer 5'GGACT*AAGCTT*TTATTTAACGGGGTATGTATAAGTGA 3'and cloned into the *Nco*I and *Hind*III restriction sites of the pTriEx-4 vector, resulting in the pHMUY11 plasmid. ER2566 *E.coli* was transformed with the pHMUY11 plasmid and grown at 37 °C for 16 h in Ap LB. The overnight culture (10 ml) was used to inoculate 1 L of Ap LB medium, which was incubated at 37 °C (shaking) until cell growth reached OD_{660}~0.5. HmuY synthesis was induced by addition 0.5 mM IPTG, and cells were cultured at 20 °C for 5 h, collected by centrifugation, resuspended in 50 ml 50 mM Tris/HCl, pH 7.6, and sonicated on ice. The supernatant was collected by centrifugation (20,000 x g for 20 min) and the protein was purified using ion-exchange chromatography (DEAE-Sephacel; Amersham Pharmacia). The unbound fraction containing HmuY was further purified by gel filtration chromatography (Sephadex G-75; Amersham Pharmacia) in gel filtration buffer A (20 mM sodium phosphate buffer, pH 7.4, containing 240 mM NaCl). The samples were dialyzed against PBS containing 1 % glycerol and then against PBS.

2.2.3. Gingipain purification and isolation.

2.1.3.1. Acetone precipitation of gingipains.

Arg- (HRgpA, HRgpB) and Lys- (Kgp) specific gingipains were kindly donated by Professor Jan Potempa (Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland). These had been isolated and purified by the department from spent culture supernatants *P. gingivalis* as previously

described (Pike et al., 1994). In brief, culture fluid of *P. gingivalis* HG66 was obtained by centrifugation of whole culture (20,000 x g, 20 min, 4 °C). The temperature of the supernatant was reduced and maintained below 0 °C using an ice/salt bath and chilled acetone (60 % final concentration) was added over a 15 min period and mixed for a further 30 min. The precipitate was collected by centrifugation (25,000 x g, 30 min, -10 °C), reconstituted in ice cold dithiodiopyridine disulfide buffer (20 mM Bis-Tris-HCl, 150 mM NaCl, 0.02 % (w/v) NaN_3, pH 6.8 supplemented with 1.5 mM 4,4'-dithiodiopyridine disulfide) and extensive dialysed in dithiodiopyridine disulfide buffer and gel filtration buffer B (20 mM Bis-Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$, 0.02 % (w/v) NaN_3, pH 6.8). The dialyzed fraction was clarified by ultracentrifugation (100,000 x g, 1 h, 4 °C), concentrated to 20 ml by ultrafiltration using an Amicon PM-10 membrane and cleaned further by an additional round of ultracentrifugation.

2.2.3.2. Separation and purification of gingipains.

Gel filtration, using a Sephadex G-150 column equilibrated with gel filtration buffer B, was used to separate RgpB from HRgpA and Kgp. The resultant fractions were assayed for activity against *N-a*-benzoylcarbonyl-l-lysine-*p*-nitroanilide (Ac-Lys-*p*NA) and Bz-L-Arg- pNA (L-BAPNA). The fraction with activity only against Ac-Lys-*p*NA (containing RgpB) was further purified by ion exchange chromatography as discussed later. Fractions possessing both Rgp and Kgp activity were pooled, concentrated using ultrafiltration, dialyzed against Affi-buffer (50 mM Tris-Cl, 1 mM $CaCl_2$, 0.02 % (w/v) NaN_3, pH 7.4) and clarified by syringe filtration through a 0.2-μm filter. This fraction was applied to an arginine-Sepharose chromatography column equilibrated with Affi-buffer and washed with several volumes of Affi-buffer at 4 °C. The column was washed with Affi-buffer containing 0.5 M NaCl until the OD_{280} baseline was 0 and then re-equilibrated with Affi-buffer. A linear gradient of 0 - 750 mM L-lysine (350 ml), followed by 150 ml 750 mM L-lysine was then applied. The column was then re-equilibrated with Affi-buffer and a linear gradient of 0 – 100 mM L-arginine was then introduced. Collected fractions were then assayed for Kgp and Rgp activity and pooled on the basis of this activity. The pooled fractions were then concentrated and dialyzed against gel filtration buffer B at 4 °C.

The RgpB containing fraction from the earlier gel filtration step was dialysed against ion-exchange buffer (20 mM Bis-Tris-HCl, 1 mM $CaCl_2$, 0.02 % (w/v) NaN_3, pH 6.4), clarified by a 0.2-μm syringe filter and then purified by ion-exchange chromatography using

a DE-52 (Whatman) matrix equilibrated with ion exchange buffer. The column was washed in ion-exchange buffer and a NaCl gradient (0 – 200 mM NaCl in a total of 200 ml ion-exchange buffer) was then applied. Rgp activity was assayed with L-BAPNA. Samples containing Rgp were pooled, concentrated by ultrafiltration, dialysed against Affi-buffer and clarified with a 0.2-μm syringe filter. The fraction was then further purified using an arginine-sepharose column. Fractions containing activity against BAPNA were pooled, concentrated and dialysed against gel-filtration buffer at 4 °C. Before use in experiments, stock gingipain buffer was replaced with 0.14 M NaCl, 0.1M Tris-HCl (pH 7.5).

2.2.4. Gingipain and interpain activity assays.

Before use in digestion experiments, the activities of Kgp and Rgp were routinely tested against the lysine containing substrate Ac-Lys-*p*NA (0.5 mM) and arginine containing L-BAPNA (0.5 mM), respectively (both prepared in DMSO) which were examined by monitoring A_{405nm}. The hydrolyses were performed in gingipain assay buffer (200 mM Tris-HCl, 150 mM NaCl, 5 mM $CaCl_2$ and 0.02 % (w/v) NaN_3, pH 7.6). InpA activity was assayed with Boc-Val-Leu-Lys-MCA (0.5 mM, prepared in DMSO) by monitoring changes in fluorescence (λ_{ex} 360 nm; λ_{em} 460 nm) using a microplate spectrofluorimeter SpectraMax Gemini EM (Molecular Devices) or L-BAPNA as above. The hydrolyses were performed in 20 mM Tris-HCl supplemented with 10 mM dithothrietol (DTT) pH 7.8 at 37 °C .

2.3. LIPOPOLYSACCHARIDE (LPS) PREPARATION.

2.3.1. Lipopolysaccharide extraction.

LPS was extracted by the hot phenol water method (Westphal and Jann, 1965). *P. gingivalis* (W50; WPH35) and *P. intermedia* (NY653) were grown in Schaedler broth, which contains a basal 10 mg L^{-1} iron protoporphyrin IX chloride [Fe(III)PPIX]Cl], for 3 days and cells were harvested by centrifugation (5,000 x g, 45 min, 5 °C) and washed twice in Tris/NaCl buffer (pH 7.5) to remove growth medium constituents. The bacteria were suspended in equal volumes of hot aqueous phenol (90 %, w/v) heated to 67 °C and vigorously stirred frequently. The mixture was then allowed to separate overnight at 4 °C into lower phenol and upper aqueous layers. Centrifugation at 5,000 x g for 5min was used to aid separation when necessary. The upper aqueous phases were removed and the extraction procedure was repeated for the remaining phenol phases. The extracted aqueous phases were

pooled, extensively dialysed and lyophilized to give the crude LPS extract. This extract was resuspended (at 10 mg ml^{-1}) in 0.14 M NaCl, 0.1M Tris, pH 7.5 and sequentially treated with 300 µl of 1 mg ml^{-1} deoxyribonuclease I (Sigma; 3,750 Kunitz units/mg) and then ribonuclease A (Sigma; 86 Kunitz units/mg) at 37 °C . Lastly, LPS was treated with proteinase K (Sigma; 41 units/mg) at 1.5 mg ml^{-1} at 60 °C for 2 h with periodic mixing. The extracts were then boiled for 15min to denature any remaining active protease, dialysed and then recovered by freeze-drying. Where appropriate, crude samples were then dissolved in water and centrifuged at 5000 x g for 5 h at 5 °C to separate supernatant and pellet fractions. Before use, *P. gingivalis* and *P. intermedia* LPS was dissolved in buffer in a sonication water bath.

2.3.2. Hexuronic acid assay.

Hexuronic acid assay was performed using the method of Bitter and Muir (1962). Test tubes containing 2.5 ml 25 mM sodium tetraborate in concentrated sulphuric acid were cooled to -70°C. 0.5 ml of D-glucuronic acid standards (200 to 5 µg ml^{-1}) or LPS in distilled water, were layered onto the sulphuric acid mixture in an ice bath and gently vortex mixed. The mixture was heated at 100 °C for 10 min and then cooled to -70 °C, before addition of 0.1 ml of 0.125 % (w/v) carbazole in ethanol and heating to 100 °C for 15 min. The A_{530nm} was then read after cooling to room temperature. A calibration curve of the D-glucuronic acid standards (5 to 200 µg ml^{-1}) was used to calculate the hexuronic acid concentrations of LPS samples.

2.4. PREPARATION OF OXYHAEMOGLOBIN AND HAEMOGLOBIN DERIVATIVES.

2.4.1. oxyHb and metHb preparation.

OxyHb was extracted from fresh horse erythrocytes as previously described (Smalley et al., 2002). In brief, erythrocytes from fresh horse blood were pelleted by centrifugation at 5000 x *g* for 5 min at 5 °C, washed three times in NaCl/Tris buffer to remove plasma components, and lysed by re-suspension in 1 mM Tris/HCl, pH 7.0, for 20 min at 20 °C. The cell membranes were removed by centrifugation (2000 x *g* for 20 min at 5 °C) and the Hb-containing supernatant re-centrifuged to remove any residual cell remnants. The Hb was stored as a concentrated solution (approximately 1mM) at -80 °C in 0.14 M NaCl, 0.1 M

Tris-HCl, pH 7.5, until required. Stock preparations of metHb were prepared by treatment of oxyHb with $NaNO_2$ at a 1:32 molar ratio as previously described (Smalley et al., 2008) or by auto-oxidation for 24 h in specified buffered conditions. MetHb formation was confirmed by UV-visible spectroscopy.

2.4.2. AzidometHb preparation.

Azide-methaemoglobin (AzidometHb) was formed by incubating metHb (produced as above with $NaNO_2$) with 0.4 mM NaN_3 in for 18 h at 37 °C. Azide-methaemoglobin formation was confirmed using spectra data (417 nm Soret band λ_{max}, 541 and 571 Q bands [Antonini & Brunori, 1971]).

2.4.3. Preparation of haemoglobin haemichrome.

MetHb (3.75 μM), prepared by auto-oxidation for 48 h at 37 °C in 0.14 M NaCl buffered with 0.1 M Tris-HCl buffer at pH 7.5, was converted to haemichrome by incubation with 8 M Urea at 37 °C for 24 h (Harrington *et al.*, 1993).

2.4.4. Haem-free apo-globin preparation.

Haem-free globin was prepared as described by Ascoli *et al.* [1981]. Briefly, a 3 % w/v solution of freshly prepared horse oxyhaemoglobin was slowly added to 30-fold volume cold (-20°C) acetone containing 5 mM HCl and was vigorously stirred. Precipitated globin was collected by low speed centrifugation (5,000 x g,10 min, 0°C), treated once more with cold acid-acetone until the red colour was completely discharged, re-suspended in a minimum volume of water, dialysed against sodium bicarbonate (0.1 g l^{-1}), and then against 0.01 M phosphate buffer, pH 7.2. The denatured globin precipitating during this step was removed by centrifugation (10,000 x g 15 min at 5°C), and the soluble globin fraction was recovered by freeze-drying after dialysis against water at 4 °C.

2.4.5. Preparation of bis(3,5-dibromosalicyl)fumarate cross-linked haemoglobin.

OxyHb was incubated at a 1:1 molar ratio with bis(3,5-dibromosalicyl)fumarate (DBBF; Sigma) in 0.2 M Tris-HCl (pH 7.2) for 2 h at 37 °C, shaking. An equimolar concentration of glycine (pH 8.8/NaOH) was added to consume any remaining reagent, and the reaction was mixed at 20 °C for 5 min. The sample was dialysed against pH 7.5 (0.14 M NaCl, 0.1M Tris-HCl) before use (Chatterjee *et al.*, 1986).

2.5. FERRIHAEM PREPARATION.

Iron(III) protoporphyrin IX was prepared as previously described (Smalley et al., 2006) by dissolving haemin (iron(III) protoporphyrin IX chloride (Fe(III)PPIX.Cl; Sigma) at 1 mM in 0.1 M Tris, pH ~ 10, containing 0.1 M NaCl. HCl was then used to adjust the pH to 7.5. Iron(II) protoporphyrin species were prepared from the above ferrihaem solution by reduction with 10 mM $Na_2S_2O_4$ (Smalley et al., 1998).

2.6. HAEMOPROTEIN PREPARATION.

2.6.1. Albumin and haemalbumin.

Purified human albumin (Sigma A-8763) and bovine albumin (Sigma A-3294) were dissolved in pH 7.5, 6.0, or 5.5 buffer where appropriate. Ferrihaem-complexed albumin was prepared by equilibrating haem with albumin for 24 h at 25°C to give haem to protein molar ratios of 1:0.9-, 2:0.9-, 4:0.9- and 8:0.9-haemalbumin. Haemalbumin formation was confirmed using UV-visible spectroscopy.

2.6.2. Haemopexin and haem-haemopexin preparation.

Haemopexin from male human plasma was kindly donated by Professor Potempa (Department of Microbiology, Faculty of Biochemistry, Biophysics and Biotechnology, Jagiellonian University, Krakow, Poland), and was purified sequentially by hemin-agarose affinity chromatography followed by a variety of ion exchange, gel filtration and affinity fractionations. Haem-haemopexin was prepared by incubating haem at 37°C with haemopexin at a 1:0.9 molar ratio. The reaction was followed by monitoring the Soret band until no further change in 414 nm (haem-haemopexin Soret λ_{max} [Morgan *et al.*, 1976]) was observed.

2.7. UV-VISIBLE SPECTROSCOPY.

UV-visible spectra were recorded using a Ultrospec 2000 scanning spectrophotometer (Pharmacia Biotech) at room temperature in 1ml quartz or plastic semi-micro cuvettes (Eklay UltraVu), with 1 cm pathlength. Where appropriate, absorbance spectra of all samples examined were routinely corrected by subtraction of control buffer.

2.7.1. Spectroscopic analysis of oxyHb and metHb concentrations.

Concentrations of oxyHb and metHb were determined spectroscopically from A_{577nm} and A_{631nm} using the following equation from Winterbourne *et al.*, (1976):

$$A_{577nm} = \varepsilon_{met577nm}.[\text{metHb}] + \varepsilon_{oxy577nm}[\text{oxyHb}] \quad (1)$$

$$A_{631nm} = \varepsilon_{met631nm}.[\text{metHb}] + \varepsilon_{oxy631nm}.[\text{oxyHb}] \quad (2)$$

The millimolar extinction coefficients (ε) at 577 and 631 nm for oxyHb ($\varepsilon_{oxy577nm} = 65$, and $\varepsilon_{oxy631nm} = 1$) and for metHb ($\varepsilon_{met577nm} = 15$, and $\varepsilon_{met631nm} = 14$) were determined in 0.14 M NaCl, 0.1 M Tris-HCl at pH 7.5 from 4 μM and 20 μM horse Hb reference samples on a tetramer basis (Smalley *et al.*, 2007). The above simultaneous equations were rearranged and solved to give the following expressions which were used to calculate [oxyHb] and [metHb] (Smalley *et al.*, 2007):

$$[\text{oxyHb}] = 0.0156.A_{577nm} - 0.0168.A_{631nm} \quad (3)$$

$$[\text{metHb}] = 0.0726.A_{631nm} - 0.0011.A_{577nm} \quad (4)$$

To determine relative rates of metHb formation, difference spectra of the Soret band region were produced by subtracting the initial time zero spectra from those at subsequent time points. These spectra essentially show the net change in absorbance in the Soret region during conversion of oxyHb to metHb (Kelm et al., 1997; Zhang et al., 1996). The integrated areas of the resulting peaks (348 to 411 nm) and trough (411 to 472 nm) thus give accurate measures of the amounts of metHb formed.

Where appropriate, to obviate any complications relating to changes in Soret area due to proteolytic degradation and haem loss particularly at a lower pH, haemoglobin oxidation was also calculated using the change in A_{577}nm versus time to give linear plots of the oxidation rate (Tsuruga & Shikama, 1997). Thus, plots of $-\ln ([HbO_2]_t / [HbO_2]_0)$ versus time t, where the change in the absorbance of the alpha band (577 nm) were used to monitor the ratio of oxyHb concentration at time t compared to time $t = 0$ were created.

2.7.2. Quantification of haem loss from haemoglobin.

Rate of haem loss from metHb in the presence of LPS or protease was calculated spectroscopically using the change of A_{Soret} nm versus time. Consequently, linear plots of –ln

($[HbO_2]_t / [HbO_2]_0$) versus time *t*, where the change in absorbance intensity of the Soret band was used to monitor the amount of haem at time *t* compared to $t = 0$, were created.

2.7.3. Reduction of samples with sodium dithionite.

Sodium dithionite ($Na_2S_2O_4$) is a strong reducing reagent used to simultaneously deoxygenate and reduce samples of haemoglobin. Sodium dithionite was added to samples to a final concentration of 10 mM. Fresh solutions were always made and used within 1 h of preparation to avoid decomposition due to atmospheric oxygen. Absorption spectra of solutions containing sodium dithionite were corrected by subtracting the spectra obtained from a control containing buffer and sodium dithionite.

2.8. ELECTROPHORESIS.

SDS-PAGE was used for the separation of proteins based on molecular weight as previously described (Laemmli, 1970). Samples were solubilised at 37 °C for 1 h or by boiling for 5 min in reducing or non-reducing LSB as appropriate. SDS-electrophoresis was carried out on 10 or 15 % polyacrylamide separating gels. For native PAGE, 10 % continuous separating gels were made but omitting SDS. The method used a slightly modified sample application buffer containing 55 % (v/v) stack gel buffer, 20 % (v/v) glycerol (BDH), 30 mM bromophenol blue (SIGMA). Molecular weight markers 14 kDa (bovine α-lactalbumin), 20 kDa (soybean trypsin inhibitor), 24 kDa (bovine trypsinogen), 29 kDa (bovine carbonic anhydrase), 36 kDa (rabbit glyceraldehyde-3-phosphate dehydrogenase), 45 kDa (Ovalbumin), 66 kDa (bovine serum albumin) were used.

2.8.1. 3,3' 5,5' tetramethylbenzidine(TMB)/ H_2O_2 staining of haem-proteins.

This was employed for the detection of haem-associated pseudoperoxidase activity in haem-containing/carrying proteins after PAGE. Note that non-reducing LSB was used during PAGE as reducing agents destroy H_2O_2. Following electrophoresis, gels were equilibrated at 4°C in the dark in TMB fixing solution (60 % (v/v) 0.25 M sodium acetate buffer, pH 5.0 (BDH), 30 % (v/v) methanol (BDH)) for a minimum of 1 h and transferred into TMB staining reagent (70 % (v/v) 0.25 M sodium acetate buffer, pH 5.0 (BDH), 20 % (v/v) methanol (BDH) with 6.3 mM 3,3' 5,5' tetramethylbenzidine (TMB; Sigma)) and gently agitated on ice for 35 min, in the dark. H_2O_2 (to final concentration of 30 mM) was then

added, followed by a further gentle agitation for 35 min (shaking; on ice; in the dark). Finally the gels were transferred to developing solution (80 % (v/v) 0.25 M sodium acetate buffer, pH 5.0 (BDH) and 20 % (v/v) iso-propyl-alcohol) for a minimum of 10 min with shaking at room temperature. Areas of the gel containing haem-binding proteins appeared blue. The gels were then counter-stained with Coomassie Blue to locate the specific peptide bands possessing the pseudoperoxidase activity.

2.8.2. Protein staining with Coomassie Blue.

To identify polypeptides/proteins, gels were stained with 0.1 % (w/v) Coomassie Brilliant Blue (Sigma) in 50 % (v/v) methanol (BDH), 7 % (v/v) acetic acid (BDH) for a minimum of 2 h and then diffusion destained with 5 % (v/v) methanol, 10 % (v/v) acetic acid.

2.8.3. Silver staining of LPS chains.

Silver staining was performed according to the method of Tsai & Frasch (1982). After SDS-PAGE, gels were first placed in oxidation solution (40 % (v/v) ethanol, 5 % (v/v) acetic acid, 0.7 % (w/v) periodic acid) for 5 min with gentle agitation and then washed in several changes of distilled water over an hour. Staining reagent was then prepared by combining 20 ml 20% (w/v) $AgNO_3$ with 580 ml of 19 % (v/v) 0.1 M NaOH with 1.4 % (v/v) ammonium hydroxide. The final wash was replaced with fresh staining reagent and gently agitated for 10 min and then washed with three replacements of water over 30 min. Development solution (50 mg L^{-1} citric acid and 0.5 ml L^{-1} formaldehyde) was added with gentle agitation (~5-10 min) and development was stopped by several washes with water over 30 min once yellow background staining of gel appeared.

2.8.4. Densitometry of gels.

Densitometry was carried out on Coomassie blue-stained bands using UVIband gel analysis software (UVItech Ltd., Cambridge, UK) after digital image capture using a UMax Powerlook 1000 flatbed transmission scanner. Volumes of Coomassie and TMB-H_2O_2-stained bands were used as indirect measures of protein and haem loss from a sample (calculated as a percentage of the time = 0 sample).

2.9. IMMOBILIZATION OF SAMPLES ON NITROCELLULOSE SHEETS.

2.9.1. Dot blotting.

The S&S Minifold I 96-Place Spot-Blot System (Scleicher & Schuell) was used to filter and immobilize samples to a nitrocellulose sheet as described in the user manual. Wells were loaded with 50µl samples and suction was applied for 3 min. The nitrocellulose sheets were then air dried.

2.9.2. Western blotting.

Transfer of samples from polyacrylamide gels to nitrocellulose membrane was carried out in Towbin buffer containing 25 mM Tris, 192 mM Glycine, 20 % (v/v) and methanol, pH 8.3 (Towbin *et al.*, 1979) using a Bio Rad Trans-Blot SD semi-dry electrophoretic transfer cell.

2.9.3. Detection of haem binding by immobilized samples.

Haem pick up by immobilized samples was identified using a modified version of the TMB-H_2O_2 stain described earlier (Section 2.7.1). The membranes were transferred to TMB staining reagent containing H_2O_2 (30 mM) for 5 min with gentle agitation in the dark to reveal haem associated peroxidase activity.

To determine haem binding by immobilized samples, the nitrocellulose sheet was blocked with aqueous gelatin (2% w/v) for 1 h, transferred to a separate container and washed several times in distilled water, then incubated (shaking) for 1h with 50 µM haem (in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5 or phosphate buffer, pH 6.4 shaking). Haem binding was verified with TMB-H_2O_2 staining as above.

2.10. PROTEOLYTIC HAEM RELEASE FROM HAEMOGLOBIN- AND HAEMALBUMIN-AGAROSE.

2.10.1. Preparation of haemoglobin-agarose beads.

Dilution of haemoglobin results in increased dissociation of the tetrameric protein into $\alpha^1\beta^1$ subunits (Bunn & Forget, 1986; Nagel & Gibson, 1971). Haemoglobin-agarose beads (Sigma; H8756) were repeatedly washed in a large excess of buffer until

the UV-spectrum of the supernatant did not reveal free haemoglobin. This was done to remove any un-conjugated haemoglobin subunits.

2.10.2. Preparation of haemalbumin-agarose beads.

Bovine albumin-agarose beads (Sigma A3790) were washed extensively in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5, until no un-conjugated free albumin could be detected (by 280nm absorbance) in the supernatant. The albumin was then complexed with haem (1:0.9 haem : protein molar ratio) by gentle agitation at 4 °C for 18 h. The beads were then washed with buffer until all unbound haem was removed, as determined spectroscopically.

2.10.3. Pyridine-haemochrome assay of free haem.

The pyridine haemochrome assay, as first described by Gallagher and Elliot (1965), was used to quantify haem release from samples of haemoglobin- or haemalbumin-agarose beads. The reduced Fe(II) form of haem will react with pyridine. For this reason, sodium dithionite was used to convert the haems to the Fe(II) state prior to exposure to pyridine. Pyridine competes with distal bound ligands for binding with the iron (II) of the haem molecule, which results in the formation a pyridine-haemochrome complex with an intense red colour and high extinction coefficients of the Soret 419 nm band ($\varepsilon = 117$) and 525 and 555 nm Q bands ($\varepsilon = 15.5$ and 30 respectively). This enables more sensitive detection of haem at relatively low concentrations. For the purpose of this investigation, the Soret 419 nm band was used as it has the strongest absorbance.

2.10.4. Quantification of proteolytic haem release from haemoglobin and haemalbumin.

Initially, a standard calibration curve of known haem concentrations against pyridine-haemochrome absorbance at 419 nm was produced. A fresh solution of haem (made up as previously described) was diluted in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5 to give a 200-1 μM range. These were assayed for pyridine-haemochrome formation by adding 500μl volumes of diluted haem (or control buffer samples) to a 500 μl solution containing 20 mM sodium dithionite and 200 mM pyridine. The absorption spectra of each of the samples were measured between 300 and 600 nm and corrected by subtraction of control (buffer) spectra.

1M pyridine is capable of bringing about haem dissociation from haemoglobin (Smalley et al., 2003b), whereas at a 10-fold lower concentration (0.1 M) this effect is not observed. For this reason, 300 μl volumes of the haemoglobin- or haemalbumin-agarose

supernatant were removed and mixed with 10 mM $Na_2S_2O_4$ and 0.1M pyridine, so that pyridine-haemochrome formation would be representative only of free haem in the supernatant.

2.11. MALDI (MATRIX-ASSISTED LASER-DESORPTION/IONIZATION)-TOF (TIME-OF-FLIGHT)-MASS SPECTROMETRY.

Mass spectrometry was kindly performed by Mark Prescott and Dr Deborah Ward of the Proteomics Laboratory, School of Biological Sciences, University of Liverpool. Samples were analyzed using a Micromass M@LDI mass spectrometer, using an α-cyano-4-hydroxy-cinnamic acid matrix (Sigma Chemicals). The spectra were recorded in the positive ion mode and the mass range scanned was 800 to 4000 Daltons. The spectrometer was calibrated using a mixture of authentic peptide samples. The observed peptide masses were compared to those predicted by theoretical cleavage using the PeptideMass program (ExPASy).

2.12. GEL FILTRATION.

Analytical gel filtration of haemoglobin preparations were performed using a Superdex 75 10/300 GL column (Tricorn) coupled to an FPLC (Fast protein liquid chromatography) system (Amersham Pharmacia).

2.13. CIRCULAR DICHROISM.

Circular dichroism measurements were kindly performed by Drs E. Yates and T. Rudd, School of Biological Sciences, University of Liverpool and carried out using a Chirascan-plus spectrometer (Applied Photophysics, Leatherhead, UK). Near-UV spectra were recorded at 293 k in a quartz cell with a path length of 1 cm (Hellma UK Ltd, Southend Sea, UK) in the range 250-340 nm (step (nm) = 1, bandwidth (nm) = 2, integration time (s) = 4). Each scan took approx. 6 min and 4 scans were recorded. Far-UV CD spectra (180-260 nm) were recorded at beamline 23, Diamond Synchrotron, using quartz cuvettes (0.2 mm, QS cuvette, Hellma). Spectra were normalised at 260 nm and smoothed using the Savitzky-Golay method, 12 point window, using a second order polynomial (Rudd *et al.*, 2010). Near and Far

circular dichroism of LPS-Hb interactions were performed by Dr H. Wojtowicz (Laboratory of Biochemistry, Institute of Biochemistry and Molecular Biology, University of Wroclaw, Poland) using a Jasco J-715 spectrometer (bandwidth (nm) = 2) at a scanning speed of 50 nm/min.

2.14. X-RAY CRYSTALLOGRAPHY.

2.14.1. Cystallization methods.

All crystals were grown by hanging-drop vapour diffusion methods. Trials for crystallization conditions were performed using the PACT Premier kit (Molecular Dimensions Ltd, UK) which contains 96 reagents arranged as a cation/polyethylene glycol (PEG) screen, an anion/PEG screen and a pH/PEG screen. This was selected as InpA crystallization had previously been achieved in PEG containing buffer (Mallorquí-Fernández et al., 2007). Droplets containing protein (1 µl) were equilibrated over wells containing 100 µl precipitation solution at 22 °C.

2.14.2. Data collection, refinement, and model building.

Data collection was kindly performed by Dr S.V. Antonyuk (Molecular Biophysics Group, University of Liverpool) at PROXIMA1 beamline at Soleil synchrotron using an ADSC Q315r detector. Indexing, intergration and scaling of diffraction data was carried out in Mosflm with scala (Leslie, 1992). Structures were solved using MOLREP (Vagin & Teplyakov, 1997) and partially refined using the maximum likelihood method as implemented in REFMAC5 (Murshudov *et al.*, 1997) in the CCP4i suite. Model building between cycles of refinement was performed with COOT (Emsley & Cowtan, 2004). Structures were elucidated under the close supervision of Dr S.V. Antonyuk. All 3D-protein structures presented in this thesis were created using Pymol.

CHAPTER 3

Role of interpain A in breakdown and release of haem from haemoglobin

3.1. INTRODUCTION

The concerted proteolytic actions of the arginine- and lysine-specific gingipains of *P. gingivalis* are essential for haem pigmentation as they work sequentially to first oxidise and finally degrade haemoglobin (Smalley *et al.*, 2002; 2004; 2007; 2008). Haemoglobin breakdown has been demonstrated for several species of *Prevotellae* (Zwickel *et al.*, 1992; Leung *et al.*, 1998) and the culture supernatant of *P. intermedia* can degrade haemoglobin over a wide pH range, but optimally at pH 5(Guan *et al.*, 2006). The precise mechanism of haemoglobin breakdown by *P. intermedia* has yet to be verified.

The cysteine protease interpain A (InpA) is produced by strains of *P. intermedia* and has been identified as a potential virulence factor by degrading complement factor C3 which has the effect of lowering the bactericidal capacity of the host (Potempa *et al.*, 2008). Cysteine proteases, such as SpeB of *Streptococcus pyogenes* (of which Interpain A is an orthologue) and the gingipains of *P.gingivalis*, have also been established as important virulence factors possessing immuno-modulatory functions (Potempa & Pike, 2005). Due to the similarities of the related pathogenic cysteine proteases produced by both *P. gingivalis* and *P. intermedia*, as well as the unique pigmentation profile that both of these organisms share, the interaction of haemoglobin with interpain A was investigated. Preliminary unpublished studies in this laboratory carried out at neutral pH (as a comparison with gingipains) revealed that InpA was a potent catalyst in oxyHb oxidation, suggesting that it may serve a similar role as R-gingipains which "primes" oxyHb for subsequent attack by Kgp, resulting in haem release (Smalley *et al.*, 2007; 2008).

3.2. METHODS

3.2.1. Incubation of InpA with haemoglobin.

Stock solutions of InpA were incubated with 4 μM oxy- and metHb preparations to working concentrations between 2 and 0.00064 μM at 37 °C in 0.14 M NaCl, 0.1 M Tris-HCl (pH 7.5) to partly emulate the conditions of the periodontal pocket (Bickel & Cimasoni, 1985). At this pH, InpA increased haemoglobin oxidation but effected little breakdown. Incubation in 0.2 M phosphate buffer (pH 6.0) or 0.06 M $KHC_8H_4O_4$

(potassium hydrogen phthalate), 0.02 M NaOH (pH 5.5) buffer, closer to the growing pH of *P. intermedia* cells (Shah & Williams, 1987; Takahashi & Yamada, 2000) resulted in greater proteolysis. To examine the importance of haem dissociation in breakdown, 4 μM azidometHb and haem free globin at 16 μM (to match the concentration of haemoglobin monomers in the tetramer) were treated with InpA.

UV-visible spectra were recorded hourly in 1 cm pathlength semi-micro cuvettes as previously described. Spectra were also taken after 24 h and 48 when appropriate. For SDS-PAGE on 15 % polyacrylamide gels, samples of the incubation mixture were removed and incubated with 0.5 mM of the irreversible protease inhibitor E-64 for 30 min at room temperature. Enzyme- haemoglobin samples were solubilised at 37 °C for 1h in non-reducing LSB.

3.2.2. Proteolytic haem release from haemoglobin-agarose beads.

In assays to determine haem release from haemoglobin, and to eliminate the problem of overlapping spectra of Hb and haem, haemoglobin-agarose was employed. Suspensions of beads (~ 10 μM Hb, determined from the Soret absorbance) were extensively pre-washed in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5 buffer to encourage dissociation of any unconjugated Hb αβ dimers, and incubated with InpA at 2 μM at 37 °C end-over-end mixing in the same buffer. Samples (300 μl) of the suspension were taken hourly, whilst agitating continually to maintain homogeneity and concentration. The beads were centrifuged (5000 x g for 1 min) and the supernatant solution was assayed for haem by the pyridine haemochrome method.

3.2.3. Gel Filtration.

To investigate Hb-InpA interactions, samples collected for analytical gel filtration chromatography (see section 2.11) were incubated in 0.1 M Tris-HCl (pH 7.5). The column was equilibrated with 0.1 M Tris-HCl (pH 7.5). Sample volumes of 100 μl (4 μM oxyHb) were injected onto the column and eluted at a flow rate of 0.5 ml/min. Hb elution was followed by absorbance measurements at 280 nm and 410 nm.

3.2.4. MALDI-TOF-MS.

To identify sites of InpA cleavage by MALDI-TOF-MS, oxyHb samples were incubated in 10 mM Tris-HCl (pH 7.5) and sampled periodically. Samples were analysed directly without any further processing.

3.3. RESULTS AND DISCUSSION

3.3.1. InpA-mediated metHb formation.

Prevotella intermedia inhabiting the periodontal pocket will likely be exposed from time to time to Hb as oxyHb from fresh bleeding (Hanioka, 2005). During incubation of oxyHb with InpA, at pH 7.5 to mimic the slightly alkaline conditions prevailing in the periodontal pocket (Bickel & Cimasoni, 1985) spectroscopic changes typically associated with auto-oxidation to the methaemoglobin form were seen. These including blue-shift in Soret band λ_{max} from 412 to 405 nm, gradual increase in 500 and 631 nm absorbance, and decrease in 577 nm and 541 nm Q bands (Winterbourne *et al.*, 1976), (Fig 3.1). Isosbestic points were observed at 475, 525 and 592 nm, indicative of direct transformation of oxyHb to the met- form without the production of any intermediates. However, compared to control auto-oxidation, InpA-mediated metHb formation occurred more rapidly. The amount of metHb formed was calculated from $A_{577\,nm}$ and $A_{631\,nm}$ values employing a multi-component analysis expression derived by Winterbourne *et al* (1976), and using the extinction coefficients previously determined for horse haemoglobin (Smalley *et al.*, 2007). After 7 h, InpA and oxyHb control incubations contained 50 % and 17.5 % metHb, respectively. This suggested that InpA was increasing oxidation of haemoglobin to ferric Fe (III) metHb, specifically hydroxymethaemoglobin, the prevalent form at pH 7.5.

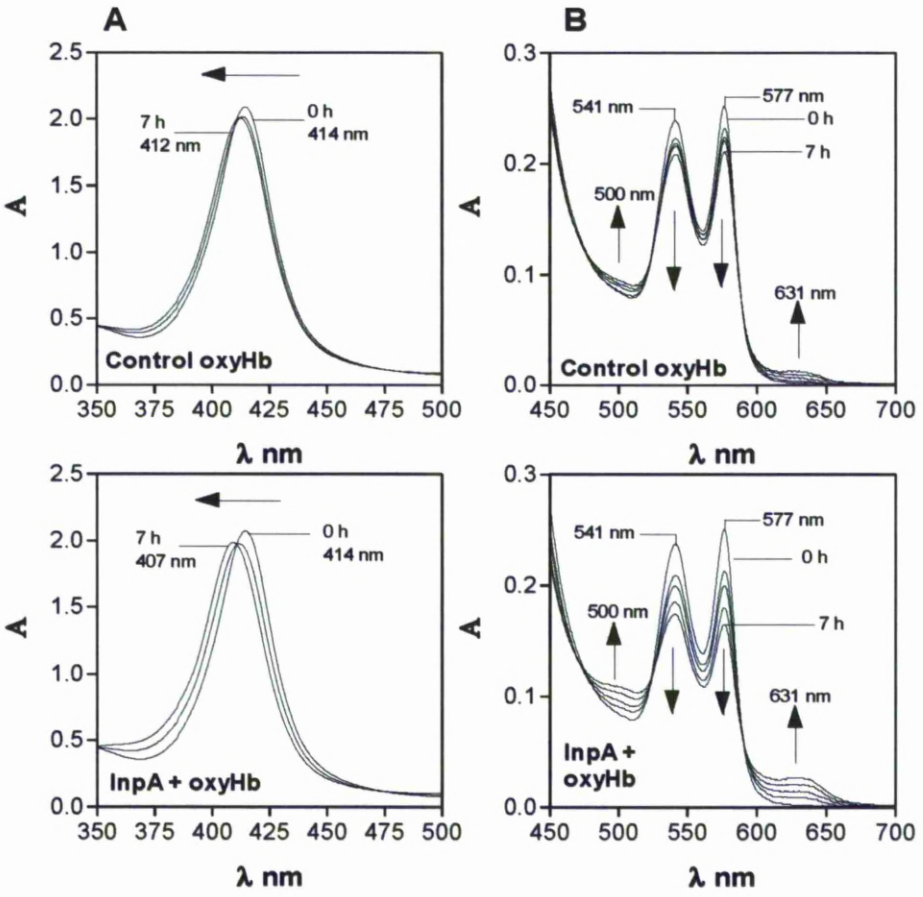


Fig 3.1. UV-visible spectra of oxyHb oxidation induced by InpA. Arrows in panels B denote changes in absorbance intensities with time.

After 7 h, 10 mM $Na_2S_2O_4$ was added to the InpA-Hb incubation to simultaneously reduce the haemoglobin haem iron and deplete the O_2 in the buffer. This revealed deoxyhaemoglobin spectrum, characterised by a Soret λ_{max} at 429 nm and 555 nm Q band peak (Fig 3.2). This verified that metHb had originally been formed by the action of InpA.

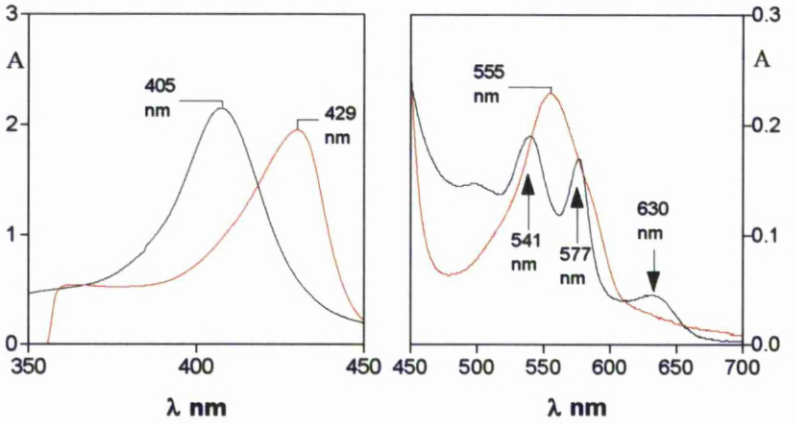


Fig 3.2. UV-visible spectrum of oxyHb incubation with InpA after 7 h (solid line). Red line shows the effect of adding 10 mM $Na_2S_2O_4$.

To determine relative rates of metHb formation, difference spectra of the Soret band region were produced by subtracting the initial time zero spectra from those at subsequent time points. These spectra had peak maxima and minima at 402 nm and 420 nm respectively, with a zero cross-over point at 411nm (Fig 3.3) and essentially show the net change in Soret band during conversion of oxyHb to metHb (Kelm *et al.*, 1997; Zhang *et al.*, 1996). The integrated areas of the resulting peaks (348 to 411 nm) and trough (411to 472 nm) give a measure of the amount of metHb formed (Kelm *et al.*, 1997; Zhang *et al.*, 1996). This showed that InpA elevated the rate of oxyHb oxidation above the auto-oxidation rate in a concentration dependent manner at pH 7.5 (Fig 3.4).

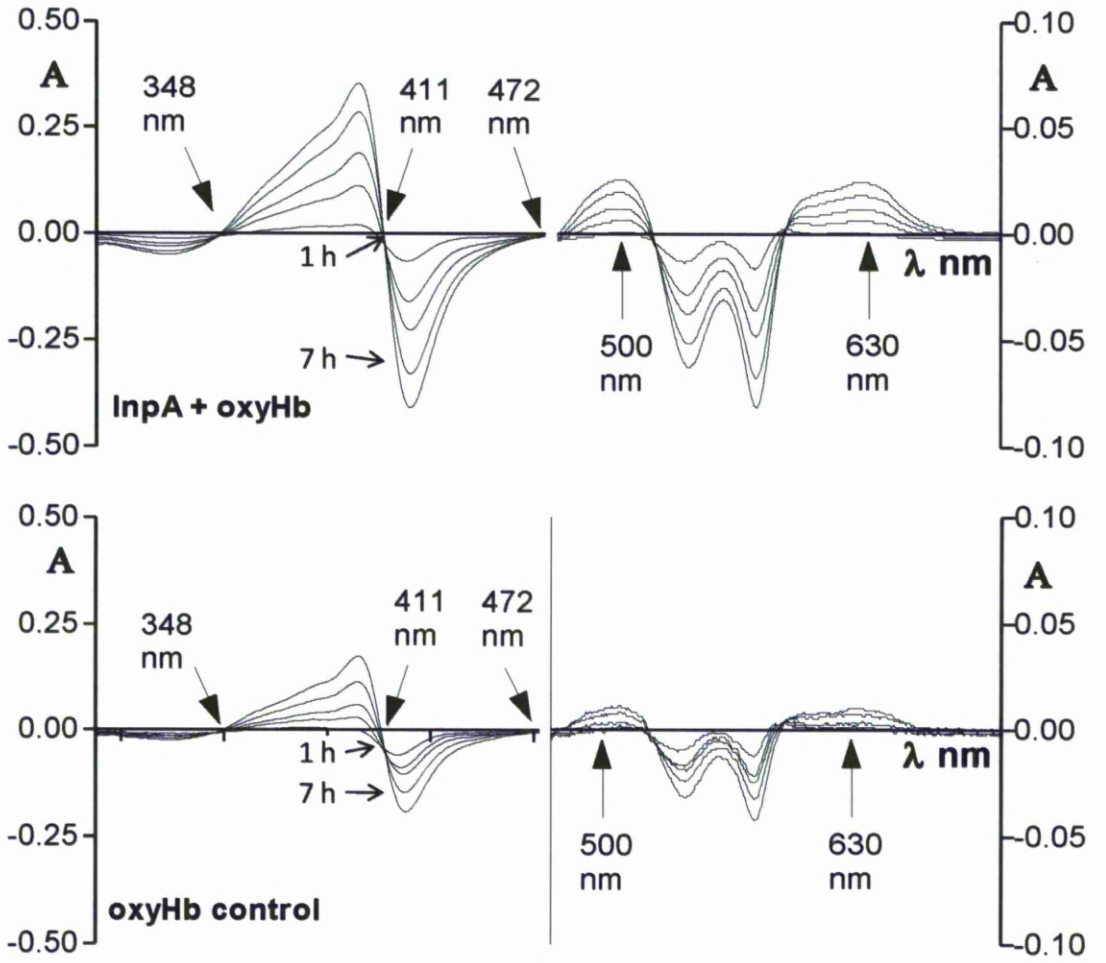


Fig 3.3. Difference spectra derived from data in Fig 3.1 showing progressive changes in the Soret band region of oxyHb over 7 h during InpA-mediated oxidation, and during auto-oxidation. Inner

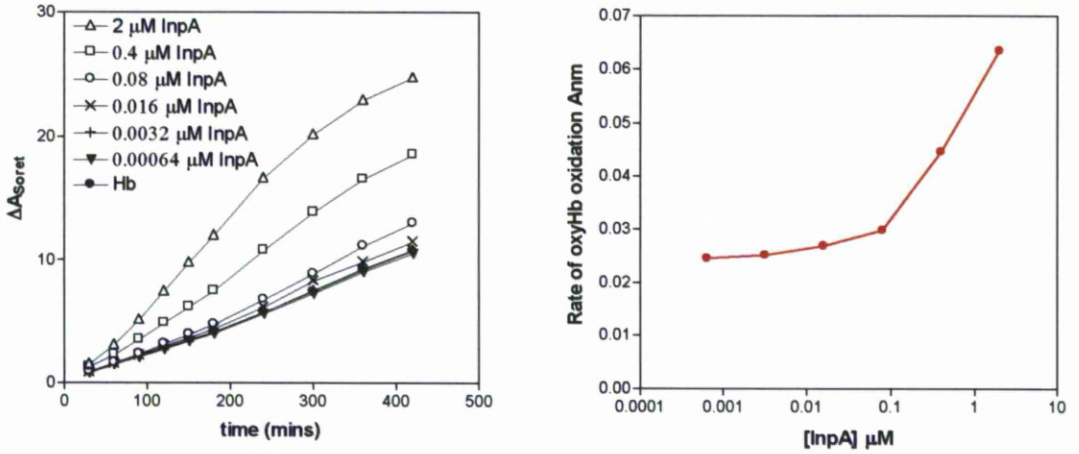


Fig 3.4. OxyHb oxidation by InpA. UV-visible spectra were used to create difference spectra (Fig 3.3), from which the area of the two peaks (between 390 and 430 nm) was used to calculate the oxyHb oxidation rate with different concentrations of InpA.

To confirm that InpA-induced metHb formation was proteolytic in nature, InpA and oxyHb incubations were carried out in the presence of the irreversible cysteine protease inhibitor, E-64 (0.5 mM), which is known to completely inhibit InpA protease activity against chromogenic *p*-nitrolanilide-derivatized peptide substrates (Potempa, unpublished data). Difference spectra were derived as above and from these the oxidation rates were calculated (Fig 3.5). Under these conditions E-64 reduced InpA-mediated metHb formation by ~ 80 %.

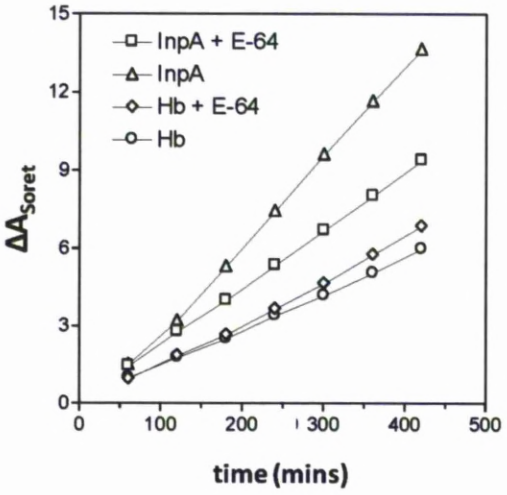


Fig 3.5. Inhibition of InpA-mediated metHb formation with E-64. Rate of metHb formation was determined using difference spectra in the Soret band region as described previously (section 2.7.1)

SDS-PAGE and densitometry analysis of Hb-InpA incubations at pH 7.5 showed only limited protein breakdown (~ 20 %) during the time in which oxidation was occurring (Fig

3.6). This indicated that although some proteolytic cleavage of the globin chain had occurred, the gross structure had remained stable. Indeed, the complex structure of haemoglobin is maintained by numerous interactions between individual residues within the globin chain and with the haem moiety, as well as interfacial interactions between the globin chains of haemoglobin tetramers and dimers. These interactions not only enable haemoglobin to alter shape in response to oxygen loading, but in this case may also have prevented complete unfolding of the globin chains in response to incisions made by InpA.

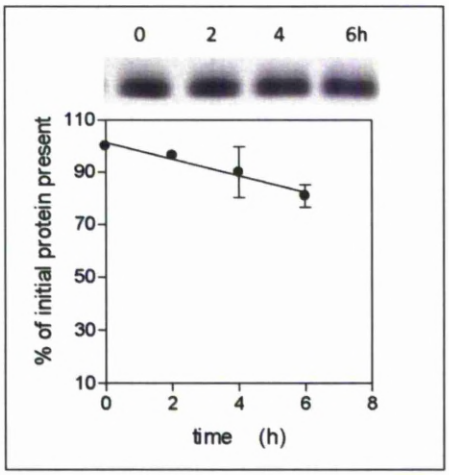


Fig 3.6. SDS-PAGE and densitometric determination of haemoglobin breakdown by InpA at pH 7.5. The data points are the mean and SD of three separate experiments.

To eliminate the possibility that InpA-mediated oxidation was a result of contaminant metal ions, which are well known to bring about oxidation (Summerfield & Tudhope, 1978), repeat incubations were performed in the presence of Ethylenediaminetetraacetic acid (EDTA; 10 μM), and the rate of metHb formation was determined using the change in $A_{577\,nm}$ versus time (as described section 2.7.1) over a 5 h period. The rate of oxidation was the same in both the presence and absence of EDTA under these conditions (Fig 3.7). It was concluded from this that the InpA-mediated oxidation was unrelated to the presence of metal ions.

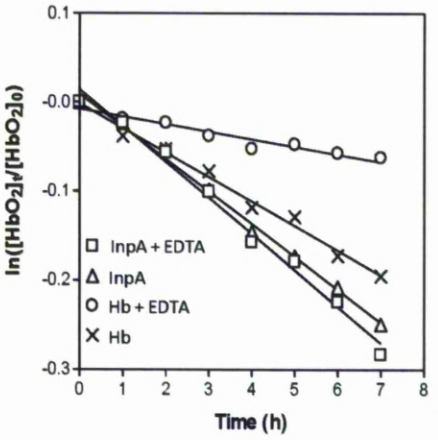


Fig 3.7. OxyHb oxidation rates in the presence of InpA and EDTA. Rates were measured as a function of A_{577nm} (see section 2.7.1.for details).

3.3.2. MS analysis of haemoglobin proteolysis during InpA-mediated oxidation.

InpA is not unique in its ability to proteolytically mediate oxyHb oxidation at pH 7.5 where natural auto-oxidation is at its lowest (Shikama, 1998). Smalley *et al.* (2004) initially demonstrated that whole cells of *P.gingivalis* could bring about oxidation as an initial step of oxyHb degradation (Smalley *et al.*, 2004). They subsequently attributed the R-gingipains as the responsible agents (Smalley *et al.*, 2007). Arginine residues are located in the C terminus of horse Hb at position 31and 92 of the alpha chain and at positions 30, 40, and 104 of the β chains (Antonini & Brunori, 1971). Rgp may attack these susceptible surface located arginine residues, bringing about structural changes leading to oxidation. Although InpA has a preference for cleavage at P1 K-residues, it will (although less efficiently) cleave substrates with P1 R-residues, and also has specificity for A- and F-residues (J. Potempa, K. Wawrzonek & A. Jaworska, unpublished work). Cleavage at R-residues by InpA could induce haemoglobin oxidation in a manner resembling that proposed for Rgp. Equally, cleavage of other residues, such as K, F, and A, could also produce structural changes.

To determine which residues are cleaved during oxidation at pH 7.5, oxyHb was incubated with InpA, sampled periodically, protease activity inhibited with E-64, and the whole digests were subjected to MALDI-TOF-MS analysis. Numerous peptides indicated C-terminal cleavage at K, R, A and F residues from both the α and β chains (Table 3.1), in

keeping with the specificities for hydrolysis of chromogenic *p*–nitrolanilide-derivatized peptides and processing sites of N- and C-terminal pro-fragments cleaved during the auto-proteolytic maturation of the pro-interpain respectively (J. Potempa, K. Wawrzonek & A. Jaworska, unpublished work). Scission by InpA at these sites, while not leading to gross breakdown, could mediate oxidation. It is noteworthy that all the digestion fragments were generated during the oxidation process, and importantly, when gross protein breakdown was not apparent (as revealed from the data in Fig 3.6).

Table 3.1. Digestion fragments identified as arising from proteolysis of α- and β-oxyhaemoglobin chains during InpA-mediated oxidation. ↓ denotes position of cleavage by InpA.

Fragment size (Da) and time of appearance	Corresponding predicted fragment size (Da)	Chain designation and residue numbers	Amino acid sequence
1126.75 (5 h)	1126.56	β-96-β-104	K↓L^{96}HVDPENFR^{104}
1356.92 (4 h)	1356.65	β-46-β-59	F↓G^{46}DLSNPGAVM GNPK^{59}
1417.92 (2 h)	1417.72	α-111-α-123	A↓V^{111}HLPNDFTPA VHA^{123}
1902.36 (8 h)	1902.94	α-8-α-26	K↓T^{8}NVKAAWSKVG GHAEGEYGA^{26}
2227.66 (4 h)	2227.08	β-41-β-61	R↓F^{41}FDSFGDLSNPG AVMGNPKVK^{61}
	2227.10	α-41-α-60	K↓T^{41}YFPHFDLSHGS AQVKAHGK^{60}
2037.65 (2 h)	2037.97	β-86-β-103	F↓A^{86}ALSELHCDKLH VDPENF^{103}

Even modest changes in haemoglobin structure can result in rapid oxidation. The distal histidine of both haemoglobin and myoglobin controls solvent access to the ligand pocket and plays a crucial role in auto-oxidation (Romero-Herrera *et al.*, 1981). Replacement of the distal His with smaller residues (e.g. L), by site directed mutagenesis in sperm whale myoglobin, creates a gap that permits solvent access, resulting in increased auto-oxidation (Springer *et al.*, 1989). Structural changes also impel the distal histidine of protozoan myoglobin and mutants of sperm whale myoglobin to behave as an endogenous

nucleophile, thereby enhancing auto-oxidation (Springer *et al.*, 1989; Tsubamoto *et al.*, 1990). Proteolytic attack by InpA and Rgp could exploit the dependency of haemoglobin and myoglobin for the distal histidine to bring about oxidation.

Detection of one prominent fragment (1356.92 Da), highlights a potential mechanism for InpA mediated oxidation. This fragment, appearing after ~ 4 h, was predicted to originate from the β chain due to cleavages at Phe-45 and Lys-59. Phe-45 of haemoglobin orientates toward the distal histidine in a similar manner to the highly conserved Phe-46 residue of myoglobin (see Fig 3.8) which plays an important role in modulating oxidation (Lai *et al.*, 2004). It was demonstrated that, owing to its close proximity to the distal histidine, Phe-46 affects ligand binding and auto-oxidation. The steric hindrance imposed on the distal histidine (His-64) by van der Waals contact between C ζ of Phe-46 and Cβ of His 64 was removed by replacing Phe-46 with a smaller amino acid residue. This enabled the imidazole side chain of His-64 to rotate about the Cβ-Cγ bond towards the space previously occupied by the native Phe-46 side chain. Not only is this movement thought to decrease the affinity of bound ligands by disrupting hydrogen bonding to His-64, but it also opens the distal pocket to water molecules, increasing oxidation. Cleavage by InpA at Phe-45 could therefore induce oxidation by disrupting the distal pocket and releasing the steric hindrance imposed on the distal histidine.

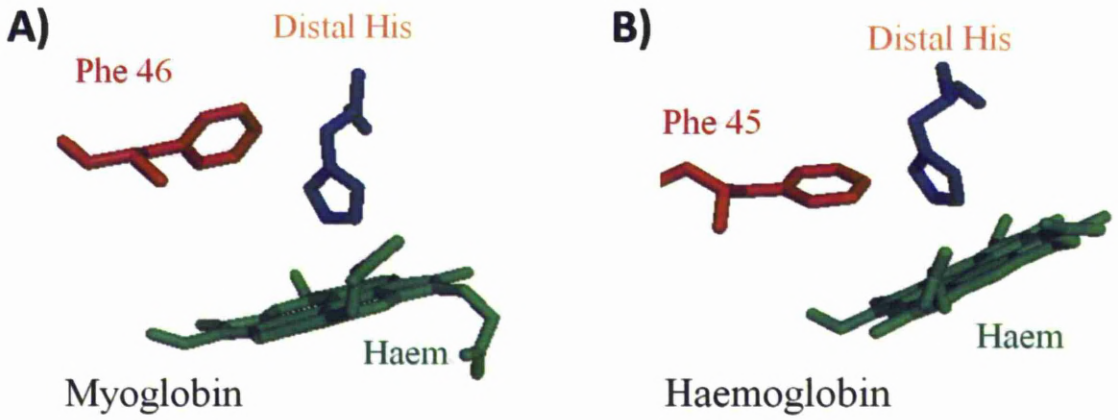


Fig 3.8. Orientation of Phe-46 of myoglobin (A) and Phe-45 of the β chain of haemoglobin (B) in relation to the distal histidine. Diagram created using Pymol (PDB access codes and 1MBO and 1MHB).

OxyHb digestion also yielded a prominent 2227 Da fragment, predicted to originate from both α and β chains, with InpA cleaving at Lys-40 and Lys-60 for the former, and Arg-40 and Lys-61 for the latter chain. Importantly, both of these peptide fragments would contain the distal histidine (Fig 3.9). InpA cleavage of β Lys-61and α Lys-60, which both are in close proximity to the distal histidine, would probably disrupt the distal histidine and lead to oxidation. Importantly, β Lys-61 is situated as a surface located residue and would be immediately available to InpA.

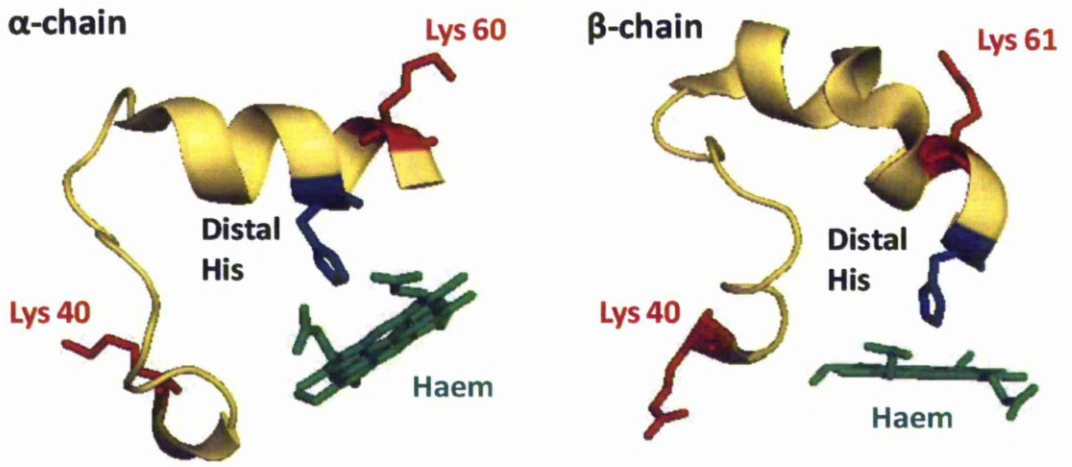


Fig 3.9. Position of InpA cleavage sites in relation to the distal histidine. Diagram created using Pymol (PDB access code 1MHB).

Several other mutations of myoglobin also engender structural changes that lead to increased auto-oxidation. Electrostatic interactions involving Thr-67, Lys-45, Asp-60, His-64, surface water molecules, and the haem-6-propionate help stabilize the closed door position of the distal histidine (Brantley *et al.*, 1993, Carver *et al.*, 1990). Disruption of this hydrogen bonding lattice was used to explain enhanced rates of myoglobin auto-oxidation observed with mutation of Thr-67 (Brantley *et al.*, 1993). Brantley *et al.* (1993) also demonstrated that replacement of Lys-45, Val-68, and Leu-29 with smaller uncharged residues, such as serine, increases the volume of the distal pocket and opens up the exposed face of the haem, resulting in a 7-fold increase in oxidation (Brooks 1935). It is also noteworthy that the mutant M haemoglobins, which have amino acid substitutions in or near the haem pocket, also auto-oxidized more easily (Percy *et al.*, 2005). InpA cleaves around the distal pocket which disrupt the confinement of the haem within its hydrophobic crevice will increase accessibility of H_2O to serve as a nucleophile to iron, or as a source of protons and solvation, and encourage oxidation (Brantley *et al.*, 1993).

Although MALDI-TOF-MS showed numerous InpA cleavage fragments, SDS-PAGE demonstrated negligible protein breakdown over 6 h but only after the sample was fully denatured by heating at 100 °C (Fig 3.6). It can be hypothesised that the complex internal architecture of haemoglobin protects it from unfolding even during InpA attack, and would explain why peptides were only seen after MALDI or denaturing PAGE, where the analysis conditions promote complete release of digestion fragments from the protein. The stability of haemoglobin attacked by InpA under non-denaturing conditions was confirmed using gel filtration. The elution point of haemoglobin increased from 10.9 ml to 11.5 ml after 7 h indicating only modest reduction of protein size (Fig 3.10). The absorbance intensity of the haemoglobin peak at 410 nm and 280 nm also decreased indicating a small amount of haem loss and protein breakdown (Fig 3.10).

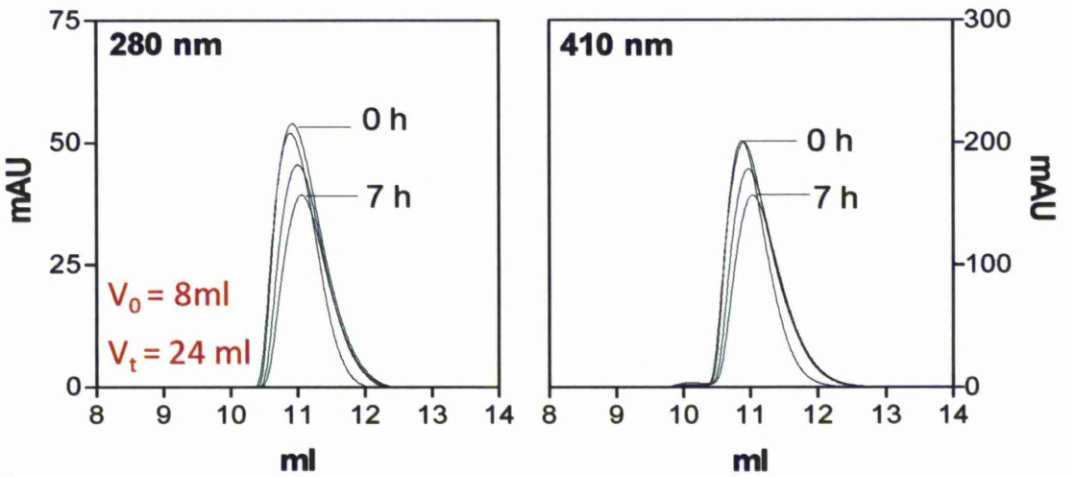


Fig 3.10. Analytical gel filtration chromatography of oxyHb incubated with InpA. Absorbances recorded at 280 and 410 nm.

3.3.3. Formation of haemoglobin haemichrome.

Short-term incubation of oxyhaemoglobin with InpA at pH 7.5 resulted in oxidation to the hydroxymetHb form. However, longer incubations periods (24 h) gave rise to a haemoglobin haemichrome (Soret λ_{max} 409 nm and a broad 535 nm Q band) (Fig 3.11). Addition of 10 mM $Na_2S_2O_4$ resulted in a haemochrome spectrum (424 nm Soret band and Q bands at 530 and 558 nm [Rifkind *et al.*,1994; Rachmilewitz *et al.*, 1971]) (Fig 3.11) confirming the original formation of a iron(III) haemichrome. Haemichrome formation by InpA was also completely inhibited by the protease inhibitor E-64 (data not shown).

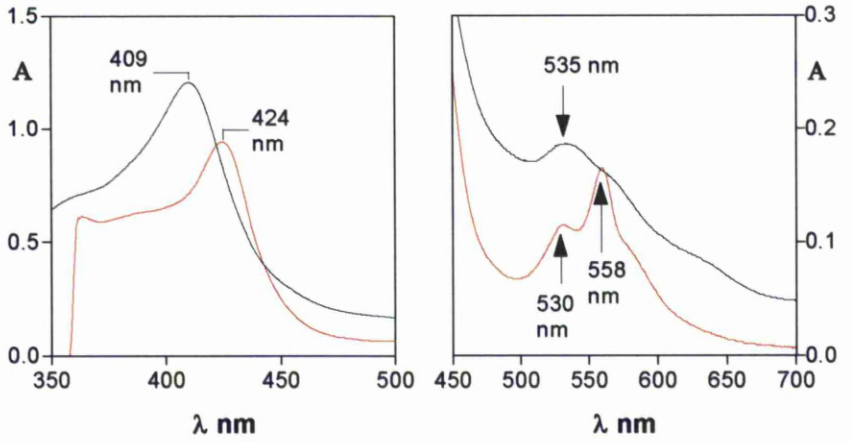


Fig 3.11. UV-visible spectra showing formation of a haemoglobin haemichrome by InpA. Red line shows the effect of adding 10 mM $Na_2S_2O_4$ after 31 h.

Haemoglobin haemichromes are formed by drastic structural changes within the globin chain which distort the haem pocket geometry such that the haem iron coordinates with amino acid ligand other than the proximal histidine. This can be brought about via protein denaturants (urea) and by fatty acids (Rifkind *et al.*, 1994; Rachmilewitz *et al.*, 1971). Haemoglobin haemichrome is also generated by the action of *P. gingivalis* Kgp (Smalley *et al.*, 2004, 2007), which may be caused by structural changes brought about by proteolytic cleavage. Analogous structural change may also occur through the action of InpA since, like Kgp, it will also cleave at K residues (J.Potempa, unpublished data).

3.3.4. Susceptibilities of hydroxy- and aquomethaemoglobin to degradation by InpA.

Smalley *et al.* (2007, 2008) demonstrated that haemoglobin is susceptible to proteolytic attack by Kgp once it has been converted to the Fe(III) state (either by $NaNO_2$ or by pre-treatment with HRgpA), and remains relatively refractory to proteolysis in the oxygenated form. Compared to oxyHb, metHb is also more susceptible to trypsin (Smalley *et al.*, 2008). Proton assisted nucleophilic displacement of O_2^- by H_2O accelerates metHb (as aquometHb) production by a factor of more than 10^6/mol (Tsuruga *et al.*, 1998). However, Shikama *et al.* (1988) demonstrated that the proton-catalyzed auto-oxidation of MbO_2 was inherently a very fast reaction reduced to negligible levels due to the low concentrations of protons (corresponding to 10^{-7} M H^+) at physiological pH. As such, the rate of auto-oxidation increases with the high hydrogen ion concentrations prevailing at low pH in sperm

whale and bovine myoglobin (Shikama & Sugawara, 1978; Shikama & Matsuoka, 1986). In this context it is noteworthy that *Prevotella* species display acid terminal growth pHs (Shah & Williams, 1987; Takahashi & Yamada, 2000) and haemoglobin degradation and binding by the bacterium also increases at acid pH (Guan *et al.*, 2006, 2004). Accordingly, pH-dependency of oxyHb oxidation and breakdown by InpA was investigated.

For oxidation rate measurements, the changes in A_{577}nm versus time were used (Tsuruga & Shikama, 1997) as this obviated any complications relating to changes in Soret area due to proteolytic degradation and haem loss at low pH (which would be manifest as a loss of Soret absorbance). This showed that InpA could increase the oxidation rate at pH 7.5, where the natural auto-oxidation of both the alpha- and beta-globin chains is lowest (Shikama, 1998), as well as at pH 6.5 and 6.0, where naturally occurring auto-oxidation is high (Fig 3.12).

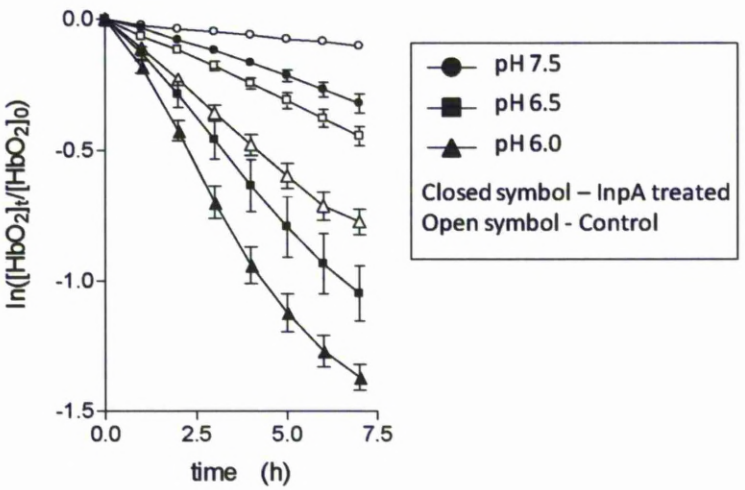


Fig 3.12. Oxyhaemoglobin oxidation mediated by InpA. Rates were measured as a function of A_{577nm} (as described in section 2.7.1). Data points are mean ± SD (n = 4, except at pH 7.5, where n=7).

After 24 h incubation InpA brought about a greater reduction in Soret band intensity (indicative of haem loss) at pH 6.0 compared to pH 7.5 (Fig 3.13), and at pH 6.0 the λ_{max} remained at ~ 405 nm showing that the final product was largely metHb (Fig 3.13). Addition of 10 mM $Na_2S_2O_4$ produced a 429 nm Soret and 555nm Q band typical of deoxyHb (Fig 3.14A). Moreover, at pH 6.0 there was no evidence of a haemichrome (peaks at 530 and 558 nm [Fig 3.14A]).

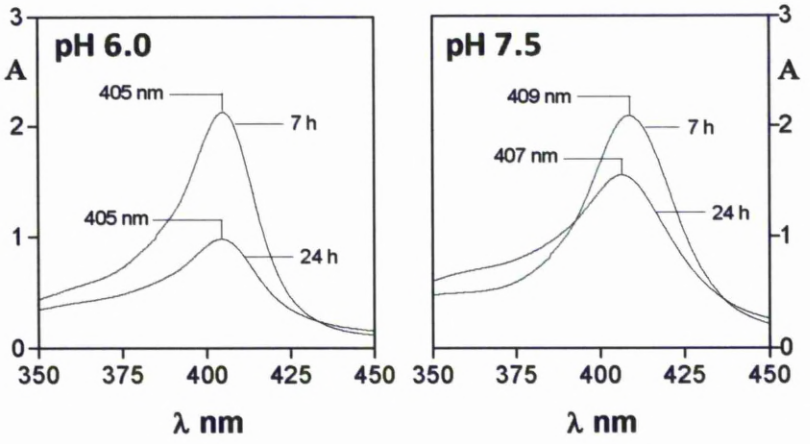


Fig 3.13. UV-visible spectra showing the effect of InpA on oxyHb at pH 7.5 and 6.0.

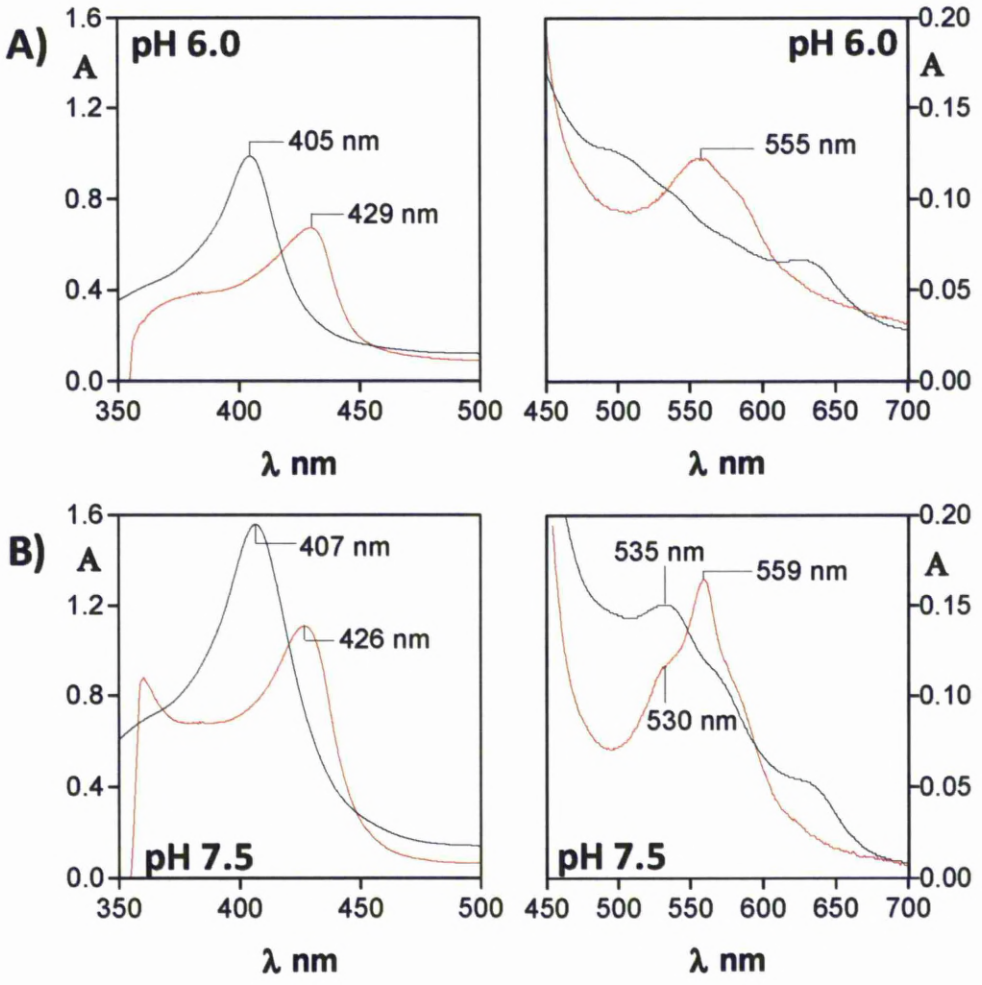


Fig 3.14. Spectra of the products formed from oxyHb by InpA at pH 7.5 and 6.0 after 24 h. Red line shows the effect of adding 10 mM $Na_2S_2O_4$ after 7 h.

Increased breakdown of haemoglobin cannot merely be attributed to enhanced enzyme activity at acidic pH, considering that a different product, namely protease-resistant haemichrome, is formed by proteolysis at pH 7.5. This must therefore imply that pH-dependent changes of Hb dictate the outcome of InpA proteolytic attack. Formation of hydroxymetHb and aquometHb at high and low pH respectively could explain this. In vivo, auto-oxidation involves the entry of solvent OH^- or H_2O molecules into the haem pocket which evoke the nucleophilic displacement of a superoxide anion, O_2^- (Shikama 1990; Tsuruga & Shikama, 1997), and which then remain bound to the iron atom at the sixth co-ordinated position, forming aquometHb (with bound H_2O at acid pH) or hydroxymetHb (with bound OH^- at alkaline pH).

To test the relative susceptibilities of hydroxy- and aquometHb to InpA breakdown, oxyHb was auto-oxidised for 24 h at pH 7.5, 6.0, 5.5 and 5.0, to form the hydroxy- form (pH 7.5) and the aquo-forms (pH 6.0, 5.5, 5.0). Under these conditions approximately 95 % of the sample had been converted to the hydroxy- and aquo-metHb forms. The metHb preparations were then exposed to InpA and sampled periodically for SDS-PAGE. Densitometry showed progressive digestion of the α- and β-chains of aquometHb at pH 6.0 and 5.5 (Fig 3.15). In addition, TMB staining indicated that proteolysis was accompanied by loss of haem, and this was most rapid at pH 5.5 (Fig 3.15). Reliable densitometry of the aquometHb digestion at pH 5.0 was not possible due to naturally occurring precipitation of haems (data not shown). It should be noted that at acid pH there was also modest haem loss in the control, attributed to enhanced haemin dissociation. In contrast, there was little or no globin chain degradation or haem loss from hyroxymetHb pH 7.5 (Fig 3.15).

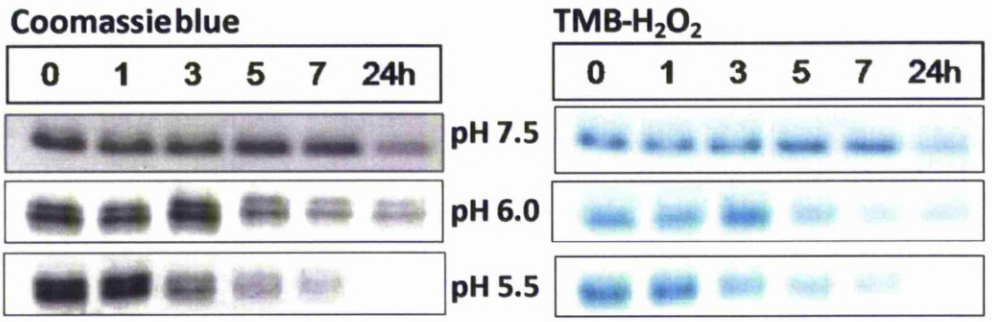


Fig 3.15. Effect of incubating aquo- and hydroxy-metHb with InpA.

UV-visible spectra of incubations of InpA with metHb also indicated more breakdown and haem release (reduction in Soret intensity), for aquometHb (pH 5.5) than hydroxymetHb (pH 7.5) (Fig 3.16).

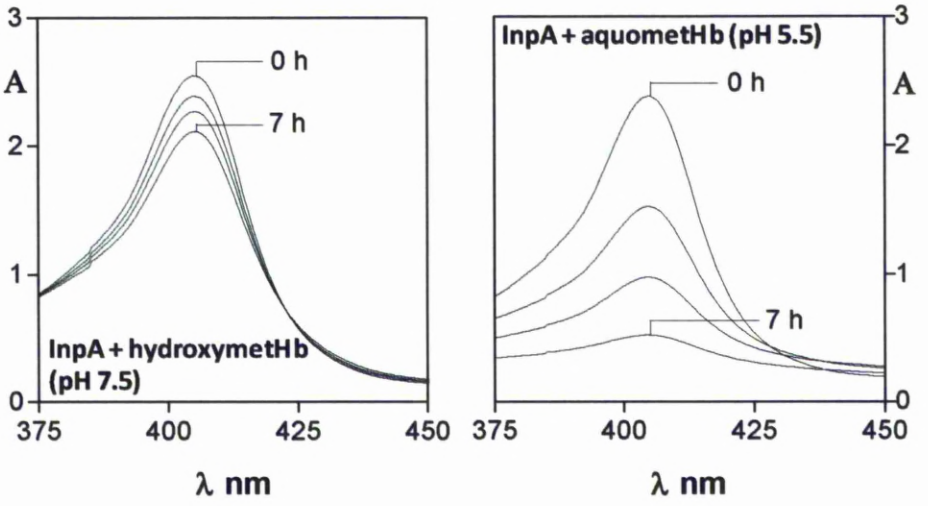


Fig 3.16. UV-visible spectra of InpA incubated with aquo- and hydroxy-metHb.

In contrast to InpA, trypsin effected complete breakdown of hydroxymetHb at pH 7.5 (collapse in Soret intensity and globin chain degradation; Fig 3.17, A). InpA released only 20 % of total haem released from hydroxymetHb- agarose by trypsin over 7 h (Fig 3.17, B). In this method, haem proteolytically freed from immobilized hydroxymetHb substrate was detected as a pyridine haemochromogen.

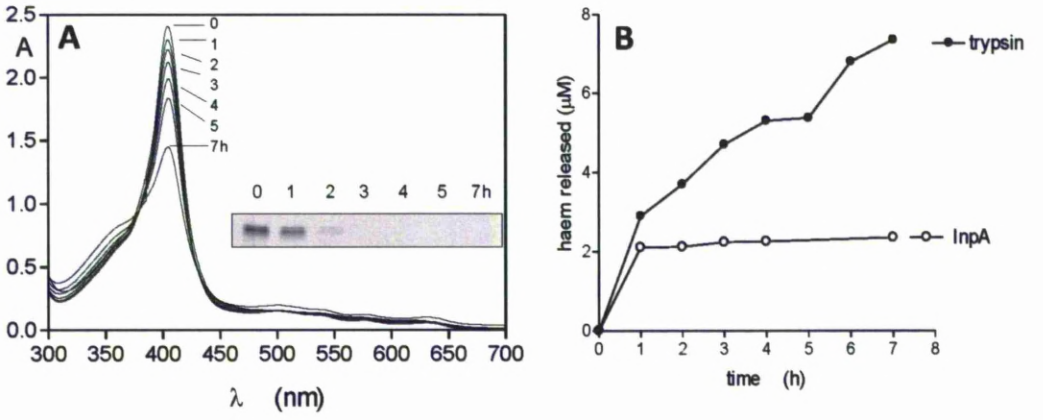


Fig 3.17. A, UV-visible spectra and SDS-PAGE of hydroxymetHb incubated with trypsin at pH 7.5. B, Proteolytic haem release from hydroxymetHb-agarose by trypsin and InpA.

These results demonstrate that aquometHb is more susceptible to InpA than hydroxymetHb. The pH of a dental plaque can vary between physical niches because of the presence of bacteria with different metabolic specificities. Growth of *P.intermedia* in liquid culture is accompanied by a pH drop as a result of saccharolytic metabolism and the production of acetate, formate and succinate (Shah & Williams, 1987; Takahashi & Yamada, 2000). MetHb forms naturally through auto-oxidation within erythrocytes, but is rapidly reduced back to oxyhaemoglobin by membrane-associated methaemoglobin reductase as well as intracellular reducing agents glutathione and ascorbate (Jaffe & Neuman, 1964). Hb liberated from erythrocytes by prevolysin of *P. intermedia* (Takada *et al.*, 2003) or by low pH (which increases the osmotic fragility of RBCs [Jacobs & Parpart, 1931; Parpart *et al.*, 1947]), will readily oxidise in the absence of these intra-cellular reductants and reductases. Release of Hb into the acidic environment surrounding *P. intermedia* cells would result in even higher rates of oxidation and aquometHb formation.

In addition to directly catalysing metHb formation, change in the activity of protons at low pH results in protonation of the globin chain which increases oxyHb susceptibility to oxidation (Myshkin, 1984). At neutral pH, the distal histidine (His E7) is in the 'down conformation' and prevents ligand molecules from entering the distal pocket. Protonation of His(E7) at low pH causes a rotation about its C^{α}-C^{β} bond (observed as an upwards movement residue) rendering the haem pocket more accessible to entering ligands, *de-facto* increasing aquometHb production (Yang & Phillips, 1996; Ringe *et al.*, 1984; Johnson *et al.*, 1989). Additionally, accumulation of positive charges around the α^1-β^1 contact by protonation of prototropic groups (e.g. imidazole and carboxylate groups) increases Coulombic repulsion of the haemoglobin subunits leading to an appreciable shift in the tetramer-dimer equilibrium which favours formation of α-β dimers (Katz *et al.*, 1973; Myshkin, 1984). Even in a weakly acidic medium (pH 6.0), there will be an appreciable concentration of α-β dimers (Katz & Crissman, 1973). Most importantly, α-β dimers are known to auto-oxidise more rapidly than tetramers (Zhang *et al.*, 1991; Benesch & Kwong, 1995). Furthermore, dissociation of haem is exacerbated when αβ dimers are freed from the tetramer (Gattoni *et al.*, 1996).

Haemoglobin degradation has been reported for several periodontal pathogens including *P.gingivalis, P. nigrescens and Prevotella loescheii* (Lewis *et al.*, 1999; Sroka *et al.*, 2001; Zwickel., 1992). Guan *et al* (2006) demonstrated that culture supernatants of

P. intermedia digested haemoglobin over a wide pH range, particularly at acidic pH. Their research also asserted that an enzyme was responsible for proteolysis as heat treatment and incubation with protease inhibitors (N-ethylmaleimide, leupeptin and N-p-tosyl-L-lysine chloromethyl ketone) reduced degradation (Guan *et al.*, 2006). Investigations by Smalley *et al* (2003a) also concluded that *P. nigrescens* and *P. intermedia* degrade haemoglobin to form $[Fe(III)PPIX]_2O$ as an intermediate, which then accumulates as the monomeric iron protoporphyrin IX, (Fe(III)PPIX.OH). The findings presented in this study implicate InpA as the key mediator of haemoglobin breakdown. Furthermore, the acid pH developed by *P. intermedia* cells may play an integral role in this process by facilitating rapid rates of aquometHb formation.

3.3.5. Azide ligation prevents InpA breakdown of aquomethaemoglobin even at pH 6.0.

Haemoglobin was efficiently degraded by InpA at acidic pH, and this could be attributed, in part, to the enhanced rate of proton-mediated oxidation and formation of the more susceptible aquometHb species. The ability of InpA to more efficiently degrade aquometHb compared to oxyHb and hydroxymetHb is, at first sight, perplexing due to the fact that, unlike oxyHb and deoxyHb which differ in their structure and conformation, the structures of oxyHb and the metHb are scarcely different (Perutz, 1970). MetHb differs from oxyHb only in its inability to carry O_2 and its reduced haem-globin affinity which encourages the dissociation of haem (Gatoni *et al.*, 1996). Indeed, in the body Fe(III) haem is released from metHb and becomes sequestered by albumin and haemopexin (Adams & Berman, 1980; Gatoni *et al.*, 1996). For this reason, the importance of haem dissociation in determining InpA breakdown of haemoglobin was investigated.

Should haem loss be an important factor for haemoglobin breakdown, then reducing haem dissociation by ligation of the haem with N_3^- ought to have the predicted effect of reducing or abrogating globin breakdown completely, as has been demonstrated for Kgp (Smalley et a., 2008). Indeed, SDS-PAGE and the spectra indicated that InpA was unable to digest α- and β-globin chains of aquometHb when the haem was ligated with azide, even at pH 6.0 where InpA proteolysis would normally be high (Fig 3.19, A). In stark contrast, InpA completely degraded haem-free globin within a few minutes even at pH 7.5 (Fig 3.19, B).

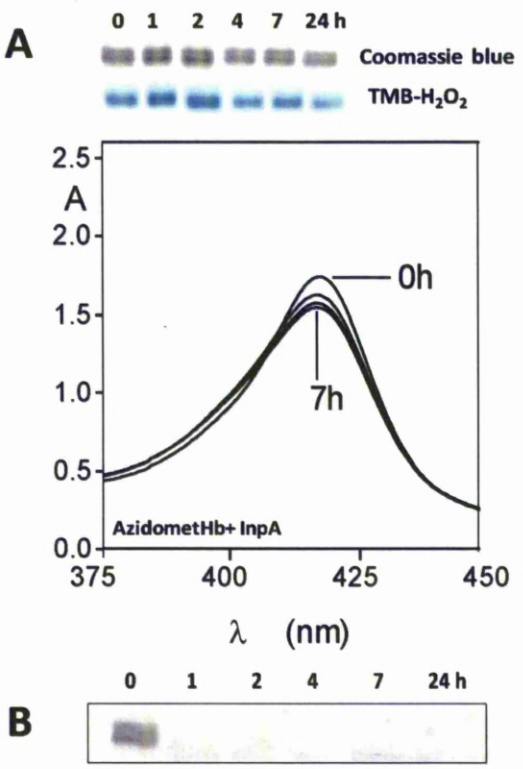


Fig 3.19. A, UV-visible spectra and SDS-PAGE of azidometHb incubated with InpA at pH 6.0. B, SDS-PAGE of haem-free globin incubated with InpA.

The structures of both haemoglobin and myoglobin are effectively stabilised in the presence of haem (Crumton & Polson, 1965; Kawahara *et al.*, 1965), and the stability is therefore determined by the affinity of haem for the globin molecule (Hargrove & Olson, 1996). Haemoglobin and myoglobin exhibit extremely high affinities for haem in the Fe(II) configuration, with dissociation constants in the region of 10^{-12} to 10^{-15} M (Hargrove *et al.*, 1996). Loss of the haem moiety from the holoprotein renders the resultant apohaemoglobin more susceptible to protease activity due to the collapse of the globin structure (Antonini & Brunori, 1971). In fact, trypsin will also degrade metHb far more rapidly than oxyHb (Smalley *et al.*, 2008). Smalley *et al.* (2007) demonstrated that while oxyHb was refractory to breakdown by *P. gingivalis* Kgp, the metHb form was highly susceptible. Ligation of the ferrihaem with the low spin azide N_3^- ligand effectively stabilised the globin by preventing haem loss (Bunn & Jandl, 1968) and therefore breakdown by Kgp, whereas the haem-free globin was easily degraded (Smalley *et al.*, 2007). Thus, InpA, like Kgp, demonstrates a pre-requisite for haem-dissociation from the oxidized Hb form.

This is of significance as haem dissociation is also increased by departure from neutral pH (Hargrove *et al.*, 1994; Bunn & Jandl, 1968). Furthermore, Hargrove *et al.*, (1994) observed greater haem dissociation in aquometHb than hydroxymetHb. *In vivo* the strength of the Fe-His93 bond is a function of the iron oxidation state, the sixth coordinate ligand and pH (Momenteau & Reed, 1994; Hargrove *et al.*, 1994). Bond stability is increased by coordination with ligands that accept π-π^* back-bonding from the iron, such as CO and CN, and destabilised with poor π acceptors, such as phenoxide and hydroxide (Momenteau & Reed, 1994; Hargrove *et al.*, 1994). As such, formation of hydroxymetHb at neutral and alkali pH is associated with enhanced haem dissociation. At low pH, protonation of the Fe-His93 bond causes it to break in both deoxyMb and aquometMb which results in haem dissociation as well as denaturation of the protein (Yang & Phillips, 1996; Barrick *et al.*, 1994). This protonation event results in the movement of the His93 C^{α} atom, and rotation of the imidazole ring, culminating in the concomitant elongation and disruption of the Fe-His93 bond. This creates more space on the proximal side of the globin, enabling a solvent water molecule to diffuse into the pocket and compete with His93 for binding to the haem (Yang & Phillips, 1996; Sage *et al.*, 1991; Barrick *et al.*, 1994). The covalent bond between His93 and the Fe atom is one of the key structural features preventing haem dissociation in myoglobin (Hargrove *et al.*, 1996). Dissociation of haemoglobin into $\alpha1\beta1$ and $\alpha2\beta2$ dimers at low pH (Katz *et al.*, 1973; Myshkin, 1984) also increases oxidation and thus haem dissociation (Zhang *et al.*, 1991; Benesch & Kwong, 1995).

Degradation was also observed to be slightly improved for β-chains than for α chains (Fig 3.20). This is noteworthy as β-chains, which (unlike α-chains) lack His-H bonding with the haem-6-propionate at the CD3 residue, exhibit greater haem dissociation (Hargrove *et al.*, 1994).

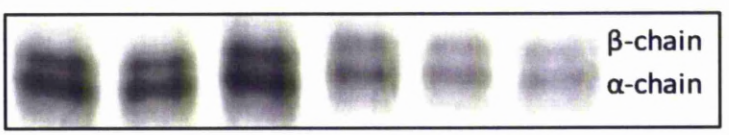


Fig 3.20. AquometHb (pH 6.0) chain digestion by InpA.

InpA displays a broad pH activity profile with a pH 8.0 optimum for synthetic chromogenic peptide substrates bearing P1 Lys or Arg residues, but an acidic pH optimum (~

pH 6) towards protein substrates azocoll, azoalbumin and azocasein (Potempa *et al.*, unpublished data). InpA efficiently degrades aquometHb but is relatively ineffective against hydroxymetHb. As these two metHb forms are structurally indistinguishable, it could be argued that the difference in susceptibility is due to an increase in enzyme activity at the lower pH at which aquometHb forms. However, if haemoglobin breakdown was simply related to the pH-dependent activity of the enzyme, then the extent of proteolysis of azide-liganded aquomethaemoglobin would be commensurate with that observed for the non-ligated substrate at pH 6, but this was not evident. Thus, it may be concluded that the enhanced Hb breakdown by InpA at acid pH is a consequence of proton assisted haem dissociation and increased oxidation rates, which also weaken the haem-iron proximal histidine bond. The reason that gross breakdown of either oxyHb or hydroxymetHb does not occur is likely that proteolytic attack, in the absence of haem dissociation, results in the production of a stable haemichrome largely resistant to further breakdown by the enzyme. Interestingly, InpA incubation with azometHb displayed no evidence of peptide release when analysed with MALDI-TOF-MS. It would be expected that many of the same amino acids residues that are cleaved by InpA to result in haemoglobin oxidation and haemichrome formation, but not those that result in haem loss and gross breakdown, would still be susceptible to cleavage. It is likely that only the most readily available surface amino acid residues were still susceptible to cleavage by InpA due to the increased stability of azometHb, which resulted in the formation of peptide fragments that were too large to be detected by mass spectrometry. This further emphasises the importance of haem dissociation for InpA mediated breakdown of haemoglobin.

3.3.6. Physiological relevance of haemoglobin oxidation.

It could be argued that InpA mediated oxidation of oxyHb is an artefact of "attempted" proteolytic degradation by the protease. The high rates of auto-oxidation and haem dissociation encountered at acidic pH generated by *P. intermedia* would obviate the requirement for the bacterium to induce oxidation proteolytically and would therefore enable efficient degradation of Hb by InpA. However, like Rgp (Smalley *et al.*, 2004), InpA may serve to oxidize oxyHb at neutral to slightly alkaline pH, as found in the diseased gingival crevice and periodontal pocket (Eggert *et al.*, 1991; Bickel & Cimasoni, 1985) where the natural oxidation rate of oxyHb is at its lowest (Shikama *et al.*, 1988), and thus "prime"

haemoglobin for further breakdown by another *Prevotella*-encoded enzyme or even loosen up the haem-globin linkage for haem capture by a haem-binding protein. The genome of *P. intermedia* 17 contains three open-reading-frames which encode SpeB-related cysteine proteases, one of which is Interpain A (Mallorquí-Fernández *et al.*, 2008; Potempa *et al.*, 2005). Given the similarities of pigment formation and haem acquisition that exist between *P. gingivalis* and *P. intermedia*, it is possible that one of these cysteine proteases may be part of an enzyme system with InpA to sequentially degrade haemoglobin, much like the Rgp and Kgp gingipains (Smalley *et al.*, 2007; 2008).

In conclusion, these studies implicate InpA as one of the key enzymes involved in haemoglobin degradation, and may therefore play a crucial role in pigmentation. This is the first description of a protease of *P. intermedia* involved in haemoglobin oxidation, breakdown and subsequent haem release. In addition, it has been demonstrated that changing conditions in the periodontal pocket, such as pH, may have a profound influence of the effectiveness of BPA virulence factors in haem acquisition from haemoglobin.

CHAPTER 4

Further characterisation of InpA-oxyHb interactions

4.1. INTRODUCTION

In the previous chapter the interaction between InpA and Hb was discussed. It was revealed that InpA proteolysis of oxyHb resulted in the formation of metHb, a phenomenon previously described for *P. gingivalis* Rgp (Smalley *et al.*, 2002; 2004). It was determined that InpA mediated metHb formation was largely a proteolytic process and MS analysis revealed key residues susceptible to InpA cleavage which provided an insight into the possible oxidation process. However, the irreversible protease inhibitor E-64 failed to completely abolish InpA-mediated oxidation, suggesting that InpA-Hb binding may be sufficient to instigate oxidation. The following chapter will further characterise the interaction between oxyHb and InpA, with particular emphasis on the mechanism of InpA-Hb binding and how this impacts on oxidation. These experiments involved an InpA variant in which the catalytic active centre, Cys154, had been mutated with alanine (C154A), termed InpA C154A.

InpA C154A is representative of the zymogenic form of InpA which differs from the mature processed enzyme in that it possesses an N-terminal pro-domain (Ala39-Asn111) (Mallorquí-Fernández *et al.*, 2007). This pro-domain is removed during auto-catalytic maturation and activation of InpA. InpA C154A retains the pro-domain as it lacks catalytic activity. In discarding the pro-domain during maturation, the catalytic His305 undergoes major spatial reorientation so as to produce a functional thiolate-imidazolium ion pair with Cys154 within the active site of the catalytic domain (Mallorquí-Fernández *et al.*, 2007). Other than this rearrangement, the catalytic domain of both the mature enzyme and the zymogen are highly structurally related (239 out of 248 common C_α atoms show a root-mean-square deviation [*rmsd*] of 0.82Å; Fig 4.1) (Mallorquí-Fernández *et al.*, 2007). Furthermore, the zymogen pro-domain does not block access to the substrate-binding cleft (Mallorquí-Fernández *et al.*, 2007). As such, it was anticipated that InpA C151A would interact with oxyHb in much the same way as mature InpA. So as to obviate complications relating to proteolysis by the active enzyme, InpA C154A was used to further elucidate interactions between oxyHb and InpA.

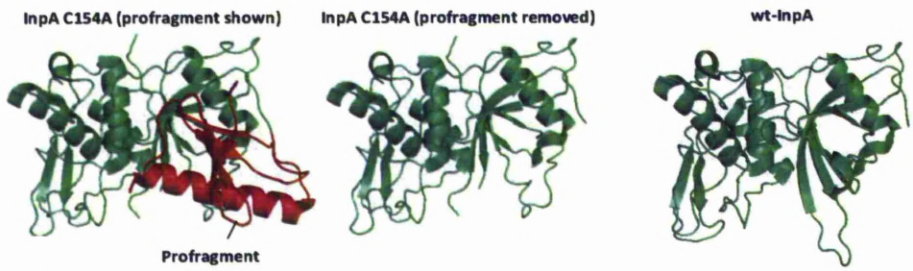


Fig 4.1. Similarities between InpA C154A- (PDB access code 3BB7) and wild type (wt) InpA (3BBA) structures. Images created using Pymol.

4.2 METHODS

4.2.1. Incubation of InpA C154A with oxyHb.

Incubation of InpA and InpA C154A with oxyHb, were carried out at 37 °C at 1:2 enzyme : substrate ratio in pH 7.5 buffer. Incubations were monitored spectroscopically as described in the previous chapter.

4.2.2. Circular dichroism (CD).

CD was performed to monitor interactions between oxyHb and InpA/InpA C154A and identify changes in Hb globin structure. InpA and InpA C154A (at 0.5 mg ml^{-1}) were extensively dialysed against H_2O and then lyophilized to remove Tris, which has a high absorbance in the far UV. The proteins were then reconstituted in dH_2O to a concentration of 2 mg ml^{-1} (74 μM). To obviate oxidation which would occur as a result of the lengthy dialysis and lyophilisation, stock oxyHb at 64 mg ml^{-1} (1 mM) in 1 mM Tris (pH 7.5) was diluted to 2 mg ml^{-1} (31 μM) to reduce the concentration of Tris to 31 μM (which had minimal UV absorbance at this concentration). For near-UV CD, spectra were recorded for oxyHb (1.25 μM) with 0.7 and 1.5 μM InpA and 0.5 and 1 μM InpA C154A. Far UV CD was performed on 62.5 μM oxyHb incubated with 74 and 50 μM InpA and InpA C154A respectively over 24 h. CD was kindly performed by Dr Ed Yates, School of Biological Sciences, University of Liverpool.

4.2.3. Gel Filtration.

To investigate Hb-InpA binding, samples for gel filtration were applied to a high performance Superdex 75 10/300 GL column equilibrated with 20 mM HEPES, 150 mM NaCl, pH adjusted to 7.5 with HCl. Sample volumes of 100 μl (4 μm InpA/InpA

C154A/oxyHb) were injected onto the column and eluted at a flow rate of 0.5 ml/min. Hb elution was followed by $A_{280\ nm}$ and $A_{410\ nm}$.

4.3. RESULTS AND DISCUSSION

4.3.1. Incubation of InpA C154A mutant with Hb results in oxidation.

The interaction between InpA C154A (2 μM) and oxyHb (4 μM) was first characterised using UV-visible spectroscopy. Confusingly, despite possessing no proteolytic activity, incubation with InpA C154A evoked spectral changes characteristic of oxyHb oxidation (Soret λ_{max} blue shift from 412 to 405 nm, increase in $A_{500\ nm}$ and $A_{631\ nm}$, and decrease in $A_{577\ nm}$ and $A_{541\ nm}$ Q bands (Fig 4.2A), similar to those for wt-InpA inhibited by E-64 (Chapter 3). Isosbestic points at 475, 525 and 592 nm indicated direct transformation of oxyHb into hydroxymetHb. After 7 h, InpA C154A had oxidized 40% of Hb, compared to 64% for wt-InpA and 24 % for control oxyHb. Assessment of $\Delta A_{577\ nm}$ versus time (see Methods) confirmed a greater rate of metHb formation by InpA C154A compared to control (Fig 4.2B). Importantly, InpA C154A displays no catalytic activity toward Hb as judged by absence of any Soret band intensity loss (Fig 4.2A). Furthermore, treatment with E-64 had no effect on InpA C154A-mediated metHb formation (data not shown) which confirmed that oxidation was unrelated to proteolysis.

After 24 h, addition of 10 mM $Na_2S_2O_4$ to InpA C154A-Hb revealed deoxyHb (429 nm Soret and 555 nm Q band) which is evidence for metHb formation (Fig 4.3A). This was in contrast to InpA-treated samples which resulted in haemochrome (424 nm Soret and Q bands at 530 and 558 nm) (Fig 4.3B). This confirmed that haemichrome formation by InpA was a proteolytic process (Chapter 3, section 3.3.3).

It is not clear how InpA C154A facilitates oxidation of Hb in the absence of proteolytic activity. Binding of oxyHb by InpA C154 is likely to be analogous to that by the mature enzyme considering that they possess virtually identical catalytic domains (Mallorquí-Fernández *et al.*, 2007). These findings may therefore be indicative of the transient nature of any InpA-oxyHb complex. Thus, initial complex formation between InpA C154A/wt InpA and oxyHb may induce conformational changes that disrupt the haem pocket, thus provoking oxyHb oxidation, and which may be essential to expose susceptible amino acid residues for cleavage by mature InpA.

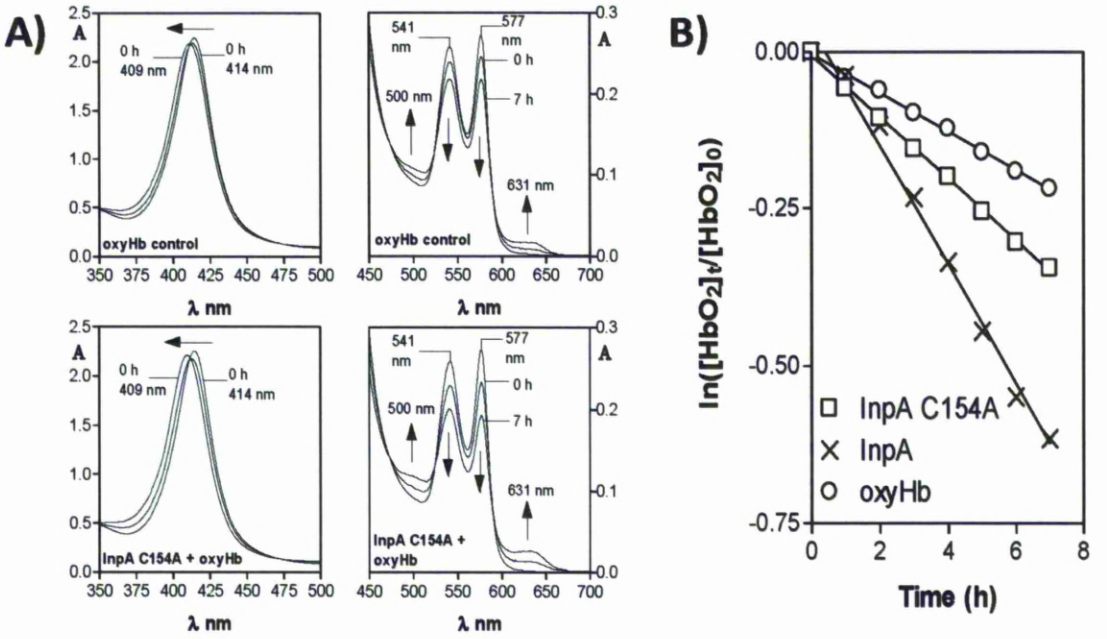


Fig 4.2. OxyHb oxidation by InpA C154A. Oxidation was assessed by ΔA_{577}nm (B).

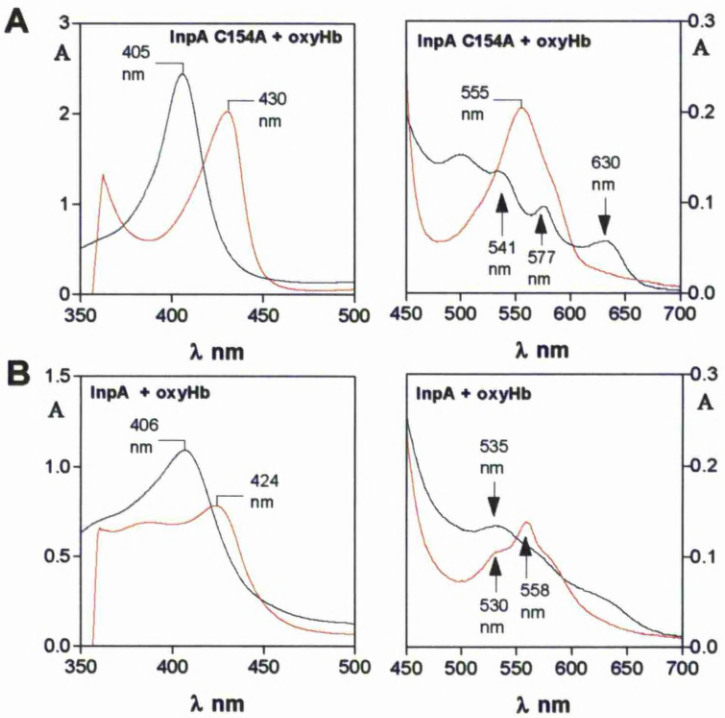


Fig 4.3. Spectrum of oxyHb after 24 h incubation with InpA and InpA C154A.

Red lines show the effect of adding $Na_2S_2O_4$.

4.3.2. Preliminary investigations to confirm InpA C154A-oxyHb interactions.

SDS- and native-PAGE resulted in separation of both InpA C154A and wt-InpA from oxyHb (data not shown). This suggested that even the mild separation conditions of native-PAGE failed to preserve the weak protein interactions. As a less disruptive technique, gel filtration was performed by using absorbance at 410 nm to monitor oxyHb elution. Although InpA had little effect on the oxyHb elution position (K_{av} = 0.145; Fig 4.4A), InpA C154A slightly increased the 410 nm peak elution point (K_{av} = 0.153; Fig 4.4B), suggesting elution of dissociated Hb αβ-dimers in association with InpA C154A, a complex with a predicted size of ~60 kDa, and thus similar elution point as tetrameric oxyHb. In this regard it is noteworthy that dissociated αβ-dimers oxidise at a greater rate than the tetramer (Zhang *et al.*, 1991). However, the ability of gel filtration to reliably differentiate between two molecules of such similar mass is doubtful. It should also be observed that the reduced absorbance of InpA C154A-treated oxyHb is an artefact resulting from a lower sample loading and is not related to the effect of InpA C154A.

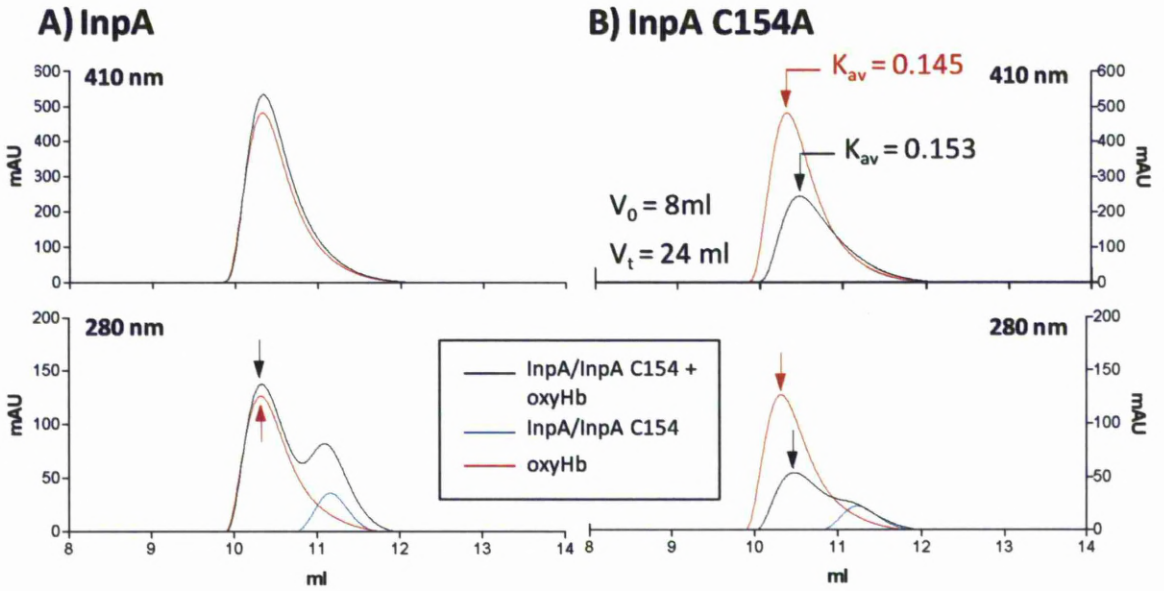


Fig 4.4. Analytical gel filtration chromatography of oxyHb incubated with InpA and InpA C154A. Note that the reduced absorbance of InpA C154A treated oxyHb (B) is a result of lower sample loading onto the column.

As a more sensitive technique, circular dichroism (CD) was used to investigate InpA C154A-oxyHb complex formation. CD is a spectroscopic method which measures the differential absorbance of left and right circularly polarized light by optically active, chiral molecules capable of providing information regarding protein secondary structure (Far-UV, 180-250 nm) and changes affecting aromatic amino acid side chains (such as Trp, Phe and Tyr), disulfide bonds and haem groups (Near-UV >250 nm).

Interactions of InpA with oxyHb (1.25 µM) were manifest in the spectra as an extended peak at ~295 nm and diminished intensity of the 260 nm region (Fig 4.5A). Diminishing intensity of the broad 260 nm region is indicative of change in the haem environment (Ruckpaul *et al.*, 1970; Perutz *et al.*, 1974), which in this case would likely be a consequence of iron oxidation (Li & Johnson., 1969). The peak at 295 nm is unrelated to oxidation of the haem iron since only very minor differences occur between met and oxyHb in this region (Simon & Cantor, 1969). Instead it is more likely that the peak reflects changes in the chiral environment of aromatic side chains either directly as a consequence of protein interactions, or as a result of binding-induced quaternary structure transitions of Hb which are also manifest in the 270-300 nm region (Perutz *et al.*, 1974; Jin *et al.*, 2004), and may account for non-proteolytic Hb oxidation. Changes in absorbance intensity were proportionate to the concentration of wt cd-InpA, with an isosbestic point at ~280 nm (Fig 4.5A). This unambiguously confirmed an interaction between the proteins. The spectra also suggested an interaction between InpA C154A and oxyHb, but the lack of isosbestic points with increasing InpA-C154A concentration suggested a more complex interaction (Fig 4.5B).

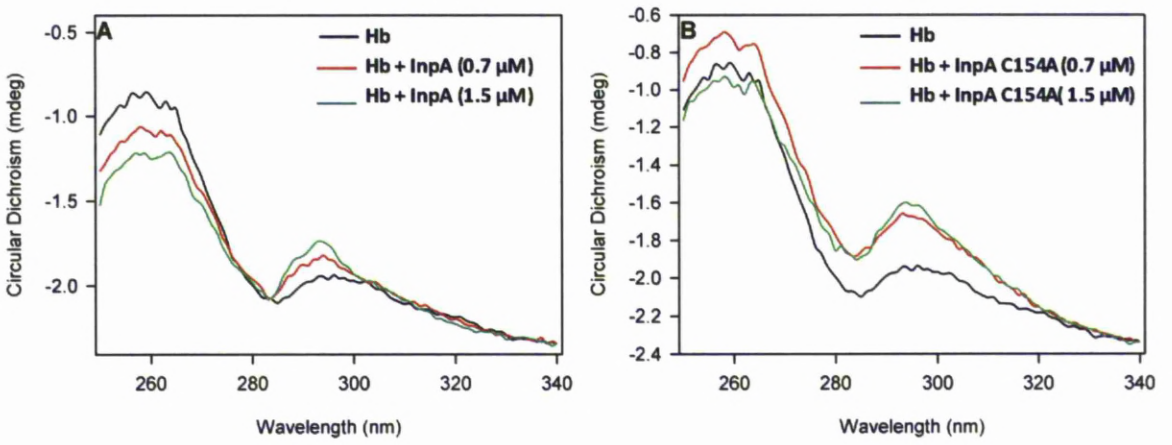


Fig 4.5. Near-UV CD spectra of interaction oxyHb with InpA and InpA C154A.

In the far UV region, interaction of oxyHb with InpA had a limited effect on the strongly alpha helical secondary structure of oxyHb (i.e. negative bands near 222 and 208 nm and intense positive band near 190 nm [Holzwarth & Doty, 1965]) (Fig 4.6). However, InpA C154A resulted in a notable reduction in α-helix content (reduced positive 190 nm band, and negative 222 and 208 nm bands) after 24 h (Fig 4.6). It seems paradoxical that InpA, which proteolytically induces metHb formation (Byrne *et al.*, 2010; Chapter 3), has little or no influence on secondary structure compared to InpA C154A. The reason why InpA fails to elicit such an obvious change in secondary structure may reside in its retained catalytic activity. Steric pressure, resulting in changes in secondary structure, may be exerted upon binding of Hb by InpA/InpA C154A, but then released following proteolysis. As such, although the tertiary structure of Hb will become increasingly disorganised (accounting for oxidation) the secondary structure will likely remain unaffected by proteolysis. In contrast, binding by non-proteolytic InpA C154A may induce sustained steric pressure. These changes in secondary structure may also explain InpA C154A induced oxidation of Hb.

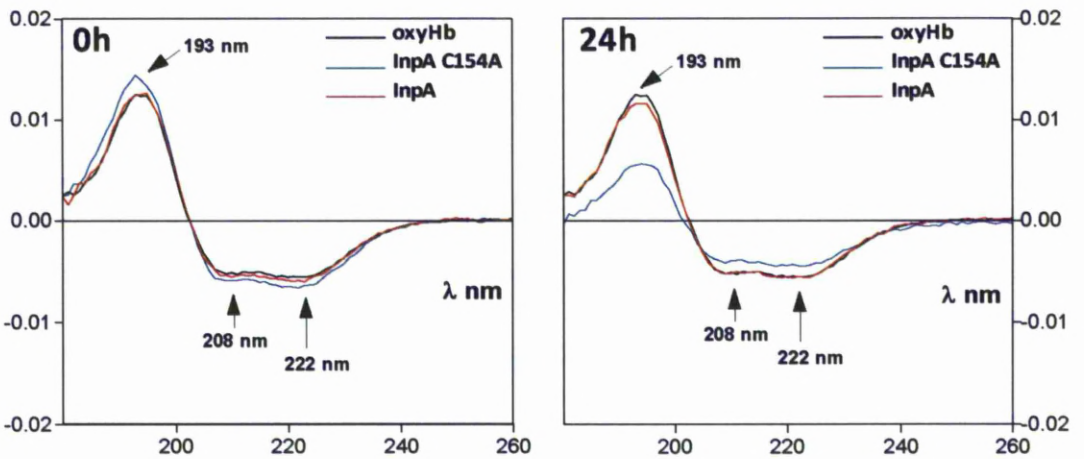


Fig 4.6. Far-UV CD spectra of InpA/InpA C154A incubation with oxyHb.

4.3.3. Preparation of fully processed InpA C154A for X-ray crystallographic investigations.

X-ray crystallography was used to elucidate the nature of the InpA-Hb complex. However, proteolysis by InpA would decrease sample homogeneity and purity by producing peptide fragments, and thus compromise X-ray diffraction experiments. As such InpA C154A, which was also shown to interact with Hb by CD (Fig 4.5), was better suited to

produce pure crystals of the protein complex whilst avoiding the complication of protein fragmentation.

InpA C154A is catalytically inert and as such is incapable of undergoing auto-processing. To examine the interaction of InpA with Hb more precisely, a fully processed mutant variant was created. Treatment of InpA C154A with catalytic InpA results in mature mutant proteins (Mallorquí-Fernández *et al.*, 2007). Unfortunately, InpA and mature InpA C154A share identical sizes and ionic potentials, and are virtually impossible to separate by conventional techniques. As each recombinant protein is modified with a C-terminal hexahistadine-tag (6xHis), removal of the tag from InpA C154A enabled separation from wt-InpA using a Fast Flow Ni-NTA Sepharose resin column.

InpA C154A was thus incubated with Carboxypeptidase A (CPA) (Sigma Chemicals, C9268) at a 100:1 ratio for 1 h at 25 °C to detach the His-tag. CPA was inactivated by treatment with 100 μM EDTA for 1 h at 25 °C and removed by ion exchange chromatography with a MonoQ column (Amersham Biosciences) equilibrated with 20 mM Tris-HCl, pH 7.5. After dialysis, His-tag-free InpA C154A was processed by InpA (10:1 substrate: enzyme ratio) for 24 h at 37 °C. SDS-PAGE confirmed the presence of processed InpA C154A (Fig 4.7A, lane 3). Both InpA variants where then separated by affinity chromatography. The Fast Flow Ni-NTA column and the sample were first equilibrated with binding buffer containing 20 mM imidazole to prevent non-specific binding of InpA C154A. The sample was then introduced to the column and the flow-through fraction, containing non bound InpA C154A (Fig 4.7B, lane 6), was collected. The column was washed with binding buffer and bound InpA was eluted using binding buffer supplemented with 500 mM imidazole (Fig 4.7B, lane 8). Both fractions were extensively dialysed and then analysed by SDS-PAGE (Fig 4.7B). Finally, to assure separation of the two InpA variants, all fractions were tested for protease activity using the fluorogenic substrate L-BAPNA (Sigma Chemicals; B3133). Crucially, proteolytic activity was only identified for the active InpA containing eluted fraction (Fig 4.7C). Fully processed InpA C154A was thus used in trials to co-crystalise InpA-C154A and Hb as a complex.

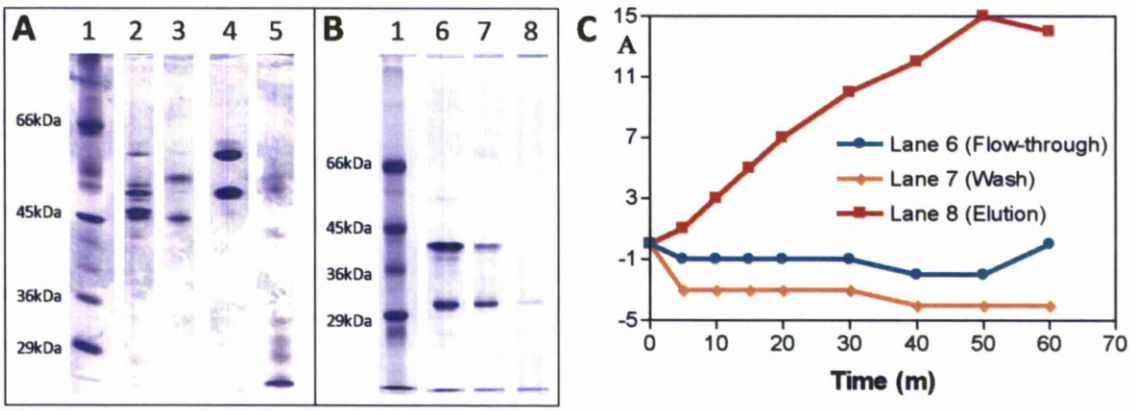


Fig 4.7. Analysis of InpA C154A processing by InpA (A), separation of the proteins by affinity chromatography (B), and identification of protease activity against L-BAPNA in the separated fractions (C). Protein standards (lanes 1); InpA mediated processing of InpA C154A after 1 h (lane 2) and 24 h (lane 3); InpA C154A (lane 4); InpA (lane 5); Fast Flow Ni-NTA column flow through (lane 6), secondary wash (lane 7) and elution (lane 8). In graph (C) absorbances have been normalised to zero and background corrected.

4.3.4. Crystallization trials for the InpA C154A-Hb complex.

Unfortunately due to a shortage of time, screening of potential crystallisation conditions for the InpA C154A-Hb complex was only examined using the PACT Premier kit (Molecular Dimensions Ltd, UK) to systematically test the effects of pH, anions and cations using PEG as the precipitant (See Section 2.13). This was selected because InpA crystallization had previously been achieved in PEG-containing buffer (Mallorquí-Fernández *et al.*, 2007). Droplets containing InpA C154A and oxyHb at a 1:1 molar ratio (~ 160 μM) in relation to the Hb dimer were assayed. Three crystal types with distinct morphologies (hexagonal, orthorhombic and needle) formed under the majority of test conditions, some of which appeared after just a few hours (Fig. 4.8). Predictably, all of crystals were red-brown in colour, which was an early indication that they were composed of Hb. A representative of each of the three primary crystal types was therefore frozen at 100 K to perform X-ray diffraction experiments. Conditions under which the harvested crystals were formed are presented in Table 4.1.

Table 4.1. Crystallization conditions.

Crystal type	Buffer Salt	pH	Precipitant
Hexagonal	0.2 M lithium chloride + 0.1 M MES	6	20 % w/v PEG 6000
Orthorhombic	0.2 M sodium citrate	-	20 % w/v PEG 3350
Needle	0.2 M potassium thiocyanite + 0.1 M Bis Tris propane	8.5	20 % w/v PEG 3350

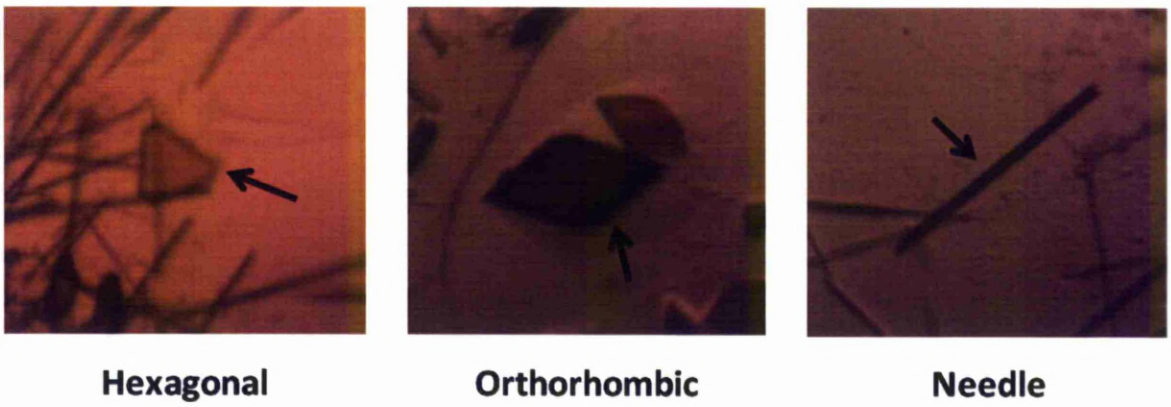


Fig 4.8. Images of crystal morphologies.

The three crystal types yielded relatively strong diffraction data and data processing revealed that each belonged to a different space group (P 3_1, C 121 & P 2_1 2_1 2_1 [Table 4.2A]). The C 121 and P 2_1 2_1 2_1 crystals were clearly composed of haemoglobin as they each belonged to related space groups and shared similar unit cell dimensions to solved haemoglobin structures in the Protein Data Bank (PDB) (Table 4.2B, highlighted). However, no horse Hb coordinates with matching space groups were found for the P 3_1 crystal (Table 4.2B). Therefore, in order to determine the composition of the P 3_1 crystal, the structure was solved by molecular replacement using the coordinates of horse haemoglobin (PDB access code 1Y8H [Table 4.2B, highlighted]). As a comparison, structures of the C 121 & P 2_1 2_1 2_1 were also solved using the coordinates of horse haemoglobin (PDB access codes 2ZLU & 1Y81, respectively). Detailed statistics on data collection and processing and the partially refined structure solutions are presented in Table 4.2A.

Table 4.2A. Crystallographic data collection and partial refinement.

Crystal type	Hexagonal	Orthorhombic	Needle
Space Group	**$P\,3_1$**	**C 121**	**$P\,2_1\,2_1\,2_1$**
Resolution range (Å) (OuterShell)	66.5-2.7 (2.9-2.7)	41.0-1.6 (1.7-1.6)	47.7-2.3 (2.4-2.3)
Completeness (%) (OuterShell)	91.61 (86.5)	99.3 (99.7)	88.8 (78.8)
R_{merge} (OuterShell)	0.067(0.127)	0.093(0.455)	0.128 (0.706)
$(I)I\sigma I$ last shell	6.4	2.2	1.8
Total no. of reflections measured	4720	21473	9259
Unique reflections	2016	6893	3222
Wilson *B*- factor (Å^2)	20.8	24.68	33.026
Unit cell (Å, °)			
a, Å	**62.8**	**107**	**62.8**
b, Å	**62.8**	**63**	**81.4**
c, Å	**132.9**	**54.6**	**117.9**
α, °	**90**	**90**	**90**
β, °	**90**	**90**	**90**
γ, °	**120**	**111.2**	**90**
Solvent (%)	48.53	54.54	48.16
No. of subunits	4	2	4
R factor	0.212	0.224	0.230
R_{free}	0.259	0.258	0.319
ESU (Å) based on *R* value	0.119	0.094	0.749

Table 4.2B. Details of horse Hb PDB files from the Protein Data Bank. Files used to solve crystal structures are highlighted.

Access codes	1Y8H	2ZLU	1Y8I	2ZLV	2ZLW	2ZLX	2D5X	1NS6	2DHB
Space Group	$P\,2_1\,2_1\,2_1$	C 2	$P\,2_1\,2_1\,2_1$	C2	C2	C2	$C222_1$	C2	$C222_1$
Unit cell (Å, °)									
a, Å	63	108	63	94	108	108	62	108	77
b, Å	83	63	81	63	63	63	107	63	82
c, Å	112	54	112	53	108	103	87	54	93
α, °	90	90	90	90	90	90	90	90	90
β, °	90	90	90	115	111	121	90	85	90
γ, °	90	111	90	90	90	90	90	90	90

The partially refined structure solutions and resultant electron density maps clearly revealed the crystals were composed of Hb (Fig 4.9). Importantly, it was evident that there were no large clusters of unexplained electron density to suggest co-crystallization with InpA. Furthermore, analysis of packing between symmetry-related Hb molecules revealed

that there were no unoccupied spaces large enough for InpA to theoretically occupy (Fig 4.10). In support of this, calculation of the Mathews coefficient suggested one molecule of Hb per unit cell (one Hb-dimer in the case of C121). As such, further refinement to improve the electron density map was not required.

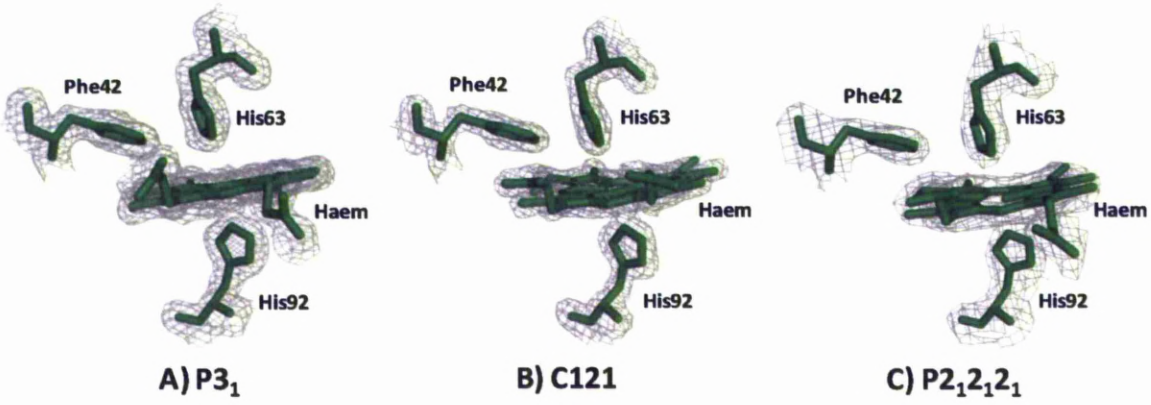


Fig 4.9. Electron density maps produced from the crystals.

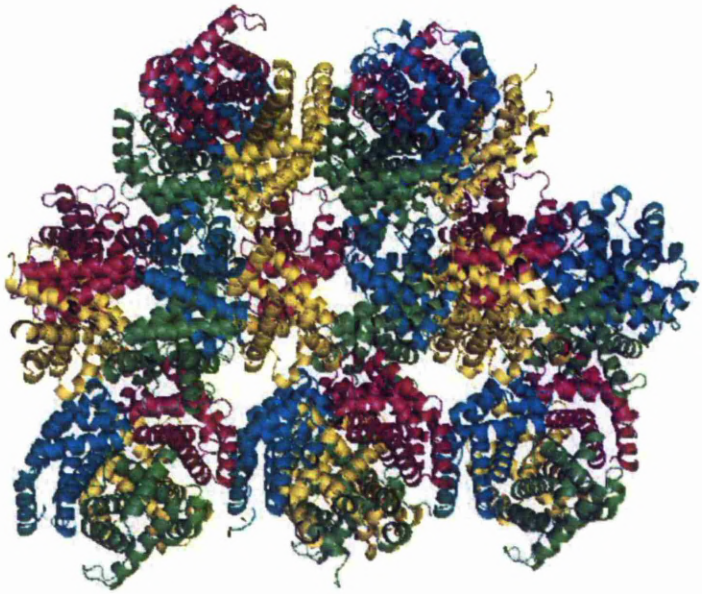


Fig 4.10. Packing of Symmetry-related Hb molecules (P3$_1$ crystal).

This unambiguously revealed that the screening conditions examined were inappropriate to generate crystals containing both InpA C154A and oxyHb. This may be accounted for by the weak nature of the complex and by the rapidity with which Hb crystals form. It is therefore clear that conditions under which the intermediary enzyme-substrate

complex is stable first need to be identified before its structure can be elucidated. However, it must be emphasised that, as judged by the data, the complex likely only forms transiently and will therefore be extremely difficult to capture in a crystal. This would constitute a significant area for further work. In summary, although this study failed to precisely define the InpA-oxyHb complex, the data collected were convincing enough to suggest that the interaction may be dependent on both proteolytic and non-proteolytic processes.

CHAPTER 5

Interactions between InpA, Rgp and Kgp in haemoglobin degradation

5.1. INTRODUCTION

In Chapter 3 evidence that haem acquisition by BPAs may be directly related to conditions prevailing in the periodontal pocket began to emerge, as InpA digestion of Hb was most effective at acid pH. Within the periodontal pocket environment, *P. gingivalis* co-aggregates with *P. intermedia* as part of a complex biofilm community (Kamaguchi *et al.*, 2003). Co-aggregation promotes the generation of complex nutrient webs connecting species with distinct metabolic capabilities. For example, streptococci produce fermentable carbohydrates, which are used by a broad range of other bacteria, by enzymic cleavage of host glycoprotein glycan chains (Byers *et al.*, 1999), and succinate production by *T. denticola* can support growth of *P. gingivalis* in co-culture (Grenier & Maryrand, 1986). In addition, isobutyric acid excreted by *P. gingivalis* can stimulate growth of *T. denticola* (Grenier, 1992). Cell extracts of both *F. nucleatum* and *T. forsythia* can also stimulate growth of *P. gingivalis* (Yoneda *et al.*, 2001). The biosynthetic activities of biofilm organisms also provide vitamin K for *P. gingivalis*, N-acetylmuramic acid for *T. forsythia*, and volatile fatty acids for *T. denticola* (Nishihara & Koseki, 2004). Relatively non-proteolytic organisms may also benefit from the potent hydrolytic activities of organism such as *P. gingivalis* to provide essential amino acids and peptides (Darenfed *et al.*, 1999).

Synergistic interactions also exist. For example, the non- or weakly-proteolytic *F. nucleatum* subsp. *nucleatum* ATC25586 binds plasminogen which is converted by *P. gingivalis* proteases and streptokinase during co-aggregation (Darenfed *et al.*, 1999), to form the active serine protease plasmin which may contribute to periodontal-tissue destruction by enabling host defence evasion and tissue invasion by *F. nucleatum*. This represents a unique form of synergy essential for *F. nucleatum* lesion formation in mice murine lesion models (Feuille *et al.*, 1996). Bacterial co-aggregation also supports the synergistic breakdown of host proteins and glycoproteins for the provision of essential nutrients. Examples of this are transferrin and bovine serum albumin degradation by S. *mutans* and other dental plaque bacteria (Homer & Beighton, 1992), casein degradation by *P. gingivalis* and *F. nucleatum* (Gharbia *et al.*, 1989), and human glycogen-associated protein (HGM) degradation by *Streptococcus oralis* and *Streptococcus sanguis* (van der Hoeven & Camp, 1991). Alone *Actinomyces naeslundii* and *S. oralis* are poor colonisers of

saliva-coated glass flowcells, but through their combined metabolic activities they produce extensive biofilms (Palmer *et al.*, 2001). Synergistic catabolism of glycoproteins (such as mucin) by several species of oral bacteria with overlapping patterns of enzyme activities (e.g. sialidase, alpha-fucosidase and endopeptidase) has also been demonstrated (Bradshaw *et al.*, 1994).

Specific interactions between BPAs in the periodontal pocket are thus also environmental considerations which must be recognised when examining haem acquisition. A degree of mutualism between *P.gingivalis* and *P. intermedia* has already been claimed to exist. For example, binding of IgG by *P. intermedia* cell-surface Fc receptor may protect *P. gingivalis* from IgG (Labbe & Grenier, 1995). Also, degradation of complement factor C3 by InpA may also advantage both organisms by inhibiting complement activation and hence the defensive sequelae associated with the inflammatory response (Potempa *et al.*, 2009). Fermentation of glutamic and aspartic acids by *P. intermedia* and *F. nucleatum* results in the generation of ammonia and a more neutral pH which benefits acid-sensitive species including *P. gingivalis* (Takahashi, 2003). It is therefore also conceivable that some cross-reactivity between the haem acquisition mechanisms of both species may exist which may also mutually benefit other periodontopathogens by providing free haem. For example, the stronger haemolytic activity of *P. intermedia* may benefit *P. gingivalis* growth by augmenting the levels of haemoglobin (Dashpand & Khan, 1999; Okamoto *et al.*, 1999).

In the previous chapters, a specific role for InpA in haemoglobin breakdown was revealed, in which the protease, like R-gingipain, crucially oxidised oxyHb as an initial step in its degradation and haem release (Byrne *et al.*, 2010; Smalley *et al.*, 2005; 2007). Kgp also achieves haem release by proteolysis of metHb (Smalley *et al.*, 2007; 2008).Considering their shared habitats and the numerous physiological, nutritional and metabolic characteristics, it is possible that *P. gingivalis* and *P. intermedia* may have developed co-operative mechanisms for haem acquisition. As such, collaborative haem acquisition from metHb and oxyHb by these enzymes was evaluated.

5.2. METHODS

5.2.1. Haemoglobin breakdown by interpain and gingipains.

Stock solutions of bacterial proteins were as follows: InpA (2 μM); HRgpA (0.2 or 1 μM); Kgp (0.6 or 1 μM). OxyHb and metHb (induced with $NaNO_2$) were used at 4 μM. Incubations were carried out at 37 °C in Tris-NaCl, pH 7.5 (pH 7.5) or 0.2 M PBS (pH 6.0) and for PAGE, InpA, Kgp, and HRgpA activities were inhibited respectively with 0.5 mM E-64, 0.2 mM T.L.C.K and 0.2 mM leupeptin (30 min at room temperature), before samples were solubilised at 37 °C for 1 h in non-reducing LSB.

5.3. RESULTS AND DISCUSSION

5.3.1. Investigating synergy in oxyHb breakdown between Kgp and InpA.

In the case of *P. gingivalis*, haem is released by Kgp from metHb formed by the action of Rgp. It is likely therefore that InpA-induced metHb is a susceptible substrate for Kgp. This co-operative action is not without precedent as synergystic iron acquisition from Hb has been shown for *E. coli* (which produces a haemoglobin protease) and *Bacteroides fragilis* during abscess formation (Otto *et al.*, 2002). In order to examine this possibility, Kgp (0.6 μM) and InpA (2 μM) were individually- and co-incubated with oxyHb; incubations being carried out at pH 7.5 to partly mimic conditions of the periodontal pocket but, more importantly, to form metHb *via* InpA activity while avoiding its gross breakdown (which would occur at lower pH [see chapter 3]).

SDS-PAGE and densitometry revealed ~60 % degradation of α- and β-globin chains when oxyHb was co-incubated with Kgp plus InpA after 24 h (Fig 5.1A), compared to the effect of either Kgp (46 %) or InpA (23 %) alone. The sum of total haemoglobin breakdown by the collective activity of both enzymes was therefore less than an additive effect. Furthermore, after 48 h individual Kgp digestion had surpassed that of the co-incubation. TMB-H_2O_2 staining also did not indicate co-operative haem release by InpA and Kgp (Fig 5.1B).

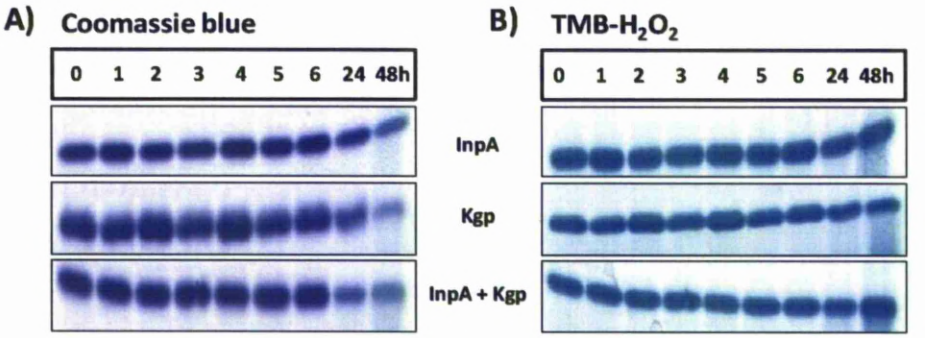


Fig 5.1. OxyHb incubation with combinations of InpA and Kgp at pH 7.5.

The inability of both Kgp and InpA (during co-incubation) to effect oxyHb breakdown is puzzling given the propensity of Kgp for metHb breakdown. However, this incongruity may simply reflect the low levels of metHb production by InpA within the time frame of the experiment. Alternatively, this may result from competition for oxyHb between InpA and Kgp, both of which individually display limited activity against oxyHb at pH 7.5. Thus, Kgp would limit metHb generation by itself promoting haemichrome formation (Smalley *et al.*, 2007, 2008).

To mitigate this problem, 0.6 μM Kgp was incubated with oxyHb which had been pre-incubated for 7 h at pH 7.5 with 2 μM InpA (referred to from now on as InpA-metHb), which increased starting [metHb] in the Hb substrate (calculated as 55 % met- and 45 % oxyHb). An auto-oxidised sample of oxyHb was used as a control (comprising 20 % metHb and 80 % oxyHb). Importantly, in the InpA-treated Hb, there was little proteolysis over the pre-incubation period (assessed by Soret band intensity; data not shown). The Soret reduction following addition of Kgp (0.6 μM) indicated haem loss from both metHb preparations (Fig 5.2), which was greatest for "InpA-metHb" (Fig 5.2A), and after 24 h there was some free haem present in the digest (395 nm Soret; 365 nm shoulder). However, the uninhibited activity of InpA also resulted in Soret reduction in the InpA-metHb control after 24 h (data not shown). It is thus likely that InpA contributed to Soret decline in the InpA-metHb samples treated with Kgp.

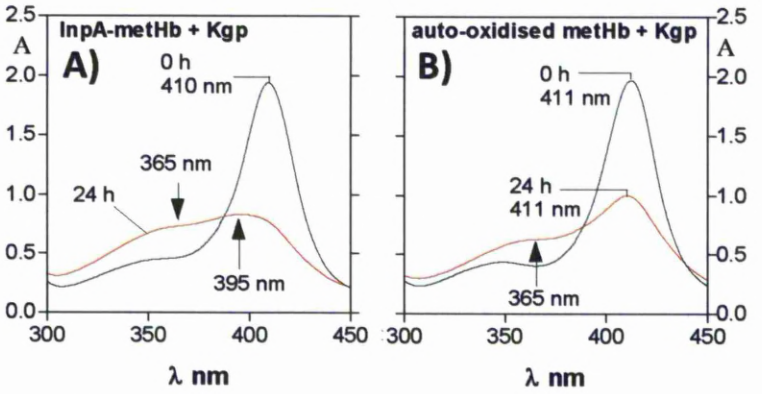


Fig 5.2. Haem release by Kgp from InpA-oxidised and auto-oxidised Hb.

The presence of free haem released by Kgp from the "InpA-metHb" was confirmed by addition of 10 mM $Na_2S_2O_4$ which yielded a 384 nm Soret (Fig 5.3A1) which agrees with the published Soret of the Fe(II) haem species (Sliver & Lukas, 1983). However, bands at 530 and 558 nm suggested that some haemichrome species was also present (Fig 5.3A2). No spectral evidence of free haem was found in the other two digests which also showed greater abundance of haemichrome (e.g. sharp 422 nm Soret and 530 and 558 nm bands [Fig 5.3B & C]). SDS-PAGE confirmed greatest breakdown for Kgp digestion of "InpA-metHb" (Fig 5.4).

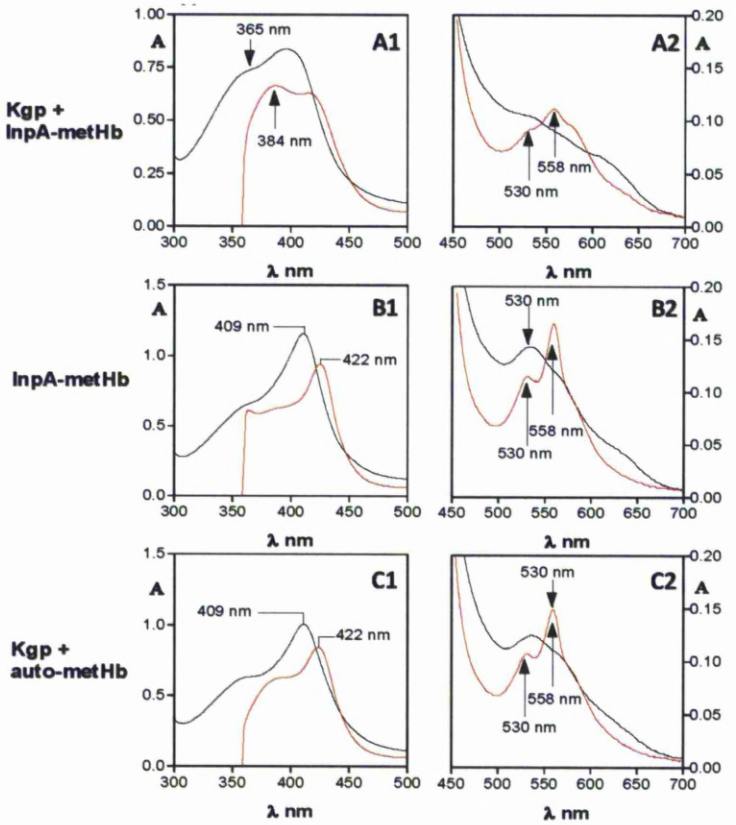


Fig 5.3. UV-visible spectra of InpA- and auto-oxidised-metHb incubated with and without Kgp for 24 h. Red line shows the effect of adding 10 mM $Na_2S_2O_4$.

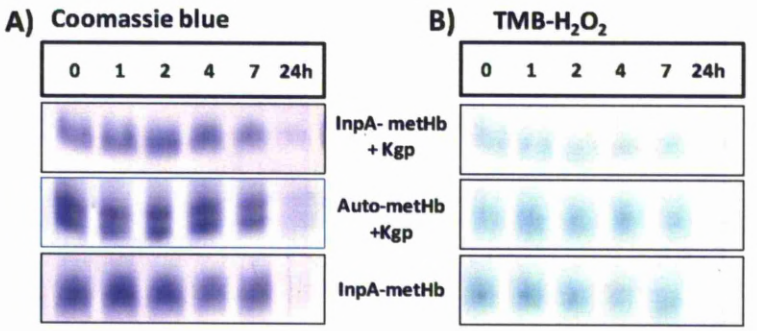


Fig 5.4 Breakdown of InpA- and auto-oxidised-metHb by Kgp.

InpA-induced metHb was also a better substrate for Kgp than that formed by $NaNO_2$, (over 7 h) as seen from the loss of Soret band absorbance (Figs 5.5A and B) despite greater concentrations of metHb in the latter (84 % compared to 60 % of total Hb, respectively). This suggested that InpA-mediated proteolysis contributed to Hb breakdown.

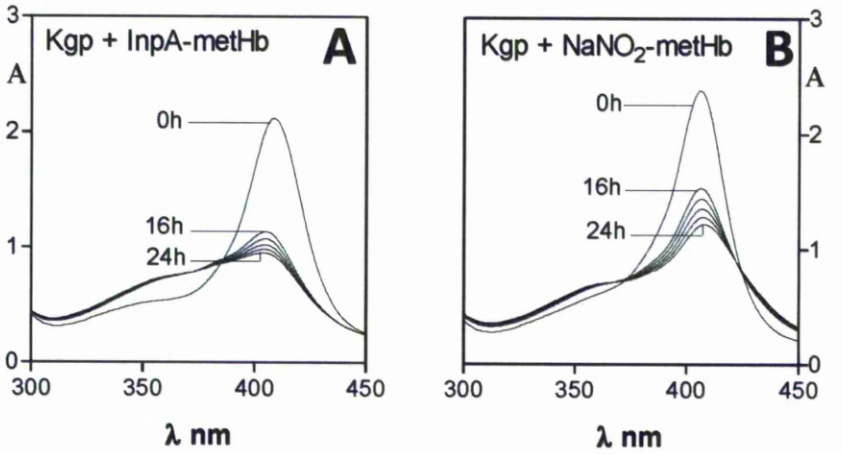


Fig 5.5. Effect of Kgp on metHb induced by either InpA (A) or $NaNO_2$ (B).

To investigate the susceptibility of InpA-metHb to Kgp, but to eliminate any additive effects of residual InpA activity, the InpA-metHb (43 % metHb) was inhibited with E-64 for 30 min prior to addition of Kgp. Crucially, E-64 has no inhibitory effect against Kgp. This incubation was compared to an auto-oxidised sample (22 % metHb) which was also treated with Kgp. Under these conditions, Kgp affected greater breakdown and haem release of auto-oxidised metHb (Fig 5.6B) than InpA-metHb (Fig 5.6A) as judged by both SDS-PAGE and UV-visible spectroscopy. This was despite greater availability of metHb in the InpA-metHb sample.

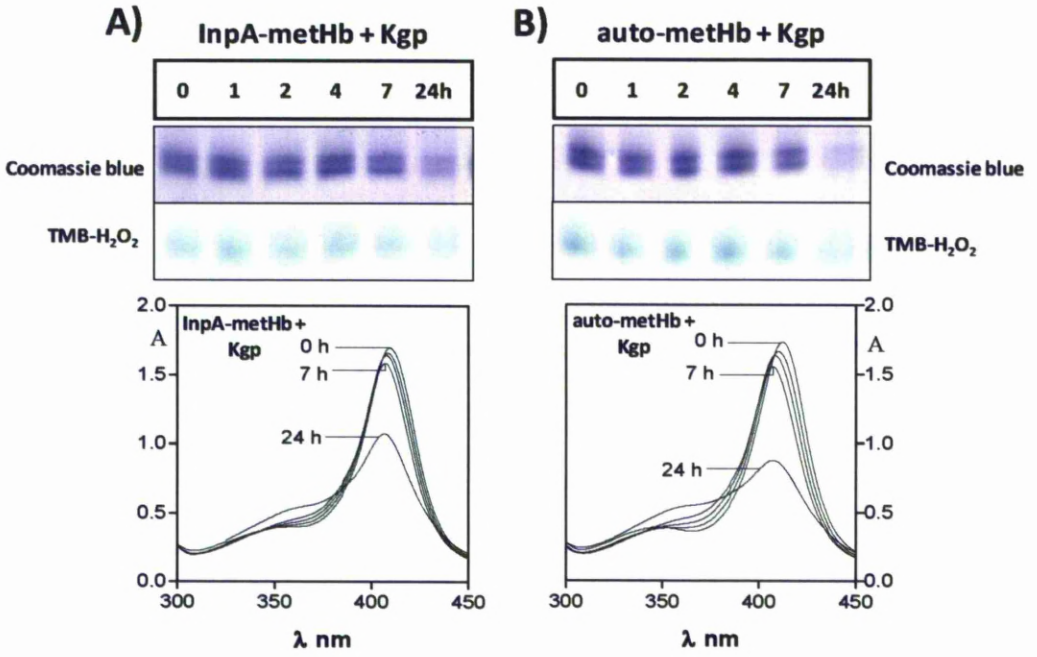


Fig 5.6. Degradation of "InpA-metHb" (A) and auto-oxidised metHb (B) by Kgp. Residual InpA activity was inhibited with E-64.

Densitometry revealed that after 24 h InpA-metHb was ~45 % less susceptible to Kgp than auto-oxidised metHb. This is paradoxical given the higher [metHb] formed by InpA and the intrinsic susceptibility of metHb to Kgp. InpA cleavage at residues around the haem pocket induces oxidation via structural changes in the α- and β-globin chains (Chapter 3; Byrne *et al.*, 2010). MetHb formed via auto-oxidation spontaneously or via proteolysis may therefore be intrinsically different. Furthermore, prolonged incubation of oxyHb with InpA (at pH 7.5) results in haemichrome formation which is resistant to further attack by InpA (Byrne *et al.*, 2010) and is also relatively stable against Kgp (Smalley *et al.*, 2004). Additionally, the modified structure of InpA-induced metHb/haemichrome may not accommodate Kgp binding or make crucial residues unavailable for cleavage, rendering InpA-metHb unsuitable for Kgp attack. This seems likely as MS analysis (Chapter 3) suggested that InpA-mediated oxyHb oxidation was a consequence of proteolytic disruption of haem pocket conformation. This could represent a unique competitive adaptation by *P. intermedia*. Several other SpeB-related cysteine proteases have been identified for *P. intermedia* (Mallorquí-Fernández *et al.*, 2008; Potempa *et al.*, 2005) and it would be of interest to determine if any of these can sequentially degrade haemoglobin with InpA in a

manner analogous to Rgp and Kgp (Smalley *et al.*, 2007; 2008). Furthermore, it has not yet been investigated whether low pH would facilitate digestion of InpA-metHb (formed at alkaline pH) by InpA since, although the diseased periodontal pocket is more likely to be slightly alkaline, *P. intermedia* may lower the local pH (Shah & Williams, 1987; Takahashi & Yamada, 2000). Haemichrome formed by InpA in the alkaline periodontal pocket may therefore become susceptible to further proteolysis at lower pH when in close proximity to *P. intermedia* cells. This, in addition to the resistance of InpA-metHb/haemichrome to Kgp, may be a way of securing haem acquisition by *P. intermedia*.

5.3.2. Investigating cooperative breakdown of metHb by Kgp and InpA.

InpA and Kgp neither sequentially nor co-operatively digest oxyHb. However, metHb is the preferred substrate of both enzymes. When Kgp (1μM) and InpA (2 μM) were co-incubated with metHb (4 μM; prepared with $NaNO_2$) at pH 7.5 and 6.0 (to emulate the optimal growing pH of *P. gingivalis* [McDermid *et al.*, 1988] and *P. intermedia* [Takahashi *et al.*, 2000] respectively) haem loss and breakdown was greater (after 24 h) than for metHb incubated with either enzyme individually (data not shown). However, it was concluded from singular enzyme incubations that InpA and Kgp contributed the majority of metHb breakdown at pH 6.0 (Fig 5.7B) and 7.5 (Fig 5.7A) respectively. This can be attributed to the respective pH optima of the two proteases. As total protein breakdown by the co-incubations of both enzymes was only equal to the sum of their parts, a synergistic relationship cannot be concluded.

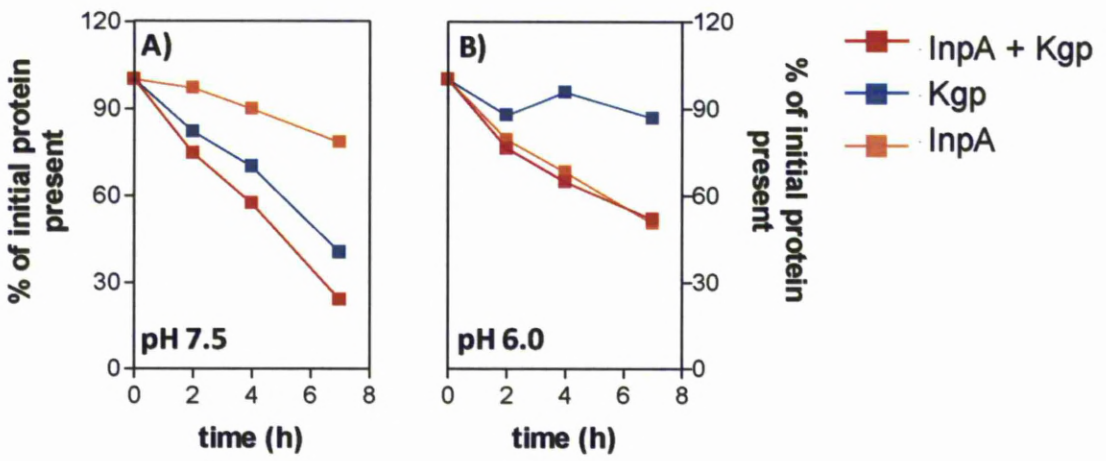


Fig 5.7. Densitometry of metHb degradation by InpA and Kgp, singly and in combination.

5.3.3. Synergy was not observed between InpA and gingipains.

Rgp promotes metHb formation from oxyHb (Smalley *et al.*, 2004; 2007; 2008) which is the prefered substrate for InpA (Chapter 3; Byrne *et al.*, 2010). Accordingly, InpA proteolysis of metHb produced by HRgpA ("HRgpA-metHb") and auto-oxidation (auto-metHb) was investigated at pH 7.5 and 6.0. Starting [HRgpA-metHb] was 54 % (at pH 7.5) and 92 % (at pH 6.0) and [auto-metHb] was 46 % (at pH 7.5) and 89 % (at pH 6.0). SDS-PAGE showed there was no synergy between HRgpA and InpA (Fig 5.8), nor was there evidence of additive proteolysis, which suggested an antagonistic effect (Fig 5.8). This can be explained either by competition between the two enzymes for substrate binding or by HRgpA induced structural modifications which render the molecule refractory to InpA. Co-incubations of the two enzymes with oxyHb revealed similar results (data not shown).

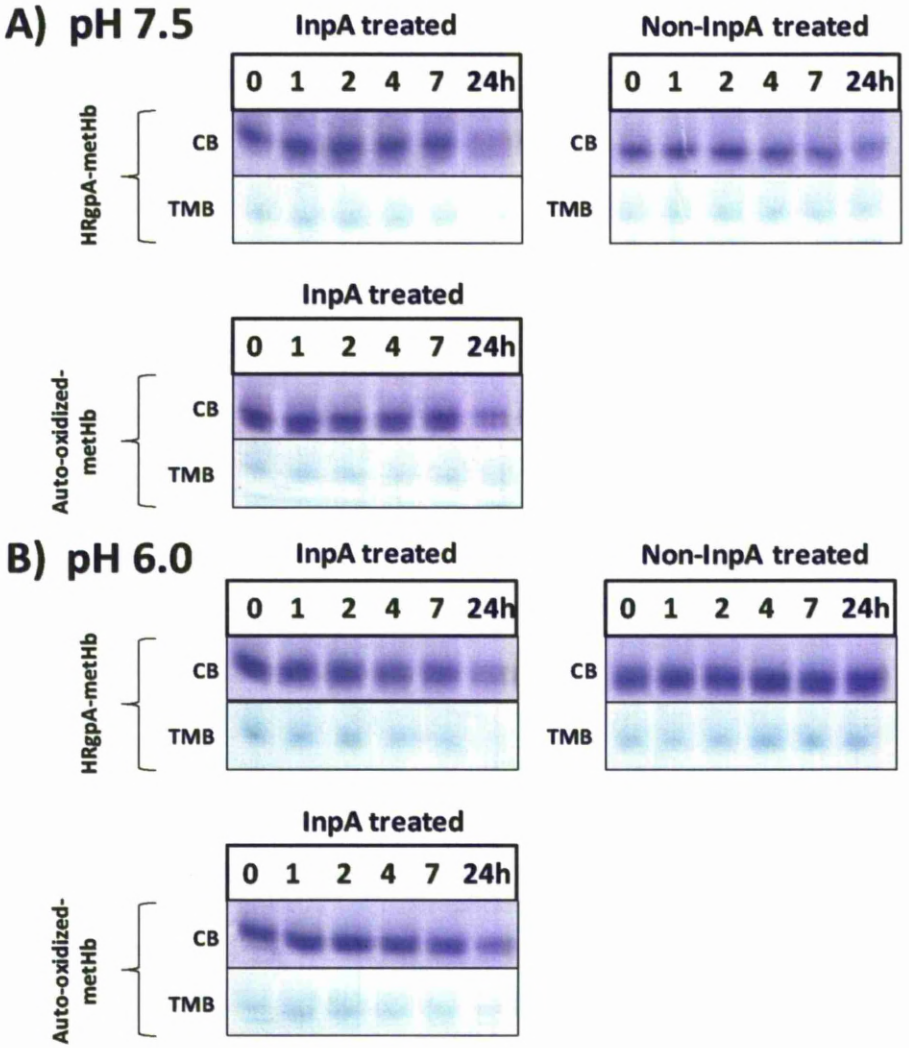


Fig 5.8 Effect of InpA on "HRgpA-metHb" and "auto-metHb".

The results presented in this chapter do not suggest that mutuality exists between InpA and either of the gingipains. Although combined proteolysis by InpA and Kgp, but not InpA and HRgpA, marginally increased digestion of haemoglobin, the increased degradation was too low to be considered additive or synergistic. Furthermore, HRgpA-metHb and InpA-metHb appeared to be refractory to attack by InpA and Kgp respectively. This suggests that there is a notable difference between metHb structures formed through auto-oxidation and proteolysis. Although the experiments performed in this chapter do not reveal synergy between InpA and the gingipains, the possibility of mutual haem acquisition between BPAs cannot be disregarded. The following chapter will elaborate on this theory by assessing the role of BPA proteases in facilitating haem extraction from haemoglobin by haem binding protein such as the *P. gingivalis* HmuY haemophore.

CHAPTER 6

The role of the HmuY haemophore of P. gingivalis in haem acquisition from haemoglobin

6.1 INTRODUCTION

One of the most extensively characterised haem acquisition systems of *P. gingivalis* is the HmuY haemophore and its cognate cell surface lipoprotein receptor, HmuR (Olczak *et al.*, 2005). HmuY is thought to transport haem to the outer membrane-located HmuR for internalisation *via* a TonB like system (Simpson *et al.*, 2000). The mature 24 kDa HmuY protein exists as a homodimer (Wójtowicz *et al.*, 2009a) but appears to form a homotetramer upon binding either Fe(II)- or Fe(III)-haem (Wójtowicz *et al.*, 2009b). Despite this change in quaternary structure, the overall HmuY structure does not change following haem binding (Wójtowicz *et al.*, 2009a).

In the metHb form, haemoglobin becomes more susceptible to breakdown by Kgp and InpA on account of reduced globin haem affinity which results from greater haem dissociation (Smalley *et al.*, 2007; 2008; Byrne *et al.*, 2010). In addition, in the ferric form, haemoglobin readily gives up haem to albumin (Bunn & Jandl, 1968). Experiments performed as part of this current investigation have shown that HmuY rapidly sequesters ferric haem from metHb (Smalley *et al.*, 2011). However, at the neutral to slightly alkaline pH of the periodontal pocket, the natural rate of auto-oxidation is at its lowest (Shikama, 1998). Given the ability of InpA in addition to R-gingipain to promote metHb formation, it was logical to investigate the role of these enzymes in aiding haem acquisition by HmuY from oxyHb.

6.2. METHODS

6.2.1. Effect of gingipain and interpain on HmuY haem pick up from haemoglobin.

In all experiments stock solutions of bacterial proteins were as follows: InpA (2 μM); HRgpA (1 μM); Kgp (1 μM), HmuY (16 μM). Haemoglobin and haemoglobin haemichrome samples were incubated at equimolar concentrations to HmuY (i.e.16 μM with respect to Hb subunit). Proteolytically-induced metHb was prepared by pre-incubation of oxyHb with either InpA or HRgpA for 7 or 24 h. Haemoglobin haemichrome was prepared by treatment of oxyHb with Kgp for 7 or 24 h, or with urea (as discussed in the methods section). The haemoglobin preparations were incubated with HmuY and sampled periodically for native PAGE and UV-visible spectroscopy. All incubations were carried out at 37 °C in 0.14 M NaCl, 0.1M Tris-HCl (pH 7.5). For analysis by native PAGE, protease activity in the samples was arrested immediately after incubation by treating with 0.5 mM E-64, 0.2 mM T.L.C.K and 0.2 mM leupeptin (30 min at room temperature) for InpA, Kgp, and HRgpA, respectively. Samples were solubilised at 37 °C for 1 h in non-reducing LSB without SDS or urea.

6.2.2. HmuY resistance to InpA proteolysis.

The effect of InpA protease pre-treatment on haem binding ability of HmuY was investigated by incubating the protein with the enzyme for 24 h and then reacting HmuY with haem (16 μM) at 37 °C. InpA proteolysis of holo- and apo-HmuY was also assessed. For the purpose of these experiments, protein and enzyme were incubated at the above concentrations at 37 °C in (pH 7.5) and sampled periodically for SDS-PAGE.

6.3. RESULTS AND DISCUSSION

6.3.1. Haem binding alters the electrophoretic mobility of HmuY.

Upon exposure to an equimolar amount of haem (16 μM) the R_f of the HmuY band (during native PAGE) increased significantly (Fig 6.1). This is likely a result of the increased negative charge imparted by the ionised carboxylate groups of the haem, which is bound biaxially via the His134 and His166 diad (Fig 6.2 [Wójtowicz *et al.*, 2009a]), and would have the effect of increasing holo-HmuY electrophoretic mobility in the absence of SDS.

Ferrihaem binding by HmuY also results in a well-defined spectrum (411 nm Soret plus 527 nm and 558 nm Q bands, Fig 6.3), representing the six-co-ordinate, low-spin HmuY-haem complex (Olczak *et al.*, 2008; Wójtowicz *et al.*, 2009a).

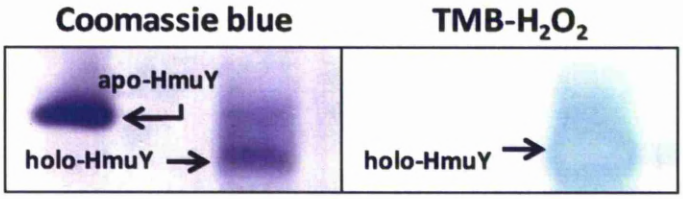


Fig 6.1. Native-PAGE of apo-HmuY and holo-HmuY.

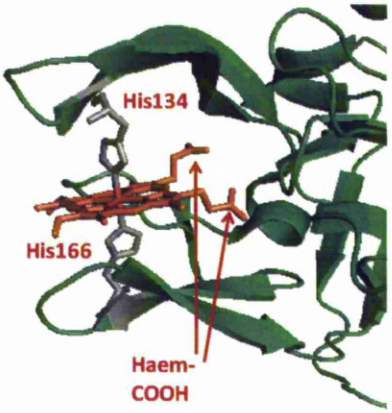


Fig 6.2. Part of the structure of holo-HmuY showing liganded haem. Diagram created using Pymol (PDB access code 3H8T).

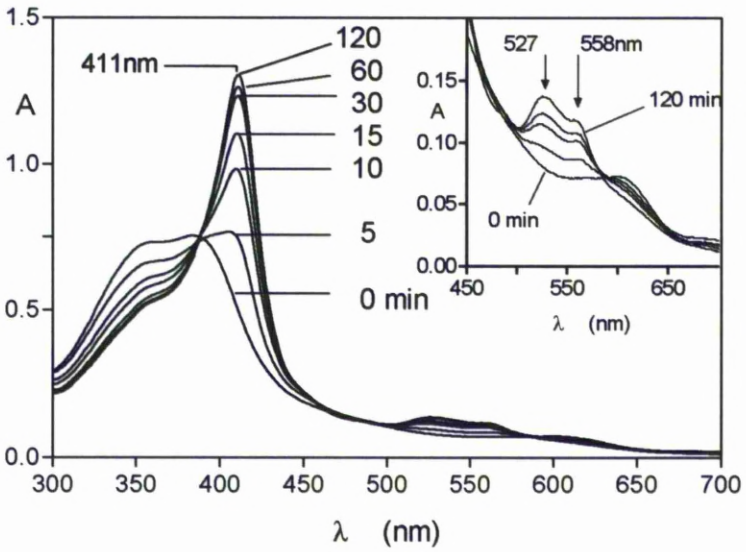


Fig 6.3. Time course of the ferrihaem-HmuY complex formation during reaction of equimolar amounts of HmuY and ferrihaem (16 μM).

6.3.2. RgpA-induced methaemoglobin is a facile substrate for HmuY haem extraction.

Preliminary investigations in this study revealed that HmuY haem extraction from haemoglobin was dependent on its oxidization to metHb (subsequently published as Smalley *et al.*, 2011). However, at slightly alkaline pH (most likely to prevail in the periodontal pocket) oxyHb oxidation is *de facto* at its lowest (Tsuruga *et al.*, 1998). Previous studies revealed that the Arg-specific gingipains of *P. gingivalis*, HRgpA and RgpB (Smalley *et al.*, 2007; 2008) and InpA of *P. intermedia* (chapter 3; Byrne *et al.*, 2010) induce haemoglobin oxidation. It is possible therefore that pick up of haem by the HmuY/HmuR system may be largely dependent on these enzymes. Crucially, both apo- and holo-HmuY, are completely resistant to trypsin, Kgp, HRgpA, and RgpB (Wójtowicz *et al.*, 2009a), making it a very durable agent to work in concert with proteases for haem extraction. MetHb formed by incubation of oxyHb with HRgpA or by auto-oxidation for 7h was examined for haem transfer to HmuY. Native PAGE revealed that α and β haemoglobin chains migrated as a diffuse band (Fig 6.4A1). When HmuY was incubated with HRgpA-metHb there was a time-dependent increase in TMB-H_2O_2 staining associated with the migrating HmuY band (Fig 6.4A2; arrowed), confirming apo-HmuY formation. There was also a reciprocal reduction in Coomassie blue (Fig 6.4A1) and TMB-H_2O_2 (Fig 6.4A2) staining of the α and β Hb bands indicating both haem and protein loss. A smaller amount of the faster migrating holo-HmuY band was observed in control HmuY-haemoglobin incubations (Fig 6.4A1), which was attributed to haem loss from oxyHb as a result of auto-oxidation. Addition of HmuY to oxyHb pre-treated for 24 h with HRgpA immediately resulted in HmuY-haem complex formation with no evidence of residual apo-HmuY (Fig 6.4B1), and was presumably attributable to a greater degree of proteolysis and/or oxidation of the oxyHb.

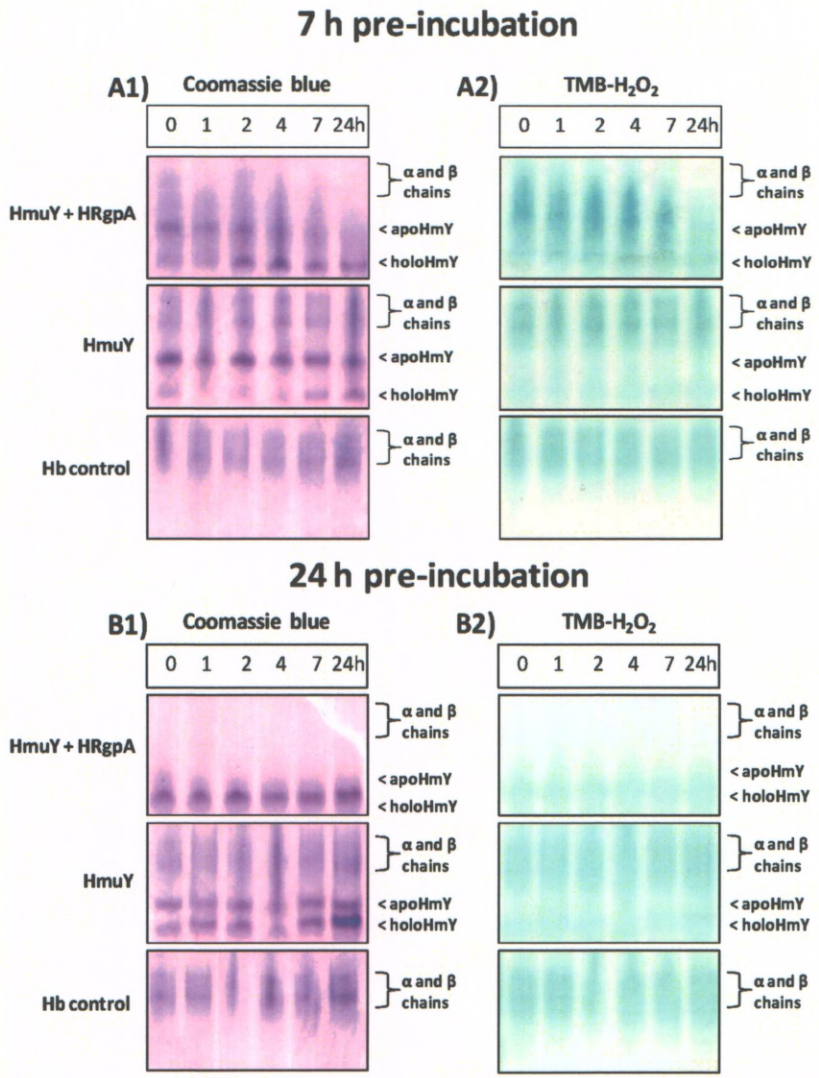


Fig 6.4. Native-PAGE showing the effect of HmuY on oxyHb with or without 7 or 24 h pre-treatment with HRgpA.

Formation of the HmuY-haem complex from HRgpA-pre-treated oxyHb (for 7 h) was corroborated by spectroscopy (Fig 6.5). This protease-mediated metHb substrate (77 % oxidised) gradually displayed a typical HmuY-ferrihaem spectrum after addition of HmuY (411 nm Soret and visible bands at A_{527nm} and A_{558nm}). A typical ferro-HmuY complex (423 nm Soret plus A_{526nm} and A_{566nm} visible bands [Wójtowicz *et al.*, 2009a]) formed upon the addition of 10 mM $Na_2S_2O_4$ (data not shown). This result unequivocally demonstrated that haem had become complexed to HmuY during incubation with HRgpA-metHb.

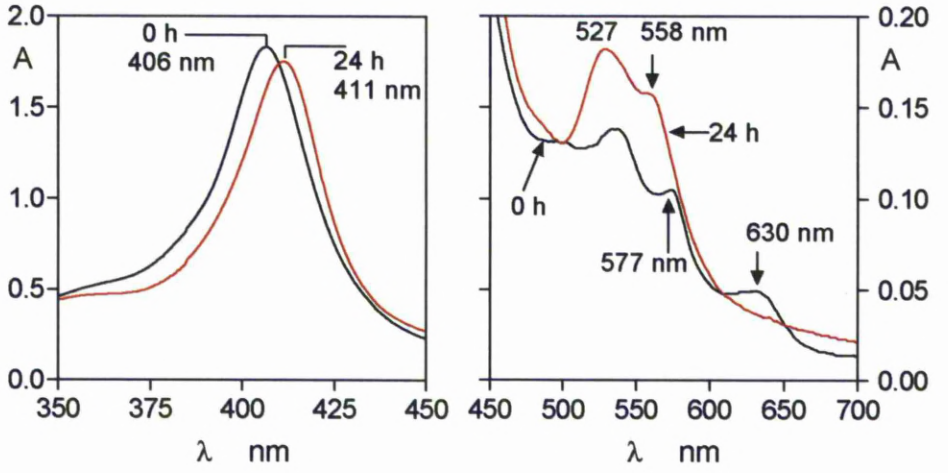


Fig 6.5. HmuY-ferrihaem complex formed from HRgpA-induced metHb. Before (black line) and 24 h after (red line) incubation with HmuY.

6.3.3. Co-incubation of oxyHb with HmuY plus HRgpA facilitates haem transfer to HmuY.

Chemostat studies show that Arg-specific protease activity and haem-binding proteins are expressed by *P. gingivalis* during haem-limitation (McKee *et al.*, 1986; Marsh *et al.*, 1994; Smalley *et al.*, 1993). Importantly, more recent work (Olczak *et al.*, 2010) has shown that HmuY is also expressed under haem restriction (in batch culture) and thus it is likely that HmuY and HRgpA are deployed together as a haem acquisition system, with HRgpA mediated auto-oxidation being a limiting factor. For the arguments discussed previously (Smalley *et al.*, 2008), both HRgpA and HmuY may often be exposed to oxyHb as a result of bleeding in the periodontal pocket. In this regard it is noteworthy that *P. gingivalis* can grow in an oxygenated environment (Diaz & Rogers, 2004). It is therefore also likely that HmuY works in concert with the Rgp to remove haem from metHb as it is formed. In respect to this, it was observed that the HmuY-haem complex formed from oxyHb faster in the presence of HRgpA compared to in its absence (Fig 6.6).

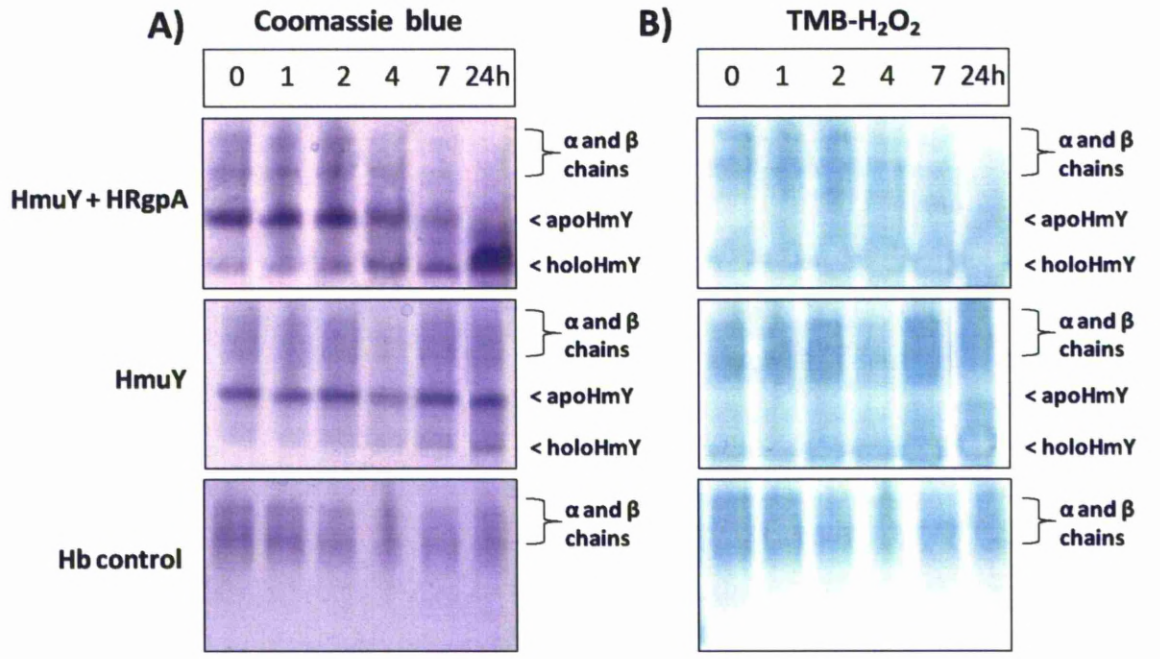


Fig 6.6. Native-PAGE showing HmuY-haem complex formation during co-incubation of oxyHb with HmuY plus HRgpA.

Taken together these data indicate that proteolytic oxidation by HRgpA facilitates the transfer of haem to HmuY. In the body, haemoglobin released by haemolysis will spontaneously oxidise such that haem can be sequestered by albumin and haemopexin (Adams & Burman, 1980). However, *P. gingivalis* may have to rely on proteolytic oxidation for haem removal to obviate the naturally low oxidation rate occurring at alkaline pH (Tsuruga *et al.*, 1998) which is considered to prevail in the periodontal pocket and gingival sulcus (Bickel and Cimasoni, 1985; Eggert *et al.*, 1991). It is thus noteworthy that gingipains have an alkaline pH optimum (Pike *et al.*, 1994; Potempa *et al.*, 1998). This is the first demonstration of a syntrophic relationship between a haemophore and bacterial protease deployed to extract haem from oxyhaemoglobin.

6.3.4. HmuY binds haem from haemoglobin haemichromes induced from oxyHb by Kgp.

Proteolytic attack by Kgp on oxyHb results in formation of a haemoglobin haemichrome (Smalley *et al.*, 2007). Haemoglobin haemichromes are formed by drastic

structural changes within the globin chain, brought about by proteolysis or denaturants, which distort the haem pocket geometry such that the haem iron coordinates with amino acid ligands other than the proximal histidine (Rifkind *et al.*, 1994; Rachmilewitz *et al.*, 1971). As seen in Figure 6.7, HmuY formed a HmuY-ferrihaem complex when mixed with the haemoglobin haemichrome (formed by treatment of oxyHb with urea); with no further spectral changes observed after 10 min. Native PAGE showed TMB-H_2O_2 staining of the HmuY band (Fig 6.8B, arrowed), unequivocally proving haem capture from the haemichrome. This was despite the fact that the high concentration of urea used to induce haemichrome formation had resulted in partial denaturation of HmuY as judged by the gradual increase in the R_f of the HmuY band which was most apparent after 24 h (Fig 6.8A).

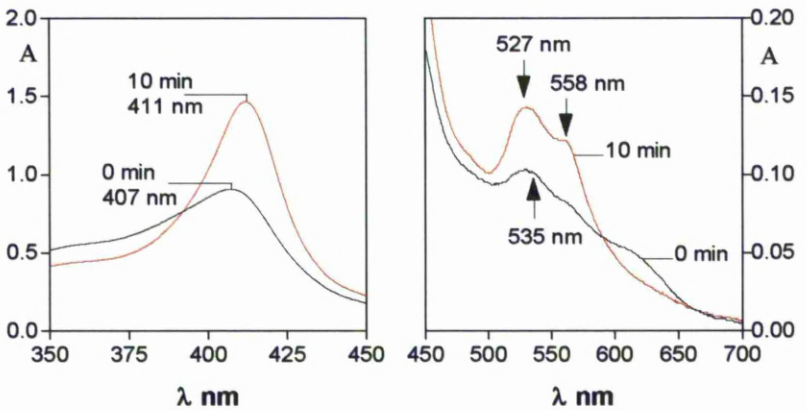


Fig 6.7. HmuY-ferrihaem complex formation from a haemoglobin haemichrome. The haemichrome (black line) was formed by treatment of oxyHb with 8 M urea for 24 h. Red line is the HmuY-ferrihaem complex.

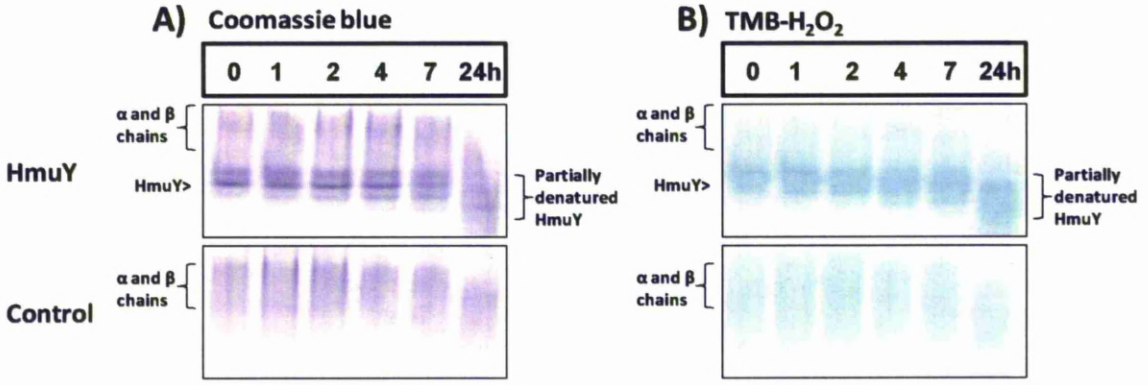


Fig 6.8. Native-PAGE showing HmuY-haem complex formed from the haemoglobin haemichrome.

Although the haemichrome product formed by Kgp proteolysis of oxyHb is stable to further degradation by the enzyme (Smalley *et al.*, 2007), the haem Fe(III) oxidation state should also theoretically render it susceptible to extraction by HmuY, as observed for the metHb species, since the haem-globin bonding is considerably weakened compared to Fe(II) haems. As such the "Kgp-formed haemichrome" was also examined. Most commonly, the haem in haemoglobin haemichromes is co-ordinated in a bis-histidine configuration (Rifkind *et al.*, 1994; Rachmilewitz *et al.*, 1971), in a similar manner to haem binding by HmuY (Wójtowicz *et al.*, 2009a). Consequently, the UV-visible spectra of the haemichrome (409 nm Soret and 535 nm visible band) and that of the HmuY-ferrihaem complex are very similar, making for difficulties in discriminating between the two haem-containing proteins. To obviate this complication, oxyHb treated with Kgp (for either 7 or 24 h) was incubated with HmuY and subjected to native PAGE. This showed increased mobility and TMB-H_2O_2 staining of the HmuY band as the holo-HmuY complex was generated (Fig 6.9.A2). With oxyHb pre-treatment with Kgp for 24 h, transfer of haem was detected immediately after mixing with HmuY (Fig 6.9B; labelled 0h), clearly revealing its susceptibility to HmuY.

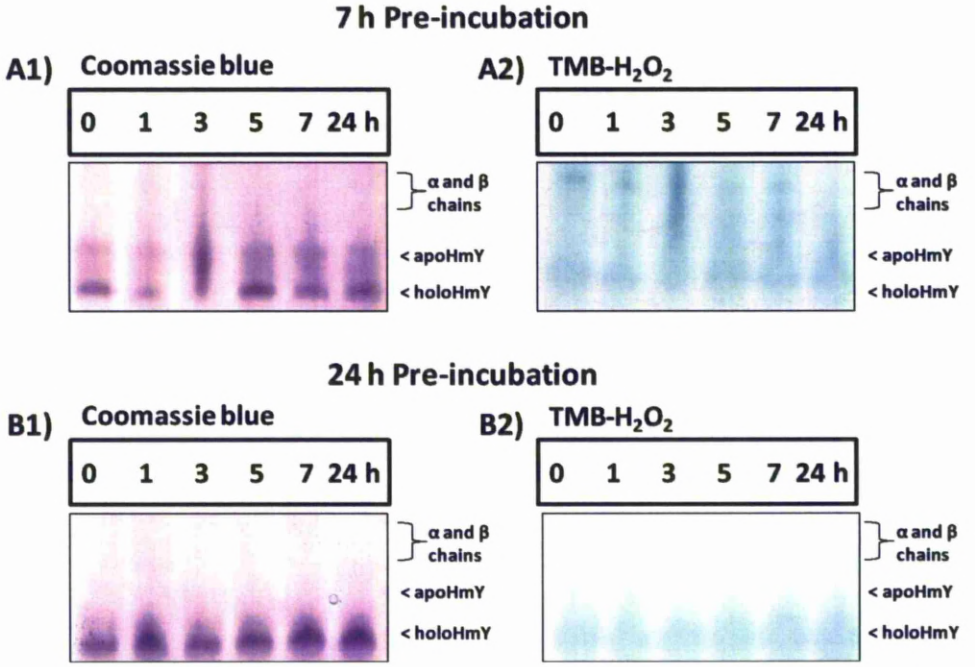


Fig 6.9. HmuY-haem complex formation from Kgp-induced haemoglobin haemichrome.

Co-incubation of oxyHb with Kgp and HmuY also resulted in holo-HmuY formation (Fig 6.10). This latter finding confirmed that Kgp may render haemoglobin susceptible to

haem extraction without the aid of the Rgp-mediated metHb formation. This points to yet another avenue through which *P. gingivalis* can acquire haem.

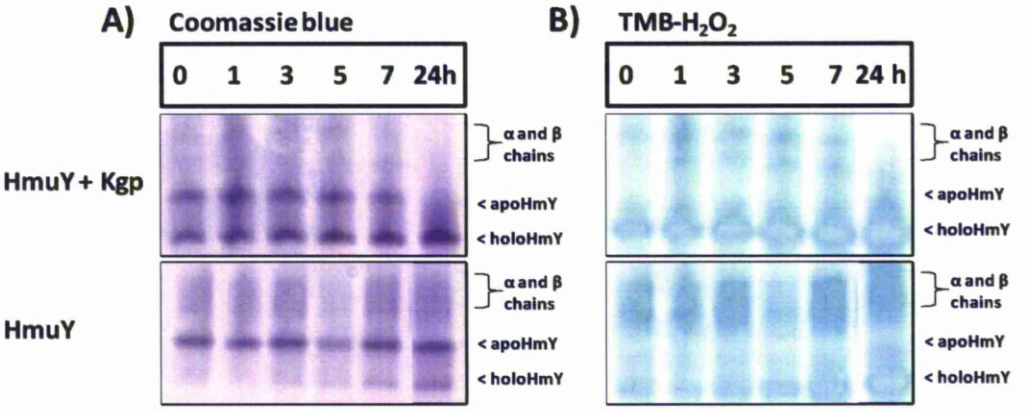


Fig 6.10. HmuY-haem formation during co-incubation of oxyHb with HmuY plus Kgp.

6.3.5. InpA-mediated methaemoglobin formation facilitates HmuY haem extraction.

The paradigm for haem acquisition by *P. gingivalis* from oxyHb involves the initial R-gingipain mediated oxidation to metHb, which *de facto* has a reduced affinity for haem, resulting in the increased degree of dissociation from the globin; the latter being more susceptible to attack by Kgp and haem release (Smalley *et al.*, 2004, 2007, 2008). Results presented in this chapter show that both auto-oxidised Hb and that formed proteolytically by HRgpA are facile substrates from which haem can be extracted by HmuY. Importantly, Rgp activity provides a means of accelerating oxyHb oxidation which is lowest at alkaline pH (Shikama, 1998), conditions which may be encountered in the inflamed gingival crevice and diseased periodontal pocket. Given the ability of InpA to mediate oxyHb oxidation over a range of pH (Byrne *et al.*, 2010), it is likely that *P. gingivalis* may benefit from InpA activity in the haem capture process. As such, potential synergy between HmuY and InpA in haem extraction from oxyHb was examined.

Importantly, both holo- (Fig 6.11, A) and apo-HmuY (Fig 6.11B) were completely resistant to InpA activity, and InpA pre-treatment (for 24h) of apo-HmuY did not affect HmuY-haem complex formation (Fig 6.12). Resistance of HmuY to

InpA would be vital in establishing a cooperative relationship between the two proteins and hence between the two microorganisms for haem capture.

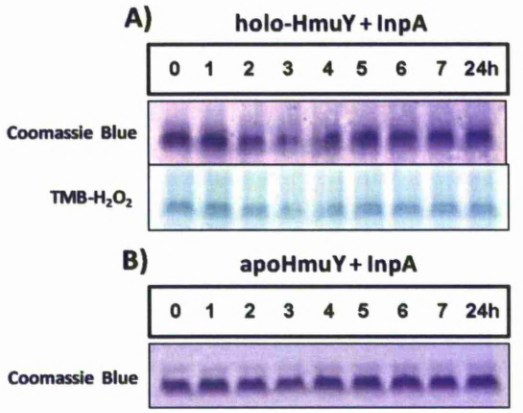


Fig 6.11. Effect of InpA on apo- and holo-HmuY.

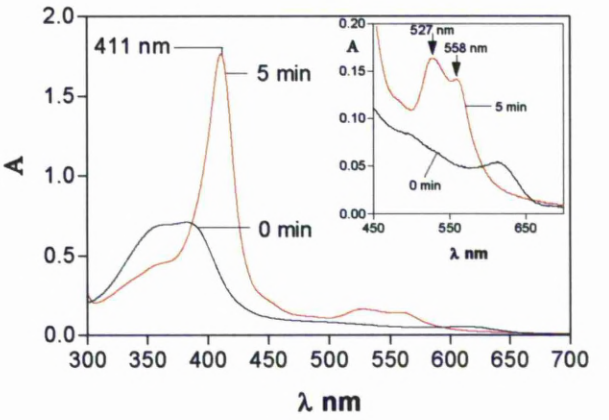


Fig 6.12. HmuY-haem formation by HmuY pre-treated with InpA for 24h.

MetHb induced by InpA pre-treatment of oxyHb for 7 h (consisting of 44 % metHb) was incubated with HmuY. This immediately resulted in a ferrihaem-HmuY spectrum which was stable over 24 h (Fig 6.13.). It is noteworthy that the InpA-induced metHb product showed little haem loss as judged by the Soret band intensity relative to auto-oxidized control (data not shown) and agrees with the previous observation of limited proteolysis at pH 7.5 (chapter 3; Byrne *et al.*, 2010). SDS-PAGE and densitometry confirmed that incubation of InpA with oxyHb at pH 7.5 resulted in immeasurably low levels of haem release after 7 h (data not show). It is unlikely that the low levels of any proteolytically liberated haem could account for the strong intensity of the ferrihaem-HmuY spectrum produced upon addition of HmuY to the InpA-Hb incubation. From this it was confirmed that holo-HmuY formation was in the majority due to the direct acquisition of haem from InpA-induced metHb.

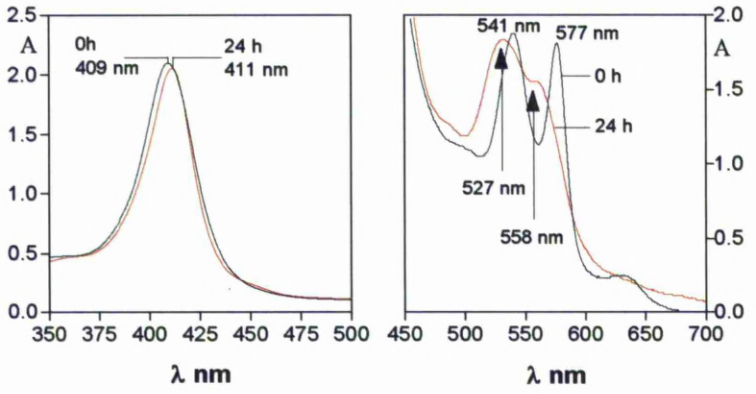


Fig 6.13. HmuY-ferrihaem formation from metHb - induced by the action of InpA on oxyHb. Black line, metHb at time zero; red line, 24 h after addition of HmuY.

During native PAGE the slightly faster migrating HmuY-ferrihaem band increased in both Coomassie blue (Fig 6.14A, arrowed) and TMB-H_2O_2 (Fig 6.14B, arrowed) staining intensity while that of the apo-HmuY displayed a reduction. This was accompanied by reduced TMB-H_2O_2 staining of the Hb bands (Fig 6.14, B), indicating loss of haem. Holo-HmuY formation occurred at a greater rate in the presence of InpA than without, which corroborated the UV-visible spectral data.

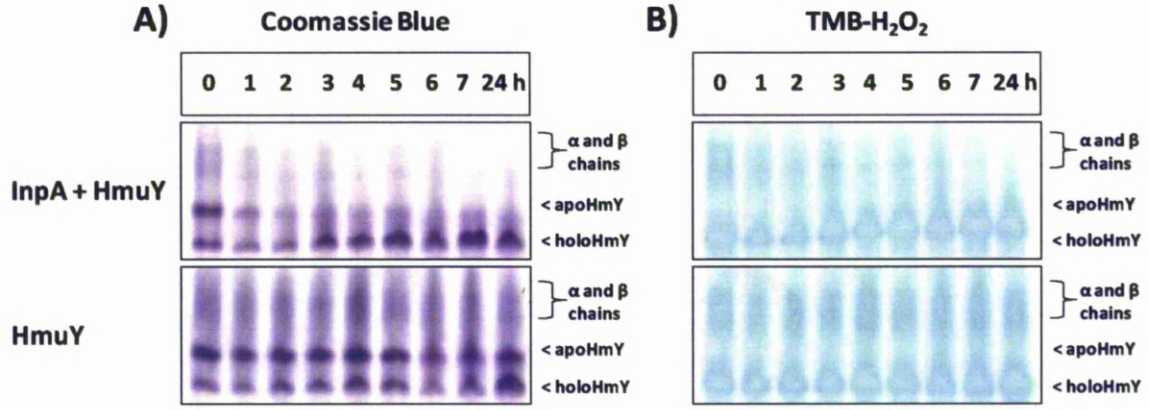


Fig 6.14. Effect of HmuY on oxyHb with or without 7 h pre-treatment with InpA.

To determine whether InpA facilitated haem extraction from haemoglobin *in situ*, InpA and HmuY were co-incubated with oxyHb, and analysed by native PAGE. This unambiguously demonstrated increased haem extraction by HmuY in the presence of InpA (Fig 6.15).

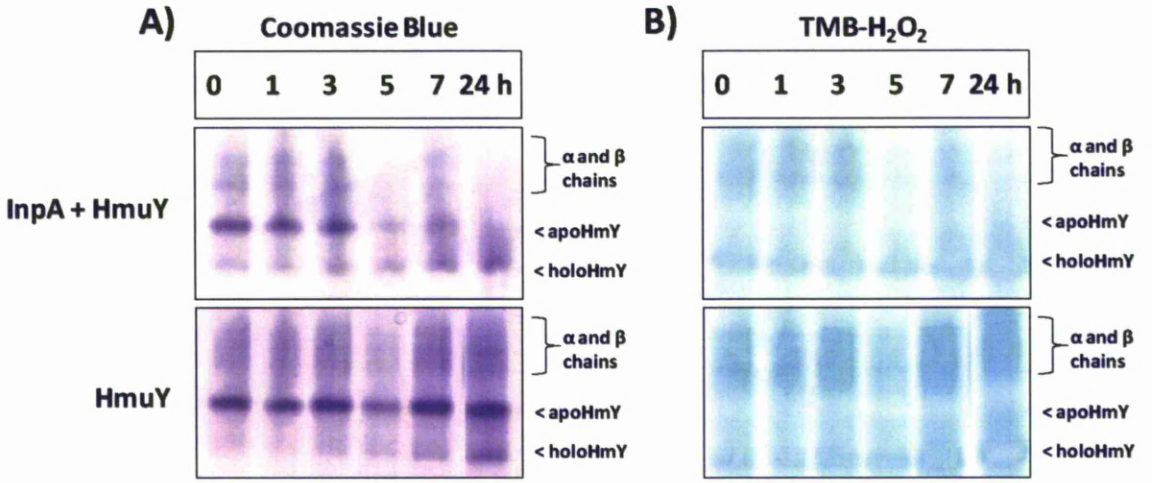


Fig 6.15. Non-denaturing PAGE of oxyHb co-incubated with InpA plus HmuY.

These findings indicate potential co-operation between *P. intermedia* and *P. gingivalis* and demonstrates how the haem acquisition mechanism of one organism may benefit another i.e., InpA being deployed by *P. intermedia* to proteolytically liberate haem from the host haemoglobin thus enabling HmuY to more effectively scavenge haem. Haem scavenging by HmuY from proteolytically (InpA)-modified haemoglobin may have developed as a consequence of the evolutionarily similar haem capture mechanisms of both organisms. It is noteworthy therefore, that a HmuY-like outer-membrane lipoprotein has been reported in *P. intermedia* strain 17, which is dramatically up-regulated in iron-deplete conditions (Yu *et al.*, 2007).

In this chapter it was shown that HmuY can extract ferrihaem from metHb produced by either InpA or HRgpA and, whilst this constitutes a potential mechanism of haem acquisition of *P. gingivalis*, it offers further insight into a possible mutualistic relationship with *P. intermedia*. Although other bacterial haemophores have been demonstrated to extract haem from Hb (Letoffe *et al.*, 1994b; Maresso *et al.*, 2008 [note that these workers used commercial preparations of haemoglobin which would probably comprise a high percentage of the oxidised form of Hb, *de facto* a very facile substrate for haem extraction!]), this is the first documented demonstration of a bacterial haemophore acting syntrophically with proteases for this purpose. In addition, this is also the first demonstration of a bacterial haemophore acting syntrophically with a specific "haemoglobinase" from a different bacterial species.

In the inflamed gingival crevice and periodontal pocket, free haem and that in the form of metHb may likely be sequestered by albumin and haemopexin which serve to limit serum haem bioavailability (Gattoni *et al.*, 1996). However, it is noteworthy that HmuY can successfully compete with serum albumin for binding both metHb derived haem and free haem, and can also extract haem directly from methaemalbumin (Smalley *et al.*, 2011). These findings are physiologically relevant as in a haem-limited environment (in the absence of overt bleeding, and thus haemoglobin) HmuY may have to extract haem directly from albumin which is the most abundant haem- sequestering component of GCF and the periodontal pocket (Curtis *et al.*, 1998). These findings further emphasise the versatility and complexity of the haem acquisition systems of both *P. gingivalis* and *P. intermedia* and highlights the ability of the periodontal pocket environment and the resident flora to influence these systems.

CHAPTER 7

Role of InpA in breakdown of albumin and haemopexin

7.1. INTRODUCTION

In the circulation, the majority of any free haem is rapidly sequestered by serum albumin (Beaven *et al.*, 1974) and haemopexin (Hrkal *et al.*, 1974) which function to limit haem bioavailability and transport it to the liver. In the "healthy" or mildly inflamed gingival sulcus, in absence of frank bleeding and hence a haemoglobin haem source, *Prevotella* species may need to depend on haemalbumin and haem-haemopexin as primary sources of haem. Albumin is the predominant protein from both healthy and inflamed periodontal sites (Curtis *et al.*, 1990, Makela *et al.*, 1991; Hanioka *et al.*, 2005). Moreover, due to the 80-40-fold greater concentration than haemopexin in serum (Muller-Eberhard & Morgan, 1975), but despite its 75-fold lower affinity for haem binding (Hrkal *et al.*, 1980), it binds more of any free haem present. It therefore constitutes a potential non-haemoglobin source of haem and amino acids for *P. intermedia* and other sub- and supragingival plaque bacteria. *P. gingivalis* can utilise haemalbumin and albumin as the sole source of carbon and nitrogen (Makela *et al.*, 1991; Smalley & Birss, 1997; Milner *et al.*, 1996), with Rgp in particular, (Grenier *et al.*, 2001c) mediating much of the breakdown. *P. intermedia* can also degrade albumin (Carlsson *et al.*, 1984a) and incubation with albumin increases both bacterial growth and proteolytic activity (Ichiro, 2000).

Unlike albumin, haemopexin can specifically bind one haem molecule (Heide *et al.*, 1964), doing so with greater affinity than albumin (Muller-Eberhard *et al.*, 1969; Drabkin, 1971). *P. intermedia* is also able to partially degrade haemopexin (Carlsson *et al.*, 1984a; Jansen *et al.*, 1994). Given the ability of InpA to proteolytically release haem from haemoglobin it is appropriate to examine the ability of InpA to degrade albumin and haemopexin.

7.2. METHODS

7.2.1. Albumin and haemopexin breakdown by InpA and gingipains.

Incubation of InpA, Kgp and HRgpA with haem-binding serum proteins and their respective haem-liganded derivatives, were carried out at 37 °C with a 1:4 enzyme : substrate ratio. Incubations were performed in pH 5.5, 6.0 or 7.5 buffers (as decribed previously). Where appropriate, 3 mM DTT was also added to examine the effects of reducing conditions, and thus partly mimic conditions in the diseased periodontal pocket (Kenny & Ash, 1969). Digestions were monitored spectroscopically and sampled for SDS-PAGE on 10 % polyacrylamide gels. For MALDI-TOF-MS analysis incubations were done as above in 10 mM Tris buffer (pH 7.5) ± 3 mM DTT.

7.2.2. Effect of InpA pre-treatment on haem binding by apoalbumin and apohaemopexin.

Haemopexin and albumin were pre-incubated with InpA for various time periods at pH 7.5 and activity then inhibited with E-64 (0.5 mM, 30 min at 20 °C), before being mixed with iron(III) protoporphyrin IX at 1:0.9 haem: protein molar ratio, for 2 h at 37 °C (pH 7.5).

7.2.3. Haem release from immobilised haemalbumin.

Haemalbumin-agarose beads (~ 45 μM with respect to haem) were incubated with gentle agitation ± 2 μM InpA and ± 3 mM DTT in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5 at 37 °C. Periodically, samples of the whole incubation mixture were removed, having first thoroughly vortex mixed the beads. Following pelleting of the beads (centrifugation at 5000 x g for 1 min at 20 °C), the supernatant was removed and any released haem was assayed by the pyridine-haemochrome method.

7.2.4. Fluorescence monitoring of structural changes to albumin.

Fluorescence of 16 μM albumin at pH 7.5 in the presence 0, 1, 2, 3, 5, 19, 20, 50 and 100 mM DTT, was measured with a Flexstation 3 Benchtop (Molecular Devices) multi-mode microplate reader using λ_{ex}295 nm and λ_{em} 310-410 nm.

7.3. RESULTS AND DISCUSSION

7.3.1. Susceptibility of albumin and haemalbumin to InpA.

In normal plasma, haemalbumin is present at a ratio of one to 5000 molecules of apoalbumin (i.e at ~ 121 nM [Walji *et al.*, 1993]). To effectively compete with the host for haem, *P. intermedia* may deploy InpA to proteolytically perturb the scavenging function of apoalbumin so as to incapacitate haem pick up. *P. intermedia* has an acid terminal growth pH (Shah & Williams, 1987; Takahashi & Yamada, 2000) and InpA has an acid pH optimum, centred around pH 6.0, towards protein substrates including azoalbumin and azocoll (Potempa *et al.*, unpublished findings). However, healthy periodontal pockets are slightly alkaline (Bickel & Cimasoni, 1985; Eggert *et al.*, 1991). In view of this InpA (4 μM) was incubated with haem-free bovine albumin (16 μM) over the pH range 7.5, 6.0, and 5.5. Under these conditions, InpA degraded less than 5 % of total albumin after 24 h at each pH (Fig 7.1A). Albumin was therefore concluded to be resistant to InpA. Although minimally susceptible towards InpA, peptide cleavage may reduce the haem binding capacity of albumin. To test this, apoalbumin (16 μM) was incubated with 2 μM InpA at pH 7.5 and samples were collected periodically and incubated with 16 μM haem. This showed that InpA had little effect on haem binding (Fig 7.1B).

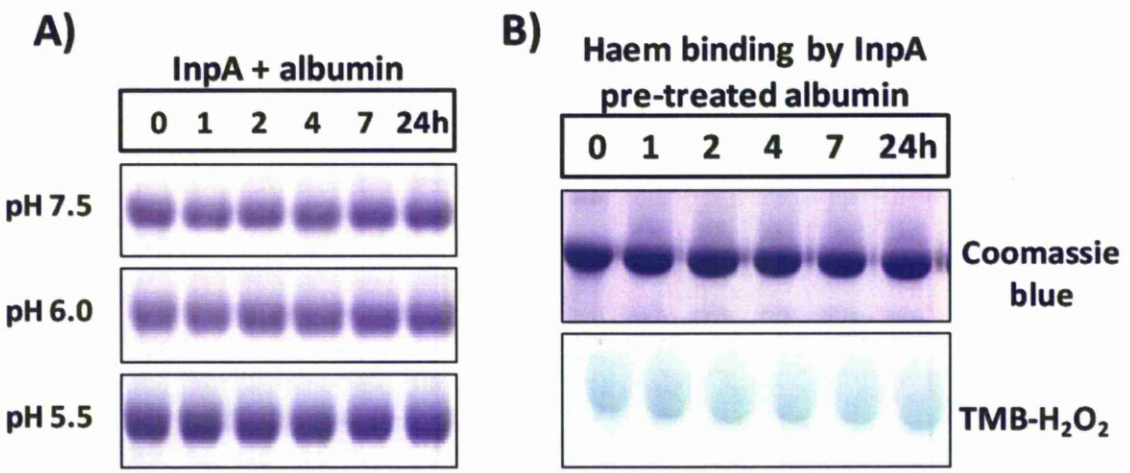
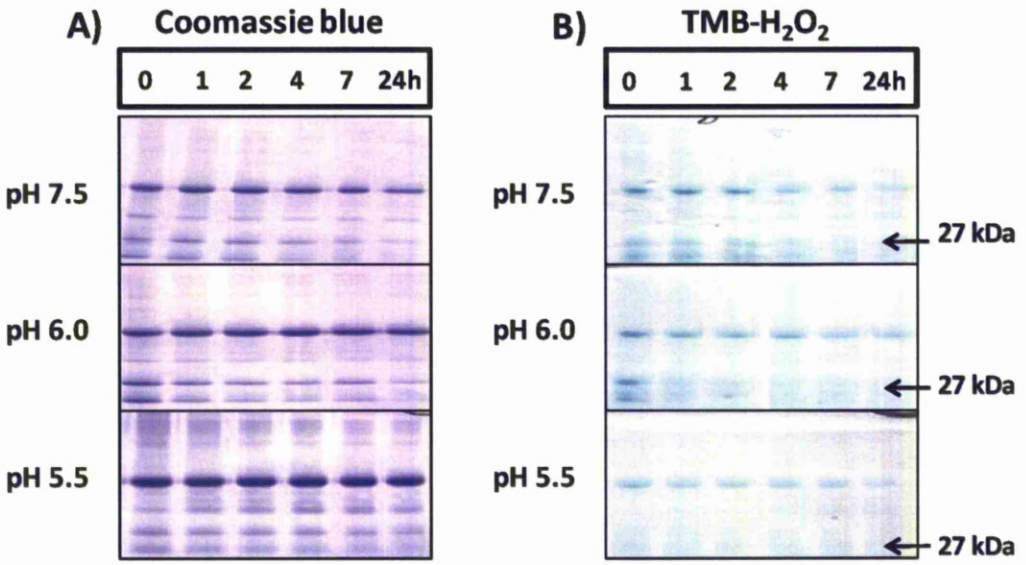


Fig 7.1. Activity of InpA towards albumin at different pH (A); Effect of InpA pre-incubation on haem binding by albumin (B).

Haemalbumin (haem : protein ratio 1:1) was slightly more susceptible to InpA than apoalbumin, particularly at pH 7.5 (Fig 7.2, A). After 24 h, the average total protein degradation by InpA was 35 % at pH 7.5, 20 % at pH 6.0, and 4 % at pH 5.5 (n=4 in all cases). InpA-mediated proteolysis of haemalbumin also produced a higher mobility peptide (M_r ~ 27 kDa) which retained haem as revealed by TMB-H_2O_2 staining (Fig 7.2 B, arrowed), and was most apparent at pH 7.5. This 27 kDa peptide was gradually degraded by InpA as observed by diminishing haem staining. Smalley and Birss (1997) also identified digestion peptides following incubation of albumin and haemalbumin by *P. gingivalis* cells. Importantly, InpA- mediated proteolysis also resulted in haem release from the main 66 kDa albumin band (Fig 7.2, B). After 24 h, albumin haem staining had decreased by 46 %, 46 % and 45 % at pH 7.5, 6.0, and 5.5, respectively (average of three experiments). It is noteworthy that the degree of total albumin haem staining lost over the course of the digest was similar at all three pH values despite variable rates of protein degradation by InpA (Fig 7.2, B2). InpA must therefore inflict specific cleavages that disrupt the haem pocket and facilitate haem release, whilst not bringing about extensive protein degradation.



	pH 7.5	pH 6.0	pH 5.5
Mean % protein loss after 24 h (n= 4)	35 (S.D = 13)	20 (S.D = 6)	4 (S.D = 6)
Mean % haem loss after 24 h (n= 3)	46 (S.D = 3)	46 (S.D = 3)	45 (S.D = 11)

Fig 7.2. Haemalbumin breakdown by InpA. TMB-H_2O_2 stained haem-bearing peptides are arrowed.

The visible spectrum of haemalbumin is characterised by a band near 620 nm indicative of high-spin haem-protein complexes (Kaminsky *et al.*, 1972) and a sharp Soret with λ_{max} at 400 nm (Rosenfeld & Surgenor, 1950; Smalley *et al.*, 2011) making it distinguishable from free haem. Reduction of Soret intensity can be used as an indirect measure for haem loss. In contrast to haem loss shown by TMB-H_2O_2 staining, UV-visible spectra of haemalbumin-InpA incubations yielded only modest reductions in Soret band intensity over 24 h (Fig 7.3). Although haemalbumin can easily be differentiated from free haem by the UV-visible region, they share overlapping spectra with similar Soret extinctions (Beaven *et al.*, 1974) and consequently, quantitative analysis of haem release by spectroscopy may not accurately reflect levels of haem lost. Notwithstanding this, there was an unmistakeable shift in Soret intensity and λ_{max} during proteolysis by InpA at every pH studied. However, the decline in haemalbumin Soret intensity at pH 5.0 (Fig 7.3, C) may be a result of light absorbance due to aggregating haem (Brown *et al.*, 1976; 1980) which become relatively insoluble below about pH 6 as the pKa of the carboxylates in approached.

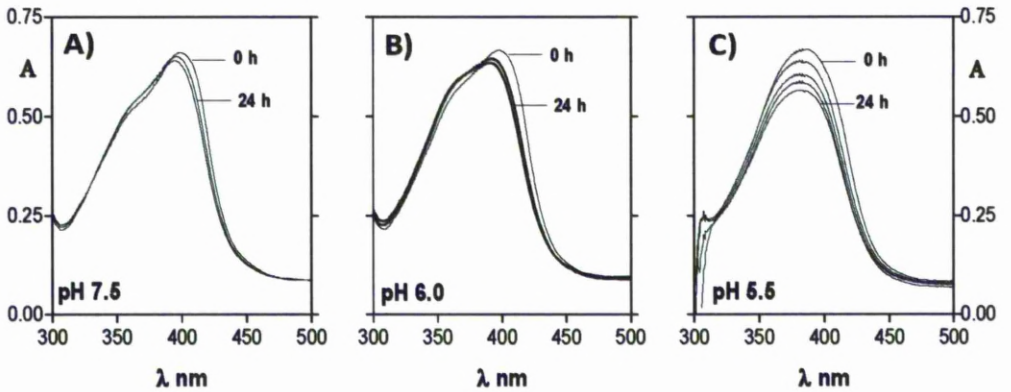


Fig 7.3. UV-visible spectra of haemalbumin during incubation with InpA.

The slight increase in susceptibility of haemalbumin to InpA compared to apo-albumin is consistant with previous observations on susceptibilty of BSA to trypsin and chymotrypsin (Shin *et al.*, 1994), but is in contrast to the findings of Smalley *et al.* (1997) who reported that haem binding stabilized albumin against proteolysis by whole cells of *P. gingivalis.* Increased susceptibility of haemalbumin towards InpA is likely a result of conformational changes occurring upon haem ligation (Walji *et al.*, 1993). The slightly greater susceptibility of haemalbumin versus apoalbumin towards InpA could enable the

protease to distinguish structurally between the two forms, and might be advantageous for *P. intermedia* by enabling differentiation between albumin and scarcer haemalbumin (Muller-Eberhard *et al.*, 1968; Miller & Shaklai, 1999; Walji *et al.*, 1993). Also of interest was the observation that haemalbumin degradation by InpA was greatest approaching alkaline pH and virtually undetectable at pH 5.5. This is somewhat paradoxical given that that InpA exhibits maximal activity against whole proteins such as azocoll, azoalbumin and azocasein (Potempa *et al.*, unpublished findings) and aquometHb (Byrne *et al.*, 2010), at acid pH. The present findings may be explained by pH-dependent changes observed for albumin in solution (Peters, 1985; Carter & Ho, 1994) at both high (Leonard *et al.*, 1963) and low pH (Sogami & Foster, 1968). Between pH 7.5 and 9.0, albumin undergoes a conformational change referred to as the N→B transition (Leonard *et al.*, 1963; Harmsen *et al.*, 1971). This would suggest that the B form may be slightly more susceptible to InpA, as it is also known that trypsin degrades albumin optimally at alkaline pH where the B form predominates (Markus *et al.*, 1967), and that albumin has greater thermal stability at pH 6.4 than pH 7.4 (Ross *et al.*, 1984). This is physiologically relevant since the diseased periodontal pockets are reported to be a slightly alkaline environment (up to pH 9 [Bickel & Cimasoni, 1985; Eggert *et al.*, 1991]).

This notwithstanding, degradation of albumin and haemalbumin by InpA was relatively low, suggesting that it is not a preferred substrate. However, InpA cleavage of haemalbumin, although insufficient to facilitate large scale haem release, may lead to proteolytic deterioration of albumin-haem affinity and assist haem extraction by the, as yet, uncharacterised haem-binding proteins of *P. intermedia*. This is not without precedence, as syntrophic extraction of haem from haemoglobin was reported between gingipains and the HmuY haemophore of *P. gingivalis* (Chapter 6; Smalley *et al.*, 2011). However, a more detailed appraisal of haem capture from host haemoproteins by haem-binding proteins of *P. intermedia* is first required before firm conclusions may be drawn.

7.3.2. Chemical reduction of albumin increases susceptibility to InpA.

P. intermedia is frequently recovered from various sites of patients suffering from oral malodour (Krespi *et al.*, 2006), a condition linked to production of volatile sulphur compounds (VSCs) such as H_2S and methyl mercaptan by periodontal bacteria, including *P. intermedia*, *P. gingivalis* and *F. nucleatum* (Morita & Wang, 2001; Persson *et al.*, 1990). VSCs also play an important role in the pathogenesis of periodontal disease by indirectly and

directly inducing periodontal tissue destruction and haemolysis (Morita & Wang, 2001), and also operate as chemical reductants, contributing to establishment of the low redox potential in the inflamed gingival sulcus and diseased periodontal pocket (Kenny & Ash, 1969). H_2S concentrations as high as ~2 mM have been recorded in GCF (Persson, 1992), and it has been detected in 90 % of periodontal pockets deeper than 4 mm, as well as in 6 % of clinically healthy pockets (Horowitz & Folke, 1973). Methyl mercaptan, has also been detected maximally at concentrations of 0.16 mM in GCF (Persson, 1992). Chemical reduction of the environment mediated by subgingival microflora has been demonstrated during batch-wise enrichment cultures of subgingival plaque inoculate in human serum, where E_h values of -70 to -550 mV were recorded over a 72 h period and H_2S production was detected (Ter Steeg *et al.*, 1987). Of significance was the observation that an E_h drop below -300 mV coincided with enrichment in numbers of *P. intermedia*.

The single polypeptide of albumin contains 17 pairs of disulfide bridges (Dugiaczyck *et al.*, 1982), disruption of which stimulates tertiary structural changes (Kang *et al.*, 2003; Naoko *et al.*, 1992). Chemical reduction of disulfide bridges by treatment with 2-mercaptoethanol (2-ME) increases the molecular flexibility of albumin leading to enhanced polymerisation by transglutaminase (Kang *et al.*, 2003) and degradation by pepsin (Guo *et al.*, 2007). Unsurprisingly, albumin degradation was greatly increased under reducing conditions (3 mM DTT) at all pHs tested, but especially at pH 7.5 (Fig 7.4). Under non-reducing conditions, InpA degraded less than 20 % of total albumin after 7 h at each pH (Fig 7.4). However, by 4 h in the presence of DTT at pH 7.5, albumin had been completely degraded (Fig 7.4, A), which may be accounted for by conformational changes and increased molecular flexibility induced by chemical reduction (Kang *et al.*, 2003). The presence of DTT also increased albumin susceptibility at pH 6.0 (86 % after 24 h [Fig 7.4, B]). However, at pH 5.5, there was little albumin breakdown (less than 10 %) even in the presence of DTT (Fig 7.4, C). The fact that least breakdown was seen at pH 6.0 and 5.5, where InpA activity towards proteins is normally high, suggests that pH-dependent conformational changes (Peters, 1985; Carter & Ho, 1994; Leonard *et al.*, 1963; Sogami & Foster, 1968) may be a key determinant in dictating the effectiveness of InpA proteolysis. In this respect it is known that accessibility of reducing agents to the disulfide bonds of albumin (which would be in the B form) increases above pH 7.0 (Katchalski *et al.*, 1957).

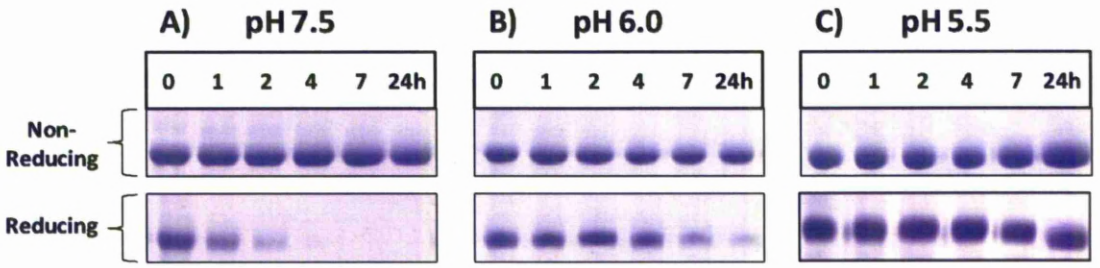


Fig 7.4. Albumin breakdown by InpA under reducing and non-reducing conditions.

Conformational changes resulting in alteration of the local environment of tryptophan can be monitored by fluctuations of intrinsic fluorescence (Steiner & Edelhoch, 1963). Treatment of albumin with increasing concentrations of DTT resulted in reduction of fluorescence intensity and moderate shift in λ_{max} of emitted light, indicative of structural changes (Fig 7.5). Above 100 μM DTT fluorescent emission was not detected which indicated that 3mM DTT, as used in the above digestions, would have been sufficient to induce structural changes, and which most likely accounted for the increased susceptibility to InpA. As InpA had already been activated by an excess of DTT prior to use in these digests, it is unlikely that increased enzyme activity accounts for the greater albumin breakdown. Interestingly, 3 mM DTT can reduce E_h to between -60 and -220 mV in *E. coli* growth medium (Kirakosyan *et al.*, 2004). This is of significance because it suggests that batch-wise enrichment cultures of sub-gingival plaque inoculated in human serum can create more potent reducing environments (E_h between -70 and -550 mV [Ter Steeg *et al.*, 1987]) than those used in this current chapter, and hence would be sufficient to reduce and denature serum albumin. Furthermore, E_h values as low as -300 mV have been reported in the periodontal pocket (Kenny & Ash, 1969).

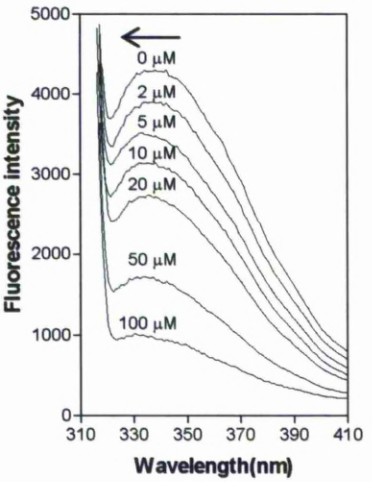


Fig 7.5. Intrinsic fluorescence spectra of BSA treated with increasing concentration of DTT at 37 °C. Arrow indicates shift in λ_{max} of emitted light.

7.3.3. InpA-mediated haem release from haemalbumin under reducing conditions.

The presence of 3 mM DTT increased the susceptibility of haemalbumin (1:0.9 haem: protein ratio) to InpA, with 80 % degraded after 24 h, compared to 30 % without reducing agent (Fig 7.6A). The amounts degraded decreased as the assay pH was lowered (Figs 7.6 B & C). Chemical reduction of haemalbumin with DTT resulted in substantial protein denaturation even in the absence of InpA at pH 7.5 (data not shown). This instability can be explained by the fact that haemin binding in HSA expands subdomain IB (Zunszain *et al.*, 2003) which is normally constrained by the disulfide bridge between Cys124 and Cys 169 on helices 7 and 9, respectively. Since the Cys123 to Cys168 disulfide bond in BSA (Katchalski *et al.*, 1957) may exert a similar steric constraint, reduction of this bond would allow greater subdomain expansion upon haem binding. This, coupled with greater susceptibility to chemical reduction of albumin at alkaline pH (Katchalski *et al.*, 1957), may explain the observed denaturation.

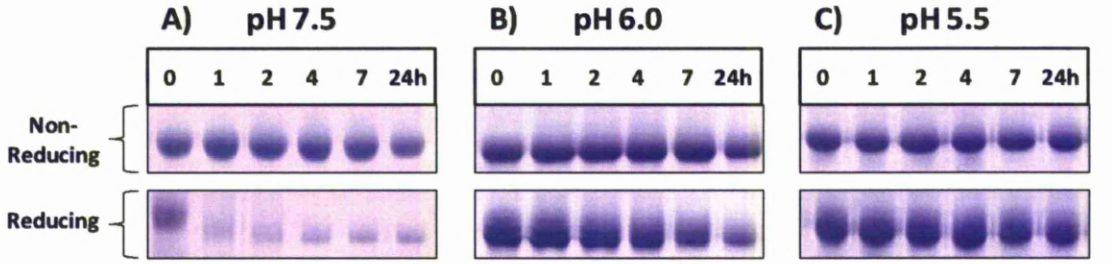


Fig 7.6. Haem-albumin breakdown by InpA under reducing and non-reducing conditions.

To determine haem release under reducing conditions, InpA was incubated with haemalbumin-agarose at pH 7.5, and supernatants assayed for the pyridine-haemochromogen. In the presence of DTT, InpA liberated ~ 300 % more haem than under non-reducing conditions after 1 h (Fig 7.7, A). Haem was also lost from control albumin under reducing conditions (Fig 7.7, B), but was less than with InpA. Haem-albumin affinity is clearly lowered under reducing conditions, which favours transfer to haemopexin in the circulation (Morgan *et al.*, 1976). This fact alone may also be of advantage to *P. intermedia* haem acquisition should the local E_h decline.

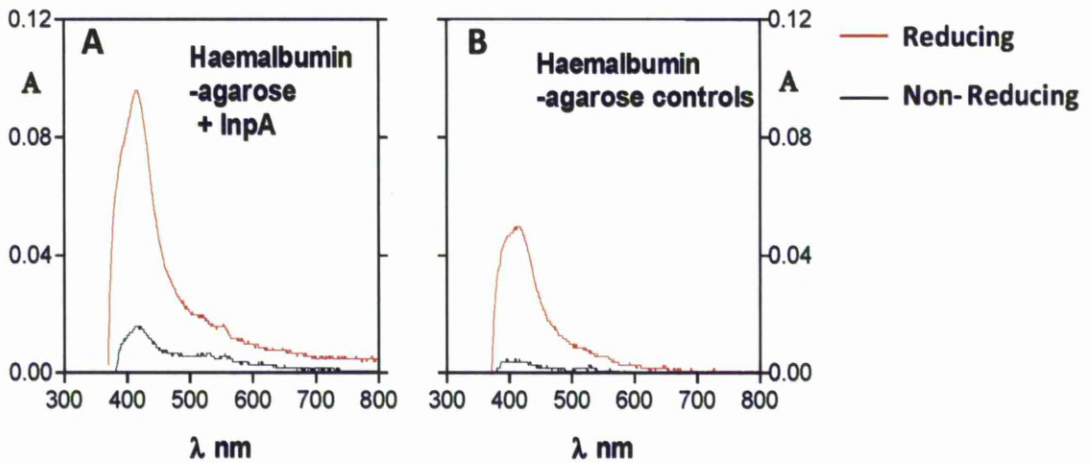


Fig 7.7. InpA-mediated haem release from haemalbumin-agarose detected as a pyridine-haemochromogen.

7.3.4. MALD-TOF-MS analysis of albumin breakdown by InpA.

InpA has specificity for K, R, A and F residues (Potempa *et al.*, unpublished findings). Prominent peptides with masses of 962.50, 964.00, 986.00, 1052.90, 1530.50, and 1844.50 Da were common to InpA digestions of BSA under both reducing (Table 7.2) and non-reducing conditions (Table 7.1). In addition, 13 and 9 peptides unique to reducing and non-reducing conditions, respectively, were also identified. Not surprisingly, the digestion fragments were far more numerous under reducing conditions, reflecting the likelihood that reduction had exposed more susceptible cleavage sites, and so all but the least prominent were reported here. It is noteworthy that digestion fragments appeared as early as the t = 0 h samples under both reducing and non-reducing conditions (also observed during SDS-PAGE of the digest; see Fig 7.2) and probably reflects the rapid rate of InpA proteolysis.

BSA shares 76 % sequence identity with HSA (Peters, 1985; Carter & Ho, 1994). However, to date, the 3-D structure of BSA has not been solved and the haem-binding domain has not been characterised. An exact correlation of InpA cleavage sites (predicted by MS) with the BSA structure, and hence the haem pocket, allows for an accurate identification of cleavages resulting in haem loss. However, to circumvent this problem, a predicted 3D model of BSA was obtained from the SWISS-MODEL Repository Database (http://swissmodel.expasy.org/repository/ [Koop & Schwede, 2006]) with the accession code P02769_C00001. InpA cleavage sites under reducing and non-reducing conditions were then mapped onto this (Fig 7.8).

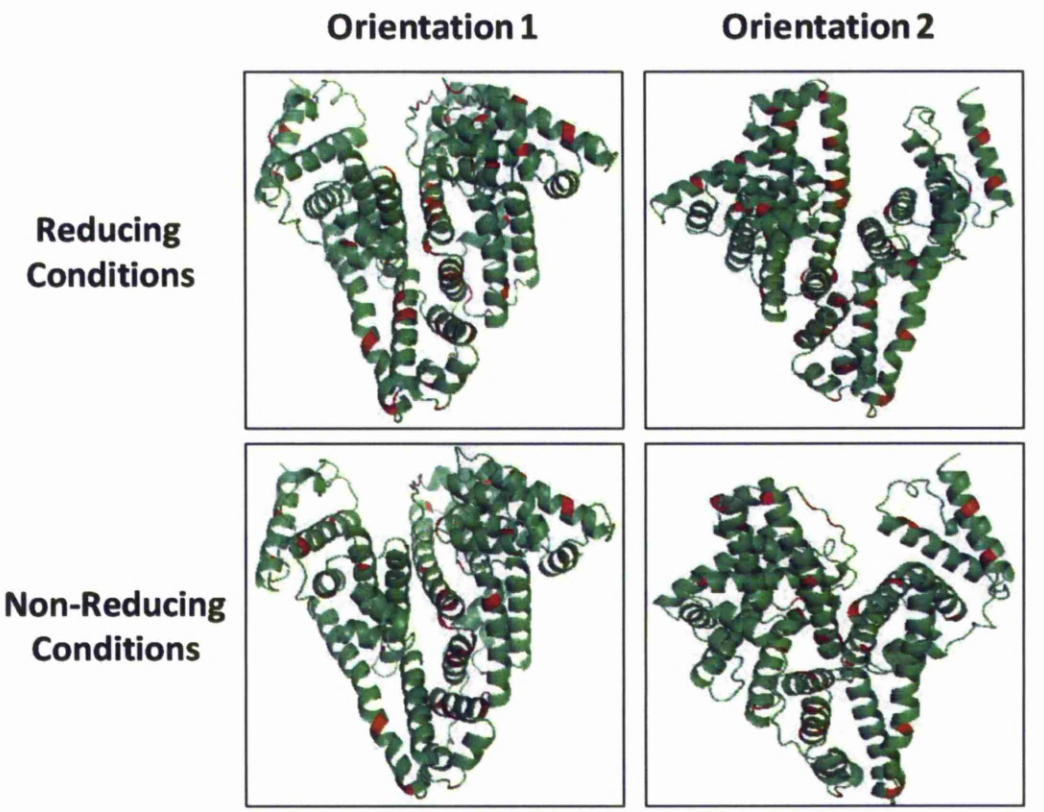


Fig 7.8. InpA cleavage sites on BSA (shown in red) as identified by MS analysis. BSA displayed in two opposite orientations. 3D model taken from the SWISS-MODEL Repository database (http://swissmodel.expasy.org/repository/ [Kopp & Schwede, 2006]), P02769_C00001.

Digestion fragments from InpA proteolysis of BSA under non-reducing (Table 1) and reducing (Table 2) conditions at pH 7.5. ↓ denotes position of cleavage by InpA.

Table 7.1: Non-Reducing

Fragment size (Da) and time of appearance	Corresponding predicted fragment size (Da)	Residue number	Amino acid sequence
909.9 (24 h)	910.4339	545-552	K↓T^{545}VMENFVA^{552}
962.5 (2 h)	962.4288	368-375	A↓C^{368}YSTVFDK^{375}
964 (0 h)	964.4986	42-49	K↓L^{42}VNELTEF^{49}
	964.521	201-208	A↓S^{201}IQKFGER^{208}
986 (2 h)	986.5054	330-336	F↓L^{330}YEYSRR^{336}
1052.9 (2 h)	1053.3976	165-173	K↓Q^{165}ECCQAEDK^{173}
	1052.4499	436-444	R↓C^{436}CTKPESER^{444}
1256.3 (4 h)	1256.7545	188-196	K↓V^{188}LASSARQRLR^{196}
1278.3 (4 h)	1278.561	117-127	K↓P^{117}DPNTLCDEFK^{127}
1343.4 (1 h)	1343.7542	206-217	F↓G^{206}ERALKAWSVAR^{217}
1347.7 (0 h)	1347.7743	201-212	A↓S^{201}IQKFGERALKA^{212}
1511.8 (0 h)	1511.842	414-427	K↓V^{414}PQVSTPTLVEVSR^{427}
	1511.9519	521-533	K↓Q^{521}IKKQTALVELLK^{533}
1530.5 (1 h)	1530.6893	313-324	K↓D^{313}VCKNYQEAKDA^{324}
1752	1752.078	336-350	R↓H^{336}PEYAVSVLLRLAK^{350}
1844.5 (7 h)	1844.8483	99-114	R↓N^{99}ECFLSHKDDSPDLPK^{114}
1986.8 (2 h)	1986.1269	226-242	A↓E^{226}FVEVTKLVTDLTKVHK^{242}
2172.9 (2 h)	2172.1552	128-144	K↓A^{128}DEKKFWGKYLYEIAR^{144}
	2172.24	206-224	F↓G^{206}ERALKAWSVARLSQKFPK^{224}
2716.9 (2h)	2717.3986	128-148	K↓A^{128}DEKKFWGKYLYEIARHPYF^{148}

Table 7.2: Reducing

Fragment size (Da) and time of appearance	Corresponding predicted fragment size (Da)	Residue number	Amino acid sequence
906.40 (0 h)	906.47	181-187	K↓I^{181}ETMREK^{187}
930.90 (0 h)	930.55	210-217	A↓L^{210}KAWSVAR^{217}
962.50 (0 h)	962.43	368-375	A↓C^{368}YSTVFDK^{375}
964.00 (1 h)	964.50	42-49	K↓L^{42}VNELTEF^{49}
	964.52	201-229	A↓S^{201}IQKFGER^{208}
986.00 (0 h)	986.51	330-336	F↓L^{330}YEYSRR^{336}
999.00 (24 h)	998.68	342-350	A↓V^{342}SVLLRLAK^{350}
1052.90 (1 h)	1053.40	165-173	K↓Q^{165}ECCQAEDK^{173}
	1052.45	436-444	R↓C^{436}CTKPESER^{444}
1065.70 (0 h)	1065.59	197-229	R↓L^{197}RCASIQKF^{205}
1148.30 (1 h)	1147.61	191-200	A↓S^{191}SARQRLRCA^{200}
	1147.64	247-232	K↓P^{223}KAEFVEVTK^{232}
1190.40 (1 h)	1190.62	71-81	F↓G^{71}DELCKVASLR^{81}
1277.00 (0 h)	1276.74	200-211	A↓S^{200}IQKFGERALK^{211}
1392.40 (4 h)	1391.65	139-126	K↓K^{115}PDPNTLCDEF^{126}
	1391.71	488-500	F↓S^{488}ALTPDETYVPKA^{500}
1530.50 (1 h)	1530.69	313-324	K↓D^{313}VCKNYQEAKDA^{324}
1700.60 (24 h)	1700.79	348-362	R↓L^{348}AKEYEATLEECCAK^{362}
1844.50 (24 h)	1844.85	99-114	R↓N^{99}ECFLSHKDDSPDLPK^{114}
2171.60 (1 h)	2171.09	488-506	F↓S^{488}ALTPDETYVPKAFDEKLF^{506}
2247.50 (24 h)	2247.05	50-70	F↓A^{50}KTCVADESHAGCEKSLHTLF^{70}
	2247.13	286-306	K↓S^{286}HCIAEVEKDAIPENLPPLTA^{306}
2368.60 (4 h)	2368.13	117-136	K↓P^{117}DPNTLCDEFKADEKKFWGK^{136}
	2368.23	342-362	A↓V^{342}SVLLRLAKEYEATLEECCAK^{362}
2497.50 (4 h)	2496.94	81-102	R↓E^{81}TYGDMADCCEKQEPERNECF^{102}

The haem pocket of HSA spans 80 resides from Leu112 to Cys200. By comparison with the predicted 3D-structure of BSA it is also likely that the BSA haem pocket is located between Leu112 and Cys199. The hydrophobic ring of haem becomes buried in the core of subdomain IB while the propionate groups interact with the solvent and several basic amino acid side chains at the pocket entrance (Zunszain *et al.*, 2003). These include Lys190, which makes salt bridges with each of the propionates, and His146, Arg114 and Arg186, which each make singular electrostatic interactions with one of the propionate groups (Zunszain *et al.*, 2003). Two tyrosine residues (138 and 161) partially block the cavity in the absence of the ligand, but rotate upon haem binding to clamp the haem in place (Curry *et al.*, 1999). The hydroxyl group of Tyr138 is relocated to the exterior of the pocket where it interacts with the solvent (Zunszain *et al.*, 2003). Tyr161 directly interacts with haem Fe^{3+} by serving as the fifth coordination site (Zunszain *et al.*, 2003). The predicted haem-binding domain of BSA shares numerous similarities with HSA and several important amino acid residues involved in HSA haem ligation are present, but in slightly different positions for BSA (Fig 7.9). For instance, the equivalent residue to Lys190 is Lys189 in BSA, with an alanine residing at position 190. Under reducing conditions a 1148.30 Da peptide was detected, predicting cleavage at Ala190. Assuming that Lys189 of BSA establishes the same salt bridges with the haem propionates as Lys190 of HSA (Zunszain *et al.*, 2003), cleavage of Ala190 may very well disrupt haem ligation. In addition, the His146 of HSA appears to be located at amino acid residue 145 in BSA. Again, despite MS not predicting direct cleavage of His145, Arg144 situated immediately downstream is cleaved under non-reducing conditions (peptide 2172.9 Da). Arg186 and Tyr161 of HSA correspond to Arg185 and Tyr160, respectively, in BSA. However, MS did not suggest any cleavage that would lead to disruption of these two amino acid residues. Arg114 is replaced by Lys114 in BSA, which is expected to occupy a similar orientation to the haem and form a salt bridge with the propionate. Importantly, Lys114 is situated on a highly exposed loop and would be immediately available to InpA. As such it is of little surprise that Lys114 cleavage was indicated under both reducing (peptide 1844.50 Da) and non reducing (peptide 1986.80 Da) conditions. Thus, cleavage at this residue may account for lowering the albumin-haem affinity, thereby encouraging haem loss. MS also identified peptides of masses 2368.6 and 1278.3 Da under reducing and non-reducing conditions, respectively, which indicate cleavage at Lys116, which is situated on the same exposed loop as Lys114. Although the literature does not propose a direct role for Lys116 in haem binding, cleavage may disrupt this loop, and hence the Lys114-propionate

salt bridge. Peptides of masses 1052.90, 1256.30, 1278.30, 1986.80, 2172.90, 2716.90 (non-reducing), and 906.40, 1052.90, 1065.70, 1148.30, 1392.4, 1833.50, 2368.60 (reducing) were also identified, indicating further cleavages within the haem binding domain. InpA cleavages which disrupt the conformation and hydrophobic nature of this domain will potentially lower albumin-haem affinity.

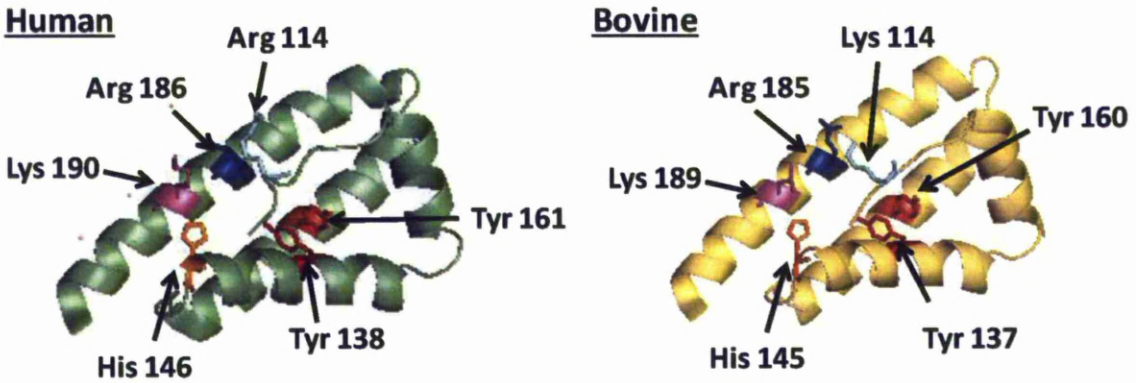


Fig 7.9. Haem pocket of HSA and that predicted for BSA based upon the SWISS-MODEL Repository database (http://swissmodel.expasy.org/repository/ [Koop & Schwede, 2006]), P02769_C00001.

The generation of different peptide fragments from albumin by InpA in the presence and absence of DTT unequivocally demonstrates that other residues became exposed and susceptible to attack under reducing conditions. Furthermore, residues were identified that may be specifically targeted by InpA to lower albumin affinity for haem. Alkaline and reducing conditions encountered in the diseased periodontal pocket would predictably result in breakdown and haem release from haemalbumin. However, under non-reducing conditions, where haemalbumin is more resistant to proteolysis (see section 7.3.3), InpA may inflict specific incisions to lower albumin affinity for haem and facilitate haem capture by *P. intermedia*. Efficient protease targeting of these susceptible residues may explain the disproportionate haem loss in comparison to protein digestion (Fig 7.2) and may obviate the requirement for gross albumin breakdown (observed only under reducing conditions) to liberate haem.

7.3.5. Reduced albumin is more susceptible to Kgp and Rgp.

Grenier *et al.* (2001c) demonstrated that Rgp and Kgp of *P. gingivalis* degrade HSA. However, in their investigation albumin degradation was monitored under reducing conditions (10 mM DTT). Therefore, it was decided to determine whether reduction of albumin and 1:0.9 haem-albumin (with 3 mM DTT) increased their susceptibility to R- and K-gingipains. As had previously been observed, albumin and haemalbumin were resistant to gingipain mediated proteolysis (Figs 7.10A & 7.10B). However, in the presence of 3mM DTT both substrates were far more susceptible (Figs 7.10B & 7.10A). After 24 h, Kgp digested 75 % of haemalbumin (Fig 7.10A2) and 100 % of albumin (Fig 7.11A4). RgpB was even more effective, resulting in near complete digestion of both substrates by the t = 0 h sample. This suggests changes in albumin structure, occurring as a result of chemical reduction, would enable degradation of this normally resistant protein by both InpA of *P. intermedia* and the gingipains of *P. gingivalis*.

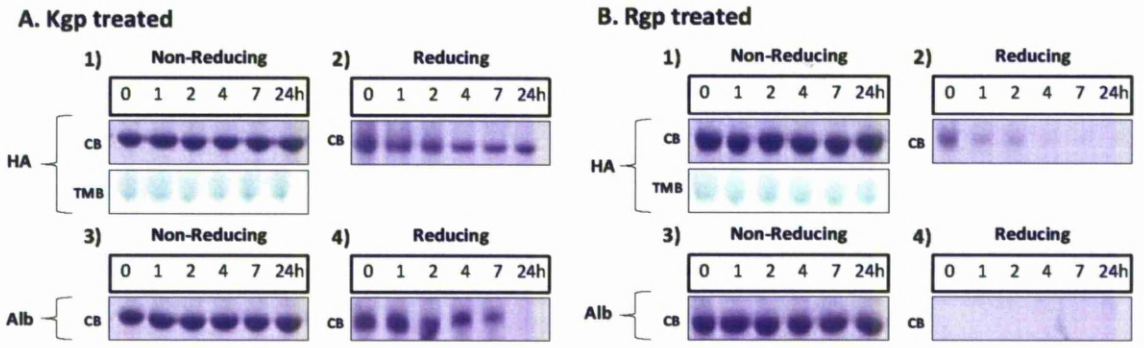


Fig 7.10. Albumin and haem-albumin (1:0.9) degradation by Kgp (A) and RgpB (B) under reducing and non-reducing conditions. HA = haemalbumin; Alb = albumin.

7.3.6. InpA degradation of haemalbumins with greater haem to protein molar ratios.

Albumin possesses a high-affinity site for haem binding ($K_a \sim 10^8\ M^{-1}$) and at least ten subsidiary lower-affinity sites (Beaven *et al.*, 1974). Haemalbumin complexes with haem to protein molar ratios 2:0.9, 4:0.9, and 8:0.9 (16 μM) were degraded by InpA (4 μM) across a range of pH. This yielded two main fragments as shown for the 4:0.9 haem:albumin complex (Fig 7.11 arrowed). One peptide (~24kDa) was gradually digested, whilst the ~32 kDa peptide appeared to be more resistant, and retained haem-staining and accumulated over the digest.

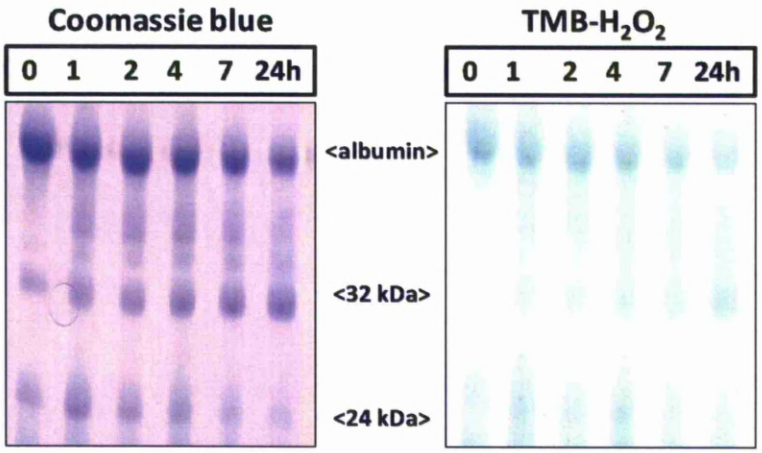


Fig 7.11.Degradation of haemalbumin (4:0.9 haem:protein) by InpA at pH 7.5.

Susceptibility of haemalbumin to *P. gingivalis* cells decreases with increasing ratio of bound haem (Smalley & Birss, 1997), whereas here, the converse was seen for InpA, particularly after 24 h (Fig 7.12). Proteolysis was lowest at pH 5.5 regardless of the haem:protein ratio, and greatest at pH 7.5 where the B form would predominate (Harmsen *et al.*, 1971). Although, staining also demonstrated haem release during the digestions (e.g. Fig 7.11), accurate estimates of haem loss were not possible at high haem : protein ratios possibly due to saturation of the bands with the TMB-H_2O_2 stain. Increasing the haem : protein molar ratio also had the effect of augmenting InpA proteolysis of haemalbumin under reducing conditions (Fig 7.13).

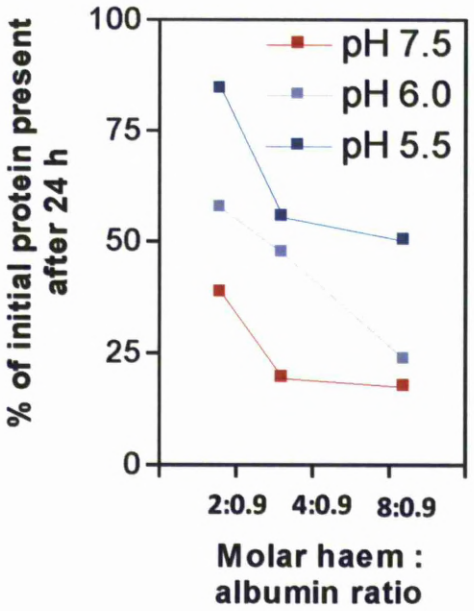


Fig 7.12. Densitometric scanning of Coomassie blue staining used to quantify degradation of haemalbumin (2:1, 4:1, and 8:1 haem : protein molar ratios) by InpA after 24 h over a range of pH.

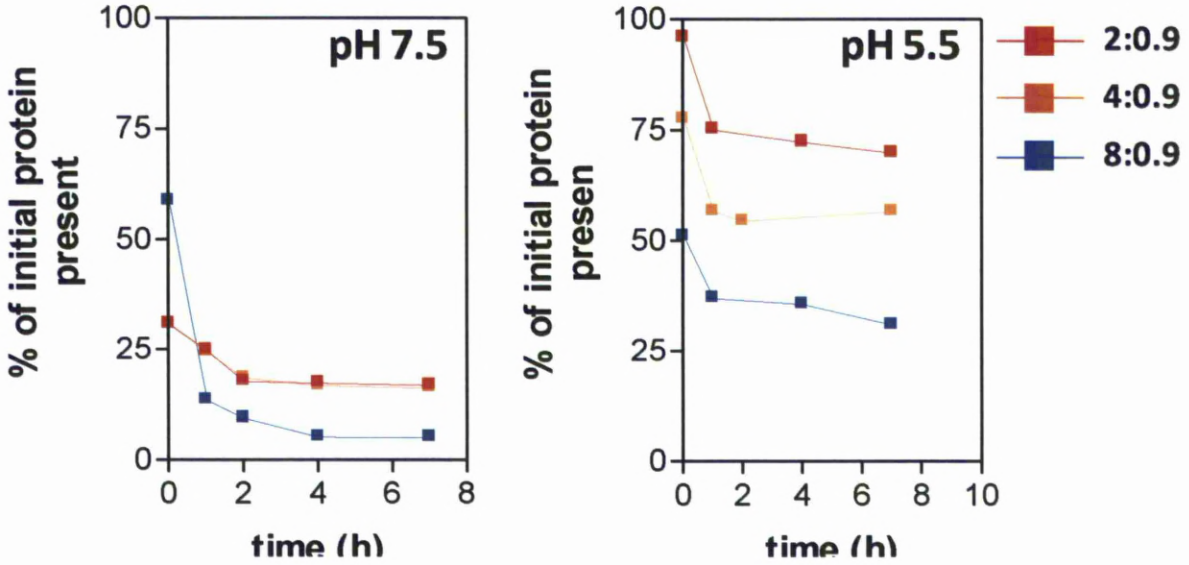


Fig 7.13. Protein loss induced by InpA from haemalbumins with 2:0.9, 4:0.9, and 8:0.9 haem : protein molar ratios under reducing conditions. Protein loss was determined by densitometry of Coomassie blue staining.

These results demonstrate that the susceptibility of haemalbumin to InpA increases with greater molar ratios of bound haem. Haem ligand-induced susceptibility to digestion has previously been observed for albumin incubated with mammalian trypsin and chymotrypsin (Shin *et al.*, 1994). These effects most probably occur as a result of ligand-induced conformational changes. This phenomenon may enable InpA to more effectively degrade albumin molecules richer in haem.

7.3.7. The diseased periodontal pocket may represent the optimum environment for albumin breakdown by InpA and enzyme of periodontal pathogens .

The preceding studies demonstrated not only that InpA can mediate haem release from haemalbumin, but revealed that, unlike haemoglobin breakdown (see Chapter 3), this was dependent on chemical reduction. Production of VSCs by oral bacteria in diseased periodontal sites has long been connected with the progression of periodontitis (Horowitz & Folke, 1973). The present chapter describes a unique mechanism by which periodontal pathogens may take advantage of the low redox potential, produced by VSCs, and the alkaline pH of the diseased periodontal pocket to aid proteolytic haem acquisition from

haemalbumin. The low redox potential may also severely perturb haem reprocessing by the host by lowering the affinity of albumin for haem (Morgan *et al.*, 1976). It may also be significant that the affinity of albumin for haem decreases approaching pH 8, owing to the transition to the B form (Reddi *et al.*, 1981). Bleeding, and hence increased abundance of haem from haemoglobin, is also associated with diseased periodontal pockets (Kenny & Ash, 1969). Normally, haem bioavailability is restricted by albumin and haemopexin, which bind both free haem and remove haem from metHb (Gattoni *et al.*, 1996). Haemolytic and proteolytic sub-gingival microorganisms may transiently elevate local haem levels in excess of the haem-carrying capacity of haemopexin, thus elevating levels of haemalbumin. This is physiologically relevant as the susceptibility of haemalbumin to InpA increases with increasing molar ratio of bound haem. As such the diseased periodontal pocket may be a perfect environment for the proteolysis of albumin. Considering that albumin is the major protein and haem sequestering component in GCF and the periodontal pocket (Curtis *et al.*, 1988; Muller-Eberhard & Morgan, 1975), this may play an important role in pigment formation by BPAs.

Exploitation of a highly reduced environment may not just be limited to *P. intermedia* and *P. gingivalis* degradation of albumin. The low redox environments of the diseased gingival sulcus and periodontal pocket may promote degradation of other GCF proteins and components of the immune response by other enzymes of periodontal pathogens. Of particular interest is haemopexin which has six disulfide bonds (Takahashi *et al.*, 1985), four of which (in the case of apohaemopexin) can be disrupted by chemical reduction resulting in irreversible abolition of haem binding (Suttnar & Hrkal, 1986). IgG also undergoes significant alterations of its quaternary structure upon reduction of its single disulfide bond (Seegan *et al.*, 1979). The reducing environment not only interferes with the physiological functions of a wide range of proteins but, may also facilitate their cleavage by proteolytic bacteria, to satisfy various nutritional requirements. These findings further underline the versatility of the protease systems of BPAs, and clearly demonstrate that *P. intermedia* and *P. gingivalis* can compete with the host to obtain haem.

7.3.8. Haem binding and pH alter the susceptibility of haemopexin to InpA.

Although InpA mediated extensive breakdown of haem-haemopexin and apo-haemopexin at pH 6.0 (70 % and 93 %, respectively; Figs 7.14, A & B), it was evident that

haem binding stabilized haemopexin to proteolysis. Despite not greatly affecting secondary structure (Bernard *et al.*, 1980; Shipulina *et al.*, 2001), haem binding leads to dramatic changes in haemopexin tertiary structure which are thought to facilitate haemopexin interaction with liver parenchymal cells (Javid *et al.* 1972; Morgan & Muller-Eberhard, 1972). These changes, which are sufficient to lower antibody binding by 30 % in immunochemical assays (Javid *et al.*, 1972), also lower haemopexin protease sensitivity (Hrkal *et al.*, 1974; Morgan & Smith, 1984) due to transition from a flexible open form to a tight association of domains (Paoli *et al.*, 1999). In apohaemopexin, the 20-residue linker that joins the two homologous domains, in particular, is highly protease sensitive (Hrkal *et al.*, 1974; Morgan & Smith, 1984). This may explain the greater digestion for apo-haemopexin compared to haem-haemopexin. However, TMB-H_2O_2 staining appeared to show little haem loss over 24 h (Fig 7.14, A), although the R_f of the main TMB-H_2O_2-stained haemopexin band increased slightly (Fig 7.14, A HPX), which was accompanied by liberation of a haem-carrying fragment (~60 kDa; Fig 7.15 A, arrowed). This suggested that degradation liberated the haem binding domain.

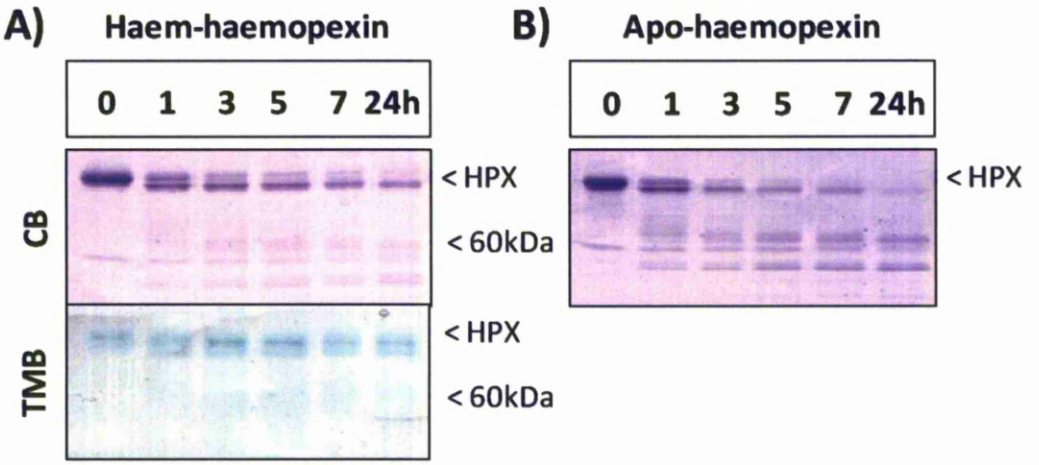


Fig 7.14. Breakdown of apo-haemopexin and haem-haemopexin by InpA.

Apo-haemopexin was most susceptible to InpA at pH 5.5 (Fig 7.15, C) and, whilst haem-haemopexin was generally more resistant than its haem free-counterpart, it also displayed dramatically increased susceptibility at lower pH (Fig 7.15). Haem-haemopexin complex formation normally results in increased thermal stability (Shipilina *et al.*, 2001) and protease resistance (Hrkal *et al.*, 1974; Morgan & Smith, 1984). However, the haem- induced protease resistance was overcome at low pH. This may be due not only to increased InpA

activity but to protonation of basic residues clustering round the haem and His213 (which co-ordinates the haem iron) (Paoli *et al.*, 1999). This would disrupt the compact haem-haemopexin complex in a manner analogous to the increased molecular flexibility resulting from the low pH environment of the endosome where haem release is facilitated (Smith & Hunt, 1990; Rosell *et al.*, 2005). Disruption of the tight haem-haemopexin complex would concomitantly result in the observed increased protease sensitivity. In this respect it must be remembered that *P. intermedia* exhibits an acid pH growth optimum. As before, the main haem-carrying haemopexin band increased in R_f (Fig 7.15, arrowed), and a TMB-H_2O_2-stained fragment (~60kDa) also appeared during digestion, being most apparent at pH 5.5, concomitant with greatest proteolysis (Fig 7.15, C arrowed).

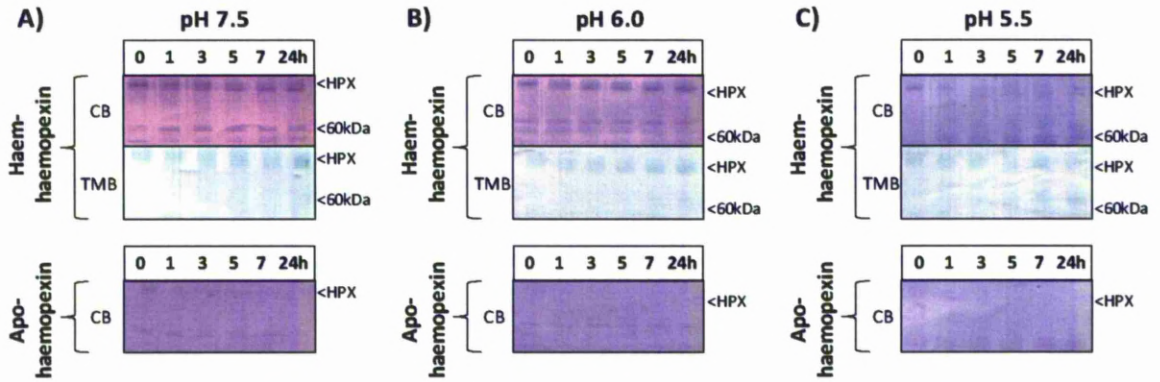


Fig 7.15. Activity of InpA towards haemopexin and haem-haemopexin at different pH.

The ability of InpA to incapacitate haem-binding by apo-haemopexin was also investigated, since it was evident (Figs 7.14 and 7.15) that the apo- form was more easily digested. Apo-haemopexin (16 μM) was incubated with InpA (2 μM) at pH 6.0. Pre-incubation of haemopexin with InpA for 2 h reduced TMB-H_2O_2 staining by ~50 % following exposure to haem (Fig 7.16), indicating decreased haem pickup. In a gingival crevice, the host retaliates against bacterial infection by invoking an inflammatory response, and *de facto* increasing levels of plasma proteins including haemopexin. If haem pickup by apo-haemopexin was attenuated by InpA, this may reduce local haem clearance and increase its bioavailability at periodontal sites, selectively advantaging *P. intermedia* and other haem scavenging bacteria. However, the 60kDa digestion fragment retained some haem staining (Fig 7.16, arrowed), and it may be concluded that InpA-mediated perturbation of haem binding by haemopexin may represent an auxiliary acquisition mechanism.

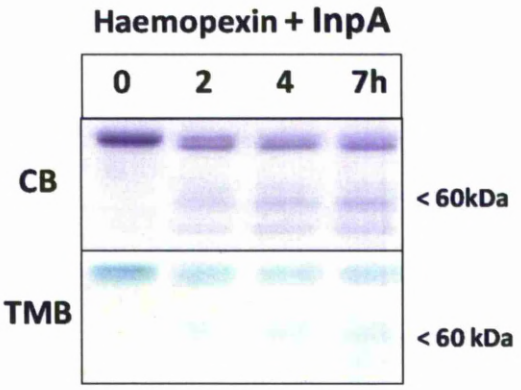


Fig 7.16. Effect of InpA pre-treatment on haem binding by apo-haemopexin.

In conclusion, InpA may function to preserve haem reserves by degrading the more protease- sensitive apo-haemopexin, in addition to efficiently degrading haem-haemopexin, especially under acid conditions. This may represent another paradigm for haem acquisition involving InpA. It would appear that InpA may play a role in pigment formation by *P. intermedia* by breaking down not only haemopexin, but albumin and haemoglobin for subsequent haem release. In a human host, free haemoglobin released from erythrocytes is rapidly captured by haptoglobin (Evans *et al.*, 1999), the circulating concentrations of which are observed to increase in response to periodontal inflammation (Ebersole *et al.*, 1997). Although there is currently no evidence to show that haptoglobin is a susceptible substrate for *P. intermedia*, the organism may also have to compete with haptoglobin for access to haemoglobin. Considering how effective InpA is at degrading all of the other major components of host haem sequestration, it is logical to hypothesize that haptoglobin may too be a target of this versatile enzyme. Preliminary work revealed partial proteolysis of haptoglobin by InpA (data not shown). Further investigations are therefore required.

CHAPTER 8

Role of P. intermedia and P. gingivalis LPS in black pigmentation.

8.1. INTRODUCTION

The ability of a pathogen to scavenge essential nutrients within the host is one of the paramount challenges in establishing an infection. In addition to the HmuR and HmuY haem binding proteins of *P. gingivalis*, other components, namely Omp26, Tla, HemR, IhtB, HBP35, HusA, RagB, and RagA, have been implicated in cell surface haem capture and binding (Bramanti & Holt, 1993; Aduse-Opoku *et al.*, 1997; Karunakaran *et al.*, 1997; Hendtlass *et al.*, 2000; Hanley *et al.*, 1999; Bonass *et al.*, 2000; Shoji *et al.*, 2010; Gao *et al.*, 2010). *P. intermedia* cell surface haem binding has also been described (Thompkins *et al.*, 1997; Leung *et al.*, 1998; Yu *et al.*, 2007), however, the precise mechanism(s) through which the bulk of haem is captured, and which contribute to the pigmentation phenotype, is far from clear. The ubiquitous nature of cell surface LPS of haem-pigmenting species makes it a potential haem binding component, and this has been hypothesized by Grenier (1991) for *P. gingivalis*. In support of this, haem-replete cells of *P. gingivalis* bind more haem than haem-starved cells (Smalley *et al.*, 2003), and it is noteworthy that haem availability influences the composition of *P. gingivalis* LPS, which binds more haem in haem-replete conditions (Cutler *et al.*, 1996). Furthermore, LPS biosynthesis-related genes are required for colony pigmentation of *P. gingivalis* (Sato *et al.*, 2009).

LPS forms complexes with haemoglobin which increases biological activities of rough and smooth LPS (Kaca *et al.*, 1994; Roth *et al.*, 1994); this interaction resulting in changes in LPS conformation (Jürgens *et al.*, 2001; Brandenburg *et al.*, 2003; Howe *et al.*, 2007) and in methaemoglobin and haemichrome production (Kaca *et al.*, 1995). LPS-induced oxidation and denaturation of haemoglobin could be an important process for the localised release of haem. This chapter describes an in-depth investigation into the mechanisms and relevance of interactions between LPS from *P. intermedia* NY653 and *P. gingivalis* W50 and WPH35, with both haem and haemoglobin in the context of haem acquisition and black-pigment formation.

8.2. METHODS

8.2.1. Investigating LPS interaction with haem and oxyhaemoglobin.

In assays to determine interactions between LPS, haem, and oxyHb, *P. gingivalis* W50 and WPH35, and *P. intermedia* NY653 LPS pellet and supernatant fractions (described in Section 2.3.1) were prepared at 0.5, 1, 2 or 4 mg ml $^{-1}$ in 0.14 M NaCl, 0.1 M Tris-HCl (pH 7.5). Haem was at 16 or 500 μM and haemoglobin and myoglobin preparations were at 4 and 16 μM respectively. All LPS fractions were observed to promote oxyhaemoglobin oxidation. Furthermore NY653 and W50 LPS liberated haem present in Hb (Section 8.3.6). The contribution made by lipid A in LPS-haem binding interactions and towards Hb oxidation was assessed by treatment of LPS with polymyxin (1 mg ml $^{-1}$). InpA of *P. intermedia* and HmuY and Kgp of *P. gingivalis* are intrinsically involved in haem release from haemoglobin (Byrne *et al.*, 2010; Smalley *et al.*, 2002; 2004; 2007; 2008). To examine potential contributory activity of LPS in these processes, bacterial proteins were incorporated as follows: InpA (2 μM); HmuY (16 μM); Kgp (0.6 μM). All experiments were carried out at 37 °C in 0.14 M NaCl, 0.1 M Tris-HCl (pH 7.5) and the UV-visible spectra recorded periodically, along with sampling for SDS- and native-PAGE on 15 % polyacrylamide gels; samples being solubilised in non-reducing LSB for 1 h at 37 °C. LPS was visualised using silver staining after SDS-PAGE on 10 % gels, with samples first solubilised in reducing LSB. Haem binding to LPS immobilized on nitrocellulose was visualized by TMB-H_2O_2 staining.

8.2.2. LPS binding to haem-conjugated agarose.

Haem-agarose beads (equivalent to 4 μM haem) were extensively pre-washed to remove unconjugated haem and mixed with LPS (2 mg ml^{-1}) for 1 h at 37 °C with gentle agitation, and then washed to remove unbound LPS; this initial supernatant being collected. Any haem-agarose-bound LPS was desorbed by incubation with non-reducing SDS-PAGE sample buffer for 1 h at 22 °C.

8.2.3. Titration of LPS with haem.

After confirmation of haem binding by LPS, the interaction was more closely scrutinized spectroscopically by titrating LPS (1 mg ml^{-1}) with serial additions of 1 or 5 μM haem at pH 7.5 (0.14 M NaCl, 0.1M Tris-HCl) or at pH 6.0 (0.2 M phosphate buffer). The UV-visible spectra were recorded and corrected for the background absorbance of an LPS control sample (1 mg ml^{-1}). To compensate for the reduction in LPS concentration with

subsequent additions of haem, equal additions of buffer were added to the LPS control. Difference spectra were created by subtracting the spectra of the control haem from those of the corrected LPS-haem. Where appropriate, changes in Soret absorbance and wavelength were plotted versus [haem].

8.2.4. LPS acquisition of haem from haemoglobin-agarose.

To further characterise haem release from haemoglobin, and to eliminate the problem of the overlapping haemoglobin and haem spectra, pre-washed Hb-agarose beads (~ 10 μM with respect to the Hb tetramer) were incubated with LPS (0.5 mg ml^{-1}) in distilled water with gentle agitation, and haem released into the bead-free supernatant was assayed spectroscopically. The supernatant samples were then returned to the incubation following recording of spectra.

8.2.5. Precipitation of LPS with ethanol.

Haem-LPS complexes in solution were precipitated with 3 volumes of cold ethanol containing 300 mM sodium acetate (to enhance precipitation [Apicella, 2008]) for 18 h at -22 °C. Precipitates were reconstituted in buffer (pH 7.5) and immobilized on nitrocellulose.

8.2.6. Circular dichroism of LPS-Hb interactions.

CD was kindly performed by Dr H. Wojtowicz (Laboratory of Biochemistry, Institute of Biochemistry and Molecular Biology, University of Wroclaw, Poland) to monitor changes of Hb globin chains in response to incubation with LPS. Near-UV spectra (x 3) were recorded at 2 min intervals and averaged. Centrifugal buffer exchange using a Microcon YM-30 filter was used to reconstitute stock oxyHb in H_2O and remove Tris. LPS (NY653 and W50 LPS) and oxyHb, made up in H_2O were at 1 mg ml^{-1} and 20 μM respectively in the reaction mixture.

8.3. RESULTS AND DISCUSSION

8.3.1. Confirmation of LPS-haem binding.

WPH35 LPS and W50 LPS pellet (LPS-P fraction), but not LPS supernatant (LPS-S fractions), immobilized on nitrocellulose and incubated with haem at pH 6.4 and 7.5 stained positively with TMB-H_2O_2 (Fig 8.1). *P. intermedia* NY653 LPS (not separated into

supernatant and pellet) also bound haem at both pH 7.5 and 6.4 (Fig 8.1 A). LPS (1 mg mL^{-1}) incubated with haem (500 μM; 37 °C for 1 h) in aqueous solution was precipitated with ethanol. TMB staining indicated haem in association with precipitated LPS (Fig 8.1, B). Ethanol treatment of LPS-free controls did not result in equivalent haem precipitation (Fig 8.1, B Control).

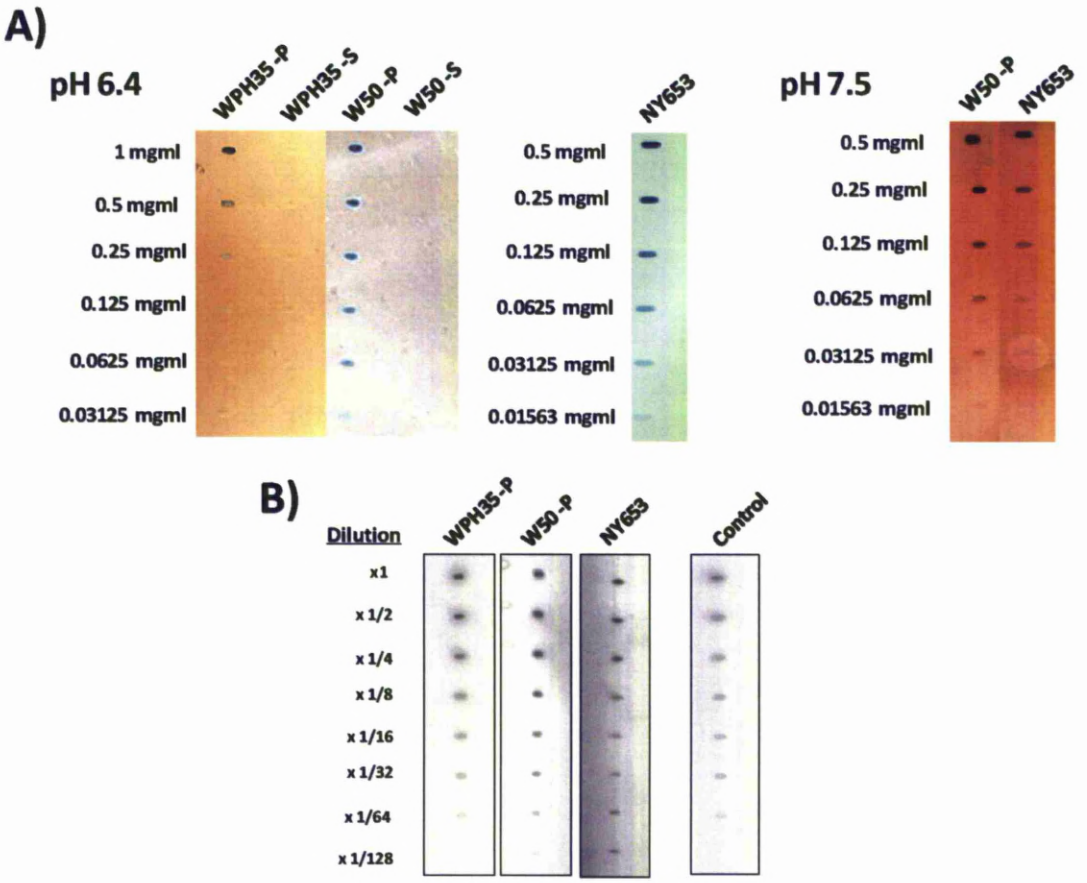


Fig 8.1. Haem pick up by LPS bound to nitrocellulose (A); Ethanol precipitation of haem in LPS-containing and LPS-free solutions (B).

Haem binding by both W50 LPS-P and –S fractions was confirmed using haem-agarose. After allowing LPS to bind to haem-agarose, bound LPS was removed by adding non-reducing LSB, and SDS-PAGE /silver staining revealed an LPS-like ladder banding pattern (Fig 8.2), similar to that for the LPS which did not bind to the haem-agarose and to an unfractionated W50 LPS sample. The indication that W50 LPS-S is also capable of binding haem (in the form of haem-agarose) is somewhat confusing as this did not pick up

haem when immobilised on nitrocellulose. This may simply reflect the lower overall capacity of the fraction to hold haem, or may be a result of low non-specific absorption to agarose matrix. NY653 LPS was bound to, and could also be desorbed from haem-agarose (Fig 8.2). Unlike W50 LPS which forms distinct ladder patterns NY653 LPS yielded diffuse staining devoid of a ladder-like pattern (Fig 8.2), suggesting that NY653 LPS is of the R-type lacking in the O-polysaccharide chains (Galanos *et al.*, 1977; Rietschel *et al.*, 1982). In this scenario, haem binding might be mediated by lipid A or core oligosaccharide chains.

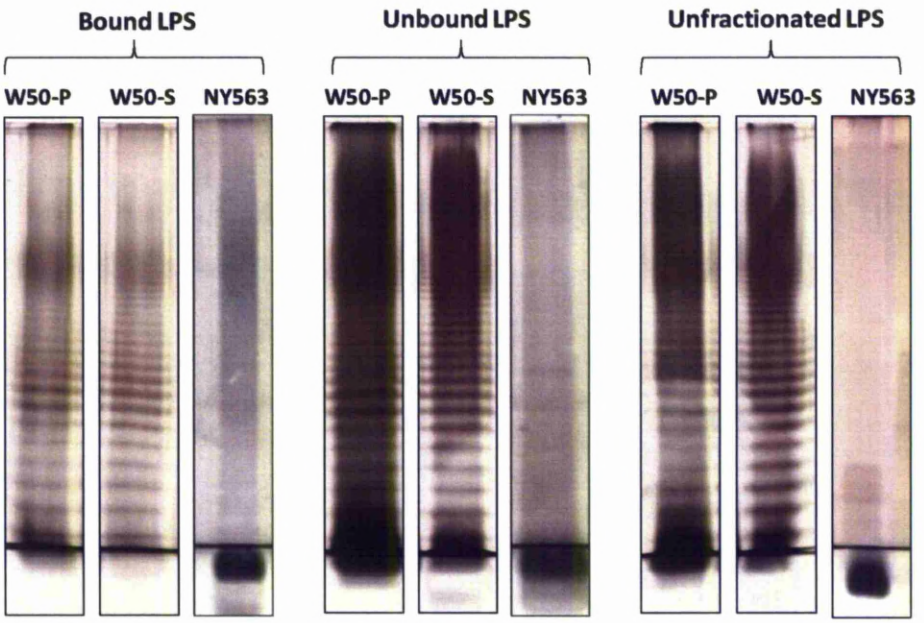


Fig 8.2. SDS-PAGE/silver-stained haem-agarose-bound and unbound W50 and NY653 LPS fractions.

The above data support the hypothesis that LPS binds haem in solution. However, SDS-PAGE of "LPS-haem complex" (formed in solution) did not reveal distinct TMB-H_2O_2-stained bands (data not shown), implying that this interaction was loose and disrupted by electrophoresis, either during solubilisation or electrophoresis *per se*. Displaced haem then migrated independently due to its negatively-charged propionate groups. Supporting this, faintly TMB-H_2O_2-stained bands were seen on a gel (after enhancement of brightness and contrast using PaintShop Pro x2) on which W50 LPS was electrophoresed and then flooded with 50 μM haem at 21 °C for 1 h and then excess haem washed off (Fig 8.3).

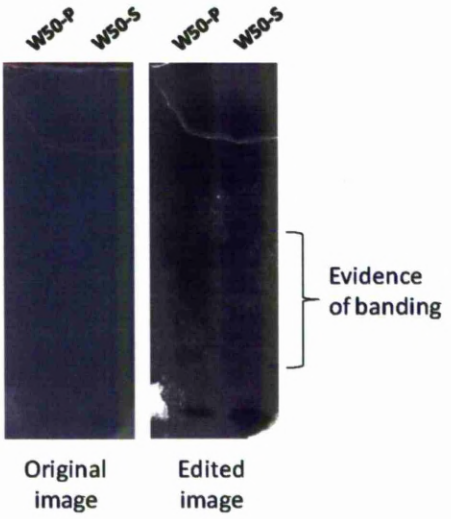


Fig 8.3. Evidence of LPS-haem binding revealed by flooding gel with haem after electrophoresis. Brightness and contrast adjusted (using PaintShop Pro x2) to reveal banding

A loose association between LPS and haem would not necessarily negate its role in cell-surface binding as a repository for haem. Extending beyond the outer membrane, LPS could serve as sites onto which haem is deposited. In addition, a low affinity interaction may also permit the ready mobilisation of haem for haemophore-mediated internalisation by, for example, the HmuY-HmuR system. However, a low specificity and affinity could render this interaction biologically insignificant in the presence of abundant high-affinity host plasma haem binding proteins, although it should be remembered that *P. gingivalis* and *P. intermedia* could "preserve" the haem deposit by degrading haem-binding proteins, particularly in the haem-free form (Jansen *et al.*, 1994; Carlsson *et al.*, 1984a; Fishburn *et al.*, 1991; Milner *et al.*, 1996).

It is unclear why the immobilised *P. gingivalis* LPS-P fraction binds haem, whereas LPS-S fractions do not. The LPS-P fraction nominally contains more uronic acid (~ 9 and 11 % for WPH35 and W50 LPS, respectively) than LPS-S (~1.5 and 2.4 % for WPH35 and W50 LPS, respectively). As such it seems unlikely that the small uronate component accounted for increased haem binding. One possibility is that LPS-P and LPS-S represent compositionally distinct fractions of a heterogeneous LPS population. The composition of *P. gingivalis* LPS is known to change when it is grown under different environmental conditions (e.g. haem repletion and limitation [Cutler *et al.*, 1996; Al-Qutub *et al.*, 2006]). The LPS extraction protocol could result in enrichment of different LPS types in the pellet and supernatant, with

more water soluble populations containing more charged polysaccharides partitioning in supernatant fractions. This possibility is supported by the identification of two types of *Proteus mirabilis* LPS differing in amounts of polysaccharide in crude LPS supernatant and pellet fractions (Greiner, 1975) and the discovery of heterogeneous lipid A (with regard to the number, type and placement of fatty acids) in a single strain of *P. gingivalis* (Jain & Darveau, 2010). However, in the present study, SDS-PAGE/silver staining revealed similar ladder patterns in both P and S W50 LPS fractions (Fig 8.2). Although this technique reveals different O chain length (Palva & Mäkelä, 1980; Tsai & Frasch, 1982) it is not reliable for distinguishing LPS varying in polysaccharide composition. Lack of haem binding for supernatant fractions may therefore be explained by contrasting LPS isoforms binding to the nitrocellulose differently (because of charge differences) and hence not having available haem-binding motifs. Charges would also affect interactions between the charged haem propionate groups. This could be investigated by separating out charged LPS fractions by techniques such as cellulose acetate electrophoresis, which could then be examined for haem binding.

8.3.2. Spectroscopic analysis of LPS-haem interactions.

LPS-haem interaction at pH 6.4 and 7.5 was confirmed spectroscopically. Sequential additions of haem to WPH35 LPS-P (1 mg ml^{-1}) initially resulted in Soret red-shift from 365 and 385 nm (signatures typical of π-π dimer at both pH 6.4 and 7.5, respectively) to 403 nm (Fig 8.4). Further additions of haem then blue-shifted the Soret back towards 365 nm and 385 nm, which could be interpreted as LPS saturation and the masking of the "haem-LPS complex" spectrum by excess haem (Fig 8.7).

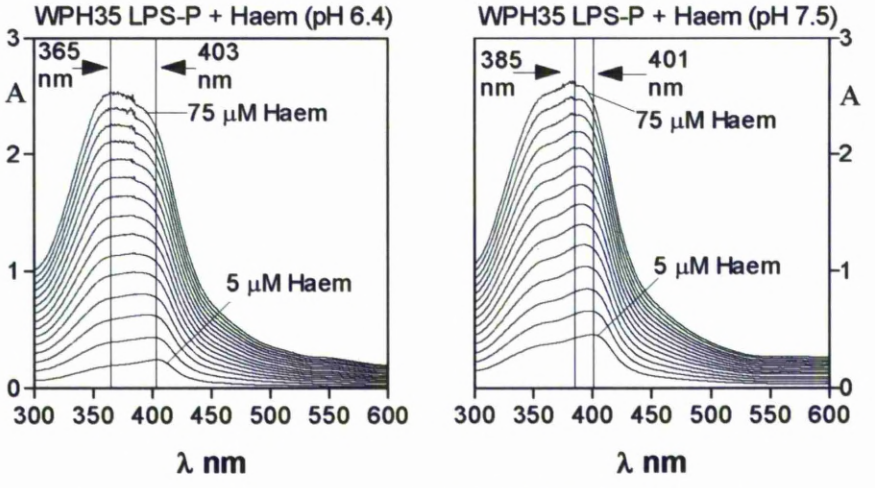


Fig 8.4. UV-visible spectra of WPH35 LPS titrated with haem.

Difference spectra were constructed (by subtracting the control spectra from those of the haem-treated LPS samples) to more precisely define the total haem-binding capacity of WPH35 LPS-P (Fig 8.5, A and B). The maximum absorbance and wavelength of resultant Soret peaks were plotted (Fig 8.5, panels C to E). The Soret λ_{max} exhibited a blue shift at both pH 6.4 and 7.5 with increasing haem concentration (Fig 8.5, panels D and F). Although this phenomenon suggests progressive change in haem speciation indicative of LPS saturation, the precise saturation point was not obvious. However, although the δA_{Soret} increase at pH 6.4 plateaued at ~20 μM haem, which could be interpreted as saturation, there was no evidence of this at pH 7.5 (Fig 8.5, panels C and E, respectively). Considering the same LPS fraction was used, it is unlikely that saturation was only achieved at one pH and not another. The linear increase in absorbance of the difference spectra at pH 7.5 may instead be explained by exaggerated artifactual discrepancies resulting from subtraction of the control haem spectra which were concomitantly aggregating (manifest as a small but gradual collapse of the Soret band).

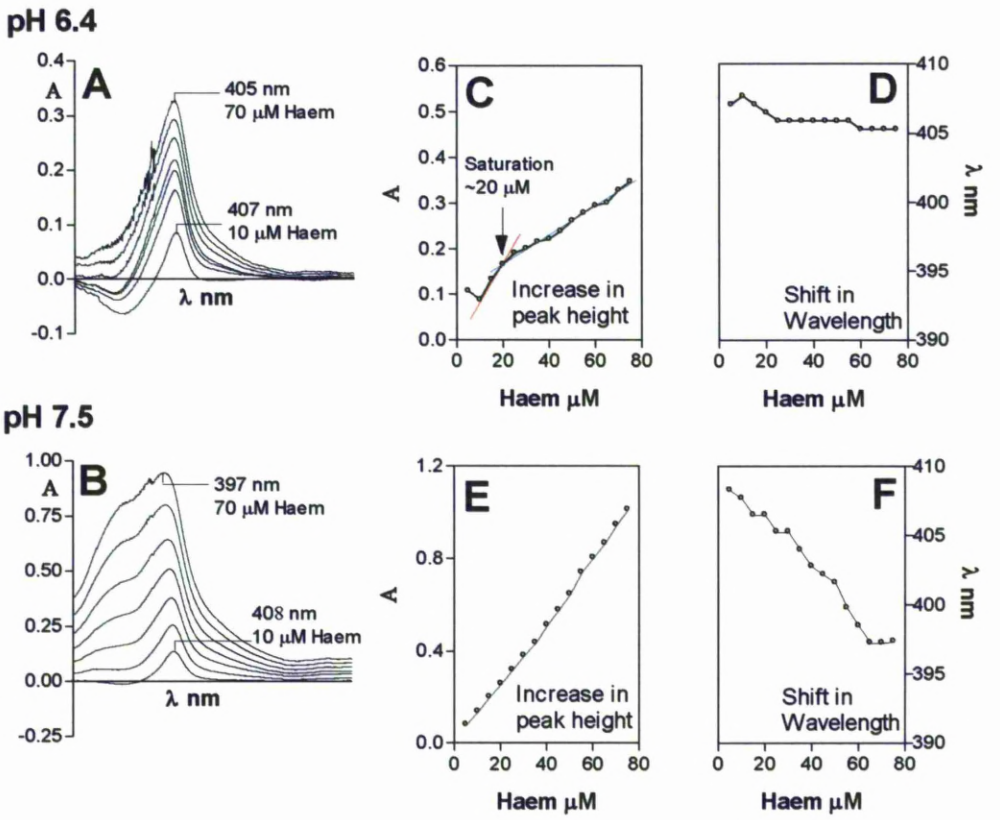


Fig 8.5. Difference spectra of WHP35 LPS-P titration with haem (A and B); δA_{Soret} versus [haem]. Theoretical saturation point at pH 6.4 is arrowed (C and E); Shift in Soret λ_{max} versus [haem] (D and F).

Spectral changes during haem titration of W50 LPS-P at pH 7.5 and 6.4 were more pronounced, also giving a Soret red shift to 400 nm (Fig 8.6). In this case, difference spectra suggested a more definite saturation point (~20 μM) at both pHs (Fig 8.7, C and E).

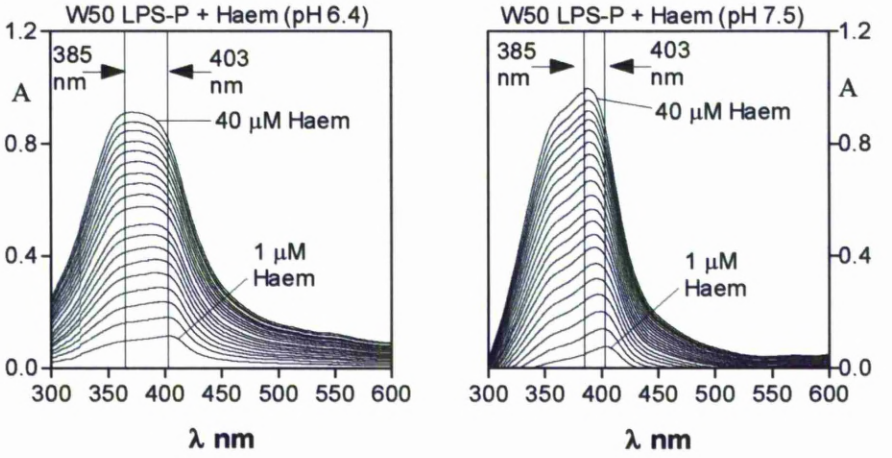


Fig 8.6. UV-visible spectra of W50 LPS-P titration with haem.

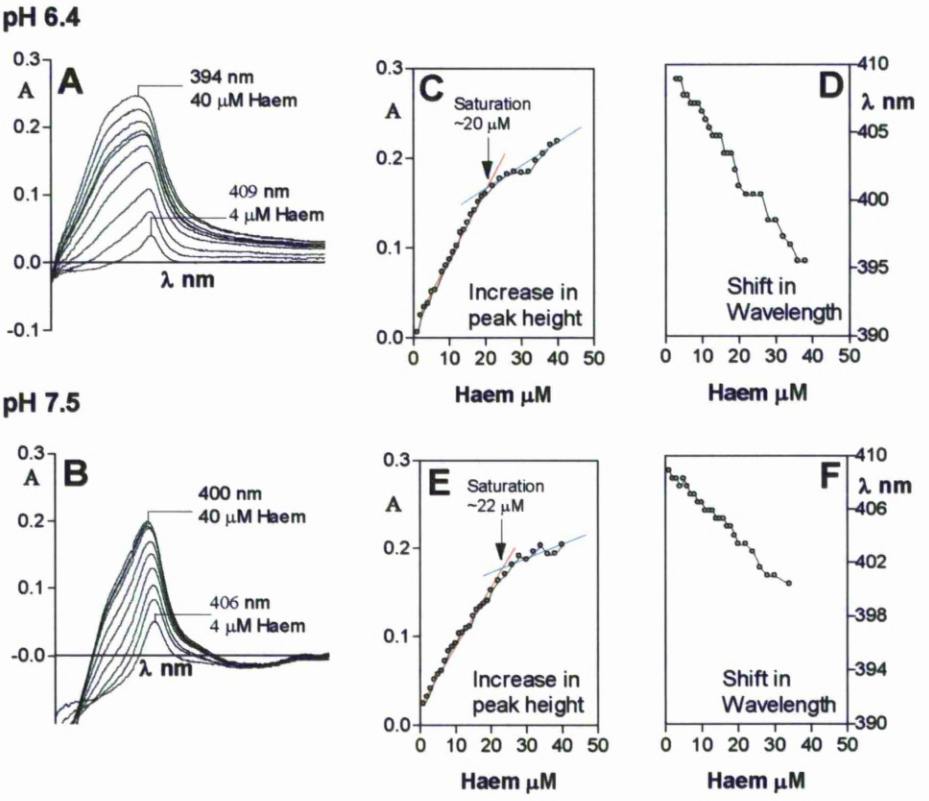


Fig 8.7. Difference spectra of W50 LPS-P titration with haem (A and B); δA_{Soret} versus [haem]. Theoretical saturation points are arrowed (C and E). Shift in Soret λ_{max} versus [haem] (D and F).

Unfractionated NY653 LPS (1 mg ml^{-1}) also revealed initial Sorets at 403 nm (pH 6.4) and 406 nm (pH 7.5) (Fig 8.8). Difference spectra could be interpreted as demonstrating saturation for 1 mg ml^{-1} NY653 LPS at ~10 μM haem (Fig 8.9).

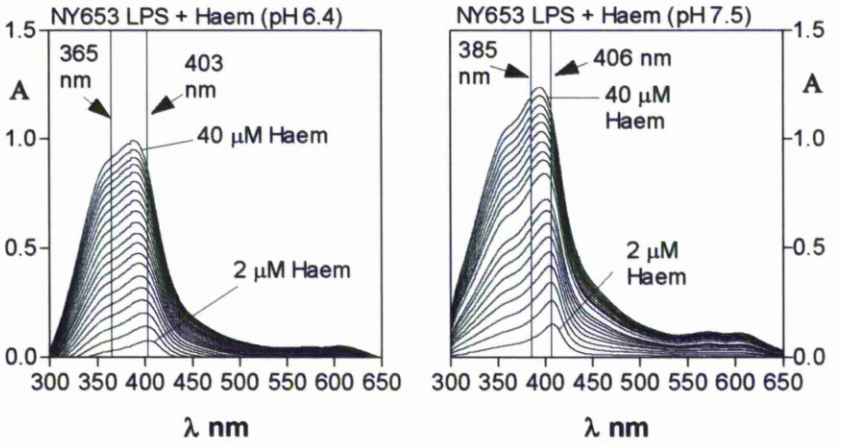


Fig 8.8. UV-visible Spectra of NY653 LPS titration with haem.

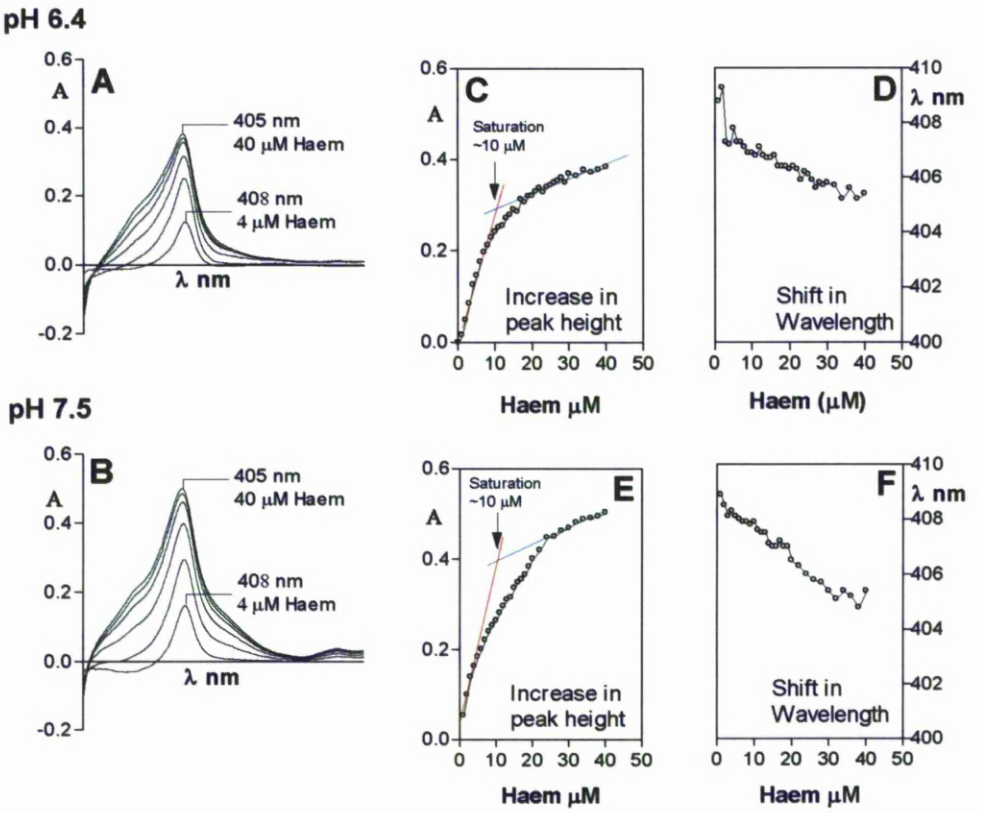


Fig 8.9. Difference spectra of NY653 LPS titration with haem (A and B); δA_{Soret} versus [haem]. Theoretical haem saturation points are arrowed (C and E). Shift in Soret λ_{max} versus [haem] (D and F).

Closer inspection of the difference spectra provides an insight into the speciation of bound haem. Each LPS type exhibited similar spectral changes with haem addition (suggesting similar interactions with haem) which were most obvious for NY653 LPS at pH 7.5 (Fig 8.10). In water, even at relatively low haem concentrations, the π-π dimer and not the monomer is overwhelmingly dominant regardless of pH (Asher *et al.*, 2009) and this was observed for haem controls (data not shown). However, at low haem concentration in the presence of LPS, difference spectra revealed sharp Soret bands at ~ 400 nm (Fig 8.10) typical of haem monomers. Although of low intensity due to low haem concentration, the bands at ~ 620 nm and 500 nm were also indicative of the presence of monomers (Asher *et al.*, 2009). Further haem addition resulted in the Soret band blue-shift, its reduction in sharpness relative to the 365 nm shoulder, and replacement of 500 and 620 nm bands with those at 575 and 600 nm (Fig 8.10), which is typical of the μ-oxo dimer (Asher *et al.*, 2009; de Villiers *et al.*, 2007). Based on these observations, the changes in the δA_{Soret} vs [haem] plot may represent a saturation point where the formation of the μ-oxo dimers from the monomer is favoured.

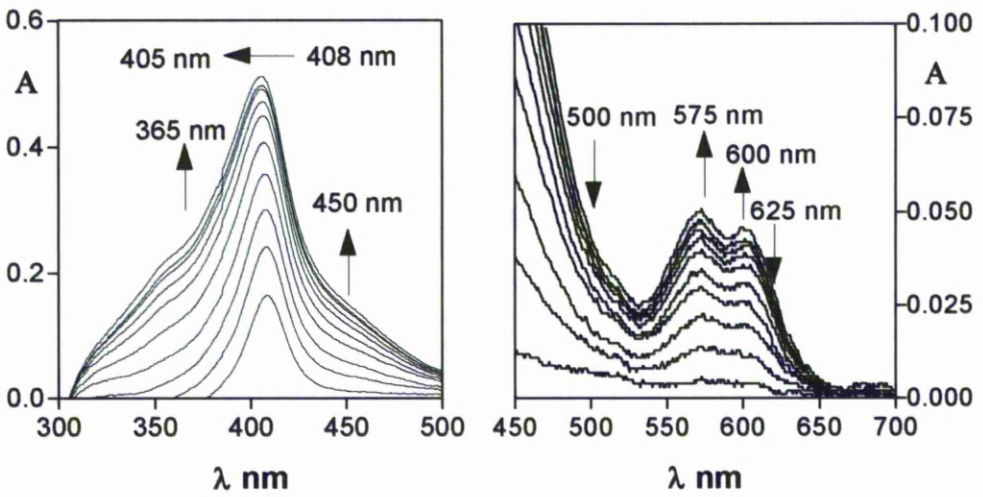


Fig 8.10. Difference spectra of titration of NY653 LPS with haem at pH 7.5.

In aqueous solution, π-π dimers comprising two five-coordinated iron porphyrins with outwardly directed H_2O/OH axial ligands are the predominant species (de Villiers *et al.*, 2007). Disruption of the axial solvation sphere in miscible aprotic solvents or in high salt concentration, which competes for hydration, will favour μ-oxo dimer formation (de Villiers *et al.*, 2007; Asher *et al.*, 2009). Mμ-oxo dimers also form within micelles of certain lipids (e.g. TWEEN-20 and

Dodecyltrimethylammonium bromide, DTAB), presumably by similar mechanisms of hydration exclusion (Casabianca *et al.*, 2009). It is likely that lipophilic haem spontaneously embeds (or is ensnared by specific interactions with LPS moieties) within hydrophobic LPS micelles and aggregates, where the equilibrium between μ-oxo and π-π dimers favours the former due to the inability to form an axial hydration sphere (Casabianca *et al.*, 2009). Under conditions such as this which disfavour π-π dimer formation, both monomers and μ-oxo dimers will probably exist at low and high haem concentrations, respectively. This would implicate LPS as the predominant cell surface haem binding entity for the pigment of *P. gingivalis*, which predominantly comprises the $[Fe(III)PPIX]_2O$ dimer (85-90% of total pigment) (Smalley *et al.*, 1998).

The existence of monomeric haematin (Fe(III)PPIX.OH) as the main haem component of the pigments of *P. intermedia* and *P. nigrescens* (Smalley *et al.*, 2003a) may also be explained by the action of LPS, since any haem initially bound to it as the μ-oxo dimer will split into Fe(III)PPIX.OH monomers following protonation of the oxo-bridge (Asher *et al.*, 2009; Casabianca *et al.*, 2009; Silver & Lukas., 1983). This is highly possible since, unlike *P. gingivalis*, *P. intermedia* produces an acid growth endpoint (Shah & Williams, 1987; Takahashi & Yamada, 2000), and importantly, pigment formation is associated with a reduction in pH during growth on blood agar (Smalley *et al.*, 2003a). It is this acid environment which favours monomer formation. In this regard it is noteworthy that difference spectra of NY653 LPS titrated with haem at pH 5.5 revealed monomer and not μ-oxo dimer (Sharp ~ 400 Soret band with no prominent 365 nm shoulder and ~ 620 nm and 500 nm bands) even at 40 μM haem (Fig 8.11). More informative techniques such as FTIR (Fourier Transform Infrared Spectroscopy) or Raman spectroscopy may confirm these hypotheses.

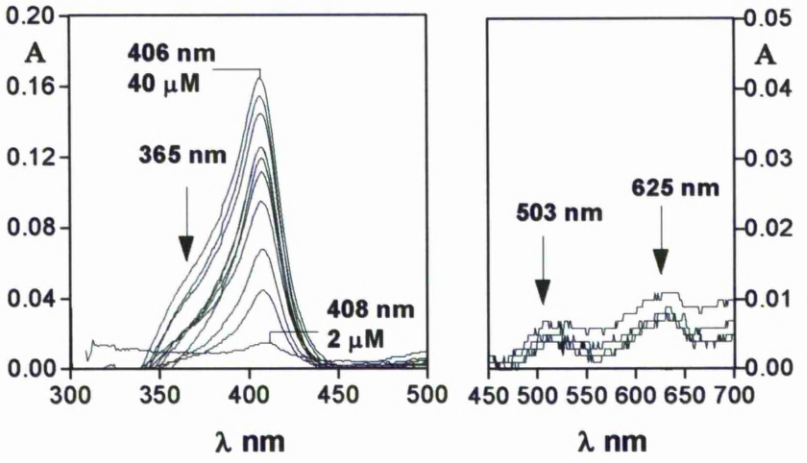


Fig 8.11. Difference spectra of titration of NY653 LPS with haem at pH 5.5. For sake of clarity not all spectra are displayed in the right hand side panel.

8.3.3. Identification of potential LPS moieties involved in haem binding.

Although variable, the composition of LPS from several strains of *P. gingivalis* has been determined (Jain & Darveau, 2010; Paramonov *et al.*, 2001; Rangarajan *et al.*, 2008). Lipid A is responsible for the majority of biological activity of LPS, and is the most conserved structure (Loppnow *et al.*, 1990). The structure of penta-acylated monophosphorylated lipid A of *P. gingivalis* is similar to that of *P. intermedia* (Hashimoto *et al.*, 2003; Jain & Darveau, 2010) and also undergoes modification in response to changes in local haem concentration (Al-Qutub *et al.*, 2006). As such, and considering that all the LPS types used in this study bound haem, lipid A was investigated as a candidate haem binding moiety. Polymyxin, which binds to lipid A (Morrison & Jacobs, 1976), only partially reduced pick up of haem by NY653 immobilized on nitrocellulose (Fig 8.12). The inability of polymyxin to abolish LPS-haem binding could suggest that other non-lipid A components also play a role. Although lipid A may indeed facilitate haem binding *in vivo* (by LPS micelles or outer-membrane vesicles for example), it is not clear how the moiety could possibly function in mediating pigmentation as this hydrophobic region anchors LPS to the outer membrane (LuÈderitz *et al.*, 1982) and therefore may not be exposed to haem outside the cell. Equally, if surface bound lipid A was responsible, haem may embed itself into the membrane and potentially result in damaging oxidant formation. Binding may instead be reliant on other surface-exposed features of the LPS molecule.

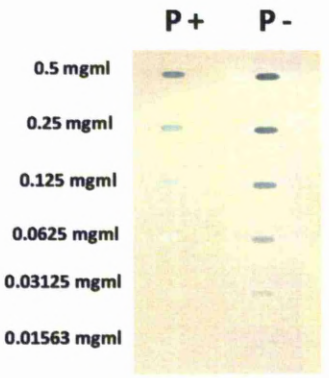


Fig 8.12. Haem pick up by NY653 LPS immobilised on nitrocellulose with (P+) and without (P-) polymyxin.

If the O-polysaccharide chain were responsible for haem pickup, then presumably longer chains possessing more repeating units would be expected to bind more haem. This could be easily tested by subjecting haem-treated LPS to SDS-PAGE and examining whether TMB-H_2O_2 staining was primarily attributed to LPS with greater O-chain lengths. Unfortunately, as discussed earlier, the LPS-haem interaction is too weak to survive the SDS-PAGE procedure. To counteract this, 4 mg ml^{-1} W50 LPS was fractionated with SDS-PAGE, transferred to a nitrocellulose sheet using western blotting and submerged in 50 μM haem for 1 h. However, TMB-staining did not reveal haem binding (data not shown). It seems unusual that LPS transferred directly to nitrocellulose from solution retains the ability to pick up haem (Fig 8.1), whereas LPS first separated by SDS-PAGE does not. It could be that the arrangement of LPS, separated by SDS-PAGE and attached to nitrocellulose, may be such that haem binding motifs are unavailable. Another more plausible possibility is that the interaction of haem with LPS is entirely dependent on association within hydrophobic LPS micelles as one might find for suspensions in an aqueous environment. SDS-PAGE, which separates LPS based on O-polysaccharide chain length, would disrupt this hydrophobic micellular environment and thus prevent haem binding. As such the accumulation of haem by LPS may only occur in solutions containing large volumes of LPS.

In summary, the precise mechanism of haem binding by LPS cannot be ascertained on the basis of the experiments carried out here. However, this preliminary work does not rule out involvement of both lipid A and/or specific residues of the O-polysaccharide. More informative spectroscopic techniques, such as FTIR, which has previously been used to locate

LPS binding of lactoferrin to the phosphate group within lipid A (Brandenburg *et al.*, 2001), may identify binding interactions between haem and specific functional groups of LPS. Equally, experiments using separated lipid A and O-antigens would provide a more comprehensive assessment of their individual behaviours. Furthermore, although this investigation did not evaluate LPS-uronic acid, it must not be entirely disregarded as a haem-binding moiety. Assessment of interactions with uronate-free LPS, produced by treatment with a chondroitinase enzyme, with haem is also required before conclusions can be drawn.

8.3.4. LPS of *P. intermedia* and *P. gingivalis* induces oxyHb oxidation.

LPS of several bacterial species binds haemoglobin in an interaction which affects the properties of both molecules, resulting in lethal toxicity in rodent models (Kaca *et al.*, 1994a; 1994b; 1995; Su *et al.*, 1997). This interaction varies between LPS types. Binding of *E. coli*, *Proteus mirabilis* and *Salmonella minesota* LPS to oxyHb leads to metHb and haemichrome formation (Kaca *et al.*, 1995). This may be physiologically relevant as the complete digestion of haemoglobin by cysteine proteases of *P. gingivalis* and *P. intermedia* is dependent on metHb formation (Smalley *et al.*, 2007; 2008; Byrne *et al.*, 2010). As such LPS may complement enzyme activity by facilitating oxyHb oxidation. LPS of *P. gingivalis* WPH35 (1 mg ml^{-1}) induced oxidation of oxyHb (4 μM) at pH 7.5, above the normal auto-oxidation rates (Fig 8.13; blue-shifted Soret from 412 to 405nm, increased $A_{500\,nm}$ and $A_{631\,nm}$ and decreased $A_{577\,nm}$ and A_{541nm} Q bands). Isosbestic points at 475, 525 and 592 nm indicated direct transformation from oxyHb to metHb. Multi-component spectral analysis showed that after 7 h, WPH35 LPS and oxyHb control incubations contained 57 % and 32 % metHb, respectively. Longer incubations did not result in haem loss (which would have been manifest as a reduction in Soret absorbance [data not shown]). Addition of 10 mM $Na_2S_2O_4$ to Hb-LPS samples yielded a deoxyHb spectrum (429 nm Soret and 555 nm Q band) confirming that metHb had originally been produced (Fig 8.14).

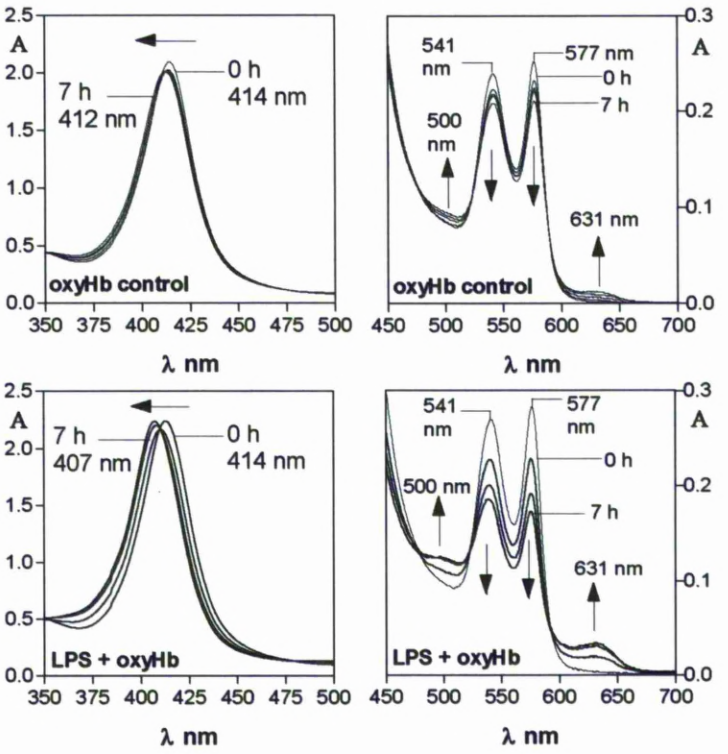


Fig 8.13. LPS-mediated oxidation of oxyHb. Arrows denote absorbance changes.

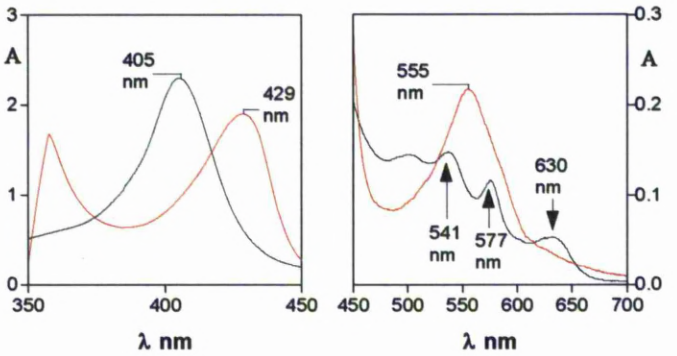


Fig 8.14. UV-visible spectra of LPS-oxyHb incubation product before (black line) and after (red line) addition of 10 mM $Na_2S_2O_4$.

The rate of metHb formation (calculated using integrated peak and trough areas of difference spectra as in Chapter 3, section 3.3.1; Byrne *et al.*, 2010) confirming that LPS elevated oxyHb oxidation rates above the control in a concentration-dependent manner (Fig 8.15). *P. gingivalis* W50 and *P. intermedia* NY653 LPS (both at 0.5 mg ml^{-1}) also enhanced oxyHb oxidation at pH 7.5 (Fig 8.16) yielding 66 % and 63 % metHb after 7 h, respectively. Note that NY653 LPS-mediated oxidation was faster than that for W50 (Fig 8.16).

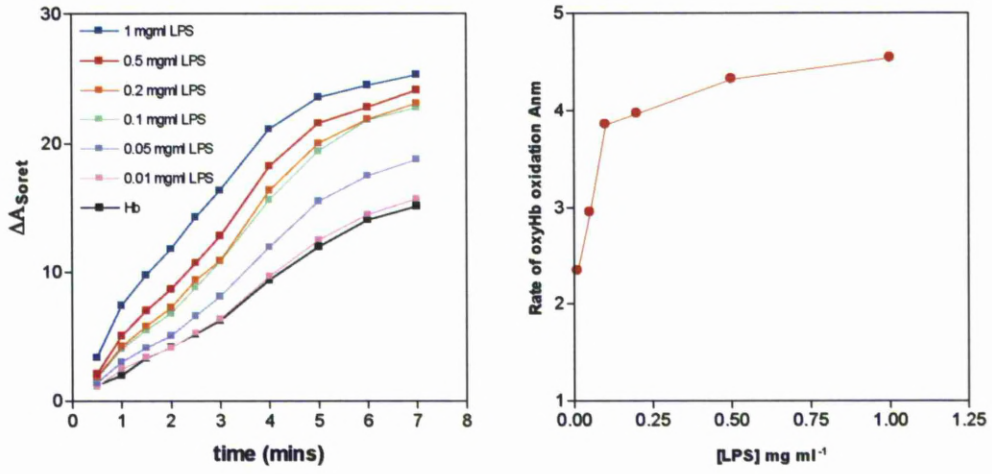


Fig 8.15. OxyHb oxidation by WPH35 LPS. Graphs created from difference spectra as described previously (see section 2.7.1 for details).

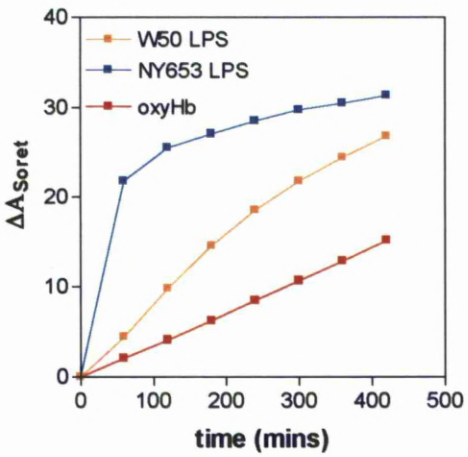


Fig 8.16. Oxidation rates of oxyHb in the presence of *P. gingivalis* W50 and *P. intermedia* NY653 LPS. Graph created from difference spectra as described previously (see section 2.7.1 for details).

The general mechanism of LPS-mediated oxidation of oxyHb is not clear, although Currell & Levin (2002) have suggested that LPS induces dissociation of oxyHb into αβ-dimers, which oxidise at a greater rate than the intact tetramer (Zhang *et al.*, 1991). To examine this possibility WPH35 LPS (0.5 mg ml^{-1}) was incubated with myoglobin (16 μM). Although Hb and myoglobin are structurally similar, myoglobin only exists as a monomer. LPS did not enhance the myoglobin oxidation rate (data not shown), suggesting that LPS would not induce oxidation of individual Hb subunits, and could therefore indicate that the

effect of LPS may be to cause dissociation into αβ-dimers with greater susceptibility to oxidation (Zhang *et al.*, 1991). However, it could be argued that LPS fails to notably increase already rapid natural rates of myoglobin oxidation. It follows that if subunit dissociation is key to this process, then cross-linked oxyHb oxidisation rates should be unaffected by the presence of LPS. To investigate this, oxyHb subunits were cross linked using 3,5-(dibromosalicyl)fumarate which crosslinks between the Lys91 and Lys127 (Yu *et al.*, 1997). However, the auto-oxidation rate of this substrate was extremely high, precluding a reliable comparison with LPS-mediated oxidation. This can be attributed to decreased oxygen affinity of cross-linked Hb and the resultant increase in deoxyHb concentration (Chatterjee *et al.*, 1986). Gel filtration, which would identify dissociation of haemoglobin dimers, would be better suited to test this. As such it was not clear if LPS mediated oxyHb was the result of haemoglobin dissociation.

In Chapter 3 it was discussed that even minor structural changes to the haemoglobin haem pocket can lead to oxidation. This phenomenon is most apparent with abnormal haemoglobin and myoglobin mutants carrying slight modifications of the haem pocket, which are far more susceptible to oxidation (Lai *et al.*, 1995; Percy *et al.*, 2005). The current picture emerging of the way in which InpA- and HRgpA- mediate oxidation of oxyHb is that of a proteolytic modification of haemoglobin structure (Byrne *et al.*, 2010; Smalley *et al.*, 2007; 2008). As LPS lacks proteolytic activity it is unable to cleave and alter the haemoglobin structure bringing about oxidation in this manner. Instead, formation of the stable LPS-Hb complex (Kaca *et al.*, 1994a; 1994b) may be sufficient to induce structural changes and therefore oxidation. Haemoglobin tertiary and quaternary structure is regulated by several allosteric effectors including 2,3-bisphosphoglycerate (BPG [Arnore, 1972]), *myo*-inositol hexakisphosphate (IHP [Benesch & Benesch, 1969]) and several phosphorylated saccharides (Fylaktakidou *et al.*, 2011). These bind to Hb and regulate oxygen binding affinity by shifting the Hb equilibrium towards the deoxy (T or Tense) or oxy (R or Relaxed) states. This is relevant because the more deoxy/tense the haemoglobin molecule is, the more oxidation occurs. Therefore, the presence of BPG and IHP increases the concentration of deoxy molecules and enhances the rate of auto-oxidation (MacDonald & Carache, 1982; Dong *et al.*, 1995). It is possible then that in binding oxyHb, LPS behaves as an allosteric effector, instigating a transition to the deoxy/T state with the consequent increase in auto-oxidation.

Far-UV CD performed in this study (Fig 8.17A) and IR analysis from Jügrens *et al.*, (2001) reveals that binding of LPS does not result in changes of oxyHb secondary structure. However, Kaca *et al.* (1995) identified localised globin conformational changes induced by LPS, which were hypothesized to destabilise the haem pocket such that the rate of metHb formation would increase. Although near UV CD of NY653 and W50 LPS incubated with oxyHb (Fig 8.17B) did not suggest Hb quaternary structure transitions (Perutz *et al.*, 1974; Jin *et al.*, 2004), it is possible that the effects of LPS binding on globin conformation are not detectable with this technique, which only detects changes of aromatic amino acid residues. A more sensitive technique, such as NMR or X-ray crystallography, is therefore necessary to investigate disruption of the globin chain as a possible cause of metHb formation. It should be noted that NY653 and W50 LPS absorbed quite strongly in the near UV region so data presented in Fig 8.17B are of difference spectra (LPS-Hb spectra – LPS spectra).

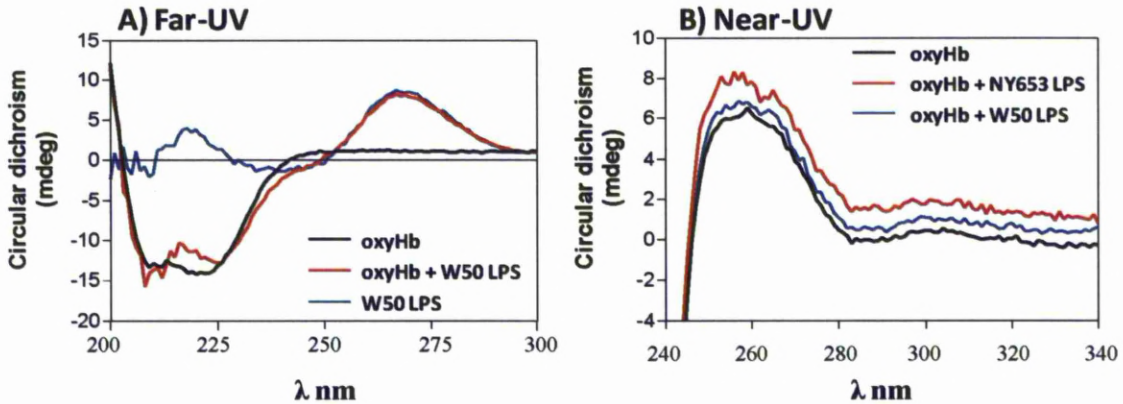


Fig 8.17. Near- and Far-UV CD spectra of oxyHb incubated with NY653 and W50 LPS.

The precise nature of the LPS-haemoglobin complex is also perplexing, since to date, the identity of the LPS component(s) involved in binding and oxidation remain elusive. However, a role for lipid A has been prominently cited (Currell & Levine, 2002; Belanger *et al.*, 1995; Kaca *et al.*, 1994a), and in support of this, pre-treatment of W50 and NY653 LPS (0.5 mg ml^{-1}) with polymyxin (1 mg ml^{-1}) partially reduced metHb formation (Fig 8.18). It is possible that insufficient polymyxin was used to completely inhibit all lipid A moieties in the reaction mixture, as Belanger *et al.* (1995) previously demonstrated that polymyxin nullifies haemoglobin-LPS interactions, which would be expected to abolish LPS-mediated oxyHb oxidation. However, it should be noted that the majority of *P. gingivalis* lipid A lacks

phosphate groups at both the C1 and C4' positions, which reduces electrostatic interactions with polymyxin and confers upon the bacterium resistance against the cationic antimicrobial peptide (Coats *et al.*, 2009). As such only a proportion of the heterogeneous lipid A population may have been susceptible to polymyxin. It is also possible that other LPS components play a role in the process. Indeed, potential roles for the KDO moiety of core oligosaccharide (Kaca *et al.*, 1994a) and the O-saccharide chains (Currell & Levin, 2002) have been postulated. Furthermore, it is clear that a unified paradigm for LPS-mediated oxidation is not attainable given the high degree of behavioural and conformational variation observed between that from different bacterial strains. For example, oxyHb oxidation induced by different preparations of lipid A did not yield a consistent pattern (Kaca *et al.*, 1994a). As such any assumptions made here can only be related to the species and strains of bacteria used in these experiments, and a more detailed appraisal of oxidation by the different LPS components is required.

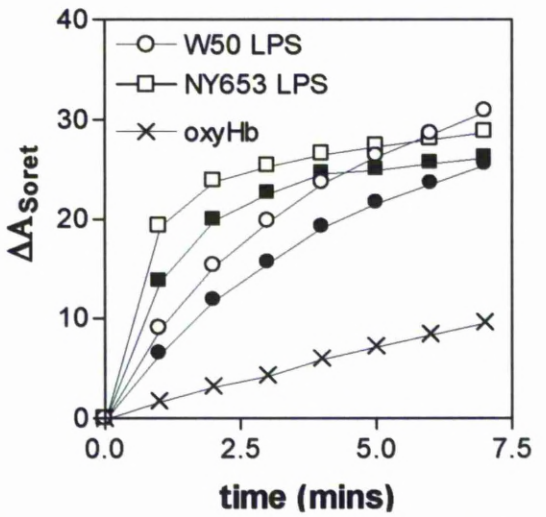


Fig 8.18. OxyHb oxidation by polymyxin B pre-treated LPS (closed symbols) and untreated LPS (open symbols). Graph created from difference spectra as described previously (see section 2.7.1 for details).

8.3.5. Examination of the potential role of LPS-mediated oxyHb oxidation.

Oxidation is the essential pre-requisite step for Kgp- and InpA-mediated breakdown of oxyHb and subsequent haem release (Smalley *et al.*, 2007; 2008; Byrne *et al.*, 2010). Accordingly, since LPS mediates oxyHb oxidation, it is likely that this may also facilitate proteolysis by promoting metHb formation. Moreover, Kgp, which is required for *P.*

gingivalis haem pigmentation (Smalley *et al.*, 2004), can form a 660 kDa cell-associated gingipain complex consisting of catalytic and haemagglutinin domains associated with LPS (Takii *et al.*, 2005). In order to substantiate the assumption that LPS may play a role in facilitating pigmentation, oxyHb (4 μM) was incubated at pH 7.5 with Kgp (0.6 μM) plus *P. gingivalis* strain WPH35 LPS (1 or 0.5 mg ml^{-1}), which was selected because, unlike NY653 and W50 LPS (as discussed later), its actions do not facilitate direct haem release from haemoglobin. Unexpectedly, despite inducing more oxidation, LPS inhibited breakdown of oxyHb by Kgp as judged by changes in Soret band intensity (Fig 8.19). This effect was least prominent with 0.5 mg ml^{-1} LPS (Fig 8.19, panel B). Interestingly, hydrolysis of *N-a*-benzoylcarbonyl-l-lysine-*p*-nitroanilide (Ac-Lys-pNA) by Kgp (20 nM) was reduced by 73 and 23 % for 1 and 0.5 mg ml^{-1} LPS, respectively (Fig 8.20). This suggested that LPS inhibited Kgp activity in a concentration dependent manner.

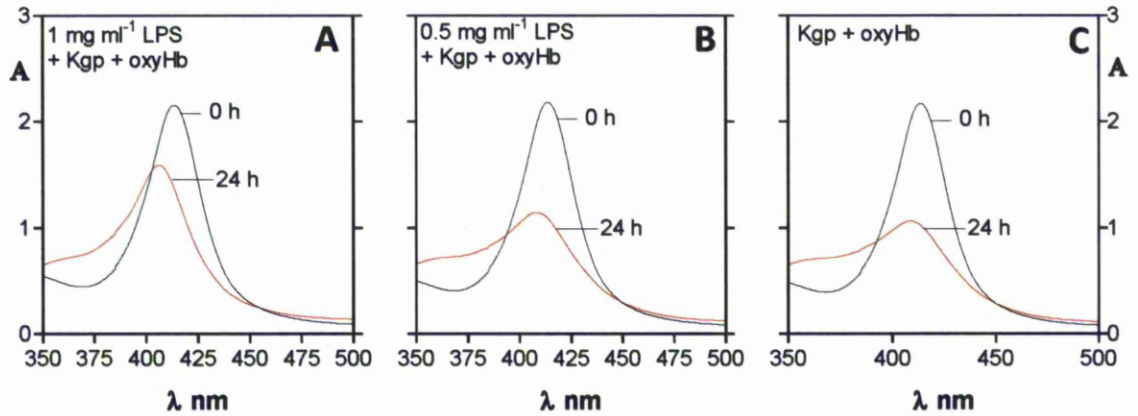


Fig 8.19. UV-visible spectra showing inhibition of Kgp-mediated breakdown of oxyhaemoglobin in the presence of *P. gingivalis* WPH35 LPS.

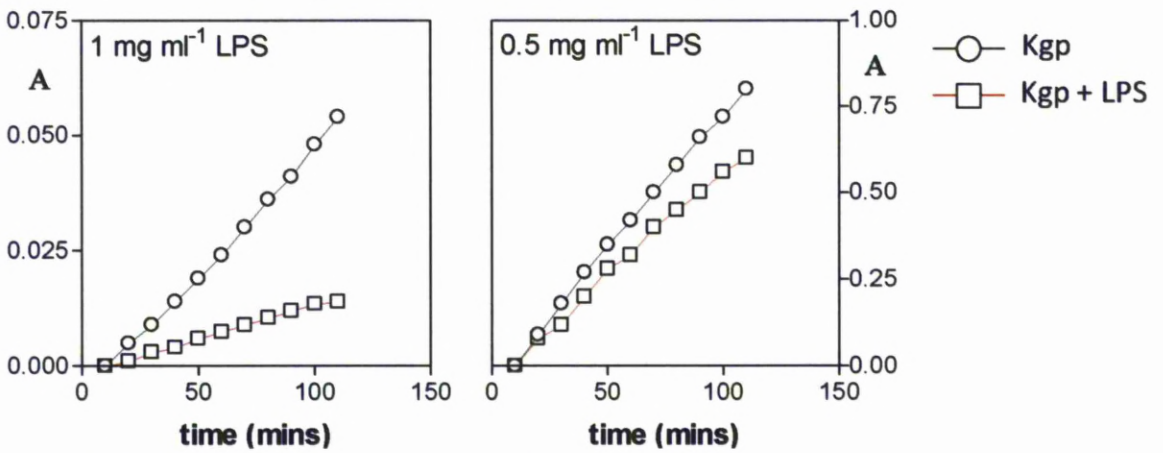


Fig 8.20. Hydrolysis of Ac-Lys-*p*NA by Kgp in the presence and absence of *P. gingivalis* WPH35 LPS.

To examine the above phenomenon under conditions where LPS was minimally inhibitory, Kgp was incubated with metHb, which was pre-formed by treatment of oxyHb for 24 h with lower levels of LPS (0.01 and 0.005 mg ml^{-1} which gave 76 and 70 % metHb, respectively), and compared to auto-oxidised and $NaNO_2$-oxidised Hb controls (64 and 92 % metHb, respectively). After 24 h the greatest level of Hb digestion by Kgp was observed for $NaNO_2$-oxidised metHb (Fig 8.21, panel C). Importantly, however, both LPS-treated samples displayed more breakdown than the auto-oxidised metHb-control (Fig 8.21, panels A and B), showing that LPS at concentrations sufficient to induce oxidation, but not to inhibit Kgp, facilitated greater Kgp-mediated digestion. Large concentrations of membrane- free LPS self-aggregate into supramolecules of different shapes and sizes by virtue of their amphipathic nature (Santos *et al.*, 2003). Kgp activity may be greatly inhibited by association with these aggregates (Takii *et al.*, 2005). Santos *et al.* (2003) proposed that, below a critical micelle concentration (CMC_a) of *E. coli* LPS (14 μg ml^{-1}), aggregations do not form, and this could account for the limited inhibition of Kgp activity at 10 and 5 μg ml^{-1} LPS (Fig 8.21).

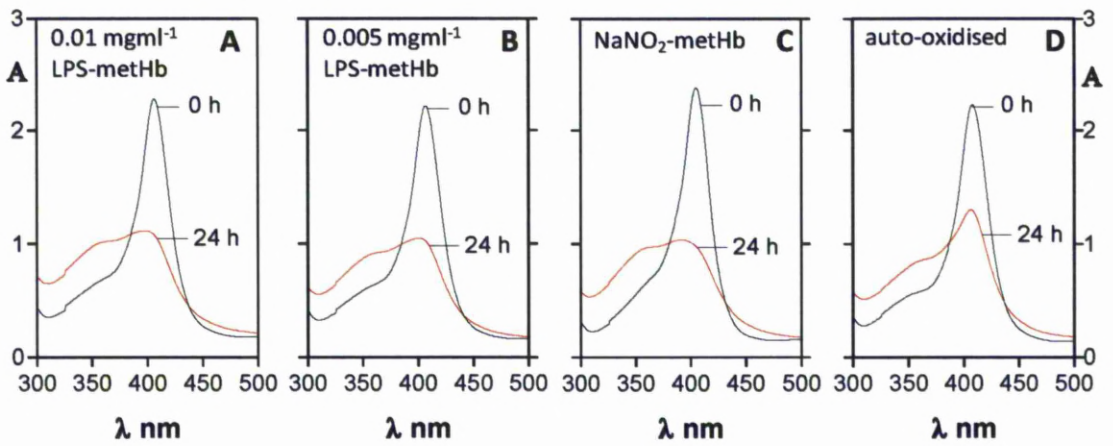


Fig 8.21. UV-visible spectra showing degradation of metHb by Kgp.

Kgp proteolysis of a 7 h auto-oxidised haemoglobin sample (37 % metHb) resulted in a haemichrome after 70 h digestion as revealed when it was reduced to the haemochrome state with $Na_2S_2O_4$ (Fig 8.22, A). However, haemoglobin oxidised by 0.5 mg ml^{-1} LPS (~ 60 % metHb) yielded less haemichrome after digestion by Kgp (Fig 8.22, A) presumably by inducing the formation of a more protease- susceptible metHb. SDS-PAGE confirmed more protein breakdown (68 % compared to 54 % after 24 h) and *de facto* less protease resistant haemichrome formation for haemoglobin treated with LPS (Fig 8.22, B).

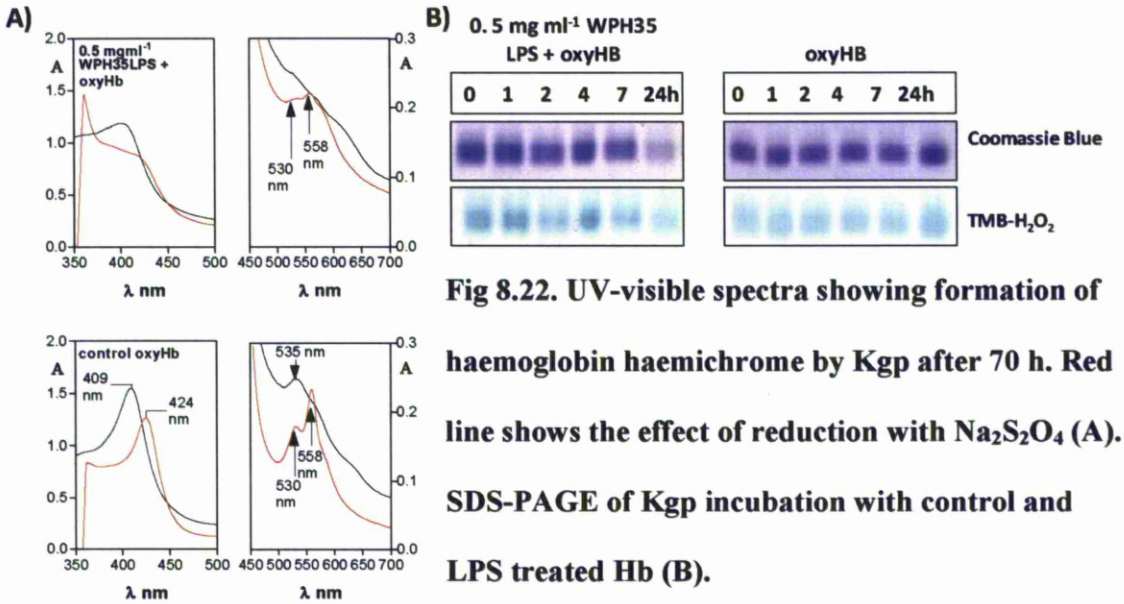


Fig 8.22. UV-visible spectra showing formation of haemoglobin haemichrome by Kgp after 70 h. Red line shows the effect of reduction with $Na_2S_2O_4$ (A). SDS-PAGE of Kgp incubation with control and LPS treated Hb (B).

Thus, low concentrations of WPH35 LPS stimulate Kgp-mediated Hb breakdown by inducing oxidation. A similar dynamic may also exist between LPS and other analogous enzymes requiring oxyHb pre-oxidation (e.g. InpA [Byrne *et al.*, 2010]). LPS oxidation of oxyHb may therefore supplement Rgp mediated metHb formation. However, LPS is normally anchored to bacterial outer membranes via unexposed lipid A (LuÈderitz *et al.*, 1982). These experiments may not accurately reflect the *in vivo* situation in which the lipid A region may not be available to interact with Hb

8.3.6. *P. gingivalis* W50 and *P. intermedia* NY653 LPS sequester haem from haemoglobin.

After 24 h incubation with W50 and NY653 LPS (but not WPH35 LPS) the Soret intensity of oxyHb decreased sharply suggesting haem release. Spectroscopic changes typical of μ-oxo dimers (Soret blue shift to 393 nm and low extinction bands at 575 and 600 nm [Fig 8.23]), which closely resembled those observed in the LPS-haem titrations (Fig 8.10), also indicated binding of the free haem by the LPS. Furthermore, addition of $Na_2S_2O_4$ to the Hb-LPS sample yielded neither a deoxyHb (429 nm Soret and 555 nm Q band) nor a haemichrome spectrum (422 nm Soret and 530 and 558 nm Q bands), but gave a 393 nm Soret with weaker 546 and 568 nm bands (Fig 8.24). The clear absence of deoxyHb and haemichrome may confirm the presence of Hb-free haem. It should however be noted that

free ferrohaem, has a Soret at 384 nm (Silver & Lukas, 1983). It is unclear what the spectrum observed in Fig 8.24 represents, but may be a mixture of LPS-bound haem and residual haemoglobin.

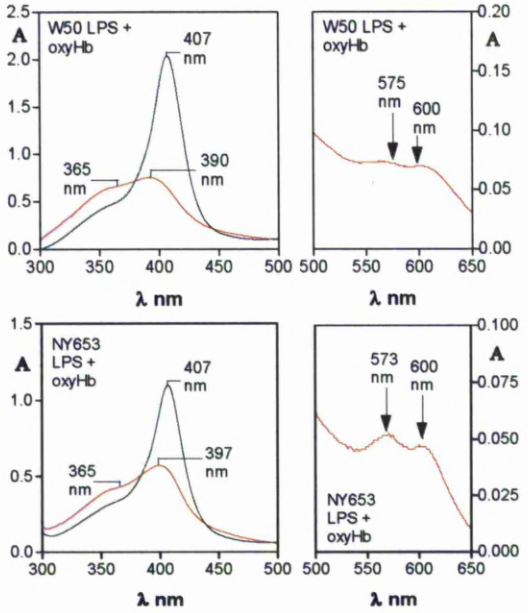


Fig 8.23. Spectra showing oxyHb after incubation with LPS for 7 h (black line) and 72 h (red line).

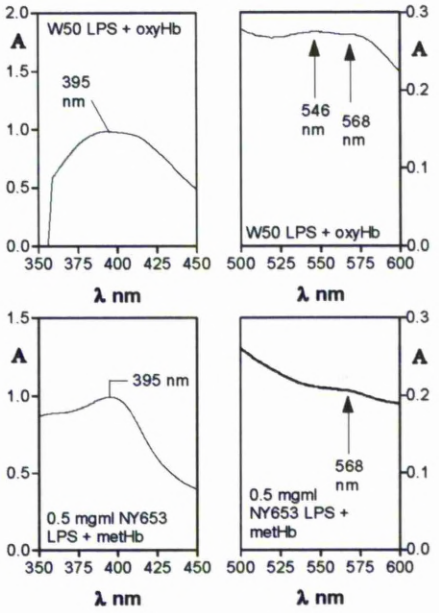


Fig 8.24. Spectra of 72 h LPS-oxyHb incubations (as in Fig 8.23) following reduction with $Na_2S_2O_4$.

Oxidation of oxyHb mediated by LPS, or other agent, would *de facto* reduce haem-globin affinity, encouraging haem dissociation (Gatoni *et al.*, 1996), and thus may permit haem sequestration by LPS. Accumulation of haem within hydrophobic LPS micelles would likely favour the μ-oxo dimer species (as discussed earlier). Susceptibility of metHb (4 μM) to this process of LPS-mediated haem extraction was confirmed by incubation with W50 and NY653 LPS (0.5mg ml^{-1}) (Fig 8.25). Decrease in Soret absorbance intensity and blue shift towards 395 nm were indicative of the μ-oxo haem dimer (Fig 8.25, B). TMB-H_2O_2 staining after SDS-PAGE confirmed haem loss and Coomassie blue staining also suggested denaturation of metHb (Fig 8.25, A), which is explainable by the inherent instability of haem-free globin (Crumton & Polson, 1965; Kawahara *et al.*, 1965) rather than direct LPS-mediated breakdown of the haemoglobin structure.

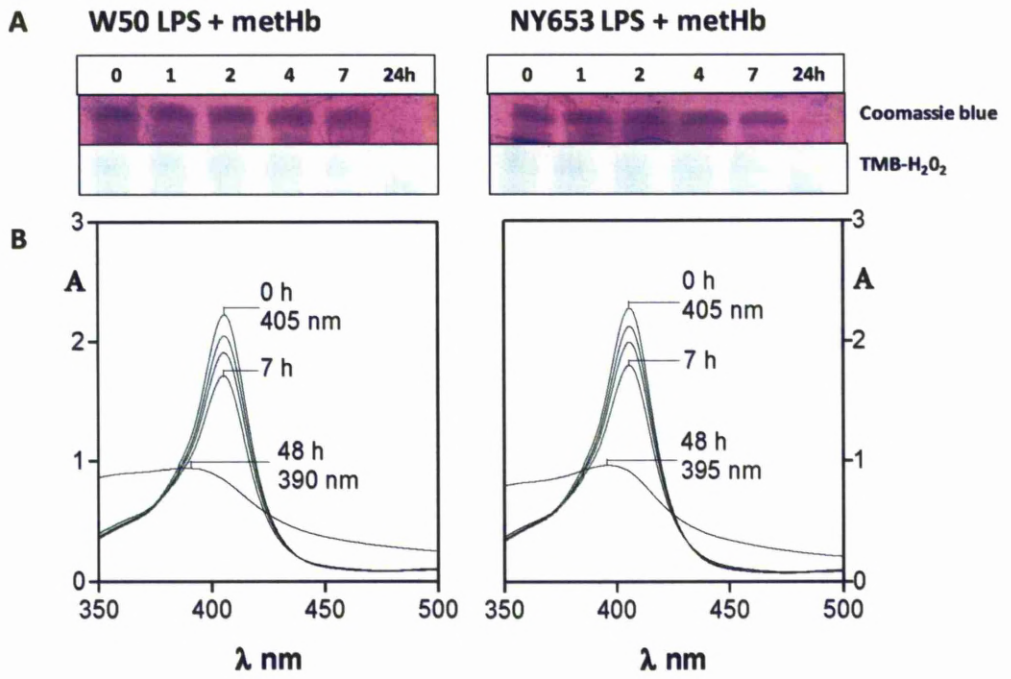


Fig 8.25. SDS-PAGE and UV-visible spectra showing the effect of incubating metHb with *P. gingivalis* W50 and *P. intermedia* NY653 LPS.

To confirm haem removal and to obviate complications due to masking of the spectra by the presence of residual metHb, W50 and NY653 LPS was incubated with metHb-agarose and the spectrum of the agarose-free supernatant recorded periodically. This revealed that haem was liberated by both LPS types (Fig 8.25), with the spectra displaying changes resembling those observed during LPS-haem titrations (as in Fig 8.10, section 8.3.2). Initially, the spectra showed sharp 406 and 407 nm Soret bands, but which were increased in intensity and blue-shifted with time (Fig 8.26). In this experiment, saturation of the LPS component(s) responsible for liberation and binding of haem would be expected to result in cessation of haem release from metHb. Therefore the observed blue shift of the Soret band may not be representative of accumulation of non LPS-bound haem due to LPS saturation which, although conceivable during LPS titration with haem (Section 8.3.2.), is not applicable here. It could therefore be argued that the highly similar changes in Soret wavelengths observed here (Fig 8.2, A) and previously for LPS titration with haem (Section 8.3.2, Fig 8.10) are indeed the result of accumulation of haem μ-oxo dimer relative to the haem monomer (discussed in greater detail in section 8.3.2.) and not LPS saturation. Furthermore, spectroscopic changes typical of μ-oxo dimer formation (blue-shifted Soret

towards 394 nm, and gradual formation of 575 nm and 600 nm bands [Fig 8.26, B]) appeared to be in agreement with this hypothesis (Asher *et al.*, 2009; de Villiers *et al.*, 2007). Again, more informative techniques, such as FTIR and Raman, are required to confirm the haem speciation.

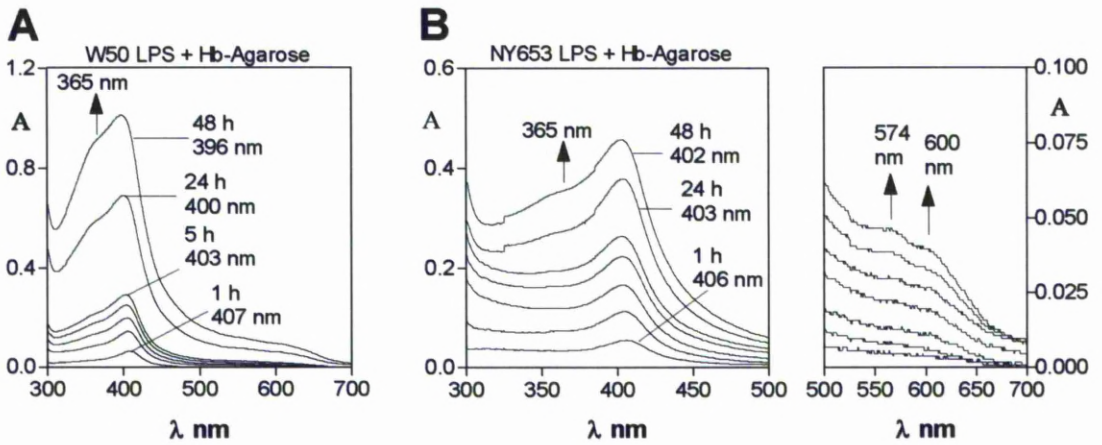


Fig 8.26. Spectra of haem release from hydroxymetHb-agarose by LPS.

From the Soret λ_{max} versus [haem] plots of NY653 and W50 LPS-haem titration taken from section 8.3.2. (Fig 8.7, panel E and Fig 8.9, panel E respectively) it was estimated that W50 and NY653 LPS bound ~ 40 μM and 10 μM haem from haemoglobin-agarose, respectively by 48 h. The partial abrogation of haem release from oxyHb (Fig 8.27) and metHb (Fig 8.28) by pre-treatment of LPS-with polymyxin also indicated the involvement of lipid A in the haem liberation process.

By first binding to oxyHb and encouraging the formation of metHb, LPS can then extract and bind to haem. LPS may thus represent an important surface store of haem and also contribute directly to haem acquisition. These findings strongly implicate LPS as an important binding site for haem pigment generation. Furthermore, in creating a hydrophobic cell surface environment, LPS may facilitate the formation of μ-oxo haem dimers (and haem monomers at low pH) which could explain the species of haem observed in the pigments of *P. gingivalis* and *P. intermedia*. This is of significance as facilitating μ-oxo dimer formation would tie up oxygen and promote anaerobiosis (Smalley *et al.*, 1998; 2002). LPS-mediated haem release from oxyHb as a consequence of binding may have further repercussions in addition to the facilitation of pigment formation. Interactions between haemoglobin and LPS increase the bioactivity and toxicity of the latter (Roth *et al.*, 1994; Roth, 1996) and it is

possible that this toxicity may, in part, result from LPS-mediated haem release, since free haem is a potent inducer of inflammation (Wagener *et al.*, 2001).

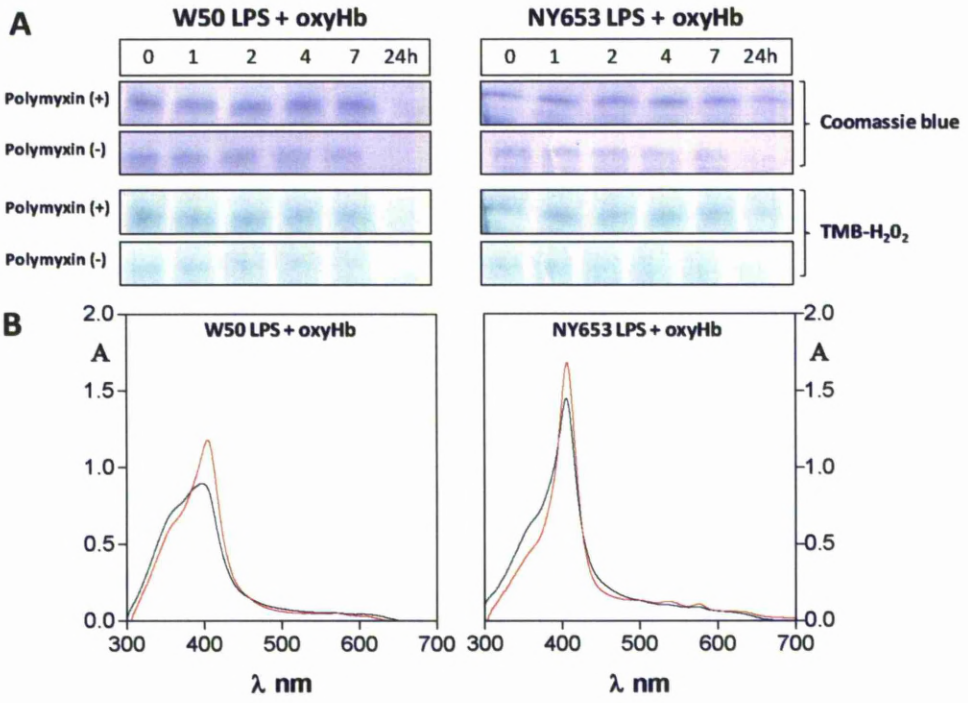


Fig 8.27. Effect of incubating oxyHb with LPS in presence (+) and absence (-) of polymyxin. Spectra recorded after 24 h; red line shows effect of adding polymyxin.

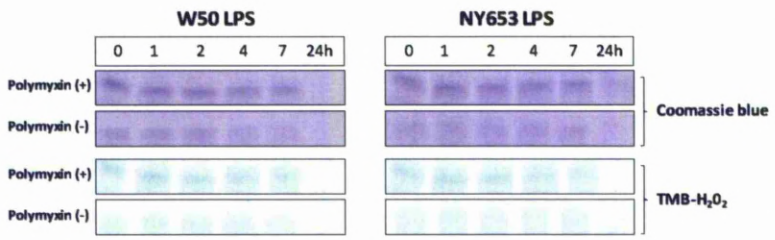


Fig. 8.28. SDS-PAGE of LPS incubated with metHb in presence (+) and absence of (-) of polymyxin.

8.3.7. Mobilization of LPS-haem by HmuY.

Haem bound to LPS at the cell surface may have to be mobilized for internalisation by, for example, the HmuY/HmuR system. Earlier evidence (section 8.3.1.) suggested that haem is only loosely associated with LPS, a factor which may facilitate this process. Thus, LPS would need to readily relinquish bound haem to HmuY for internalisation. To examine this possibility, a haem-LPS complex was made by pre-treating Hb-agarose with W50 LPS (0.5 mg ml^{-1}). LPS bound haem was rapidly expropriated by HmuY (Fig 8.29), developing the distinctive haem-HmuY spectrum (sharp 411nm Soret and Q bands at 527 nm and 558

nm (Smalley *et al.*, 2011; Wójtowicz *et al.*, 2009a). The presence of the HmuY-haem complex was confirmed by SDS-PAGE (data not shown). These data indicate that surface LPS-bound haem would be a facile 'substrate' from which HmuY could obtain haem. In this regard it is noteworthy that haem, attached to outer-membrane preparations from *P. gingivalis*, can satisfy the haem requirement of the organism in a haem limited environment.

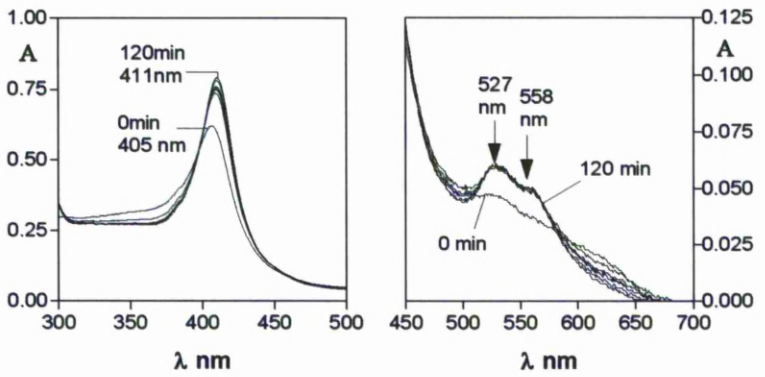


Fig. 8.29. Spectroscopic demonstration of HmuY-ferrihaem complex formation from LPS-bound haem.

In addition to binding haem, LPS also forms a complex with haemoglobin (Kaca *et al.*, 1994, Roth *et al.*, 1994) which may become attached to the bacterial surface. This interaction would not only facilitate metHb formation (Kaca *et al.*, 1995) but also bring haemoglobin into contact with the cell surface and the HmuY haemophore, which readily sequesters haem from metHb (Smalley *et al.*, 2011). Moreover, when either NY653 or W50 LPS was incubated with oxyHb in the presence of HmuY, greater amounts of holoHmuY were formed with a concomitant reduction in oxyHb-TMB-H_2O_2 staining as shown by native PAGE (Fig 8.30, tracks A, B, and C). Haem acquisition by the HmuY/HmuR system is dependent on gingipain-mediated oxidation of haemoglobin (Smalley *et al.*, 2011). Here it is demonstrated that ferrihaemoglobin formed by LPS also facilitates rapid haem acquisition by HmuY. In addition, haem extracted directly by LPS would be readily mobilized by HmuY. These findings reveal a unique syntrophic mechanism of haem acquisition by LPS and HmuY, further highlighting the importance of LPS in pigment formation.

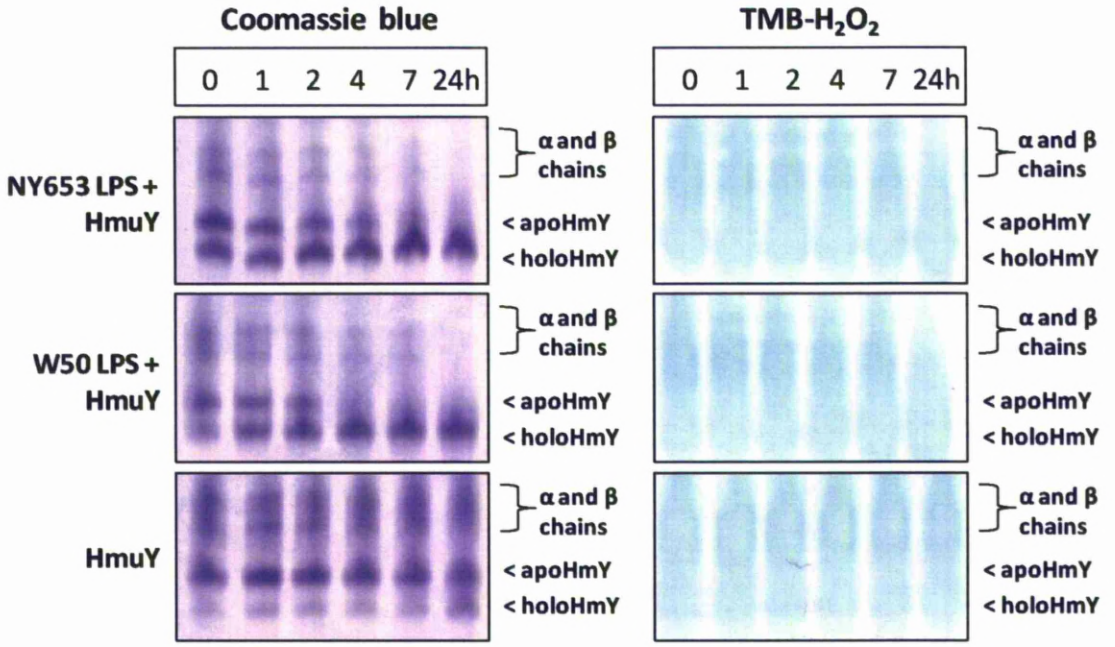


Fig 8.30. HmuY-haem complex formation during co-incubation of HmuY with oxyHb and LPS.

8.3.8 LPS may serve as a multifaceted contributor to pigmentation.

This study is the first to present evidence linking interactions of LPS with haem/haemoglobin with the formation of haem pigments by *P. intermedia* and *P.gingivalis*. LPS may not only serve as the primary attachment site for haem at the surface of the bacterium, facilitating the accumulation of haem and thus pigment formation, but may also directly contribute to haem acquisition by oxidizing haemoglobin and extracting the haem from the resultant metHb. Furthermore, metHb produced by LPS may serve as a facile substrate for haem transfer to haemophores, such as HmuY. This chapter, and much of the literature, also proposes a role for the hydrophobic lipid A region of LPS in facilitating interactions with haem and haemoglobin, although ideally these experiments should be repeated with isolated lipid A fractions. *P. gingivalis* also produces large quantities of surface 'blebs' and extracellular vesicles (Smalley *et al.*, 1993b; Okuda *et al.*, 1981; McKee *et al.*, 1986). Extracellular membrane-bound vesicles, derived from blebbing of the outermembrane, are considered a source of cell-free lipopolysaccharide (Russell, 1976). These may also serve as a binding site for haem/haemoglobin. It is of note in this regard that haem limitation results in the production of greater amounts of surface vesicle and extracellular vesicle material (McKee *et al.*, 1986).

CHAPTER 9

General Discussion

The aim of this thesis has been to further characterise the mechanisms by which *P. intermedia* and *P. gingivalis* generate their haem-containing pigments. This type of investigation was deemed necessary as the black pigments behave as virulence factors by destroying neutrophil originating H_2O_2 (Smalley *et al.*, 2000) and, under certain conditions, consuming oxygen and promoting anaerobiosis (Smalley *et al.*, 1998; 2002). As the majority of host haem is contained within intracellular haemoglobin, this tetrameric protein is the most important source of haem for periodontal pathogens which are frequently exposed to bleeding in the gingival crevice and periodontal pocket. Acquisition of haem from oxyHb by *P. gingivalis* is a sequential process, which requires its prior oxidation by R-gingipains to facilitate complete globin degradation by K-gingipains, as a result of greater haem dissociation from metHb (Smalley *et al.*, 2007; 2008). Importantly, the present investigations have also revealed that InpA of *P. intermedia* behaves in a similar manner to R-gingipain by inducing metHb formation.

Further characterisation of InpA-oxyHb interactions revealed that metHb formation was largely a proteolytic process. MS analysis suggested that cleavage at several specific residues in both the α- and β-globin chains disrupted the hydrophobic haem pocket and facilitated oxidation. Cleavage at Phe-45 of the β-chain, in particular, highlights a potential route through which InpA may facilitate this event, as it is this residue which imposes steric constraints upon the distal His-64 and thus limits auto-oxidation. Surprisingly however, treatment of InpA with the irreversible protease inhibitor E-64 only abrogated metHb formation by 80 %. Furthermore, a Cys154Ala mutant of InpA (InpA C154A) also induced oxyHb oxidation above the basal auto-oxidation rate. This indicated that transient complex formation between InpA and oxyHb may be sufficient to promote oxidation. Circular dichroism not only confirmed such an interaction but also revealed that alterations of the globin structure may account for metHb formation.

X-ray crystallographic studies using fully processed InpA C154A (lacking N-terminal pro-domain) were undertaken to better understand the nature of the InpA-oxyHb complex and how its formation affects Hb globin chain structure and therefore oxidation. Unfortunately, successful co-crystallisation of the complex could not be achieved and this was attributed to rapid Hb crystal formation and the possible unstable nature of the intermediary complex. A more detailed appraisal of suitable co-crystallisation conditions is therefore required before conclusions about the InpA-Hb complex can be made. This would constitute a significant

amount of further work, and would be hugely assisted by identification of conditions (e.g. pH) in which the complex is most stable. Biophysical techniques such as isothermal titration calorimetry (ITC) and fluorescence anisotropy (FA) titration could be used to determine the most suitable conditions for complex formation (Xiao *et al.*, 2007). ITC is a thermodynamic technique which measures heat generated or absorbed during enzyme-substrate reactions to generate accurate estimates of binding constants (K_B), reaction stoichiometry (*n*), enthalpy (ΔH), and entropy (ΔS), whereas FA titration is useful for determining binding constants and has the benefit of requiring smaller amounts of protein. This information could be used to optimize screening conditions and improve the chance of successfully generating crystals containing both proteins.

Haemoglobin oxidation induced by R-gingipain is an important first step in its degradation by *P. gingivalis* as it greatly weakens the haem-globin affinity. This study revealed that Rgp facilitated greater extraction of haem from oxyHb by the haemophore HmuY of *P.gingivalis*. Importantly, Rgp provides a means of accelerating oxyHb oxidation which is naturally lowest at alkaline pH conditions, which are known to prevail in the periodontal pocket. HmuY was also capable of extracting haem from Kgp-treated oxyHb. Interestingly, metHb produced by InpA was also a facile substrate for HmuY haem scavenging. This is of particular significance as *P. gingivalis* co-aggregates with *P. intermedia* in the periodontal pocket biofilm (Kamaguchi *et al.*, 2003) and suggests that cooperative haem acquisition between these two bacteria could potentially occur *in vivo*. Haem acquisition by haemophore systems have been described for many species of bacteria, but this present study is the first to describe a potential role for proteases in this process. It would be interesting to examine whether this feature is unique to the *P. gingivalis* HmuR/HmuY haem uptake system or is common to other bacterial haemophore systems.

Despite promoting HmuY haem acquisition, the "metHb" product of InpA proteolysis of oxyHb was refractory to Kgp. This further supports the hypothesis that InpA-mediated proteolytic oxidation occurs as a consequence of the disrupted globin chain structure, which equally may not accommodate binding by Kgp or make key susceptible residues unavailable for cleavage. InpA-induced "metHb" may therefore differ structurally from metHb structures that are susceptible to Kgp, such as those produced by auto-oxidation, and treatment with $NaNO_2$ or Rgp. Clear distinctions must therefore be made when describing naturally occurring metHb, which differs

from oxyHb only in haem-globin affinity, and proteolytically induced metHb which probably exhibits a modified structure. In support of this, proteolysis of oxyHb by Rgp also had an inhibitory effect on InpA mediated breakdown. Despite the lack of any apparent cooperative interaction between InpA and the gingipains in haemoglobin breakdown, *P. intermedia* and *P. gingivalis* may still indirectly benefit from the ability of each other to liberate haem from haemoglobin *in vivo*. To examine this hypothesis, growth and pigment formation by gingipain deficient *P. gingivalis* mutants on blood containing media could be investigated in the presence of InpA.

These investigations also emphasised how alterations in the environment of the periodontal pocket might profoundly influence haem acquisition by these organisms. Enrichment of pathogenic bacteria will exacerbate tissue destruction, the inflammatory immune response and bleeding, resulting in greater haem availability. Furthermore, growth of certain bacteria will alter the local biofilm pH. InpA was ineffective at inducing gross breakdown and haem release from hydroxymetHb at alkaline pH, instead resulting in haemichrome formation. However, aquometHb (the predominant species found in an acid environment) was highly susceptible to InpA, which was attributed, in part, to the known greater haem dissociation compared to hydroxymetHb. Limited InpA degradation of azide-liganded metHb (in which the haem is greatly stabilised against dissociation) confirmed this hypothesis.

Haemopexin, which undergoes drastic structural changes associated with increase molecular flexibility at low pH, was also highly sensitive to InpA under acid conditions. These findings are of physiological relevance because growth of *P.intermedia* in liquid culture is accompanied by a pH drop as a result of saccharolytic metabolism (Shah & Williams, 1987; Takahashi & Yamada, 2000). Conditions which lower the pH of the periodontal pocket or which lead to enrichment of acidogenic bacterial species may also be risk factors for development of periodontitis, in that this may expedite haem acquisition by *P. intermedia*.

Alkaline conditions (which are observed in diseased periodontal pockets) were also demonstrated to aid InpA-mediated haem acquisition by promoting the transition of haemalbumin to the more protease sensitive B form. Furthermore, diseased periodontal pockets are often highly reduced as a consequence of VSC production by resident flora, and evidence presented in this study indicated that

chemical reduction of albumin is crucial in enabling proteolysis by InpA. It was noteworthy that digestion of albumin by Kgp or Rgp was also hugely improved by chemical reduction. The diseased periodontal pocket may provide an ideal environment for albumin degradation by periodontal pathogens. As albumin is considered the major protein in haem sequestration in the GCF and the periodontal pocket, its degradation is likely an important factor in pigment generation and in haem acquisition, expecially under conditions of haem limitation (i.e. in a minimally inflamed pocket/gingival sulcus environment in the absence of frank bleeding). It was also noteworthy that albumin degradation by InpA was positively correlated with increasing molar ratio of bound haem, most probably as a result of haem induced conformational changes.

These studies have revealed several new pathways of haem acquisition for both *P. intermedia* and *P. gingivalis* and emphasise how effective these are at competing with the host for haem. In addition, this study has highlighted InpA as the first identified protease of *P. intermedia* to play what may be a central role in haem acquisition from a variety of sources. It can be concluded that InpA functions both to directly liberate haem from haem-containing proteins and to perturb host recycling of haem by degrading haemoproteins which the host employs to reduce haem bioavailability to microbes. In this regard it should be pointed out that the apo-form of haemopexin was more susceptible to InpA than its haem liganded counterpart.

However, the objective of this investigation was to explicate the mechanisms through which haem pigments are formed. Although characterisation of the systems deployed to wrest haem from the host is an important piece of the puzzle, it does not explain how accumulation of haem, manifest as a cell-surface pigment, occurs. The information presented in this study strongly implicates LPS, which is ubiquitous at the cell surface, as a mediator of haem binding. A number of experiments overwhelmingly demonstrated haem binding by both *P. intermedia* and *P. gingivalis* LPS. Furthermore spectroscopic analysis indicated that the nature of this presumably hydrophobic association may explain the differential haem speciation found in the respective pigments of *P. intermedia* (i.e. Fe(III)PPIX.OH; haematin monomer [Smalley *et al.*, 2003a]) and *P. gingivalis* ($[Fe(III)PPIX]_2O$; μ-oxo dimer [Smalley *et al.*, 1998]) by favouring the μ-oxo dimer and, under acid conditions, haem

monomers. However, it must be emphasised that this was concluded on the basis of UV-visible spectroscopy. A more informative technique such as FTIR, which will detect stretching of μ-oxo bridge of the μ-oxo dimer (Brown *et al.*, 1969; Smalley *et al.*, 2006), would be required to confirm this hypothesis. FTIR analysis was originally planned as part of these studies but unfortunately it was not possible to procure access to suitable equipment. Also noteworthy was the fact that haem which was loosely associated with LPS was readily mobilized and picked up by HmuY. This places LPS as an important cell surface haem storage molecule which can be tapped to provide haem for internalisation.

Additional roles for LPS in haem acquisition were also revealed. LPS-mediated oxidation of oxyHb predictably facilitated HmuY-haem extraction and, at low concentrations of LPS, induced a slight increase in Hb degradation by Kgp. Furthermore, LPS from *P. gingivalis* (W50) and *P. intermedia* (NY653) were shown to directly extract and bind haem from metHb although the exact mechanism is not clear.

This present study was successful in identifying and partially characterising InpA and LPS as active agents in pigment generation, although further work is vital to understand their importance during bacterial infection. For example, experiments with protease-null mutants have shown that gingipains are essential for haem pigmentation by *P. gingivalis* (Shoemaker *et al.*, 1986; Chen *et al.*, 2000; Smalley *et al.*, 2004). Although it can be concluded that InpA may play an important role in haem acquisition by *P. intermedia*, the relative contribution of the enzyme to pigment generation can only be speculated. It is therefore vital to produce an isogenic mutant of *P. intermedia* deficient in InpA synthesis to assess its growth, pigmentation on blood containing media and ability to utilise different haem-containing substrates. Unfortunately, at the time of writing no InpA deficient mutants had successfully been produced.

It is highly likely that *P. intermedia* produces other enzymes involved in haem acquisition. As discussed earlier, the genome of *P. intermedia* 17 encodes two other SpeB-related cysteine proteases (Mallorquí-Fernández *et al.*, 2008; Potempa *et al.*, 2005) which may supplement InpA activity. Just as optimum pigment formation by *P. gingivalis* requires complete R- and K-gingipain activity (Smalley *et al.*, 2004), it would not

be unexpected if *P. intermedia* pigmentation was dependent on a synergistic combination of other related enzymes. Purification and characterisation of these cysteine proteases in relation to proteolytic haem liberation would greatly benefit our understanding of this organism.

Compared to *P. gingivalis*, little is known regarding haem acquisition by *P. intermedia*. For example, at the time of writing, although a specific haem binding site has been speculated (Tompkins *et al.*, 1997), no haem-binding proteins or haemophores have been characterised or even identified! As such, a great deal of further work would be required to identify and characterise other virulence factors involved in haem acquisition. In addition, it is not known under which conditions InpA is maximally expressed. Considering how intimately InpA activity is related to factors such as pH, its expression may be limited to situations which promote optimum haem acquisition (e.g. acid pH for oxyHb and haemopexin degradation). A detailed appraisal of the InpA expression profile would help characterise its role *in vivo*.

The crystal structure of the InpA-Hb complex could identify structural components that mediate the interaction between the two proteins and provide further insight into the mechanisms of proteolytic oxidation. Equally, identification of the structural components of LPS that facilitate association with Hb and haem would greatly benefit our understanding of these interactions. Although preliminary evidence is presented here implicating lipid A as a mediator of haem binding and metHb production (e.g. experiments involving polymyxin) further work is required to prove this and to examine the involvement of the O-chains and core oligosaccharides. Isolation of these components would be required to more accurately assess their relative contributions to haem acquisition.

One other fundamental puzzle is that whilst the majority of Gram-negative bacteria produce LPS, only two other species (*Yersinia pestis* and *Aeromonas salmonicida*) produce haem-containing pigments, although epidemic strains of *Burkholderia cenocepacia* have been shown to accumulate some cell-surface μ-oxo dimer (Smalley *et al.*, 2003b). It could quite reasonably be argued that LPS is not exclusive enough to *P. gingivalis* and *P. intermedia* to be responsible for this phenomenon. If LPS from black pigmenting bacteria is truly responsible for the bulk of haem pick up, then further work must be undertaken to elucidate the unique characteristics that differentiate it from the LPS of non-pigmenting species. For this purpose the complete structure of *P. intermedia*-LPS would also need to be solved.

In summary, work presented in this study has been successful in identifying several novel mechanisms of haem acquisition of *P. gingivalis* and *P. intermedia* and has thus deepened our understanding of the pigmentation process. However, it is becoming increasingly apparent that these organisms deploy a wide and highly versatile range of virulence factors for the express purpose of amassing the large quantities of haem required to generate their characteristic black pigments. Further experimental work is essential to understand the full library of virulence factors utilised by these pathogens for this purpose, which could potentially enable development of new preventative treatments for periodontal diseases with which these organisms are associated.

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BJ Structure

Biochem. J. (2010) 425, 257–264 (Printed in Great Britain) doi:10.1042/BJ20090343

Role of the cysteine protease interpain A of *Prevotella intermedia* in breakdown and release of haem from haemoglobin

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The Gram-negative oral anaerobe *Prevotella intermedia* forms an iron(III) protoporphyrin IX pigment from haemoglobin. The bacterium expresses a 90 kDa cysteine protease, InpA (interpain A), a homologue of *Streptococcus pyogenes* streptopain (SpeB). The role of InpA in haemoglobin breakdown and haem release was investigated. At pH 7.5, InpA mediated oxidation of oxyhaemoglobin to hydroxymethaemoglobin [in which the haem iron is oxidized to the Fe(III) state and which carries OH^- as the sixth co-ordinate ligand] by limited proteolysis of globin chains as indicated by SDS/PAGE and MALDI (matrix-assisted laser-desorption ionization)–TOF (time-of-flight) analysis. Prolonged incubation at pH 7.5 did not result in further haemoglobin protein breakdown, but in the formation of a haemoglobin haemichrome (where the haem Fe atom is co-ordinated by another amino acid ligand in addition to the proximal histidine residue) resistant to degradation by InpA. InpA-mediated haem release from hydroxymethaemoglobin–agarose was minimal compared with trypsin at pH 7.5. At pH 6.0, InpA increased oxidation at a rate greater than auto-oxidation, producing aquomethaemoglobin (with water as sixth co-ordinate ligand), and resulted in its complete breakdown and haem loss. Aquomethaemoglobin proteolysis and haem release was prevented by blocking haem dissociation by ligation with azide, whereas InpA proteolysis of haem-free globin was rapid, even at pH 7.5. Both oxidation of oxyhaemoglobin and breakdown of methaemoglobin by InpA were inhibited by the cysteine protease inhibitor E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane]. In summary, we conclude that InpA may play a central role in haem acquisition by mediating oxyhaemoglobin oxidation, and by degrading aquomethaemoglobin in which haem–globin affinity is weakened under acidic conditions.

Key words: haem, haemoglobin, interpain A, methaemoglobin, *Prevotella intermedia*, protease.

INTRODUCTION

The genera *Prevotella* and *Porphyromonas* belong to a group of Gram-negative black-pigmenting oral anaerobes which are associated with periodontal diseases in humans and other animals [1]. The black pigments, which develop upon prolonged incubation on blood-containing media, are composed of iron(III) protoporphyrin IX, Fe(III)PPIX, and are derived from the breakdown of haemoglobin. The haem pigment produced by *Porphyromonas gingivalis* is composed of Fe(III)PPIX in the μ-oxo bishaem or dimeric form, $[Fe(III)PPIX]_2O$ [2], whereas that from both *Prevotella intermedia* and *Prevotella nigrescens* is in the form of monomeric haematin, Fe(III)PPIX·OH [3]. These pigments serve a defensive role as they make use of the intrinsic ferrihaem catalase activity to destroy H_2O_2 [4]. In addition, the formation of the μ-oxo dimer by *P. gingivalis* from ferrous haemoglobin is a chemical mechanism which can tie up oxygen and thus promote anaerobiosis [2,5].

The mechanism of protease-mediated haem release from haemoglobin by *P. gingivalis* involves the concerted sequential action of both the arginine- and lysine-specific cysteine protease gingipains [5–7]. Haemoglobin breakdown has also been demonstrated for *Prevotella* species [8–10], but the way in which haem is released from haemoglobin by these bacteria is unclear. Guan et al. [10] have shown that the culture supernatant of *P. intermedia* could degrade haemoglobin over a wide pH range, but with optimal activity at around pH 5. Whereas di- and tri-peptidyl peptidases and general thiol-dependent proteolytic activity have been partially characterized from *P. intermedia* and *Prevotella nigrescens* [11,12], little is known regarding the individual endopeptidases of these organisms. The only endopeptidase characterized from *Prevotella* spp. is InpA (interpain A) [13], a cysteine protease which is an orthologue of streptopain of *Streptococcus pyogenes* (SpeB) and periodontain of *P. gingivalis* (PrtP) [14]. Several other InpA orthologue genes are apparently present in the genomes of the *Bacteroidetes* [15]. However, in strict contrast with SpeB, gingipains and many other bacterially derived cysteine proteases [16], little is known about the role of interpain-like enzymes in bacterial pathogenicity and/or haem acquisition.

The gingipains of *P. gingivalis* have been shown to play an essential function in formation of haem pigmentation of *P. gingivalis*, where, at slightly alkaline pH, they work sequentially to first oxidize oxyhaemoglobin to methaemoglobin [where the haem iron is in the Fe(III) state and OH^- is the sixth co-ordinate ligand], and, finally, to degrade this species to release Fe(III)PPIX, which becomes dimerized and incorporated into the pigment [6,7,17]. To elucidate the mechanism of haem acquisition by *P. intermedia*, we have examined the role of InpA in haemoglobin breakdown. In the present study, we demonstrate that, at pH 7.5, InpA mediates hydroxymethaemoglobin formation from oxyhaemoglobin and that, under acidic conditions, which may be

Abbreviations used: AMC, 7-amino-4-methylcoumarin; DTT, dithiothreitol; Fe(III)PPIX, iron(III) protoporphyrin IX; InpA, interpain A; MALDI, matrix-assisted laser-desorption ionization; TMB, 3,3′,5,5′-tetramethylbenzidine; TOF, time-of-flight.

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generated as a result of their saccharolytic metabolism, the aquo- (or acid) form of methaemoglobin is rendered susceptible to InpA enzymic attack and haem release.

EXPERIMENTAL

InpA isolation and purification

InpA was expressed as a recombinant protein in *Escherichia coli* and purified by affinity chromatography on Fast Flow Ni-NTA (Ni^{2+}-nitrilotriacetate)–Sepharose (Qiagen) followed by anion-exchange chromatography (MonoQ, GE Healthcare) as described previously [13]. The amount of active enzyme in wild-type InpA preparations was determined by active-site titration using inhibitor E-64 [*trans*-epoxysuccinyl-L-leucylamido-(4-guanidino)butane] (Sigma). Briefly, recombinant protein was activated at 37 °C for 15 min in 0.1 M Tris/HCl and 5 mM EDTA (pH 7.5), freshly supplemented with 2 mM DTT (dithiothreitol) and then pre-incubated with increasing concentrations of E-64 for 30 min at room temperature (20 °C). Residual enzyme activity was determined by measurement of fluorescence ($\lambda_{ex} = 380$ nm and $\lambda_{em} = 460$ nm) of AMC (7-amino-4-methylcoumarin) released from Boc-Val-Leu-Lys-AMC (where Boc is t-butoxycarbonyl) (PeptaNova) added to the reaction mixture at 250 μM final concentration and using the microplate spectrofluorimeter SpectraMax Gemini EM (Molecular Devices). The concentration of active InpA was determined by active-site titration using an appropriate dilution of a standardized 1 mM aqueous solution of the inhibitor E-64 needed for total inactivation of the proteinase. Before use, InpA was pre-activated by incubation for 15 min in 0.1 M NaCl and 0.1 M Tris/HCl (pH 7.5), supplemented with 2 mM DTT. For use in haemoglobin degradation assays, this was replaced with the above buffer without DTT by ultrafiltration using 10 kDa cut-off Microcons (Amicon).

Haemoglobin preparations

Oxyhaemoglobin was prepared from fresh horse erythrocytes as described previously [5] and stored as a concentrated solution (approx. 1 mM) at −80 °C in 0.14 M NaCl and 0.1 M Tris/HCl (pH 7.5) until required. Stock preparations of methaemoglobin were prepared from the oxygenated protein by treatment with sodium nitrite as described previously [7]. Azidomethaemoglobin was formed from methaemoglobin by incubation with 0.4 mM sodium azide in 0.2 M phosphate buffer (pH 6.0) for 18 h at room temperature [7]. Bovine haemoglobin–agarose was obtained from Sigma Chemical Company. In assays to determine haem release from haemoglobin subunits, haemoglobin–agarose beads were first washed in 0.14 M NaCl buffered with 0.1 M Tris/HCl buffer (pH 7.5), to remove any unconjugated haemoglobin. The beads were then incubated with InpA and at various time periods, samples of these were removed, pelleted by centrifugation at 5000 *g* for 1 min and the supernatant solutions were carefully removed. Fe(III)PPIX released from the protein was assayed using the pyridine haemochromogen method as described by Gallagher and Elliot [18], after first reducing the sample with 10 mM sodium dithionite, followed by the addition of 0.1 M pyridine.

Haem-free globin

Haem-free globin was prepared as described by Ascoli et al. [19]. Briefly, a 3 % (w/v) solution of freshly prepared horse oxyhaemoglobin was added slowly to a 30-fold volume of vigorously stirred cold (−20 °C) acetone containing 5 mM HCl. Precipitated globin was collected by low-speed centrifugation (5000 *g* for 10 min at 0 °C), treated again with ice-cold acid/acetone until no red colour remained, resuspended in a minimum volume of water, dialysed against sodium bicarbonate (0.1 $g \cdot l^{-1}$), and then against 0.01 M phosphate buffer (pH 7.2). The denatured globin precipitating during this step was removed by centrifugation at 10000 *g* for 15 min at 5 °C, and the soluble globin fraction recovered by freeze-drying after dialysis against water at 4 °C.

SDS/PAGE and tetramethylbenzidine staining for haem-associated peroxidase

SDS/PAGE was carried out as described previously [20] on 15 % polyacrylamide gels. For haem-associated peroxidase staining in the gels, samples were prepared in non-reducing sample buffer by incubation at 37 °C for 1 h. For some experiments, samples were solubilized by heating at 100 °C for 5 min as indicated.

Densitometry

Densitometry was carried out on Coomassie Blue-stained bands using UVIband gel analysis software (UVItech) after digital image capture using a UMax Powerlook 1000 flatbed transmission scanner.

Measurement of methaemoglobin formation

The change in the concentration of methaemoglobin formed as a result of oxyhaemoglobin oxidation was estimated by difference spectroscopy as a function of the change in area of the Soret band [21,22], and as a function of the Q band absorbances as described previously [17]. The rate of haemoglobin (HbO_2) oxidation was also followed quantitatively using plots of $-\ln([HbO_2]_t/[HbO_2]_0)$ against time t, where the ratio of HbO_2 concentration after time t to that at time $t = 0$ was monitored by the absorbance changes of the α band (576 nm) of oxyhaemoglobin [23].

MALDI (matrix-assisted laser-desorption ionization)–TOF (time-of-flight)-MS

Samples were analysed using a Micromass M@LDI mass spectrometer, using an α-cyano-4-hydroxy-cinnamic acid matrix (Sigma). The spectra were recorded in the positive-ion mode and the mass range scanned was 800–4000 Da. The spectrometer was calibrated using a mixture of authentic peptide samples. The peptide masses observed were compared with those predicted by theoretical cleavage using the PeptideMass program (ExPASy).

RESULTS AND DISCUSSION

For the arguments presented previously [7], *P. gingivalis* and other black-pigmenting species inhabiting the gingival sulcus or periodontal pocket, may be exposed, from time-to-time, to oxygenated haemoglobin. However, the refractory nature of the oxyhaemoglobin molecule to proteolysis and haem release is overcome by *P. gingivalis* by first promoting its oxidation to methaemoglobin through the proteolytic action of R-gingipain [5–7]. In methaemoglobin, the affinity of globin for haem in which the iron is in the Fe(III) state is greatly relaxed, and the resultant haem-free protein is rendered susceptible to proteolysis by the lysine-specific gingipain [7]. K-gingipain can also

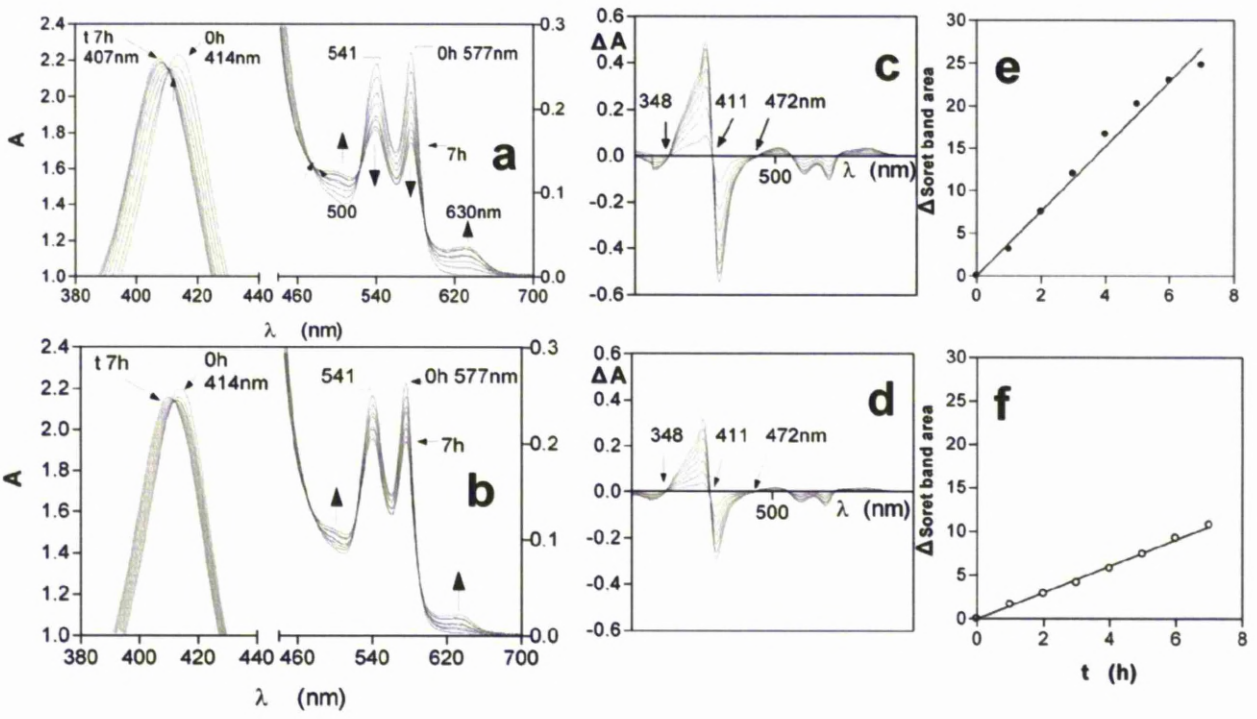

Figure 1 UV–visible spectra of horse oxyhaemoglobin during oxidation by InpA

(**a**) Oxyhaemoglobin (4 μM with respect to tetramer) was incubated with 2 μM InpA. (**b**) Oxyhaemoglobin auto-oxidation. (**c** and **d**) Difference spectra derived from data in (**a**) and (**b**), showing the changes in the Soret band region of oxyhaemoglobin during oxidation mediated by InpA (**c**) and during auto-oxidation (**d**). (**e** and **f**) Linear increases in change in Soret band region area indicative of methaemoglobin formation for InpA-mediated oxidation (**e**) and auto-oxidation (**f**). Buffer was 0.1 M Tris/HCl and 0.14 M NaCl (pH 7.5), and incubations were carried out at 37 °C.

attack hydroxymethaemoglobin produced non-proteolytically by treatment of haemoglobin with sodium nitrite [17].

Formation of methaemoglobin by InpA

In the present study, incubation of horse oxyhaemoglobin at pH 7.5 over a 7 h period in the presence of InpA (Figure 1a) resulted in spectral changes indicative of oxidation to hydroxymethaemoglobin, i.e. increases in absorption at 500 and 630 nm, and decreases at 541 and 577 nm. These were accompanied by an increase in absorbance and blue-shift in λ_{max} of the Soret band to approx. 407 nm. The same spectral changes were observed during incubation of oxyhaemoglobin in the absence of the protease (Figure 1b). Isosbestic points were observed in both sets of spectra (at 348 and 472 nm). This indicated that only two absorbing species were present and showed that InpA had mediated the direct conversion of oxyhaemoglobin into the methaemoglobin form. When 10 mM $Na_2S_2O_4$ was added to the haemoglobin samples after 7 h of incubation, to chemically reduce the haem iron and to deplete O_2 in the buffer, both were converted into deoxyhaemoglobin (Soret λ_{max} at 429 and Q band at 555 nm), confirming that methaemoglobin had originally been produced (results not shown).

By subtracting the initial zero time spectrum from those at subsequent time periods shown in Figures 1(a) and 1(b), a series of difference spectra were obtained with peak maxima at 402 nm and minima at 420 nm, with a zero cross-over point at 411 nm (Figures 1c and 1d), which is also characteristic of methaemoglobin formation [21,22]. Summation of the integrated peak areas between 348 and 411 nm, and trough areas between 411 and 472 nm, giving the total area change of the Soret band during the change from the oxy- to the met- state, allowed assessment of the extent of methaemoglobin formation [22]. This showed that InpA had mediated a 3-fold increase in oxidation rate compared with auto-oxidation (Figures 1e and 1f). Incubation of oxyhaemoglobin with InpA in the presence of the specific cysteine protease inhibitor E-64 (0.5 mM) had the effect of reducing the oxidation rate by approx. 80 % (results not shown), thus demonstrating that the ability to mediate oxyhaemoglobin oxidation was a result of its enzyme activity.

MS analysis of haemoglobin breakdown during InpA-mediated oxidation

To gain some insight into which residues were attacked during InpA-mediated oxidation of oxyhaemoglobin at pH 7.5, the enzyme was incubated with oxyhaemoglobin at 37 °C, samples were removed periodically, and the protease was inactivated by incubation for 30 min with E-64 (as above) before being subjected to MALDI–TOF-MS analysis. Table 1 shows the major digestion fragments identified as arising from α- and β-globin chains during InpA-mediated oxidation of oxyhaemoglobin. These indicated cleavages at the C-terminal sides of lysine or arginine and alanine or phenylalanine are in keeping with the specificities for hydrolysis of chromogenic *p*-nitrolanilide-derivatized peptides and processing sites of N- and C-terminal profragments cleaved during the autoproteolytic maturation of prointerpain respectively (J. Potempa, K. Wawrzonek and A. Jaworska, unpublished work).

Table 1 Digestion fragments identified as arising from proteolysis of α- and β-oxyhaemoglobin chains during InpA-mediated oxidation

Oxyhaemoglobin (4 μM) was incubated at 37 °C with 2 μM InpA, and samples were removed periodically, incubated for 30 min with E-64 (0.5 mM) to inhibit protease activity and subjected to MALDI–TOF-MS (see the text for details).

Fragment size (Da) and time of appearance	Corresponding predicted fragment size (Da)	Chain designation and residue numbers	Amino acid sequence
1126.75 (5 h)	1126.56	β-96–β-104	K↓L[96]HVDPENFR[104]
1356.92 (4 h)	1356.65	β-46–β-59	F↓G[46]DLSNPGAVMGNPK[59]
1417.92 (2 h)	1417.72	α-111–α-123	A↓V[111]HLPNDFTPAVHA[123]
1902.36 (8 h)	1902.94	α-8–α-26	K↓T[9]NVKAAWSKVGGHAGEYGA[26]
2227.66 (4 h)	2227.08	β-41–β-61	R↓F[41]FDSFGDLSNPGAVMGNPKVK[61]
	2227.10	α-41–α-60	K↓T[41]YFPHFDLSHGSAQVKAHGK[60]
2037.65 (2 h)	2037.97	β-86–β-103	F↓A[86]ALSELHCDKLHVDPENF[103]

Particularly noteworthy was the detection of peptides of masses 1356.92 and 2227.66, which indicate cleavage of residues around the distal region of the haem pocket of the α- and β-chains. Importantly, SDS/PAGE analysis of the haemoglobin samples incubated with InpA and solubilized by heating at 100 °C clearly showed a limited degree of protein breakdown (approx. 20 %) had occurred over the same time period (see Figure 3), suggesting that, although the globin structure has been proteolytically cleaved, giving rise to oxidation, its gross structure had remained stable. Indeed, the complex structure of haemoglobin is maintained by numerous interactions between individual residues within the globin chain, and with the haem moiety, as well as interfacial interactions between the globin chains of haemoglobin tetramers and dimers. These interactions not only enable haemoglobin to alter shape in response to oxygen loading, but also may prevent complete unfolding of globin chains during incisions made by InpA. This would also explain why peptides are only released from the protein under the extreme conditions of denaturation during the MALDI process or boiling.

Longer-term incubation of InpA with oxyhaemoglobin at pH 7.5 produces a haemoglobin haemichrome

Although short-term incubation of oxyhaemoglobin with InpA resulted in hydroxymethaemoglobin formation, interaction for periods of 24 h or greater resulted in a characteristic haemichrome spectrum [24] with Soret λ_{max} 409 nm and a broad Q band at 535 nm (Figure 2). The haemichrome nature of this product was confirmed by reduction to a haemochrome with sodium dithionite (424 nm Soret and 530 and 558 nm Q bands [24,25]) (Figure 2, continuous lines). Although UV–visible spectroscopy appeared to show that there had been no detectable InpA-mediated breakdown of haemoglobin over the initial 6 h, SDS/PAGE (where samples had been solubilized by heating at 100 °C) and densitometry showed that approx. 20 % of the globin chains were degraded after 6 h of incubation (Figure 3). It is noteworthy that, like the arginine-specific gingipains HRgpA [17] and RgpB (J.W. Smalley, A.J. Birss and J. Potempa, unpublished work), InpA also promoted haemoglobin oxidation at pH 7.5. This is a pH at which the natural oxidation rate of oxyhaemoglobin is lowest [26]. However, the hydroxymethaemoglobin product formed by InpA at this pH was refractory to further breakdown by InpA, and further incubation resulted in conversion into a haemichrome. This is puzzling given the numerous lysine and arginine residues present in α- and β-globin chains, the broad pH optima of InpA proteolytic activity in the pH range 5.5–8.0 and the preference of InpA for arginine, lysine, alanine and phenylalanine residues at the C-terminal side of hydrolysed peptide bonds (J. Potempa, K. Wawrzonek and A. Jaworska, unpublished work).

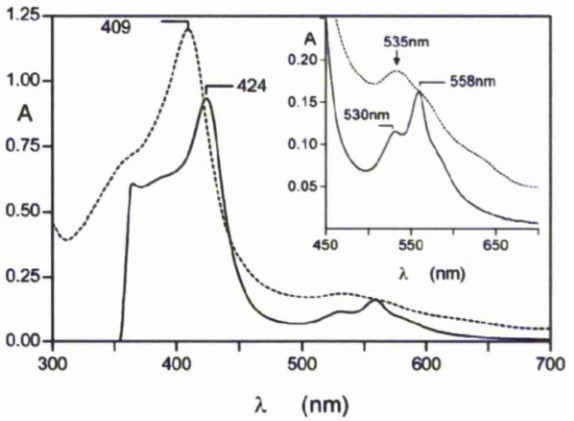

Figure 2 UV–visible spectrum of the haemoglobin haemichrome formed after longer incubation of oxyhaemoglobin with InpA at pH 7.5

The haemichrome produced after 24 h of incubation (broken line) gave rise to a haemochrome spectrum upon addition of 10 mM $Na_2S_2O_4$ (continuous line). Incubation conditions and enzyme and haemoglobin concentrations were as for Figure 1. Inset shows the Q band region.

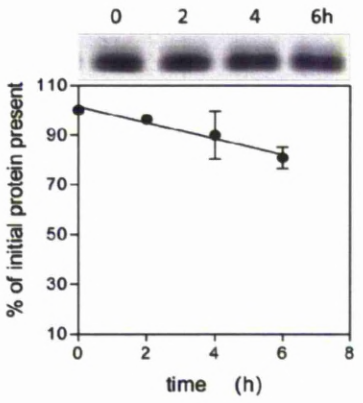

Figure 3 SDS/PAGE of oxyhaemoglobin during incubation with InpA

Incubation conditions and enzyme and substrate conditions were as described in Figure 1. The gel was stained with Coomassie Blue and scanned densitometrically to quantify the loss of protein during incubation, and expressed as the percentage of initial protein present. Samples were solubilized by heating at 100 °C for 5 min. Results are means±S.D. for three separate experiments.

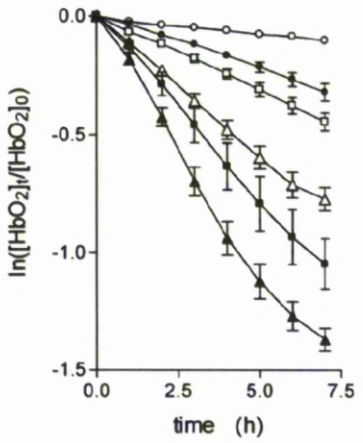

Figure 4 Oxyhaemoglobin oxidation rates in the presence of InpA

Rates were measured as a function of the change in A_{576} in the presence of InpA at pH 7.5 (○, ●), 6.5 (□, ■) and 6.0 (△, ▲). Open symbols depict the auto-oxidation rate at each pH in the absence of enzyme. Incubations were carried out at 37 °C with 4 μM oxyhaemoglobin (as tetramer) and 2 μM InpA, in 0.14 M NaCl, buffered with 0.1 M Tris/HCl (pH 7.5) or 0.2 M phosphate (pH 6.5 and 6.0). Results are means±S.D. of four separate determinations, except for those at pH 7.5, where $n = 7$.

Relative susceptibilities of methaemoglobin and aquomethaemoglobin to degradation by InpA

Prevotella species display acidic terminal growth pHs [27–29]. Both binding to the outer membrane and degradation of haemoglobin mediated by enzymes in the growth supernatant of *P. intermedia* is enhanced at acid pHs [10,30]. In addition, pigment production during growth on blood agar is associated with a fall in pH to approx. 5.8 [3]. Accordingly, we investigated the pH-dependency of oxyhaemoglobin oxidation and breakdown by InpA. To measure the rate of haemoglobin oxidation while obviating any complication relating to changes in Soret band area due to degradation and loss of haem from the protein, the rate of haemoglobin oxidation was quantified by following the change in A_{576} over time [23]. This gave linear plots of the initial oxidation rate over the 6 h incubation period (Figure 4), which clearly demonstrated that InpA increased oxidation above that of the auto-oxidation rate at each pH examined. In this context, it is noteworthy that, whereas InpA increased the oxidation rate at pH 7.5 (a pH at which the auto-oxidation rate of both the α- and β-haemoglobin chains is naturally lowest [26]), it was also able to increase the oxidation rate above the auto-oxidation rate at the acid pHs, conditions where the natural oxidation rate of oxyhaemoglobin is high [26]. This is relevant to the biology of the sub-gingival environment, since *Prevotella* species produce a potent haemolysin [31–33], and any haemoglobin released through the haemolysis of extravasated erythrocytes will be readily oxidized in the absence of intra-erythrocytic reductants and the enzyme methaemoglobin reductase.

We have shown previously that, whereas oxyhaemoglobin is not degraded by the lysine-specific gingipain, it is susceptible to breakdown by this protease when converted into the hydroxymethaemoglobin form, either through oxidation mediated by sodium nitrite or via pre-treatment with the arginine-specific gingipain HRgpA [7,17]. To test whether hydroxy- and aquo-methaemoglobin species were susceptible to breakdown by InpA, oxyhaemoglobin was first auto-oxidized by incubation for 24 h at 37 °C at pH 7.5, 6.0 or 5.5. Under these conditions, approx. 95 % of the oxyhaemoglobin was converted into the

Figure 5 SDS/PAGE showing the effect of InpA on methaemoglobin species and haem-free globin chains

Hydroxymethaemoglobin, pH 7.5 (gels a), aquomethaemoglobin, pH 6.0 (gels b) and pH 5.5 (gels c), azidomethaemoglobin, pH 6.0 (gels d), haem-free globin, pH 7.5 (gel e). Haemoglobin substrates at 4 μM (with respect to tetramer) were incubated with InpA (2 μM) at 37 °C and aliquots withdrawn at the time points indicated were subjected to SDS/PAGE analysis. The hydroxymethaemoglobin and aquomethaemoglobin species were formed by auto-oxidation of oxyhaemoglobin at 37 °C for 24 h at pH 7.5, 6.0 or 5.5, and constituted 95 % of the total haemoglobin present. (**A**) Gels stained with Coomassie Blue. (**B**) Gels stained with TMB for haem-associated peroxidise activity. Azidomethaemoglobin was prepared by incubating aquomethaemoglobin in the presence of 400 μM NaN_3. To aid comparison of the time-dependent breakdown and haem release from the haemoglobin species (gels b–e) with that of hydroxymethaemoglobin (gels a), tracks of the 2 and 4 h time samples were excised from the images of these gels. The points of excision are displayed by vertical lines.

hydroxy- and aquo-methaemoglobin forms. The methaemoglobin preparations (4 μM as tetramer) were then exposed at 37 °C to InpA (2 μM) and sampled periodically for SDS/PAGE. Gels were first stained with TMB (3,3′,5,5′-tetramethylbenzidine)/H_2O_2 to reveal haemoglobin-associated haem peroxidase activity, and then counterstained for protein with Coomassie Blue (Figure 5). Densitometry revealed that during incubation with InpA at pH 7.5, the globin chains were still intact (Figure 5A, gel a). Moreover, TMB staining (Figure 5B, gel a) showed that little or no haem had been lost from hydroxymethaemoglobin. In contrast, however, the aquomethaemoglobin preparations were degraded to a much greater degree, especially at pH 5.5 (Figure 5A, gels b and c), and proteolysis of the haemoglobin chains was also accompanied by a greater haem loss as shown by the reduced level of TMB staining (Figure 5B, gels b and c). The susceptibility of aquomethaemoglobin and hydroxymethaemoglobin to InpA was also examined spectroscopically. These substrates (~ 4 μM on a tetramer basis) were prepared by oxidation of oxyhaemoglobin with sodium nitrite in either pH 5.5 or 7.5 buffers, to give the aquo- or hydroxy-methaemoglobin forms respectively, and were then exposed to 2 μM InpA. As can be seen in Figure 6, InpA effected an almost complete breakdown of aquomethaemoglobin (Figure 6a) compared with the hydroxymethaemoglobin form (Figure 6b) over 7 h as is evident by the collapse of the Soret band. In addition, spectrophotometric analysis also confirmed that azidomethaemoglobin, in which the haem was stabilized by N_3^- ligation, was degraded minimally by InpA (Figure 6c). However, trypsin (2 μM) brought about complete breakdown of the hydroxymethaemoglobin, even at pH 7.5 (Figure 7). The trypsin-mediated breakdown of the hydroxymethaemoglobin protein chains was also reflected in the loss of Soret band intensity (Figure 7). The reduced ability of InpA to degrade hydroxymethaemoglobin and to release haem

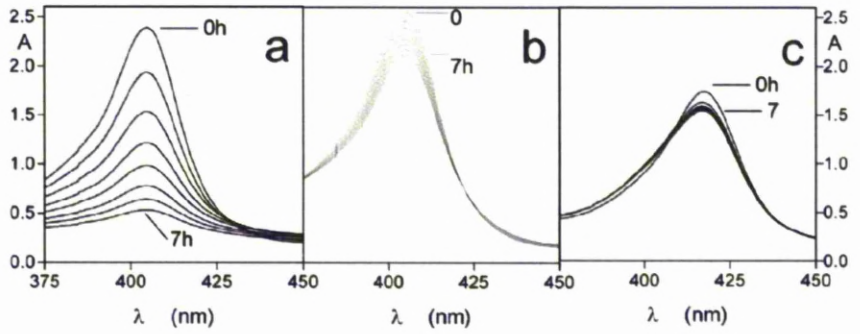

Figure 6 Soret band regions of (a) aquomethaemoglobin, (b) hydroxymethaemoglobin and (c) azidomethaemoglobin during incubation with InpA

Substrate and enzyme concentrations were 4 μM (as tetramer) and 2 μM respectively. Incubations were carried out at 37 °C.

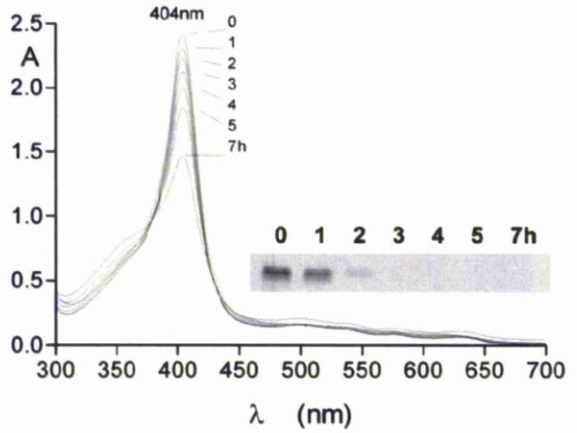

Figure 7 Trypsin-mediated breakdown of hydroxymethaemoglobin as shown by loss of Soret band intensity and by SDS/PAGE

The gel was stained with Coomassie Blue. Haemoglobin was at 4 μM and trypsin at 2 μM. Incubation was carried out at 37 °C in 0.1 M Tris/HCl and 0.14 M NaCl (pH 7.5).

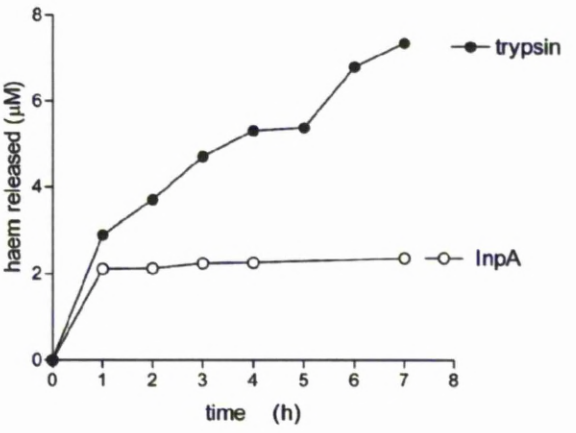

Figure 8 Proteolytic release of haem from bovine hydroxymethaemoglobin–agarose mediated by trypsin and InpA

Suspensions of methaemoglobin–agarose beads (4 μM with respect to haemoglobin tetramer) were incubated at 37 °C with trypsin or InpA (2 μM) in 0.1 M Tris/HCl and 0.14 M NaCl (pH 7.5). Beads were pelleted by centrifugation and the supernatant buffer was assayed at intervals for released free haem using the pyridine haemochrome assay.

at pH 7.5 was confirmed in a separate experiment in which hydroxymethaemoglobin–agarose was incubated with InpA or trypsin. Haem released from the immobilized haemoglobin was detected as the pyridine haemochromogen after reduction with sodium dithionite, followed by reaction with pyridine. This showed that InpA was only able to release approx. 20% of the total haemoglobin haem liberated by trypsin over a period of 7 h (Figure 8). Significantly, this observation also correlates perfectly with the 20% degradation of oxyhaemoglobin by InpA over the same time period (Figure 3).

Azide ligation prevents InpA breakdown of aquomethaemoglobin at pH 6.0

Aquomethaemoglobin was efficiently degraded by InpA. However, when the haem iron of aquomethaemoglobin was ligated with azide, the protein was resistant to breakdown by InpA at pH 6.0. Neither globin chain proteolysis nor haem loss was observed (Figures 5A and 5B, gel d). In stark contrast, haem-free globin (at the same concentration as the methaemoglobin substrates) was completely degraded within a few minutes by InpA, even at pH 7.5 (Figure 5A, gel e).

The difference in susceptibility of the aquomethaemoglobin compared with the hydroxymethaemoglobin substrate is, at first sight, puzzling, as these two forms are structurally indistinguishable. Although InpA displays a broad pH activity profile centred on pH 8 compared with the synthetic chromogenic peptide substrates bearing P1 lysine or arginine residues, it also has an acidic pH optimum (~ pH 6) towards protein substrates azocoll, azoalbumin and azocasein (J. Potempa, K. Wawrzonek and A. Jaworska, unpublished work), which would explain the efficient proteolysis at acidic pH. However, if haemoglobin breakdown was simply related to a pH effect on the enzyme, then the extent of proteolysis of azide-liganded aquomethaemoglobin would be commensurate with that observed for the non-azide bound substrate at pH 6, but this was not the case. The mass spectrometric detection of peptide fragments arising from both α- and β-globin chains during incubation of oxyhaemoglobin with InpA demonstrates that proteolytic attack occurs during oxidation. The reason that gross breakdown of either oxyhaemoglobin or hydroxymethaemoglobin does not occur is that proteolytic modification resulting in oxidation at pH 7.5, brought about the production of a stable haemichrome,

largely resistant to further breakdown by the enzyme. Rather, the underlying reason for proteolysis of aquomethaemoglobin by InpA resides in the fact that haem dissociation is greatly increased when haemoglobin is in the aquomethaemoglobin compared with the hydroxymethaemoglobin form, which in turn facilitates the proteolysis of the newly formed haem-free globin.

Mechanism of InpA-mediated oxidation

As a functional oxygen carrier, the oxyhaemoglobin haem iron exists in the ferrous (Fe^{2+}) form. However, the ferrous iron can spontaneously oxidize (auto-oxidation) into the ferric state (methaemoglobin) with the production of superoxide anion, O_2^- [23,24], and in this form is incapable of binding oxygen. Because O_2 is a poor one-electron acceptor, the Fe–O_2 bond is relatively stable, which poses a considerable thermodynamic barrier for electron transfer. For this reason, auto-oxidation via spontaneous superoxide dissociation is considered unlikely [34]. Rather, the accepted mechanism is the entry of OH^- or water molecules into the haem pocket from the solvent, to effect a stoichiometric nucleophilic displacement of O_2^- [23,26]. These nucleophiles remain bound to the iron at the sixth co-ordinate position, forming either aquomethaemoglobin (or acid form), for bound water, or hydroxymethaemoglobin, for bound OH^-. The formation of these species is indicative of the oxidation process. During InpA-mediated oxyhaemoglobin oxidation, we detected several peptides by MS, indicative of scission at sites around the haem pocket. Such cleavage events, although not leading to extensive proteolysis, may instead lead to structural changes which facilitate the displacement of O_2^- as is evident by formation of the hydroxy- and aquo-methaemoglobin species at pH 7.5 and 6.0 respectively, and in keeping with the accepted paradigm for the oxidation process [23,34,35].

Even minor structural changes to the haem pocket of haemoglobin disturb its function. For example, a F46V mutation in myoglobin markedly increases the rate of oxidation by facilitating access of water molecules [36]. This is because Phe^{46} stabilizes the distal His^{64}, and removal of this contact through mutation to valine opens the distal pocket. In this context, it is noteworthy that a prominent peptide of molecular mass 1356.92 (β-chain, $F^{45}\downarrow G^{46}$DLSNPGAVMGNPK^{59}) was observed early in the time course of haemoglobin digestion by InpA, and is evidence that InpA disrupts the distal region of the haem pocket. Given that Phe^{45} of the β-chain shares a similar orientation to the distal histidine as Phe^{46} of myoglobin, and the structural similarities between these proteins, it is possible to speculate that this residue plays a similar role in dictating the degree of auto-oxidation, and that cleavage by InpA at this site may account for the increased oxidation rate of oxyhaemoglobin. It is also noteworthy that the abnormal M haemoglobins (mutant haemoglobins which have a propensity to become easily auto-oxidized to the methaemoglobin form) have amino acid substitutions in or near the haem pocket [37].

The possibility that oxidation was a result of the presence of contaminant metal ions which are well known to bring about oxidation was also tested by conducting incubations of InpA with oxyhaemoglobin in the presence of EDTA (10 μM). Under these conditions, the rates of methaemoglobin formation brought about by InpA (determined by plots of ΔA_{576} as above) measured over a 5 h period were the same in both the presence and absence of EDTA (results not shown). It was concluded from this that the InpA-mediated oxidation was unrelated to the presence of metal ions.

Although oxidation of oxyhaemoglobin weakens the haem iron-proximal histidine bond, permitting haem dissociation [38,39], breakdown of haemoglobin by InpA at acidic pH may also be aided, in addition to the proton-assisted oxidation [26], by the low pH which enhances haem mobility and dissociation from the protein, especially from the β-chain [39]. We have shown that *P. intermedia* and *P. nigrescens* engender a low pericellular pH during pigment formation [3]. This would promote dissociation of haemoglobin tetramers into $\alpha\beta$ dimers and enhance haem loss from the latter [40]. It should also be noted that $\alpha\beta$ dimers auto-oxidize more rapidly than tetramers [41].

The present paper is the first to describe a specific role of a *P. intermedia* protease in both haemoglobin oxidation and breakdown and subsequent haem release. InpA may thus play a major role in pigment formation by this black-pigmenting organism. Together with our previous work [7,17], these findings have revealed a new paradigm for haem acquisition from haemoglobin by black-pigmenting species, which is dependent upon initial haemoglobin oxidation which facilitates haem dissociation and globin breakdown.

AUTHOR CONTRIBUTION

As senior authors, John Smalley and Jan Potempa had the initial concept and designed the experimental strategy. Katarzyna Wawrzonek and Anna Jaworska performed the initial expression and purification of the recombinant proteases, while Andrew Birss carried out the preliminary experimental studies to establish the phenomenon of InpA-mediated oxidation of oxyhaemoglobin. Dominic Byrne produced the majority of the data presented in the paper except for the MS analysis, which was carried out by Dr Deborah Ward and Mark Prescott as part of the service provided by Proteomics Laboratory, School of Biological Sciences, University of Liverpool, as stated in the acknowledgements.

ACKNOWLEDGEMENTS

We thank Mark Prescott and Dr Deborah Ward of the Proteomics Laboratory, University of Liverpool, School of Biological Sciences, who carried out the MS.

FUNDING

J.W.S. is grateful to the School of Dental Science, University of Liverpool, for financial support for D.P.B. This study was also supported in part by grants from the National Institutes of Health [grant number DE 09761], and the Department of Scientific Research, Polish Ministry of Science and Higher Education [grant number 1642/B/P01/2008/35].

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Received 25 February 2009/2 October 2009; accepted 9 October 2009
Published as BJ Immediate Publication 9 October 2009, doi:10.1042/BJ20090343

OPEN ACCESS Freely available online

PLoS one

HmuY Haemophore and Gingipain Proteases Constitute a Unique Syntrophic System of Haem Acquisition by *Porphyromonas gingivalis*

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Abstract

Haem (iron protoporphyrin IX) is both an essential growth factor and virulence regulator for the periodontal pathogen *Porphyromonas gingivalis*, which acquires it mainly from haemoglobin via the sequential actions of the R- and K-specific gingipain proteases. The haem-binding lipoprotein haemophore HmuY and its cognate receptor HmuR of *P. gingivalis*, are responsible for capture and internalisation of haem. This study examined the role of the HmuY in acquisition of haem from haemoglobin and the cooperation between HmuY and gingipain proteases in this process. Using UV-visible spectroscopy and polyacrylamide gel electrophoresis, HmuY was demonstrated to wrest haem from immobilised methaemoglobin and deoxyhaemoglobin. Haem extraction from oxyhaemoglobin was facilitated after oxidation to methaemoglobin by pre-treatment with the *P. gingivalis* R-gingipain A (HRgpA). HmuY was also capable of scavenging haem from oxyhaemoglobin pre-treated with the K-gingipain (Kgp). This is the first demonstration of a haemophore working in conjunction with proteases to acquire haem from haemoglobin. In addition, HmuY was able to extract haem from methaemalbumin, and could bind haem, either free in solution or from methaemoglobin, even in the presence of serum albumin.

Citation: Smalley JW, Byrne DP, Birss AJ, Wojtowicz H, Sroka A, et al. (2011) HmuY Haemophore and Gingipain Proteases Constitute a Unique Syntrophic System of Haem Acquisition by *Porphyromonas gingivalis*. PLoS ONE 6(2): e17182. doi:10.1371/journal.pone.0017182

Editor: Anil Tyagi, University of Delhi, India

Received September 27, 2010; **Accepted** January 22, 2011; **Published** February 17, 2011

Funding: This work was supported in parts by the Ministry of Science and Higher Education, Poland (grant No. N N303 406136 and 1642/B/P01/2008/35 to T.O. and J.P., respectively), the Jagiellonian University (statutory funds DS/9/WBBiB), the National Institutes of Health (Grant DE 09761) and the Foundation for Polish Science (Team/2009-4/8). The Faculty of Biochemistry, Biophysics and Biotechnology of the Jagiellonian University is a beneficiary of the structural funds from the European Union (grant No. POIG.02.01.00-12-064/08 - "Molecular biotechnology for health"). J.W.S. is grateful to the School of Dental Science, University of Liverpool, for financial support for D.P.B. The funders had no role in study design, data collection and analysis, decision to publish, or preparation of the manuscript.

Competing Interests: The authors have declared that no competing interests exist.

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Introduction

Porphyromonas gingigivalis is a gram-negative oral anaerobe which has been implicated as one of the major aetiologic agents of periodontitis in adults [1]. It displays a black-pigmenting phenotype which is due to accumulation of a haem-containing pigment. The pigment is formed during growth on blood containing media and the haem component of this is derived from proteolytic breakdown of the haemoglobin, and the haem-carrying proteins haemopexin and haemalbumin. The haem in the pigment is present in the so-called μ-oxo bishaem, or dimeric form of iron(III) protoporphyrin IX, $[Fe(III)PPIX]_2O$, which is stored in aggregated form on the *P. gingivalis* cell surface [2]. *P. gingivalis* has an essential requirement for haem [3] which has a profound influence on its virulence properties [4], [5]. However, *P. gingivalis* cannot synthesize this iron porphyrin and thus acquisition from exogenous sources, coupled with its binding and cell surface storage, is essential for the growth and survival of this bacterium. Whilst the agent(s) responsible for haem deposition at the cell surface have not been elucidated, binding of haem for purposes of internalisation is mediated by a dual component uptake system comprising the haem-binding protein HmuY and its cognate receptor HmuR [6]. Both HmuY and HmuR are encoded by the *hmu* operon, encompassing a total of six genes, *hmuYRSTUV*, which is regulated by the environmental levels of iron [7]. The potential importance of the *hmu* operon in haem acquisition has been demonstrated by the fact that its inactivation results in reduction of both cellular haem binding and its uptake [8]. The mature membrane-associated HmuY protein has a molecular weight of 24 kDa [9]. As a non-haem liganded apoprotein, it exists as a homodimer, whereas upon binding of haem it can form a homo-tetrameric complex [7], [9]. HmuY can also bind iron protoporphyrin IX in both Fe(III) and Fe(II) states [10]. Point mutation studies have shown that residues His134 and His166 of HmuY are involved in six-coordinate binding of haem [10]. Concurrently, solution of the crystal structure of HmuY has confirmed this arrangement and demonstrated a unique all beta-fold protein structure [9].

The emerging paradigm for proteolytically mediated haem acquisition from oxygenated haemoglobin involves the concerted action of both arginine- and lysine-specific gingipain proteases, Rgp and Kgp, [11], [12], [13]. In this process the Fe(II) oxyhaemoglobin

species is firstly oxidised by the proteolytic action of Rgp giving rise to methaemoglobin, the oxidised form of haemoglobin. The concomitant reduction in the affinity of globin for the iron protoporphyrin IX, now in the Fe(III) state, results in dissociation of the haem moiety from the globin protein, which is then rendered susceptible to degradation by Kgp [12], [13]. It is well documented that the clearance of free haem from the systemic circulation occurs following oxidation of oxyhaemoglobin, and the ferric haem thus formed is easily transferred from methaemoglobin to albumin [14], [15]. Given the propensity for more facile haem loss and the proteolytic susceptibility of ferric haemoglobins, together with the demonstrated affinity of HmuY for ferric (and ferrous) haem species [7], [10], we thus investigated the role of this haemophore protein in acquiring haem directly from haemoglobin and also the cooperative role of the *P. gingivalis* gingipain proteases in this process.

Results

Formation of ferrihaem-HmuY and ferrohaem-HmuY

To confirm the spectrum of the Fe(III) haem-HmuY complex, the protein was incubated at 37°C with an equimolar quantity of iron(III) protoporphyrin IX in NaCl-Tris-HCl buffer, pH 7.5. As seen in Figure 1, the HmuY reacted rapidly with the iron(III) protoporphyrin IX causing a red shift simultaneous with the increase of the extinction of the Soret band (λ_{max} 411 nm), plus Q bands of approximately 527 nm and 558 nm. This is in accord with the previously published spectrum for the six-coordinate, bis-histidine ligated, 1:1 HmuY-ferrihaem low-spin complex [10]. The generation of the HmuY monomeric ferrihaem complex under these conditions reached a maximum after approximately 120 min, after which time no further changes to the spectrum were observed. The series of time-course spectra depicted in Fig. 1A displayed an isosbestic point at approximately 388 nm showing the presence of only two absorbing species and indicating the direct conversion of the free ferrihaem into the HmuY-iron(III) protoporphyrin IX complex. This is in keeping with the known 1:1 stoichiometry of this interaction [7]. The binding of iron protoporphyrin IX was also monitored in the presence of 10 mM $Na_2S_2O_4$ to reduce the iron(III) protoporphyrin IX to the Fe(II) species (Fig. 1B). Reaction of the HmuY with this form of haem gave the Fe(II) haem-HmuY complex with Soret band λ_{max} of 422 nm and Q bands at 526 nm and 556 nm, consistent with the previously published spectrum of the ferrohaem-HmuY complex [10]. Under these conditions, the ferrohaem-HmuY complex formed immediately upon mixing and the Soret band attained a maximum after 45 min after which time there was no further change in the spectrum.

Effect of haem complexation on the electrophoretic mobility of HmuY

Complexation of HmuY with an equimolar amount of ferrihaem increased significantly the electrophoretic mobility of the HmuY as analyzed by the native PAGE (Fig. 2). This effect is presumed to occur as a consequence of bis-histidine ligation of haem by the HmuY, resulting in a haem-HmuY complex in which the ionised carboxylate groups of the iron porphyrin impart additional negative charge, which makes the liganded holoHmuY migrate faster than apoHmuY in the electric field during native PAGE.

HmuY binds iron(III) protoporphyrin IX from methaemoglobin

To determine whether the haemophore could form a ferrihaem complex through interaction with methaemoglobin, HmuY was incubated with immobilised methaemoglobin conjugated to agarose. Such preparations have been used previously to determine the rates of haem loss from haemoglobin [16]. This method allows the monitoring of protein-haem complex formation (in solution) separately from the haemoglobin attached to the solid phase in a way free of any compounding spectral features due to the co-presence of haemoglobin. Previously we have used this approach to demonstrate the facile breakdown and release of haem from methaemoglobin by Kgp [13] and interpain A of *Prevotella intermedia* [17]. Here we incubated HmuY with methaemoglobin-agarose and at various time intervals sedimented the beads by low speed centrifugation, and recorded the spectrum of the agarose-free supernatant. The spectral analyses revealed a typical ferrihaem-HumY spectrum with the characteristic 411 nm

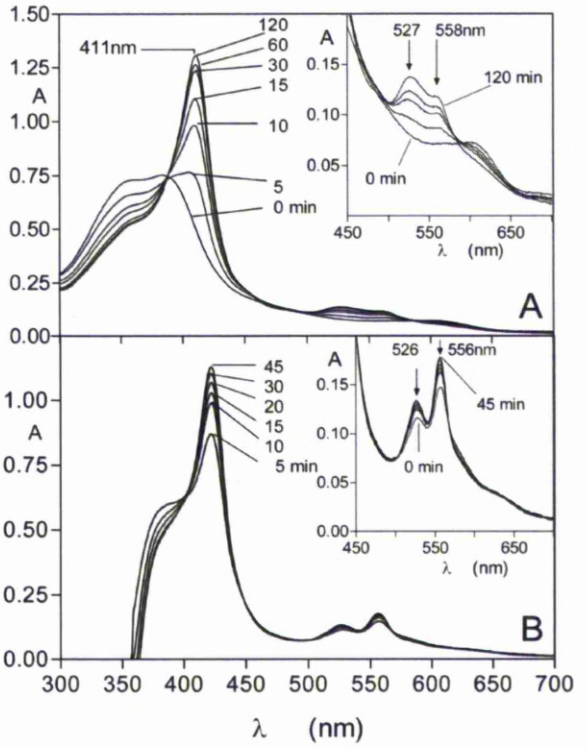

Figure 1. Formation of the HmuY-ferrihaem (A) and HmuY-ferrohaem (B) complexes during reaction of HmuY with iron protoporphyrin IX. HmuY (16 μM) was reacted with an equimolar amount of iron protoporphyrin IX. In (B) the ferrohaem species was generated by inclusion of 10 mM $Na_2S_2O_4$ in the buffer. The steep drop in absorbance below 375 nm is due to subtraction of the reference background spectrum of the dithionite-containing buffer.
doi:10.1371/journal.pone.0017182.g001

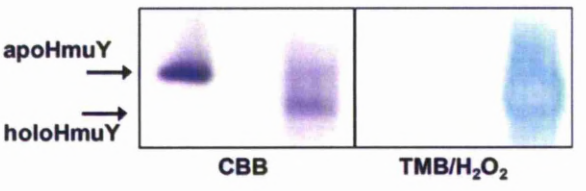

Figure 2. Effect of ferrihaem binding on the electrophoretic mobility of HmuY. The presence of haem in the complex with HmuY is confirmed by haem-peroxidise activity (bottom panel).
doi:10.1371/journal.pone.0017182.g002

Soret band and 527 and 558 nm visible bands which developed within 5 minutes of mixing (Fig. 3). Following the addition of 10 mM sodium dithionite to the incubation mixture a spectrum indicative of the ferrohaem-HmuY complex developed (a 422nm Soret and 526 and 556 nm Q bands) (data not shown).

The results of spectral analysis indicating HmuY-haem complex formation were corroborated by non-reducing SDS-PAGE analysis of samples obtained at different time intervals during incubation of HmuY with immobilized methaemoglobin. The HmuY protein band showed a time-dependent increase in TMB/H_2O_2 haem-peroxidase staining unequivocally confirming the formation of the HmuY-haem complex (Fig. 4).

Methaemoglobin formation by R-gingipain facilitates extraction of haem from haemoglobin by HmuY

In vivo, at periodontitis sites where bleeding may occur, any oxyhaemoglobin released from erythrocytes must firstly be oxidized to the methaemoglobin form before haem can be sequestered by HmuY. Therefore the process of haem acquisition and uptake by the HmuY/HmuR system should be dependent upon gingipain protease-mediated haemoglobin oxidation [12], [13]. To experimentally verify this hypothesis, an HRgpA-induced methaemoglobin preparation (total haemoglobin concentration 16 μM and comprising 77% in the oxidised form) was incubated with HmuY (16 μM). A control $NaNO_2$-induced methaemoglobin preparation (containing 83% in the oxidised form) was also incubated with HmuY as above for comparison. With time, spectral features were observed indicative of conversion of R-gingipain-induced methaemoglobin into the ferrihaem-HmuY complex, i.e., red shift of the Soret band from 406 to 411 nm, loss of $A_{500\ nm}$ and $A_{630\ nm}$, and increases in $A_{527\ nm}$ and $A_{558\ nm}$, as shown in Fig. 5. These spectral features were also observed for the incubation of HmuY with the $NaNO_2$-induced methaemoglobin preparation (data not presented).

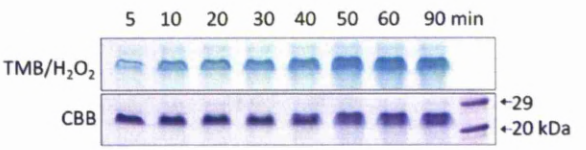

Figure 4. Haem pickup by HmuY from methaemoglobin-agarose. HmuY (16 μM) was incubated with methaemoglobin-agarose beads and after centrifugation the agarose-free supernatant was sampled and analyzed by non-reducing SDS-PAGE, and stained with TMB/H_2O_2 to reveal haem-associated peroxidise activity before being stained with Coomassie brilliant blue (CBB). Protein loading per track was 7 μg.
doi:10.1371/journal.pone.0017182.g004

To confirm that haem had been complexed by HmuY and was not still present in the form of haemoglobin, the reaction mixture was treated with 10 mM $Na_2S_2O_4$ and the UV-visible spectrum recorded. Analysis of this spectrum showed a 423 nm Soret band, plus visible bands at 526 nm and 556 nm, clearly indicating the presence of the ferrohaem-HmuY complex (data not presented). This result unambiguously indicated that haem had become complexed to HmuY during incubation with the methaemoglobin which had been formed by the action of HRgpA.

The facile transfer of iron(III) protoporphyrin IX from methaemoglobin to HmuY was further corroborated by assessment of the relative amount of the HmuY-ferrihaem complex formed from either HRgpA- or $NaNO_2$-induced methaemoglobin. To this end, difference spectra were derived by subtracting the control spectra from the tests at each time interval (Fig. 6A and B) which showed that the amount of HmuY-haem complex formed was greater from methaemoglobin proteolytically induced by HRgpA compared to that produced by $NaNO_2$ treatment.

These difference spectra were also characterised by troughs at 630 nm and 500 nm, which related to loss of methaemoglobin,

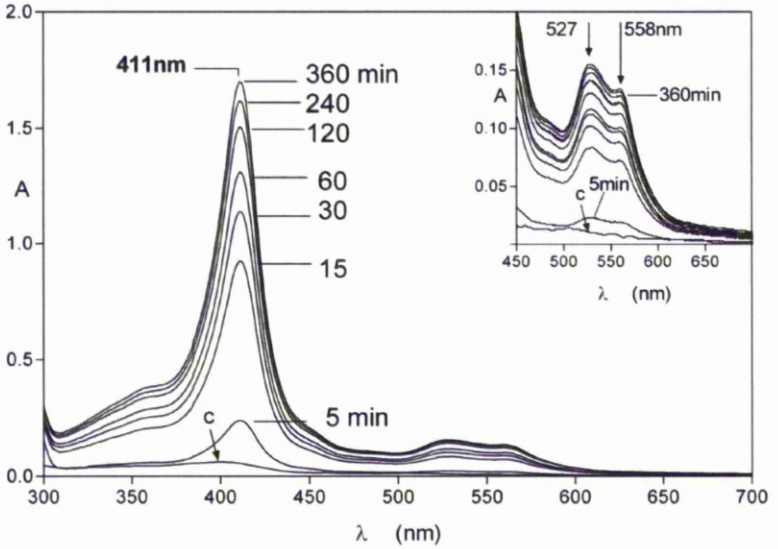

Figure 3. Spectroscopic demonstration of the formation of the HmuY-ferrihaem complex following interaction with immobilised methaemoglobin. Methaemoglobin-agarose (16 μM with respect to haemoglobin subunit) was incubated with an equimolar amount of HmuY and at various time periods the agarose beads sedimented by centrifugation and the spectra of the supernatant recorded. The spectrum denoted c represents the small amount of methaemoglobin (~0.25% of total) spontaneously released from the control methaemoglobin-agarose beads after 6 h in the absence of HmuY. The incubations were carried out at 37°C. See text for details.
doi:10.1371/journal.pone.0017182.g003

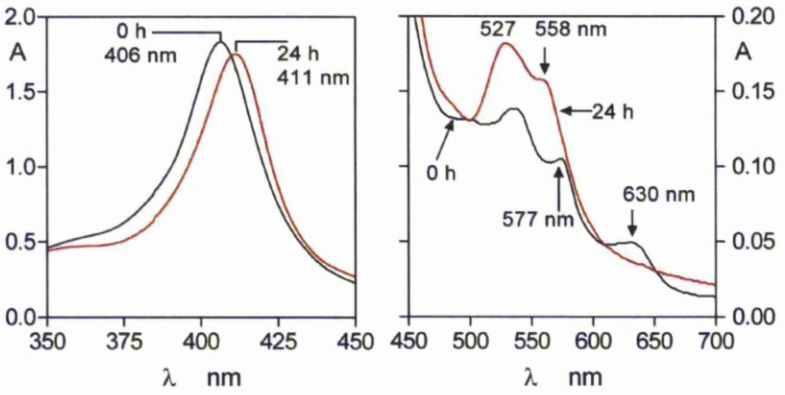

Figure 5. Demonstration HmuY-ferrihaem complex formation after exposure of HRgpA-induced methaemoglobin to HmuY. Before exposure, black line; 24 h after exposure, red line. For clarity, only the initial and final spectra are shown. HRgpA and HmuY were used at 0.4 and 16 μM, respectively. Starting concentration of haemoglobin was 16 μM (with respect to haemoglobin subunit) and comprised 77% methaemoglobin.
doi:10.1371/journal.pone.0017182.g005

and increases in absorbance at 525 nm and 558 nm, attributable to the formation of the HmuY-ferrihaem complex. Isosbestic points were observed at 510 nm, 590 nm, and 660 nm (arrowed) showing the direct conversion from methaemoglobin to HmuY-haem complex. These spectra clearly showed that the amount of HmuY-haem complex formed from HRgpA-pre-treated haemo-

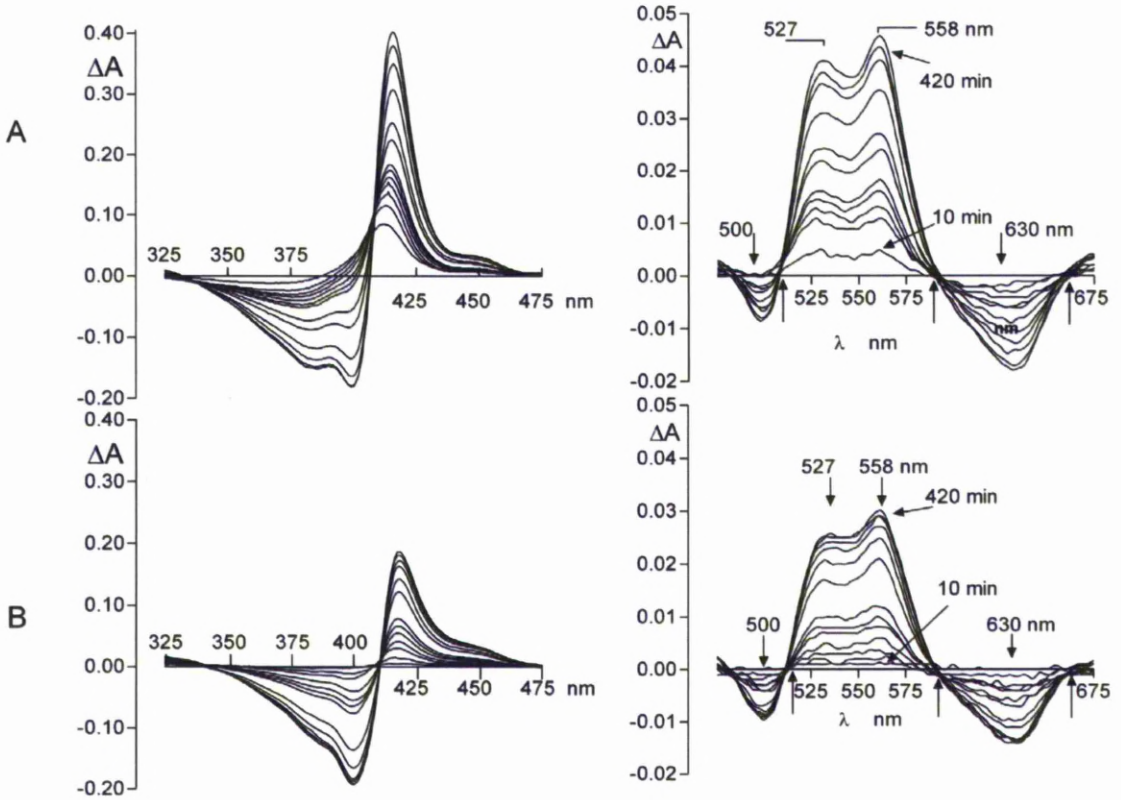

Figure 6. Difference spectra showing HmuY-haem complex formation from HRgpA-methaemoglobin (A) and $NaNO_2$-induced methaemoglobin (B). The difference spectra in the visible region show the appearance of the 525 nm and 558 nm bands attributable to formation of the HmuY-ferrihaem complex. Note that the relative extinctions at 527 nm and 558 nm are reversed compared to those in the ferrihaem-HmuY complex shown in Fig. 1, and occur as a result of subtraction of the spectra. Starting concentrations of haemoglobin were 16 μM (haemoglobin subunit basis) and contained 77 and 87% methaemoglobin for the HRgpA- and $NaNO_2$-treated oxyhaemoglobin, respectively. HRgpA and $NaNO_2$ concentrations were 0.4 μM and 64 μM, respectively.
doi:10.1371/journal.pone.0017182.g006

globin was greater than methaemoglobin formed by treatment with $NaNO_2$.

HmuY-haem complex forms during co-incubation of oxyhaemoglobin with both HRgpA and HmuY

Chemostat studies [3] have shown the presence of arginine-specific protease activity in haem-limited cultures expressing haem-binding proteins [4], [18]. Since *P. gingivalis* may concomitantly deploy the HmuY haemophore and gingipain proteases, we investigated the effect of co-incubation of oxyhaemoglobin with HmuY in the presence of HRgpA to mimic the *in vivo* situation more closely. It is noteworthy in this respect, that HmuY is absolutely resistant to degradation by either R- or K-gingipains [9]. For this, HmuY (16 μM) was incubated with 16 μM oxyhaemoglobin (with respect to haemoglobin subunit) in the presence of HRgpA (400 nM) and the haem transfer from oxyhaemoglobin to HmuY monitored spectroscopically and by native PAGE (Figs. 7 and 8, respectively). In the presence of both HRgpA and HmuY, the oxyhaemoglobin spectrum was transformed after 24 h into one typical of the HmuY-ferrihaem complex with a 411 nm Soret band and 528 nm and weak 559 nm visible bands (Fig. 7).

During electrophoresis under non-denaturing conditions, apoHmuY migrated as a single band with an R_f greater than that of the α and β haemoglobin chains (Fig. 9). During incubation of oxyhaemoglobin with HRgpA plus HmuY there was a progressive increase in the degree of CBB staining of the faster migrating holoHmuY (Fig. 8A; arrowed) which was matched by an increase in the TMB/H_2O_2 staining intensity (Fig. 8B). This was accompanied by a reciprocal decrease in both the TMB/H_2O_2 and CBB staining of the slower migrating haemoglobin bands, the latter being typical of haem-free globin chains [13]. It is noteworthy that a small amount of the faster moving HmuY-haem complex was also generated (most notably after 24 h) when oxyhaemoglobin was incubated only with HmuY (Fig. 8, gel tracks C and D). This was attributed to the HmuY pickup of haem lost from oxyhaemoglobin as a result of auto-oxidation during the period of the experiment.

P. gingivalis Lys-gingipain (Kgp) also facilitates haem acquisition by HmuY

It has been previously demonstrated that the proteolytic attack by Kgp on oxyhaemoglobin results in the formation of a haemoglobin haemichrome, stable to further degradation by the enzyme [12]. Because of the close similarities of the spectra of the haemoglobin haemichrome (409 nm Soret and 535 nm visible bands) and the HmuY-ferrihaem complex, a definitive identification of the formation of the latter from the product of oxyhaemoglobin proteolysis by Kgp is not possible by UV-visible spectroscopy alone. Therefore, the co-incubation mixtures of oxyhaemoglobin in the presence of both Kgp and HmuY were examined as above by non-denaturing PAGE. This showed both an increase in the mobility and TMB/H_2O_2 haem staining of the HmuY (Fig. 9). Indeed, there was demonstrable TMB/H_2O_2 staining of the faster-migrating liganded HmuY as soon as the mixing had taken place (at time 0), clearly showing the facile haem pick up by the HmuY from the haemichrome.

HmuY mediates ferrohaem pickup from deoxyhaemoglobin

Since *P. gingivalis* may also encounter deoxygenated haemoglobin in the anaerobic gingival sulcus or periodontal pocket, it was decided to investigate the interaction of HmuY with deoxyhaemoglobin-agarose. The haemoglobin-agarose was deoxygenated by the addition of sodium dithionite [19] and maintained anaerobically whilst periodically removing the agarose-free, HmuY-containing supernatants for which the spectra were recorded. These showed that a ferrohaem-HmuY complex developed within 5 min of exposure of the deoxyhaemoglobin to HmuY (Fig. 10).

HmuY can extract haem from methaemoglobin in the presence of serum albumin

We tested whether HmuY was capable of binding haem complexed in the form of methaemoglobin but in the presence of serum albumin. It should be noted that methaemalbumin and methaemoglobin share similar spectral features at physiological pH and that the presence of all three components in solution would yield a very complex spectrum. Therefore, we used immobilised methaemoglobin-agarose as the substrate so that the differential formation of either the methaemalbumin or HmuY-ferrihaem complexes (which are spectrally distinct) could be more easily followed. All the three components each at 16 μM (with methaemoglobin on a subunit basis as above) were incubated together at 37°C, and the supernatant solutions were removed periodically from the agarose beads, and the spectra recorded as for the two component (HmuY/methaemoglobin-agarose) system. As

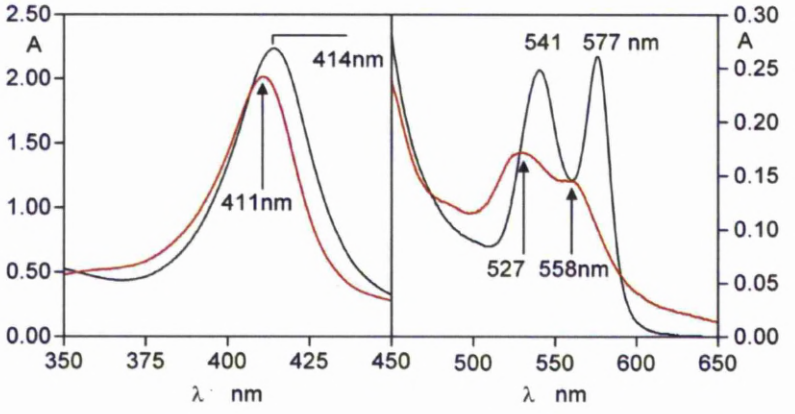

Figure 7. Spectrum showing the ferrihaem-HmuY complex (red line) formed after 24 h co-incubation of oxyhaemoglobin with HRgpA and HmuY. The oxyhaemoglobin and HmuY concentrations were both 16 μM, whilst HRgpA was at 0.4 μM.
doi:10.1371/journal.pone.0017182.g007

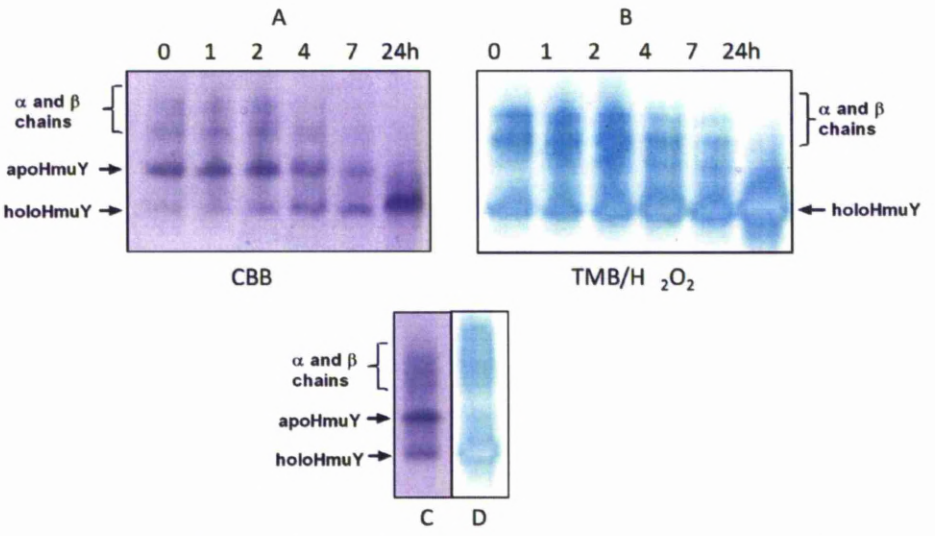

Figure 8. Native PAGE showing the HmuY-haem complex formed through co-incubation of oxyhaemoglobin with HRgpA plus HmuY. Oxyhaemoglobin was co-incubated with HmuY plus HRgpA (CBB stained gel A and TMB/H_2O_2 stained gel B). Note that a small amount of the HmuY-haem complex was formed after 24 h incubation of oxyhaemoglobin plus HmuY only (CBB stained gel track C and TMB/H_2O_2 stained gel track D), attributed to pickup of haem lost from oxyhaemoglobin as a result of auto-oxidation. The oxyhaemoglobin concentration was 16 μM (on a haemoglobin subunit basis), as was HmuY, whilst HRgpA was present at 0.4 μM.
doi:10.1371/journal.pone.0017182.g008

can be seen in Fig. 11 (panel A), in the three component mixture a spectrum was observed with a 411 nm Soret band and visible bands at 527 and 558 nm, which was indicative of the formation of the HmuY-ferrihaem species. The intensities of these bands increased with time of incubation as previously demonstrated in Figure 4 for the reaction of HmuY plus methaemoglobin-agarose only. In contrast however, for the incubation of the methaemoglobin-agarose with albumin generated spectra with a 402 nm Soret band and lower intensity bands at 500 and 620 nm, indicative of the formation of the methaemalbumin complex (Fig. 11, panel B).

HmuY binds free ferrihaem in the presence of serum albumin

To determine whether HmuY could also compete with serum albumin for free iron(III) protoporphyrin IX, a mixture of both proteins (each at 16 μM) was incubated with haem (also at 16 μM) at 37°C and the spectra were recorded periodically. As seen in Fig. 12A, there was an immediate red shift in the Soret band from a λ_{max} of 385 nm (that of haem) to 411 nm, and the appearance of visible bands at 527 and 558 nm as observed for the addition of haem to HmuY as seen in Fig. 1 for the formation of the HmuY-ferrihaem complex. Note the presence of isosbestic points at 395, 460, 512 and 580 nm indicating the direct conversion of the free haem into the HmuY-ferrihaem complex. In contrast, incubation of serum albumin with ferrihaem resulted in the formation of a Soret band with λ_{max} of 402 nm, indicative of the presence of methaemalbumin (Fig. 12B).

HmuY can extract haem from methaemalbumin

In the absence of free haem or that in the form of methaemoglobin, the HmuY haemophore may also have to compete for haem already complexed by serum haem-binding proteins. We therefore tested this by incubating methaemalbumin (16 μM) at 37°C with an equimolar quantity of HmuY and periodically monitored the UV-visible spectrum. As seen in Fig. 13, there was a progressive increase in the intensity and red shift in the Soret band from 402 nm to 411 nm. This was accompanied by a decrease in the 500 and 620 nm methaemalbumin associated

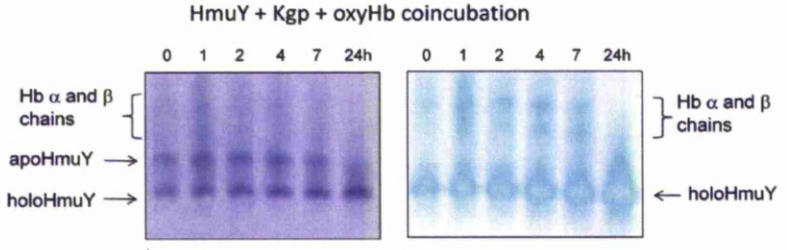

Figure 9. HmuY-haem complex formation during the co-incubation of oxyhaemoglobin with both HmuY and Kgp. HmuY and oxyhaemoglobin (both at 16 μM) were incubated at 37°C with Kgp (0.2 μM) and sampled periodically, and subjected to native PAGE. Gel tracks were loaded with ~12 μg total protein.
doi:10.1371/journal.pone.0017182.g009

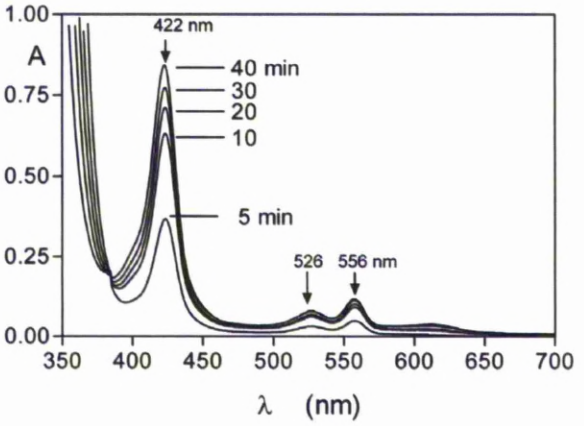

Figure 10. Formation of the ferrohaem-HmuY complex during incubation of HmuY with deoxyhaemoglobin-agarose. Haemoglobin-agarose was deoxygenated with sodium dithionite and maintained anaerobically during the reaction with HmuY. Concentration of haemoglobin was 16 μM (with respect to haemoglobin subunit) as was HmuY. See text for details. The absorbance below 375 nm is due to the presence of dithionite. A small amount of deoxyhaemoglobin (~0.1% of the total) was released from the control deoxyhaemoglobin-agarose after 40 min incubation in the absence of HmuY.
doi:10.1371/journal.pone.0017182.g010

bands, and a reciprocal increase in the absorbance at 527 nm with a shoulder at 558 nm band, features indicative of the formation of the HmuY-ferrihaem complex. Clear isosbestic points were observed at 464, 515, and 578 nm indicating that the methaemalbumin had been transformed directly into the HmuY-ferrihaem complex.

To confirm the presence of the HmuY-haem complex, a sample of the incubation mixture (after 5 h) was subjected to non-reducing SDS-PAGE and the proteins stained for the presence of haem with TMB/H_2O_2, and then counterstained with CBB (Fig. 14). As can clearly be seen, the methaemalbumin complex (track B) had been almost completely depleted of haem as revealed by TMB/H_2O_2 staining, whilst in contrast the faster moving HmuY band was heavily stained for haem.

Discussion

We have demonstrated in this study that, in addition to binding free haem, the HmuY haemophore of *P. gingivalis* can extract ferrihaem from methaemoglobin. This is a significant advancement in the understanding of the mechanisms of haem acquisition by this organism. The paradigm for proteolytically-mediated haem acquisition from oxyhaemoglobin by *P. gingivalis* involves its initial oxidation to the methaemoglobin form by the action of R-gingipain [11], [12], [13]. This results in the relaxation of the affinity of globin for the ferrihaem species (compared to that of iron(II) haem). As a consequence, this greatly facilitates the proteolytic attack on the globin protein by Kgp and subsequent haem release [12], [13]. In this respect it is noteworthy that in the body reprocessing of haemoglobin from damaged and senescent erythrocytes firstly involves the oxidation of haem such that it can be removed by either serum albumin and/or haemopexin which have association constants for ferrihaems of 10^8 and 2×10^{14} M^{-1}, respectively [20], [21]. Thus, in parallel with this paradigm was the finding that methaemoglobin formed by the proteolytic attack on oxyhaemoglobin by HRgpA was a facile "substrate" from which the HmuY molecule could easily remove haem. To our knowledge, this is the first demonstration of a bacterial haemophore acting syntrophically with proteases to extract haem from haemoglobin.

We have previously demonstrated the presence of a haem-binding protein in chemostat cultures of *P. gingivalis* run under conditions of both haem excess and haem limitation [18]. Under these conditions, these cells produce arginine-specific protease activity [4]. In addition, Olczak *et al.* [22] have shown that HmuY is expressed at higher levels by cells in batch culture in which the haem level is restricted. Therefore it is likely *in vivo* that *P. gingivalis* would concomitantly deploy a combination of protease and haemophore molecules as a syntrophic haem acquisition mechanism system. Importantly, in addition, it has been demonstrated that HmuY is completely resistant to the proteolytic effects of HRgpA, RgpB and Kgp [9], indicating that HmuY could very well function alongside these proteases in the process of haem acquisition.

The primary habitat of *P. gingivalis* and other black-pigmenting anaerobes is the diseased periodontal pocket and gingival sulcus.

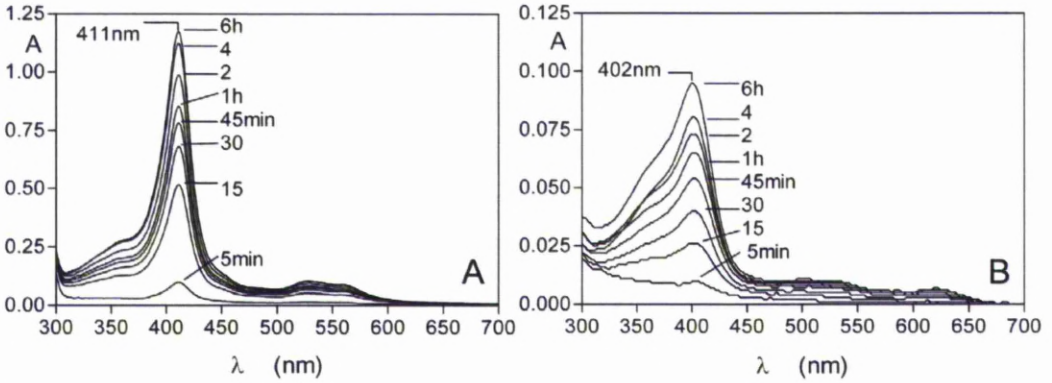

Figure 11. Formation of the HmuY-ferrihaemcomplex during incubation of methaemoglobin-agarose in the presence of human serum albumin. HmuY, albumin and methaemoglobin were each present at 16 μM (with the haemoglobin on a subunit basis). Panel A, methaemoglobin-agarose co-incubated with both HmuY and albumin. Panel B, methaemoglobin plus albumin only. The experimental protocol was the same as described for Fig. 4, and the incubations were carried out at 37°C. The spectra are background corrected to take account of the small amount of methaemoglobin released at each time period from the haemoglobin agarose during incubation.
doi:10.1371/journal.pone.0017182.g011

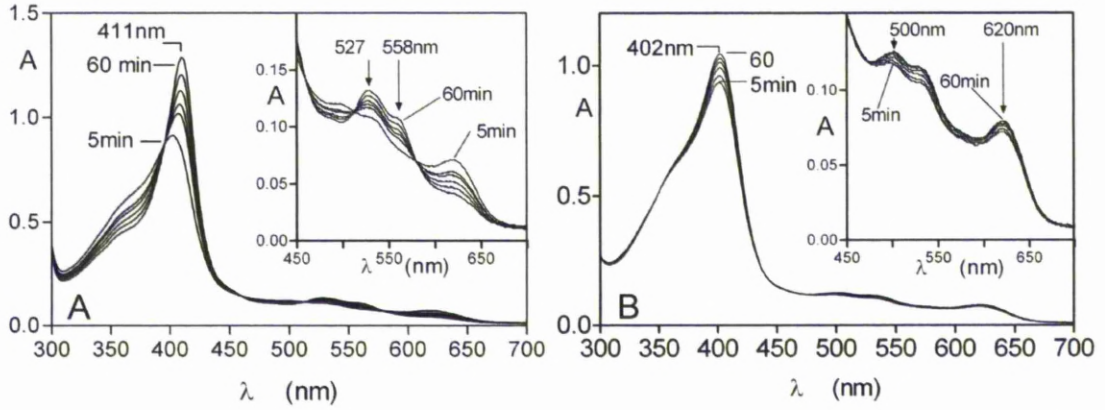

Figure 12. Spectroscopic demonstration of the formation of the HmuY-ferrihaem complex during co-incubation of HmuY and iron(III) protoporphyrin IX in the presence of human serum albumin. Panel A, spectra obtained during incubation of all three components. Panel B, incubation of albumin and haem. All components were present at 16 μM. See text for details.
doi:10.1371/journal.pone.0017182.g012

These environments have been shown to be slightly alkaline [23], [24]. The need for *P. gingivalis* to rely upon proteolytically–mediated oxidation of oxyhaemoglobin as a first step in haem removal from oxyhaemoglobin in this type of environment is critically important. This stems from the fact that at alkaline pH, the natural oxidation rate of oxyhaemoglobin is at its lowest [25]. Thus, haem acquisition by *P. gingivalis* from oxyhaemoglobin reaching this environment as a result of bleeding and haemolysis, would be reliant upon a mechanism which would promote its oxidation. In this regard, it is noteworthy that gingipains have an alkaline pH optimum [26], [27].

Although diseased periodontitis sites may experience fresh bleeding as a result of inflammation and micro-trauma, very anaerobic and reduced gingival sulcus and pocket microenvironments are also likely to contain deoxygenated haemoglobin. In view of this likelihood, we also investigated the pickup of ferrohaems from deoxyhaemoglobin-agarose which was maintained anaerobically. We unequivocally demonstrated the rapid generation of the ferrohaem-HmuY complex immediately after mixing of the two proteins. Importantly, whilst this compares starkly with the difficulty of haem pick up from the oxygenated protein (in the absence of the prior oxidation to the met-form), it nevertheless clearly shows the versatility of the HmuY haemophore which has the ability to extract haems from both the deoxy-iron(II) as well as from the oxidised haemoglobin species.

We have found that whilst Kgp is effective in degrading methaemoglobin, especially that produced by the action of HRgpA on oxyhaemoglobin [12], [13], the action of Kgp on oxyhaemoglobin results in the formation of a haemoglobin haemichrome which is resistant to further attack by the Kgp protease [12], and also by HRgpA (Smalley JW, Birss AJ, and Potempa J, unpublished findings). However, this study has shown that haem can be extracted by HmuY from the haemoglobin haemichrome and has revealed yet another route through which

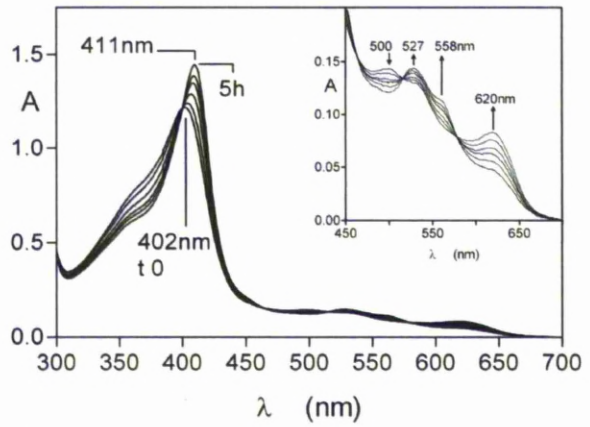

Figure 13. Formation of the ferrihaem-HmuY complex during incubation of human methaemalbumin with HmuY. Methaemalbumin (16 μM) was incubated with an equimolar amount of HmuY at 37°C. Arrows denote changes in the spectra with time at the indicated wavelengths.
doi:10.1371/journal.pone.0017182.g013

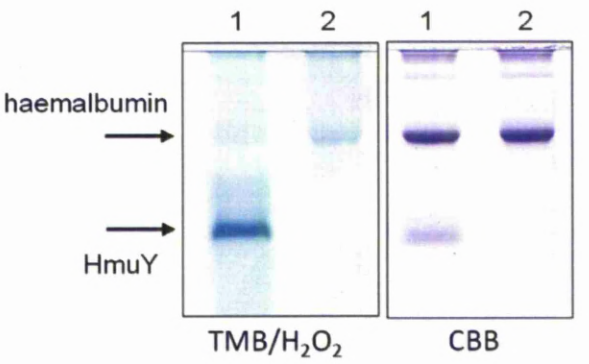

Figure 14. SDS-PAGE showing the formation of the HMuY-haem complex after incubation with human methaemalbumin. Methaemalbumin (16 μM) was with either 16 μM HmuY (track 1) or alone (track 2) for 5 h at 37°C, electrophoresed under non-reducing and then stained for the presence of haem with TMB/H_2O_2 before counterstaining for protein with CBB. Note the depletion of TMB/H_2O_2 staining for haem of the methaemalbum band in track 1 after exposure to HmuY.
doi:10.1371/journal.pone.0017182.g014

haem might be acquired as a consequence of the deployment of the gingipain proteases.

The question arises as to the capability of HmuY of gaining haem in the inflamed gingival crevice and periodontal pocket. In such environments any free haem is normally sequestered by albumin which is the major protein in gingival crevicular fluid [28] and by haemopexin, which play the important roles of restricting its bioavailability to bacteria. It is important to note that albumin also plays the role of removing haem from methaemoglobin [14], [15], [16], for the eventual transfer to haemopexin and hence to the liver for reprocessing. We therefore tested the ability of HmuY to compete with serum albumin for the haem in the form of methaemoglobin. Haem exchange experiments in which immobilised methaemoglobin-agarose was co-incubated with an equimolar amount of both albumin and HmuY, revealed the preferential rapid formation of the HmuY-ferrihaem complex. We also found that haem was preferentially bound by HmuY when it was introduced into a solution comprising equimolar amounts of both HmuY and albumin.

Of equal importance physiologically however, is the likelyhood that in a haem-limited environment in the absence of any free haem or of haemoglobin released from erythrocytes, HmuY may have to extract essential haem directly from host haem sequestering proteins. Accordingly, we tested whether HmuY could remove ferrihaem from methaemalbumin by co-incubating these two proteins in equimolar amounts. Spectroscopic analysis showed that loss of haem from the methaemalbumin and formation of a ferrihaem-HmuY complex occurred rapidly. This was confirmed by SDS-PAGE and TMB/H_2O_2 staining which showed that the haemalbumin was depleted in haem. Together, the above findings unequivocally demonstrated that HmuY can successfully compete with albumin for any available ferrihaem.

The K_a for HmuY and haem is of the order 3×10^2 M^{-1} as determined by UV-visible and fluorescence analysis (Wojtowicz, H and Olczak, T, unpublished findings), whilst that for HmuR and haem is approximately 4×10^5 M^{-1} [29]. It is thus easy to appreciate the facile haem transfer from HmuY to the HmuR receptor. However, it is noteworthy that the K_a of the albumin-haem binding system is around 10^8 M^{-1} [20]. The pickup of haem by HmuY from methaemalbumin is therefore somewhat paradoxical from a simple consideration of the relative haem binding affinities of the two proteins. It is as yet unclear how haem exchange from albumin to HmuY is achieved, but it is likely that this may involve some other process such as induction in the change in molecular conformation of the albumin.

It is noteworthy that other bacterial haemophores have been shown to extract haem from methaemoglobin [30], [31]. However, it has been demonstrated here that the HmuY lipoprotein of *P. gingivalis* can extract haem not only from methaemoglobin, but also from deoxyhaemoglobin. We have also shown here for the first time that HmuY can remove haem from oxyhaemoglobin with the aid of specific haemoglobin degrading gingipain proteases. HmuY was also found to be capable of binding both free haem and that in present in methaemoglobin even in the presence of serum albumin. Given that albumin is the major protein component of gingival crevicular fluid and the periodontal pocket where it functions to sequester and withhold this essential nutrient from microbes, it is of significance that HmuY can also wrest haem from directly methaemalbumin. These findings further underline the versatility of the haemophore and protease systems of *P. gingivalis*, and demonstrate that *P. gingivalis* is highly capable of competing with the host in its primary habitats to obtain haem.

Materials and Methods

Purification of HmuY

P. gingivalis apoHmuY lacking the first 25 residues (NCBI accession number CAM 31898) was expressed using a pHmuY11 plasmid and *Escherichia coli* ER2566 cells (New England Biolabs) and purified from a soluble fraction of the *E. coli* lysate as previously described [7]. As the soluble protein released from the cell membrane, the recombinant HmuY lacked the signal peptide and first five amino acid residues (CGKKK) of the nascent secreted protein [7], [9].

Gingipain purification

The arginine and lysine-specific gingipains HRgpA and Kgp, respectively, were isolated and purified from spent culture supernatants as previously described [26].

Ferrihaem preparation

Iron(III) protoporphyrin IX was prepared as described previously [32], [28] by dissolving haemin (iron(III) protoporphyrin IX chloride, Fe(III)PPIX.Cl, at 1 mM in 0.1 M Tris, pH~10, containing 0.14 M NaCl. The pH of this solution was then decreased to pH 7.5 by the slow addition of dilute HCl. The iron(II) protoporphyrin IX species was prepared from the above ferrihaem solution by reduction in the presence of 10 mM $Na_2S_2O_4$ [2].

Polyacrylamide gel electrophoresis (PAGE)

SDS-PAGE was carried out as previously described by Laemmli [33] and gels were stained for protein with Coomassie Brilliant Blue R-250 (CBB). Protein-bound haem was located on the gels by staining for haem-associated peroxidase activity using tetramethylbenzidine/H_2O_2 (TMB/H_2O_2), the samples being solubilised at 37°C for 1 h in Laemmli electrophoresis sample application buffer without dithiothreitol [17]. For native PAGE, urea and SDS were omitted from the separating gel, and the samples were also solubilised for 1 h in Laemmli sample application buffer without SDS, urea and dithiothreitol.

Haem exchange experiments using immobilised haemoglobin

Bovine haemoglobin conjugated to agarose (Sigma Chemical Company Ltd.) was washed extensively in buffer (0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5) to remove any free haem or unconjugated haemoglobin as previously described [17]. The amount of haemoglobin conjugated per ml of agarose was determined from the UV-visible spectrum of the haemoglobin-agarose suspension. The spectrum revealed that the conjugated haemoglobin is in the oxidized form (methaemoglobin). The methaemoglobin-agarose beads were incubated at 37°C with HmuY (16 μM) and periodically pelleted by low speed centrifugation at 2000× *g* for 2 min and the UV-visible spectrum of the supernatant containing the HmuY was recorded. The supernatant solution was then added back to the beads which were then re-incubated as above prior to the next sampling.

Haemoglobin preparations

Oxyhaemoglobin was prepared from fresh horse erythrocytes as previously described [19] and stored at -80°C in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5. Methaemoglobin was prepared from the oxygenated protein by treatment with 64 μM $NaNO_2$ in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5 [12], [13] and stored in this buffer at −80°C until required. This yielded a preparation comprising

87% methaemoglobin as calculated from $A_{577\ nm}$ and $A_{630\ nm}$ values as described previously [12]. Proteolytically induced methaemoglobin was also prepared by treatment of oxyhaemoglobin with arginine-gingipain A (HRgpA) [13]. Briefly, oxyhaemoglobin (16 μM with respect to haemoglobin subunit) was pre-incubated with 0.4 μM HRgpA for 24 h at 37°C in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5. This haemoglobin preparation comprised 77% methaemoglobin as calculated above.

Deoxyhaemoglobin-agarose was prepared by treating the haemoglobin agarose with 10 mM sodium dithionite, under an anaerobic atmosphere (80% nitrogen, 15% carbon dioxide and 5% hydrogen), in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5. Once deoxygenated, the haemoglobin agarose beads (16 μM with respect to haemoglobin subunit) were incubated with an equimolar amount of HmuY at 37°C under anaerobic conditions and at various times the beads were sedimented by centrifugation as above and the agarose-free supernatant solutions containing HmuY were sampled anaerobically, kept sealed in an optical cuvette and the spectrum recorded.

Methaemalbumin preparation

Methaemalbumin was prepared by incubating a 120 μM stock solution of human albumin (Sigma product A-8763) at 37°C in 0.14 M NaCl, 0.1 M Tris-HCl, pH 7.5, with iron(III) protoporphyrin IX at a 1 : 0.9 protein to haem molar ratio to ensure that no free uncomplexed haem remained in the preparation. The haemalbumin complex formed after 18 h displaying a Soret band at around 402 nm, along with bands at 500, 530 and 620 nm, in keeping with previous work [34].

UV-visible spectroscopy

UV-visible spectra were recorded with a Ultrospec 2000 (Biochrom Ltd) using 1 cm pathlength cuvettes.

Author Contributions

Conceived and designed the experiments: JWS. Performed the experiments: DPB HW AJB AS. Analyzed the data: JWS DPB TO JP. Contributed reagents/materials/analysis tools: JWS TO JP. Wrote the manuscript: JWS TO JP.

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