Reduction of hydrogen peroxide in gram-negative bacteria - Bacterial Peroxidases

Cláudia S. Nóbrega and Sofia R. Pauleta*

[1] Microbial Stress Lab, UCIBIO, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal.

Corresponding Author

Sofia R. Pauleta
Microbial Stress Lab, UCIBIO, REQUIMTE, Departamento de Química, Faculdade de Ciências e Tecnologia, Universidade Nova de Lisboa, 2829-516 Caparica, Portugal.
E-mail: srp@fct.unl.pt
Tel. +351212948385, ext 10967
Fax +351212948550

Running Title: Bacterial Peroxidases - H_2O_2 Respiration

Abstract

Bacteria display an array of enzymes to detoxify reactive oxygen species that cause damage to DNA and to other biomolecules leading to cell death. Hydrogen peroxide is one of these species, with endogenous and exogenous sources, such as lactic acid bacteria, oxidative burst of the immune system or chemical reactions at oxic-anoxic interfaces. The enzymes that detoxify hydrogen peroxide will be the focus of this review, with special emphasis on bacterial peroxidases that reduce hydrogen peroxide to water. Bacterial peroxidases are periplasmic cytochromes with either two or three *c*-type haems, which have been classified as classical and non-classical bacterial peroxidases, respectively. Most of the studies have been focus on the classical bacterial peroxidases, showing the presence of a reductive activation in the presence of calcium ions. Mutagenesis studies have clarified the catalytic mechanism of this enzyme and were used to propose an intramolecular electron transfer pathway, with far less being known about the intermolecular electron transfer that occurs between reduced electron donors and the enzyme. The physiological function of these enzymes was not very clear until it was shown, for the non-classical bacterial peroxidase, that this enzyme is required for the bacteria to use hydrogen peroxide as terminal electron acceptor under anoxic conditions. These non-classical bacterial peroxidases are quinol peroxidases that do not require reductive activation but need calcium ions to attain maximum activity and share similar catalytic intermediates with the classical bacterial peroxidases.

Keywords (5-10): Bacterial peroxidase, hydrogen peroxide, immune system, *c*-type cytochrome, peroxide reduction, reactive oxygen species, catalytic mechanism, activation mechanism, three-haem peroxidases, electron transfer

Abbreviations

BCCP - Bacterial cytochrome *c* peroxidase
E haem – Electron Transfer haem
FNR – Fumarate nitrate reduction
HP – High potential
LS – Low spin
MCD – magnetic circular dichroism
nIR-MCD – near-infrared magnetic circular dichroism
P haem – Peroxidatic haem
ROS – reactive oxygen species
SOD – Superoxide dismutase

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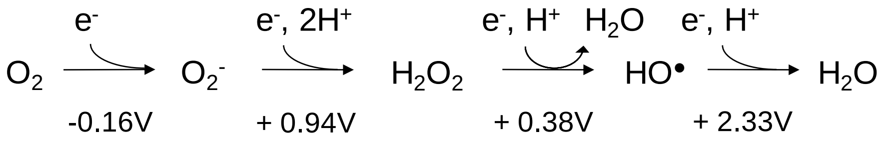
1. Oxidative stress systems

1.1 Reactive oxygen species

The first living microorganisms evolved in a world without oxygen. With the oxygenation of the planet's atmosphere a new challenge is presented to the wide variety of existing microorganisms.

Oxygen is a reactive molecule which can cross the biological membranes due to its small, non-polar nature. The ability to deal with this new molecule divided the organisms in strict anaerobes that survive in the few habitats without oxygen, microaerophiles that tolerate low levels of oxygen in the environment or aerobes that can survive in environments with high levels of oxygen.

However, molecular oxygen itself cannot react with most essential biomolecules, such as amino acids, carbohydrates, lipids and nucleic acids. The toxic molecules are the intermediates of the reduction of molecular oxygen to water (Cabiscol *et al.*, 2000), which are known as reactive oxygen species (ROS), namely superoxide (O_2^-), hydrogen peroxide (H_2O_2) and hydroxyl radical ($HO^\bullet$) (Scheme 1).



Scheme 1 – Stepwise one-electron reduction of molecular oxygen to water through formation of superoxide, hydrogen peroxide, hydroxyl radical. The reduction potentials indicated are for pH 7.0. Scheme adapted from Mishra and Imlay (Mishra and Imlay, 2012).

It should be noted that molecular oxygen has an even number of electrons in which two are unpaired, making it difficult to accept one electron at a time (the first reaction in Scheme 1 should be thermodynamically unfavourable) (Naqui *et al.*, 1986). However, molecular oxygen can receive electrons from good electron donors, such as quinones, flavins and metals or metal cofactors, such as Fe/S clusters (Figure 1). Electron carriers in the respiratory chains are rich in these type of cofactors and were shown to be one of the endogenous sources of O_2^- and H_2O_2 (Massey *et al.*, 1969). Even without respiratory enzymes it was found that these two ROS could arise from the autoxidation of non-respiratory flavoproteins *in vitro* (Figure 1). Therefore, the intracellular levels of ROS will depend on the amount of these auto-oxidizable enzymes in the cell and on the oxygen concentration in the environment (more oxygen leads to more successful encounters) (Seaver and Imlay, 2004). It was estimated that under steady-state conditions the intracellular concentration of H_2O_2 and O_2^- is 20-50 nM and 2 nM, respectively in *Escherichia coli* (Imlay, 2013).

A source of the hydroxyl radical is the Fenton reaction (Equation 1), where ferrous iron transfers an electron to H_2O_2 according to the equation:

$$Fe^{2+} + H_2O_2 \rightarrow FeO^{2+} + OH^- + H^+ \rightarrow Fe^{3+} + OH^- + HO^\bullet \qquad (1)$$

The iron that catalyses this reaction is “free iron”, which means that it is not incorporated into enzymes nor iron-storage proteins, but it is presumably bound to metabolites and to the surfaces of biomolecules (Figure 1). The $HO^\bullet$ produced in this reaction, is considered highly toxic as it can directly damage most biomolecules and it is therefore, linked to protein carbonylation, lipid peroxidation and DNA damage, that can lead to cell death (Keyer and Imlay, 1996).

Besides these endogenous sources, microorganisms can also be exposed to exogenous sources of ROS, such as those produced by the host immune system or by other microorganisms that live in the same habitats, such as the hydrogen peroxide produced by lactic acid bacteria, or redox-cycling small molecules secreted by some organisms, or the chemical oxidation of reduced compounds at oxic-anoxic interfaces (Imlay, 2018).

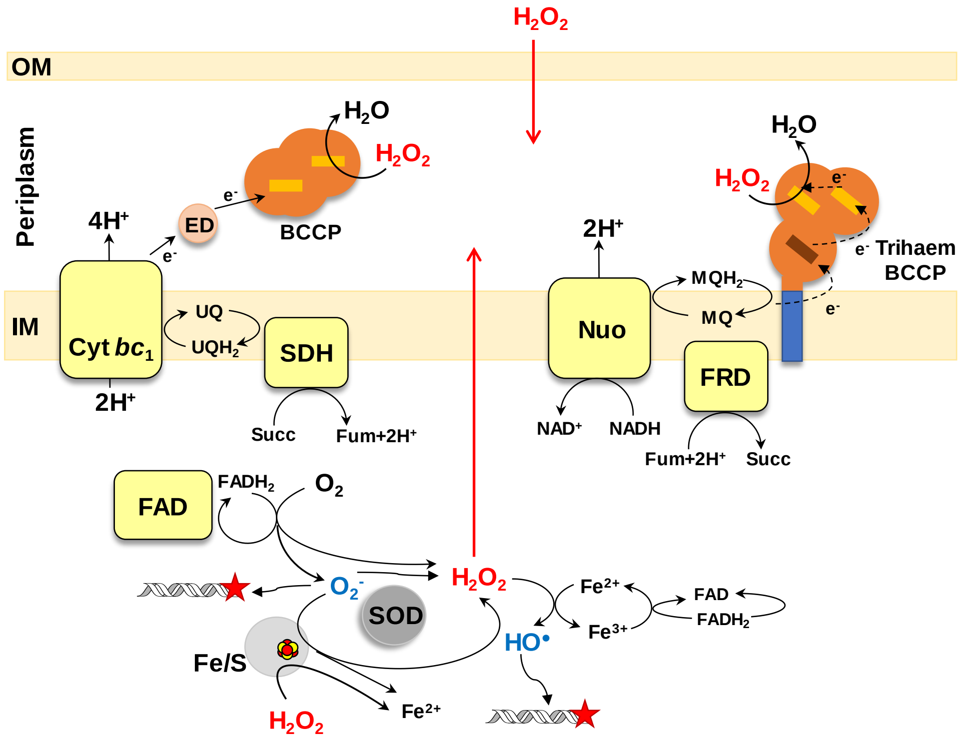


Figure 1 – Schematic representation of the physiological function of bacterial peroxidases in Gram-negative bacteria under anoxic conditions, with this enzyme using H_2O_2 as a terminal electron acceptor to support anoxic respiration. In the case of classic BCCPs electrons are delivered from small electron donor proteins (ED) from the cytochrome bc_1 complex, while non-classical BCCPs receive electron from menaquinol. Hydrogen peroxide can be produced in the cytoplasm by different mechanism, mainly from flavoenzymes that adventitiously transfers electrons from oxygen. The different pathways for ROS generation and DNA damage are schematically represented. SOD – superoxide dismutases; Fe/S – Fe-S containing proteins; FAD – flavoenzymes; FRD – fumarate reductase; SDH – succinate dehydrogenase; OM – Outer membrane; IM – Inner membrane; Nuo – NADH desidrogenase I.

1.2 Bacterial defence mechanisms

ROS are able to cause serious damage to cell components (Apel and Hirt, 2004). Thus, to survive in oxygenated environments, during infection or in certain habitats, microorganisms developed strategies to detoxify the cells from these compounds.

These strategies include specialized enzymes, such as superoxide dismutases (SOD) (Equation 2), catalases (Equation 3) and peroxidases (Equation 4) that perform the following general reactions:

$$O_2^- + O_2^- + 2\ H^+ \rightarrow H_2O_2 + O_2 \qquad (2)$$

$$H_2O_2 + H_2O_2 \rightarrow O_2 + 2H_2O \qquad (3)$$

$$RH_2 + H_2O_2 \rightarrow R + 2H_2O \qquad (4)$$

In the following sections, an overview of these enzymes and respective regulators found in bacterial systems will be briefly presented. A better understanding of the oxidative stress scavengers and regulators is crucial to comprehend the role of bacterial cytochrome *c* peroxidases, which is the focus of this review.

E. coli has been the main bacterial model to study oxidative stress, as it is a facultative anaerobe with several scavenging enzymes that respond to distinct stress signals in the cell. Therefore, it will be used as an example of what is known in terms of the molecular systems involved in oxidative stress response.

1.2.1 Oxidative stress regulators – OxyR, PerR and SoxRS

Microorganisms typically present a low basal expression of the enzymes to detoxify ROS at low levels. However, increased level of ROS exerts an oxidative stress that is sensed by specific transcription factors. These regulators are found in a wide range of bacteria reflecting their positive selection and important physiological role (Chiang and Schellhorn, 2012).

OxyR is one of these regulators, a tetrameric protein with two important cysteine residues per monomer that is usually inactive and downregulates its own expression. When the H_2O_2 levels in the cytoplasm rises to 100- 200 nM, a disulphide bond between those cysteines is formed, activating OxyR, which then promotes the transcription of several genes related to oxidative stress (some of them will be described below) (Chiang and Schellhorn, 2012; Michán *et al.*, 1999; Zheng *et al.*, 2001). The disulphide bonds are re-reduced by glutathione, which in turn is reduced by glutathione reductase, inactivating OxyR. The expression of the genes coding for glutathione reductase (GSH) and glutaredoxin (Grx1) is under the control of OxyR, thus the response is self-regulated (Zheng *et al.*, 1998).

Besides H_2O_2, this protein also senses nitrosactive stress, which leads to the activation of OxyR by S-nitrosylation followed by the expression of genes that allow the cell to adapt to reactive nitrogen species. This shows that OxyR can process different signals with distinct transcriptional responses

(Hausladen *et al.*, 1996; S. O. Kim *et al.*, 2002).

In Gram-positive bacteria there is an alternative mechanism to OxyR, the PerR, which is an iron binding protein of the Fur family. *Bacillus subtilis* PerR in its active form has two metal centres, zinc and iron. The zinc stabilizes the homodimeric form and the Fe^{2+} is necessary for the protein to acquire the correct conformation and bind DNA (Herbig and Helmann, 2001; J. W. Lee and Helmann, 2006). H_2O_2 reacts with the Fe^{2+} in the active site, forming a hydroxyl radical that then can oxidize one of the histidine residues coordinating the iron atom and inactivates PerR. This modified transcription regulator no longer binds DNA, which leads to the transcription of oxidative stress related genes (repressed by PerR). PerR not only responds to exogenous H_2O_2 but it also seems to be important in transitioning from anaerobic to aerobic environments by sensing O_2 (J. W. Lee and Helmann, 2006; Sethu *et al.*, 2016).

OxyR and PerR are two of the systems that sense H_2O_2 but O^{2-} can also increase under certain conditions. For instance, bacteria can induce production of O^{2-} in the cytoplasm of other bacteria by secreting redox-cycling organic compounds (Hassett *et al.*, 1992).

Superoxide has different properties than H_2O_2 because it is charged at physiological pH and thus cannot penetrate membranes. Therefore, this species is usually formed inside the cell. To sense O^{2-}, bacteria uses the SoxRS system which consists of two components. SoxR is a homodimer with a [2Fe-2S] cluster in each subunit, which becomes oxidized when exposed to redox-cycling compounds or O^{2-} (Gaudu *et al.*, 2000; Greenberg *et al.*, 1990; Tsaneva and Weiss, 1990). In both oxidation states, the protein binds upstream *soxS* gene but only in the oxidized form it induces its expression. SoxS induces the expression of several genes that include systems to deal with these redox-cycling compounds and O^{2-}, with its detoxification being catalysed by superoxide dismutase (Pomposiello *et al.*, 2001). In some microorganisms (such as *Pseudomonas aeruginosa* that lacks SoxS) SoxR can directly affect the transcription levels of oxidative stress related genes (Kobayashi and Tagawa, 2004; Palma *et al.*, 2005).

1.2.2 Enzymatic systems involved in ROS detoxification

1.2.2.1 Superoxide dismutase

Superoxide dismutase is a metalloenzyme capable of promoting the dismutation of O^{2-} to O_2 and H_2O_2. This enzyme is ubiquitous in both prokaryotes and eukaryotes, and is an endogenous source of hydrogen peroxide (Figure 1), which will be scavenged by catalases and peroxidases.

SOD is usually a cytoplasmic enzyme, since this is the place where superoxide is formed during aerobic growth and because it cannot cross the membranes. Nevertheless, some pathogenic bacteria, such as strains of *Brucella, Haemophilus, Legionella, Mycobacterium* and *Nocardia*, secrete SODs suggesting that this enzyme might play a role in neutralizing ROS produced by the host immune system or by other exogenous sources (Lynch and Kuramitsu, 2000).

E. coli has three types of SODs: two are cytoplasmatic with iron or manganese cofactors (FeSOD and MnSOD) and one is periplasmic with a copper-zinc centre (CuZnSOD). SODs are typically dimeric enzymes, with a single metal atom constituting its active site. In CuZnSOD each subunit contains an atom of each metal, with the copper being reduced and oxidized during catalysis, while the zinc atom does not change oxidation state, but is not merely stabilizing the copper site, as it influences the reduction potential of the redox centre and its geometry (Gort *et al.*, 1999; Nedd *et al.*, 2014).

FeSOD and MnSOD are encoded by *sodA* and *sodB,* respectively, and are both regulated in response to iron levels. FeSOD is expressed under high levels of iron and when iron levels decrease, the Fur repressor, that is blocking MnSOD synthesis (and a MntH manganese importer), is deactivated. This allows MnSOD transcription, as well as the sRNA RyhB that triggers the degradation of FeSOD transcript. This system, depending on the cofactor availability, controls which SOD is active (Massé and Gottesman, 2002; Niederhoffer *et al.*, 1990). MnSOD expression can also be stimulated by the SoxRS system, which is active in the presence of exogenous superoxide sources (Compan and Touati, 1993).

CuZnSOD encoded by *sodC* was believed to be only present in eukaryotes, but this gene has been found in some bacterial species, such as *E. coli* and *Neisseria meningitidis* (Kroll *et al.*, 1995; Wilks *et al.*, 1998)) and compared to the eukaryotic CuZnSOD, the prokaryotic enzyme appears to be highly resistant to hydrogen peroxide, which is one of the products of its enzymatic activity (Battistoni *et al.*, 1998). In *E. coli*, CuZnSOD is repressed by FNR (Fumarate and Nitrate Reduction regulatory protein, a global anaerobic regulator) under anaerobic conditions and induced in the stationary phase by the transcription regulator RpoS (stationary phase sigma factor) (Gort *et al.*, 1999).

The importance of SOD in preventing oxygen-dependent DNA damage was demonstrate in a *sodAB E. coli* mutant, in which it was observed a variety of oxygen-dependent phenotypes, including a high rate of spontaneous mutagenesis (Farr *et al.*, 1986). This fact can be explained by the ability of cytoplasmic SODs to maintain superoxide anion at sub-nanomolar concentrations (Imlay and Fridovich, 1991).

1.2.2.2 Catalase

Catalase, as well as peroxidase, plays an important role in scavenging H_2O_2. This cytoplasmic enzyme catalyses the dismutation of H_2O_2 to molecular oxygen and water and are found in most bacterial species, with a few exceptions (enterococci, streptococci and leuconostocs (Mishra and Imlay, 2012)). Catalases can be divided into three families: two are haem catalases and one is a manganese catalase (only found in some bacteria, such as *Lactobacillus plantarum* (Kono and Fridovich, 1983)). All catalases catalyse the dismutation reaction of H_2O_2 but one family of haem catalases has also peroxidase activity, and is named bifunctional catalase or catalase-peroxidase. This bifunctional enzyme is found in prokaryotes and eukaryotes, with the exception of plants and animals, while haem monofunctional catalase is widespread among various organisms (Zamocky *et al.*, 2008).

The dismutation of H_2O_2, common to both haem catalases, requires two molecules of H_2O_2, according to Equations 5 and 6:

$$\text{Por-Fe}^{3+} + H_2O_2 \rightarrow \text{(Compound I) Por}^{1+}\text{-Fe}^{4+}\text{=O} \qquad (5)$$

$$\text{(Compound I) Por}^{1+}\text{-Fe}^{4+}\text{=O} + H_2O_2 \rightarrow \text{Por-Fe}^{3+} + H_2O + O_2 \qquad (6)$$

In the first step (Equation 5) of the reaction a Por-Fe^{3+} reduces H_2O_2 forming a Por^{1+}-Fe^{4+}=O oxoferryl radical intermediate (Compound I, with a porphyrin cation radical (Hillar *et al.*, 2000)). In the second step (Equation 6), compound I oxidizes another molecule of H_2O_2, releasing O_2 and H_2O and returning to the initial Por-Fe^{3+}. This highly reactive Compound I is a potential problem within the cell when the levels of H_2O_2 decrease. In monofunctional catalases, it was shown that NADPH can be used to revert Compound I to the Por-Fe^{3+} initial state (Hillar and Nicholls, 1992).

The bifunctional catalases have a low peroxidatic activity (corresponding to 1%), by receiving electrons from exogenous electron donors (R) to reduce Compound I (from reaction in Eq. 5) to Compound II (Por^{1+}-Fe^{4+}=OH) and then back to Por-Fe^{3+}:

$$\text{(Compound I) Por}^{+}\text{-Fe}^{4+}\text{=O} + \text{RH} \rightarrow \text{(Compound II) Por-Fe}^{4+}\text{-OH} + \text{R} \qquad (7)$$

$$\text{(Compound II) Por-Fe}^{4+}\text{-OH} + \text{RH} \rightarrow \text{Por-Fe}^{3+} + H_2O + \text{R} \qquad (8)$$

The K_M values of bifunctional (3.5-8 mM) and monofunctional (40-600 mM) catalases are in the millimolar ranges although physiological concentrations of H_2O_2 are in the micromolar range, well below the K_M of both types of catalases (Chelikani *et al.*, 2004). Therefore, under physiological conditions alkyl hydroperoxidase reductase would play the major role in detoxifying the cell from endogenous cytoplasmic H_2O_2 (see Section 1.2.2.3), while catalase becomes the major scavenger when H_2O_2 concentrations drastically increase, or the cellular levels become too low that precludes the activity of thiol-based peroxidases that require NADH for activity (see Section 1.2.2.3).

The *katG* gene, encoding the bifunctional catalase KatG is usually induced by oxidative stress through regulatory systems, such as OxyR (Zheng *et al.*, 2001) or PerR (Horsburgh *et al.*, 2001), while *katE*, encoding the monofunctional catalase KatE, is overexpressed in the stationary phase under the regulation of RpoS (Schellhorn and Hassan, 1988).

Bacteria usually have more than one catalase isoenzyme and there are even cases in which pathogenic bacteria secrete bifunctional catalases to the periplasm, such as KatP in *E. coli O157:H7* (Brunder *et al.*, 1996). Nevertheless, it is not clear why both catalases, with distinct expression patterns, are needed when a *E. coli* catalase mutant, in the absence of oxidative stress is phenotypically similar to the wild-type (Mishra and Imlay, 2012).

1.2.2.3 Enzymes that reduce hydrogen peroxide

Prokaryotes have several enzymes that catalyse the reduction of H_2O_2 to water, named peroxidases. In the presence of low-levels of H_2O_2 stress, these enzymes are better scavengers compared to catalases, but just as long as there is enough reducing power (NADH). Peroxidases may not have enough catabolic substrates, for instance during starvation periods (as during these periods in the absence of a carbon source the cell is depleted of NADH), and they can saturate at H_2O_2 micromolar range concentrations. In those scenarios, catalase represents an advantage to the cells.

Some of these enzymes are thiol-based peroxidases, such as alkylhydroperoxide reductase (Ahp), bacterioferritin comigratory protein (BCP), thiol peroxidase (Tpx) (all three belong to the peroxiredoxin family), and glutathione peroxidase (Gpx) (Poole, 2005). The non-thiol-based peroxidases include rubrerythrin and bacterial cytochrome *c* peroxidase (BCCP) (Mishra and Imlay, 2012).

These peroxidases differ in their substrate specificity, localization, metal requirements, as well as in protein stability. For this reason, some bacteria (such as *E. coli*) have in their cells more than one of these molecular systems to cope with oxidative stress from various sources and different environments.

Thiol-based peroxidase

Alkylhydroperoxide reductase (**Ahp)** consists of two cytoplasmic proteins: AhpC, which forms a dimer and is the catalytic subunit containing two conserved cysteine residues, and AhpF, a flavoprotein dimer with NADH:disulfide oxidoreductase activity (Poole *et al.*, 2000). The *ahpCF* expression is regulated by OxyR. Ahp receives electrons from NADH to reduce H_2O_2 (concentrations < 20 µM). Reduction of H_2O_2 leads to the oxidation of AhpC, that forms a decameric complex (pentamer of dimers), which is reduced by AhpF.

Ahp is present in several bacteria but its importance as peroxidase became clear as an Ahp and KatG/KatE *E. coli* triple mutant was not able to scavenge H_2O_2 and the cells continuously released H_2O_2 to the medium (Seaver and Imlay, 2001b). This showed that although *E. coli* has several other peroxidases, these three enzymes are the main scavengers that maintain the low levels of intracellular hydrogen peroxide, and that allow *E. coli* to successfully grow under oxic conditions.

Bacterioferritin comigratory protein (**BCP)** is another thiol-based peroxidase with a conserved N-terminal cysteine. The physiological electron donor is unknown but the thioredoxin system is a likely candidate (Reeves *et al.*, 2011) (Scheme 2). Recombinant BCP was shown to react with *t*-butylhydroperoxide and linoleic hydroperoxide, besides H_2O_2 (Jeong *et al.*, 2000). In other microorganisms, such as *Campylobacter jejuni* and *Helicobacter pylori*, BCP mutants show sensitivity to cumene hydroperoxide or to aerated conditions but no sensitivity to H_2O_2 (Atack *et al.*, 2008; Wang *et al.*, 2005). This hints to a role as organic hydroperoxidase, which would explain why an *E. coli* Ahp$^-$Kat$^-$ mutant cell suspension, when incubated with 1.5 µM H_2O_2 does not reduce hydrogen peroxide (Seaver and Imlay, 2001a), thus presenting no peroxidase activity *in vivo*, even though BCP is produced in exponentially growing cells (Mishra and Imlay, 2012). There is also no evidence of *bcp* responding to hydrogen peroxide, as this gene is not regulated by either OxyR or PerR.

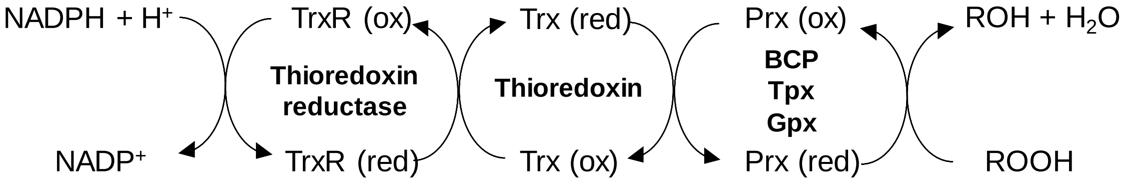
Thiol peroxidase (Tpx) is a cytoplasmatic enzyme dependent on two conserved cysteine residues (Atack *et al.*, 2008; Cha *et al.*, 2004; Tao, 2008), that is proposed to use thioredoxin as direct electron donor (Chae *et al.*, 1994; Rhee *et al.*, 2005) (Scheme 2). *E. coli* Tpx shows peroxidase activity *in vitro* with H_2O_2, *t*-butylhydroperoxide, cumene hydroperoxide and linoleic acid hydroperoxide, with dithiothreitol as electron donor. However, as observed for BCP, an *E. coli tpx* null mutant did not display a different phenotype when grown under aerobic conditions, only a slight sensitivity to organic hydroperoxides (Cha *et al.*, 2004), hinting to its role as an organic hydroperoxidase.

Organic hydroperoxidases main function is to detoxify the cells from organic hydroperoxides, which can damage the membranes, affecting their integrity and fluidity. These organic hydroperoxides can have two sources: one is the oxidation of linoleic acid and arachidonic acid by lipoxygenases or cyclooxygenases. The other is the reaction between polyunsaturated fatty acids and free radicals, also known as lipid peroxidation. In bacteria, lipid peroxidation is not well documented due to the low levels of polyunsaturated fatty acids in their membranes. However, this phenomenon has been described in some bacteria, such as *E. coli* and *Mycobacterium tuberculosis* (Master *et al.*, 2002; Pérez *et al.*, 2008) and in some cases it was demonstrated the uptake of exogenous polyunsaturated fatty acids from host organisms or the ability to produce them in some marine bacteria (Nichols, 2003). The role of hydroperoxidases (as suggested for BCP and Tpx) is crucial for cell survival.

Glutathione peroxidase (Gpx) is a thiol-based peroxidase with the ability to reduce hydroperoxides using glutathione as electron donor. This enzyme was first discovered in eukaryotes with a selenocysteine as peroxidatic residue (Flohe *et al.*, 1973). The bacterial enzyme, found by sequence homology in *E. coli* is BtuE (Arenas *et al.*, 2011) and it has a cysteine instead of a selenocysteine. *E. coli* BtuE has peroxidase activity *in vitro* with *t*-butylhydroperoxide, linoleic hydroperoxide and H_2O_2. However, *btuE* expression is not induced by H_2O_2 and even when it is overexpressed it does not confer significant peroxidase activity in a *E. coli* strain lacking Ahp and catalases, as BCP (Mishra and Imlay, 2012).

Gpx, besides using glutathione, as BCP and Tpx, also uses thioredoxin (Ohdate *et al.*, 2010) explaining how this bacterial enzyme can exist in a system that lacks the glutathione biosynthetic pathway (Scheme 2).

Overall BCP, Tpx and Gpx showed peroxidase activity *in vitro*, but there is no clear indication that this is their role *in vivo*. Also depending on the microorganism, they have shown different affinities for the mentioned substrates. For instance, in *Campylobacter jejuni*, a *bcp* mutant showed lower cell-viability upon aeration and sensitivity to cumene hydroperoxide (Atack *et al.*, 2008), and in *Enterococcus faecalis* a *tpx* mutant is sensitive to exogenous H_2O_2 and cumene hydroperoxide, but not to aeration (La Carbona *et al.*, 2007).



Scheme 2 - Proposed mechanism for the thiol-based reductases (Prx): BCP, Tpx and Gpx. These enzymes use thioredoxin (Trx) and thioredoxin reductase (TrxR) as electron donors, to reduce peroxides (ROOH). Adapted from Mishra and Imlay (Mishra and Imlay, 2012).

Rubrerythrin

Rubrerythrin is an example of an enzyme which has non-thiol-based peroxidase activity and that is widely distributed in anaerobic and microaerophilic bacteria. It has two metal centres: a rubredoxin-type iron centre [Fe(Cys)$_4$] and a di-iron site with a μ-oxo bridge (LeGall *et al.*, 1988). The di-iron centre is oxidized by H_2O_2 (forming water) and to complete the cycle it requires two consecutive electrons from the rubredoxin site that *in vivo* would receive electrons from a rubredoxin protein (which in turn is reduced by the NADH:rubredoxin oxidoreductase) (Kawasaki *et al.*, 2009). In some bacteria there is a reverse form of rubrerythrin (the position of the two domains is reversed) whose expression is regulated by PerR, suggesting a role for these enzymes in scavenging H_2O_2. *In vivo* and *in vitro* studies with the reverse rubrerythrin from *Clostridium acetobutylicum* showed that it has peroxidase and oxidase activity. Furthermore, a *C. acetobutylicum* strain overproducing this protein had higher tolerance to H_2O_2 and O_2 (Riebe *et al.*, 2009; Weinberg *et al.*, 2004).

Bacterial cytochrome c peroxidase

Bacterial cytochrome *c* peroxidases (BCCPs), or just bacterial peroxidases, constitute a family of enzymes that catalyse the reduction of H_2O_2 to water using *c*-type haem cofactors (Equation 9). Classical BCCPs contain two *c*-type haems and are homodimers located in the periplasm (Fülöp *et al.*, 1995; Goodhew *et al.*, 1990) (see Section 2) (Figure 1).

$$H_2O_2 + 2\,e^- + 2\,H^+ \rightarrow 2\,H_2O \qquad (9)$$

The eukaryotic counterpart, cytochrome *c* peroxidase (CCP), contains one *b*-type haem and accepts electrons from a small redox protein, cytochrome *c*, with the one from *Saccharomyces cerevisiae* being the most well characterized eukaryotic CCP to date. This enzyme is located in the inter-membrane space of mitochondria (Erman and Vitello, 2002). Another well studied eukaryotic peroxidase was isolated from horseradish, which has several isoenzymes, being isoenzyme C the most abundant and well-studied (Grigorenko *et al.*, 2015; Veitch, 2004). There are other haem containing peroxidases in eukaryotes, such as ascorbate peroxidase isolated from chloroplasts (Dietz, 2016) and lignin peroxidases found in fungi (Falade *et al.*, 2017).

The study of these various peroxidases, although significantly different from bacterial peroxidases, has been essential to understand many of the BCCPs features, such as its spectroscopic and kinetic properties, and its catalytic mechanism.

The bacterial peroxidase constitutes a unique family of peroxidases that will be described in the following sections, with a special focus on its structural and biochemical properties, the currently accepted catalytic mechanism and its physiological function, highlighting the questions that remain to be unanswered.

2. Bacterial Cytochrome *c* Peroxidase

2.1 Gene distribution and regulation

BCCP are encoded by the *ccp* gene. A blast search reveals that this gene is found in the genome of many (but not all) Gram-negative bacteria, with some being pathogenic species, such as *Neisseria*, *Salmonella* and *Yersinia* genus, but not in Gram-positive bacteria and archaea. Some species harbour more than one *ccp* homologous gene in their genome, as it is the case of *Geobacter sulfurreducens*, where a MacA enzyme was reported as a second BCCP (Seidel *et al.*, 2012), and of *C. jejuni* that has in its genome two putative genes encoding for BCCPs (Bingham-Ramos and Hendrixson, 2008).

The *ccp* gene expression is regulated by either FNR or OxyR transcription regulators. In most species, an FNR box is present in the promoter region of *ccp*. In the case of *Neisseria gonorrhoeae* (Lissenden *et al.*, 2000; Turner *et al.*, 2003), *P. aeruginosa* (Van Spanning *et al.*, 1997) and *Pseudomonas stutzeri* (Vollack *et al.*, 1999) this gene has been shown experimentally to be regulated by FNR (Figure 2A), while OxyR, the oxidative stress regulator (see Section 1.2.1), regulates the expression of *ccp* in *Bacteroides fragilis* (Herren *et al.*, 2003) and *Shewanella oneidensis* (Li *et al.*, 2014). In some species, this gene might be regulated by both OxyR and FNR, as it is the case of *E. coli yhjA* (Partridge *et al.*, 2007) (Figure 2B).

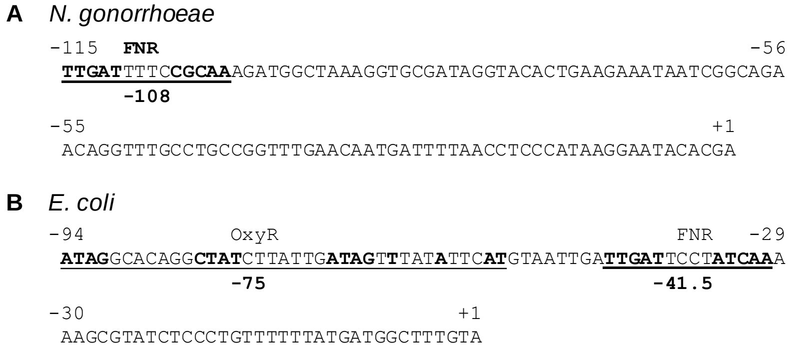


Figure 2 – Nucleotide sequence of the promoter region of (A) *N. gonorrhoeae ccp* (Lissenden *et al.*, 2000) and (B) *E. coli yhjA* (Partridge *et al.*, 2007). The putative FNR and OxyR binding sites are underlined and the nucleotides in bold match their consensus sequences.

The role of FNR in the regulation of *ccp* gene expression might be a paradox, as one would expect H_2O_2 concentrations to be higher under oxic conditions, as a product of oxidative stress. However, this might be related to its physiological role and the habits in which the Gram-negative bacteria carrying the *ccp* gene strive, as it will be discussed in Section 2.3.

2.2 Primary sequence analysis - Enzyme localization and phylogenetic groups

The analysis of the primary sequence of BCCP reveals the presence of either two or three canonical *c*-type haems binding motifs, -CXXCH-, and also the presence of a signal peptide recognized by the Sec system (type II secretion system), indicating that this enzyme will be located in the periplasm (Goodhew *et al.*, 1990). However, with the increasing number of sequenced genomes, it is observed that BCCP is most probably always located in the periplasm (as it requires the cytochrome *c* biogenesis system for haem *c* attachment to the polypeptide chain), but in some cases it is anchored to the inner-membrane (Figure 3).

Considering the number of *c*-haem binding motifs and the presence of an anchor to the membrane, these enzymes can be divided into the Classical BCCPs and the non-classical trihaem BCCPs (Figure 3).

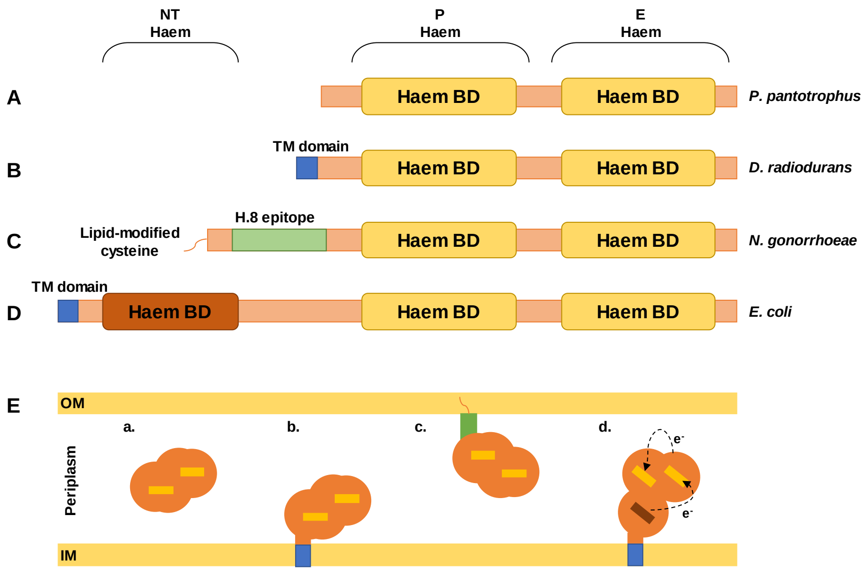


Figure 3 – Schematic representation of the BCCPs primary sequence (A-D) and their respective location in the periplasm (E). (A) *P. pantotrophus* BCCP is soluble in the periplasm and has two *c*-type haem binding domains (Haem BD). (B) The putative *D. radiodurans* BCCP has a transmembrane (TM) domain predicted as a transmembrane helix to anchor the enzyme to the inner membrane (IM). (C) *N. gonorrhoeae* BCCP is bound to the outer membrane (OM) by a lipid-modified cysteine and has a linker region (named H.8 epitope) that confers flexibility within the periplasm. (D) *E. coli* BCCP has three *c*-type haem binding domains and a transmembrane helix to anchor the enzyme to the IM. (E) The putative periplasmic localization of each BCCP is represented by the various haem domains (orange circles) with respective haem (yellow bars). BCCP is presented as a dimer except for trihaem BCCP that is a monomer. The proposed electron transfer route in trihaem is also represented by dashed lines.

The classical BCCPs have two *c*-type haems and are usually soluble periplasmic enzymes (Figure 3A). The first classical BCCP to be isolated was the one from *P. aeruginosa* (Soininen and Ellfolk, 1972; 1973), but since then it has been isolated and characterized from many different Gram-negative bacteria, such as *Paracoccus pantotrophus* (Goodhew *et al.*, 1990), *Nitrosomonas europaea* (Arciero and Hooper, 1994), *Rhodobacter capsulatus* (De Smet *et al.*, 2001), *Methyloccocus capsulatus* Bath (Zahn *et al.*, 1997), *P. stutzeri* (Timóteo *et al.*, 2003), *Marinobacter hydrocarbonoclasticus* (Alves *et al.*, 1999), *N. gonorrhoeae* (Turner *et al.*, 2003), *S. oneidensis* (Pulcu *et al.*, 2012) and *G. sulfurreducens* (Hoffmann *et al.*, 2009; Seidel *et al.*, 2012) (Table 1).

These dihaemic enzymes are not all soluble enzymes, as some are anchored to the inner-membrane by a transmembrane helix or to the outer-membrane by a lipid-modified residue, such as the ones from *Deinococus radiodurans* (Figure 3B) and *N. gonorrhoeae* (Turner *et al.*, 2003) (Figure 3C), respectively.

The non-classical BCCPs have three *c*-type haems and have not been significantly investigated until recently. Two of the C-terminal haem domains are homologous to the classical BCCPs and the third additional N-terminal haem domain is completely new (Figure 3D). These trihaemic BCCPs are anchored to the membrane by a transmembrane helix (Yamada *et al.*, 2007). To date the only non-classical BCCPs that were characterized are the ones from *Aggregatibacter actinomycetemcomitans (Abe et al., 2017; Takashima and Konishi, 2008; Takashima et al., 2010; Yamada et al., 2007), Zymomonas mobilis* (Balodite *et al.*, 2014), and *E. coli* (Khademian and Imlay, 2017; Nobrega *et al.*, 2018), presenting quinol peroxidase activity, which means they use the quinol pool reductive power to reduce hydrogen peroxide (see Section 2.5). The gene coding for trihaemic enzymes is present the genome of pathogenic bacteria, such as *Salmonella typhimurium* and *Yersinia pestis* (Atack and Kelly, 2007), but these enzymes have not been isolated to date.

There are other BCCP-like proteins, such as the orthologous MauG proteins and the extracellular rubber oxygenase RoxA from *Xanthomonas* sp. These proteins have two *c*-type haems and share structural similarities, but do not have the same physiological role.

The MauG is present in many Gram-negative bacteria that use methylammonium as a carbon and nitrogen source. This enzyme is involved in the formation of tryptophan-tryptophylquinone (TTQ), a co-factor of methylamine dehydrogenase (MADH). MauG forms TTQ by binding and activating oxygen and linking two tryptophan residues of MADH. MauG has a high sequence and structural homology to BCCP, but presents little peroxidase activity suggesting that these two enzymes share a common ancestral (Wang *et al.*, 2003; Wang *et al.*, 2005).

RoxA, a 75 kDa protein secreted by *Xanthomonas* sp., is a dioxygenase that catalyses the slow cleavage of cis-1,4-polyisoprene, present in the latex milk from rubber trees, to 12-oxo-4,8-dimethyl-trideca-4,8-diene-1-al (Braaz *et al.*, 2005). Most of the protein structure consists of unstructured loop regions, explaining the high molecular mass compared to BCCP, which is typically around 35 kDa. RoxA has low sequence homology but a portion of its core structure surrounding the haem domains, is similar to BCCP structural architecture, suggesting that they are evolutionary related enzymes (Seidel *et al.*, 2013)

2.3 Physiological role of bacterial peroxidases

As mentioned before, in a bacterial cell the enzymes that deal with hydrogen peroxide are cytoplasmic, Ahp, KatG, KatE, except for BCCP, that is located in the periplasm. The *in vivo* role of BCCPs has been described to be the protection against exogenous hydrogen peroxide and to reduce H_2O_2 produced by the electron transfer chain in the inner membrane (Pettigrew *et al.*, 2006). However, recently it was shown that in *E. coli*, BCCP confers the cells the ability to use H_2O_2 as a terminal electron acceptor, to sustain respiration in the absence of oxygen (Khademian and Imlay, 2017) (Figure 1). In fact, this explains why, as mentioned in Section 2.1, *ccp* gene would be under the regulation of FNR and thus its expression levels increase under anoxic or microoxic conditions.

Considering this hypothesis, due to the periplasmic location of BCCP this enzyme cannot be energy conserving by itself, and so there is the need of membrane complexes to be involved to establish a proton motive force, required for ATP synthesis. Under such scenario, the proton motive force might be generated by primary dehydrogenases and/or by the bc_1-type cytochrome complex (when present), which then reduces small electron carriers that are used as electron donors to classical BCCP (see Section 2.4.3) or would reduce menaquinol, the proposed electron donor to non-classical trihaemic BCCPs (as in the case of *E. coli* BCCP) (see Section 2.5) (Figure 1).

Early work in the group of G. Pettigrew using a catalase-negative strain of *Campylobacter mucosalis* had already proposed that BCCP could play a role in using H_2O_2 as terminal electron acceptor (Goodhew *et al.*, 1988). They have estimated a stoichiometry of H^+ pumping of around 0.6 H^{1+}/H_2O_2, when the bacterium was grown in the presence of formate. Under these conditions, H_2O_2 formed by formate dehydrogenase could be reduced by BCCP present in the periplasm with electrons delivered by a small *c*-type cytochrome that would be reduced by a membrane electron transport system. Other evidences for the role of BCCP in the respiratory chain came from the fact that H_2O_2 competes with nitrate for the electrons in the respiratory chain of *P. pantotrophus* and in some strains of *R. capsulatus*, under oxic and anoxic conditions (Richardson and Ferguson, 1995). This might be explained by both enzymes using the same small periplasmic electron carrier. Other studies had proposed that BCCP could function as electron carrier under anoxic conditions (Koutny *et al.*, 1999). Nevertheless, the role of BCCP in the respiration of H_2O_2 needs to be confirmed for other bacterial species, such as pathogenic bacteria that harbour the classical BCCP type, as these bacteria encounter exogenous hydrogen peroxide during oxidative burst by macrophages, tissue inflammation or when produced by lactic bacteria in common environments, as is the case of *N. gonorrhoeae*. In fact, in *N. gonorrhoeae,* a *katA* and *ccp* double mutant was much more sensitive to hydrogen peroxide in disc diffusion assays, than a *katA* mutant alone, but a *ccp* single mutant was not much more sensitive than the wild-type (Turner *et al.*, 2003). Nevertheless, it has been reported that this enzyme plays a crucial role during infection (McClure *et al.*, 2015). Moreover, in *C. jejuni*, deletion of each of the two putative *ccp* genes, encoding DocA and Cjj0382, led to a 10^5-fold and 50-fold reduction in colonization of chick cecum by these strains, respectively. This result suggested a different role for the BCCPs in this bacterium during colonization, while its catalase (KatA), seemed to be responsible for hydrogen peroxide resistance.

Recent studies on BCCPs from bacteria involved in dissimilatory metal-reduction, such as *S.*

oneidensis and *G. sulfurreducens*, showed that *S. oneidensis* BCCP plays a role as hydrogen peroxide scavenger, since a *ccp* mutant resulted in significant loss of the cells peroxidase activity (Schütz *et al.*, 2011). *G. sulfurreducens* is a micro-oxic tolerant bacteria that has two *ccp* genes that encode CcpA (Hoffmann *et al.*, 2009) and MacA (Seidel *et al.*, 2012), with the latter being propose to play a double role in the periplasm of this bacterium. MacA has peroxidase activity, and thus may be important in detoxifying H_2O_2 produced as a by-product of Fe^{3+} oxyhydroxide reduction (Aklujkar *et al.*, 2013), and it has also the ability to channel electrons to a periplasmic soluble trihaemic *c*-type cytochrome, PpcA (Seidel *et al.*, 2012), suggesting its involvement in the extracellular electron transfer required for U^{6+} and Fe^{3+} oxide reduction by this bacterium (B. C. Kim and Lovley, 2008; Shelobolina *et al.*, 2007).

In conclusion, it seems that BCCP plays a role in virulence as this enzyme is important in colonization settings, especially in pathogenic bacteria. Having said this, it is plausible that the role of BCCP is just to provide the cells with the ability to respirate, increasing ATP production, in the absence of oxygen in environments that have a high concentration of H_2O_2. In fact, the habitat of pathogenic and non-pathogenic bacteria can be anoxic and have a high concentration of H_2O_2 (Imlay, 2018), as for instance due to the presence of lactic acid bacteria, inside phagosomes, and in oxic-anoxic interfaces, in which sulphides produced by sulphate reducing bacteria can diffuse to the microoxic region, chemically reacting with oxygen and producing H_2O_2. These issues still need to be investigated in different bacteria and under different environmental settings to ascertain the physiological role of BCCP.

2.4 Classical bacterial peroxidases

2.4.1 Haem coordination and Activation mechanism

Classical BCCPs are periplasmic enzymes that bind two *c*-type haems per polypeptide chain and usually exists in a monomer-dimer equilibrium. These enzymes are usually isolated with both haems in the oxidized state, and the difference between their reduction potentials is so that it is possible to have the enzyme in a mixed-valence state (one haem reduced and the other oxidized) or in a fully reduced state (Table 1).

Therefore, since early studies the two haems were defined by their redox properties. The C-terminal haem, with a high potential, is capable of receiving electrons from sodium ascorbate or small electron donor proteins, and was named the electron transferring haem (E haem) (Pettigrew *et al.*, 1999; Ronnberg *et al.*, 1981b). The N-terminal haem, with a low-potential, can only bind exogenous substrates when E haem is reduced and was named the peroxidatic haem (P haem), being the active site. The reduction potential of each haem varies greatly among BCCPs (Table 1). For E haem it varies between + 226 mV (*P. pantotrophus* BCCP (Gilmour *et al.*, 1993)) and + 450 mV (*N. europaea*

BCCP (Arciero and Hooper, 1994)), while P haem reduction potential ranges from - 150 mV (*P. pantotrophus* BCCP (Gilmour *et al.*, 1993)) to - 330 mV (*P. aeruginosa* BCCP (Ellfolk *et al.*, 1983)). The axial ligands of each haem were first assigned using near-infrared magnetic circular dichroism (nIR-MCD) at cryogenic temperatures. In the fully oxidized state the P haem has features of a low-spin bis-histidine coordinated haem and E haem, of a low-spin histidine-methionine coordinated haem. At room temperature, E haem showed also high-spin features suggesting to exhibit a high/low-spin equilibrium that was attributed to a weak methionine-iron bond.

- Insert Table 1 in here -

When E haem is reduced (mixed-valence state), P haem becomes high-spin (featuring bands between 700-1300 nm) (Foote *et al.*, 1984; 1985). In the visible spectra of mixed-valence BCCPs there are two characteristic bands of this high-spin ferric haem, one at 350-380 nm and another less intense charge-transfer band at 600-640 nm, similar to the one observed in the visible spectrum of the penta-coordinated cytochrome *c'* (Weiss *et al.*, 2006). This mixed-valence state is the active form of the enzyme.

Therefore, to achieve maximum catalytic activity, BCCPs must be reductively activated, reduction of E haem with consequent formation of the a high-spin P haem, with the exception of *N. europaea* and *M. capsulatus* BCCPs (Arciero and Hooper, 1994; Zahn *et al.*, 1997).

The importance of calcium ions in the enzyme activation mechanism and catalytic activity was first studied in *P. pantotrophus* BCCP since without excess of calcium ions, the enzyme in the mixed-valence state had little catalytic activity (Gilmour *et al.*, 1994; Gilmour *et al.*, 1995). In fact, in *P. pantotrophus* and *P. stutzeri* BCCPs, removal of calcium ions by EGTA led not only to an inactive enzyme but also to the disappearance of the P haem high-spin bands at 380 nm and 630 nm in the visible spectrum of the mixed valent form (Gilmour *et al.*, 1994; Gilmour *et al.*, 1995; Timóteo *et al.*, 2003). This led to the conclusion that calcium ions were essential to form the high-spin P haem in the mixed-valence active form (Figure 4).

Furthermore, ultracentrifugation, differential scanning calorimetry (DSC) and chromatographic techniques demonstrated that BCCPs in solution are in a monomer-dimer equilibrium and that addition of calcium ions or a higher ionic strength in *P. pantotrophus* BCCP led to homodimerization (Gilmour *et al.*, 1995; Pettigrew *et al.*, 2003a; Pettigrew *et al.*, 2003b). *P. stutzeri* BCCP was purified as a dimer and addition of calcium ions led to the formation of a tetramer, while removal of calcium ions by EGTA led to a monomeric population in solution (Figure 4). This is the only case where formation of a tetramer has been reported. However, not all classical BCCPs present a monomer-dimer equilibrium is the oxidised form, as is the case of *N. gonorroheae* BCCP. In the oxidised form this enzyme only dimerizes in the presence of calcium ions or at high protein concentrations (10 µM) due to the lower hydrophobic character of its dimer interface. This property might be related with the localization of *N. gonorroheae* BCCP *in vivo*, as it is not freely diffusing in solution but is anchored to the membrane, and thus its dimer does not need to be so strong (Nóbrega *et al.*, 2017).

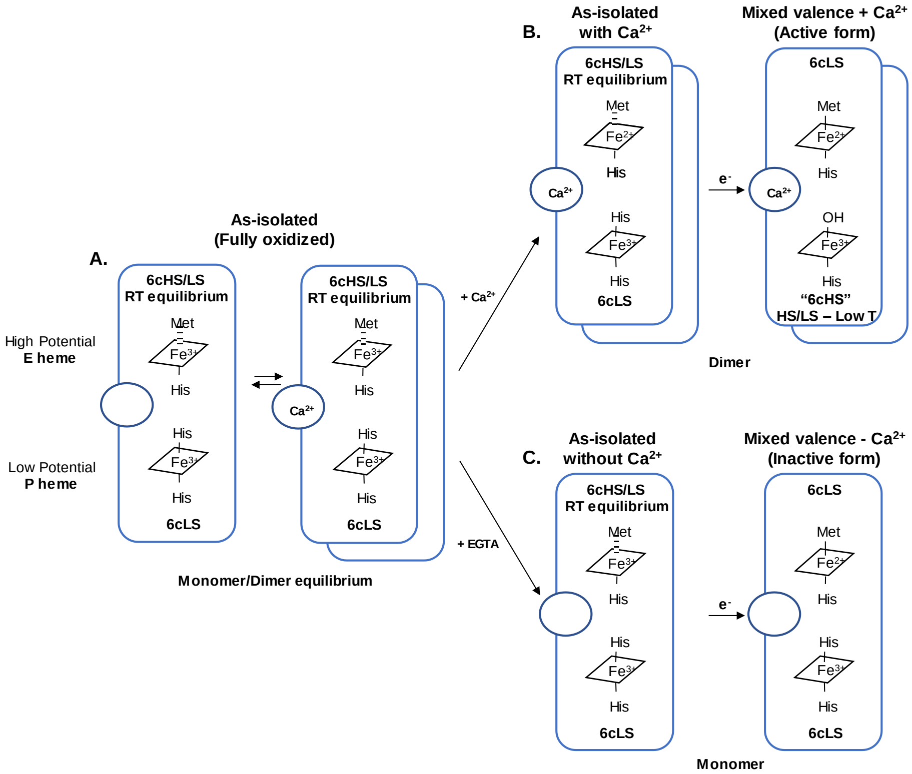


Figure 4 – Activation mechanism of classical BCCPs. (A) The as-isolated BCCP has a His/Met hexa-coordinated E haem in a high/low-spin (6cHS/LS) equilibrium at room temperature (RT) due to a loosely bound methionine residue (dashed line). The low potential P haem is hexa-coordinated and low-spin (6cLS) with two axial histidine ligands. Typically, the enzyme is in a monomer-dimer equilibrium and becomes fully dimeric in the presence of calcium ions. (B) When E haem is reduced by a small redox protein or a reducing agent, such as sodium ascorbate, in the presence of calcium ions, P haem becomes OH^-/His-coordinated ("6cHS"), in a HS/LS equilibrium at low temperatures. This the mixed-valence active form. (C) Removal of calcium ions by a calcium chelator, such as EGTA, induces monomerization of BCCP. In the mixed-valence state, the P haem continues to be His/His-6cLS and the enzyme is inactive.

To sum up, the active form of BCCP requires calcium ions, is dimeric and the P haem is high-spin. Analysis of the activation mechanism of *P. stutzeri* BCCP by mediated cyclic voltammetry (using its redox partner, cytochrome c_{551}) showed that the activation process is fast as there was no difference in the results when using the oxidized or the mixed-valence forms of the enzyme (Paes de Sousa *et* *al.*, 2011b).

2.4.1.1 Calcium binding site

Although it was known that calcium ions were essential for dimerization and for enzyme activation, prior to the crystallographic structures it was not clear how many calcium ions existed per protein nor the location of the calcium binding site was known. Early data from *P. pantotrophus* BCCP suggested two calcium binding sites (Gilmour *et al.*, 1995), one with higher affinity (site I, K_D = 1.2 µM) and usually fully occupied, and the other with lower affinity (site II, K_D = 0.52 mM) and empty or partially occupied in the oxidized form. It was proposed that calcium site II was required for the activation of the enzyme and for enzyme activity. On the other hand, *P. aeruginosa* BCCP was purified with calcium tightly bound to the protein (K_D = 0.3 nM), which is sufficient to attain a fully active, dimeric form of the enzyme (Echalier *et al.*, 2008; Soininen and Ellfolk, 1972). For this reason, there was no biochemical studies on the role of calcium ions on the activation of this enzyme until much later, when the mixed-valence structure was solved by Echalier *et al.*, showing that when calcium ions were removed the enzyme became inactive (Echalier *et al.*, 2008).

This leads to the conclusion that *P. pantotrophus* BCCP needed additional calcium ions, which were lost during the enzyme purification, probably due to its low binding affinity. It was proposed that since the enzyme was purified from the periplasm, and spheroplasts were prepared using EDTA, calcium ions were removed from the enzyme (Fülöp *et al.*, 2001). However, even if a similar procedure was used to isolate *P. stutzeri* and *P. aeruginosa* BCCPs, these enzymes still have the calcium binding site occupied due to their high calcium affinity.

Thus, calcium affinity is not a common trait in classical BCCPs. In fact, *N. gonorrhoeae* BCCP is a monomer in solution in the as-isolated form and only dimerizes in the presence of calcium ions, which also stabilizes the mixed-valence form, as it has a higher T_m value than the oxidized form (Nóbrega *et al.*, 2017). This enzyme binds calcium ions with a K_D of 8.5 ± 0.5 nM in the mixed-valence form, a high affinity binding constant when compared with the one of *P. pantotrophus* BCCP (K_D = 2.6 µM) (Gilmour *et al.*, 1995), which is explained considering the this site is partially occupied after purification.

Even if the calcium binding affinity differs between BCCPs from different bacterial species, calcium ions are always present in the *in vitro* activity assays to assure maximum catalytic activity.

When the first crystallographic structures were published, it was found that BCCPs bind one calcium ion per monomer between the two haem domains (Figure 5A) (De Smet *et al.*, 2006; Dias *et al.*, 2004; Fülöp *et al.*, 1995; Shimizu *et al.*, 2001). In these structures the calcium atom is coordinated by four water molecules and three conserved residues: the amide oxygen from Asn79 side-chain and the carbonyls from Thr256 and Pro258 (*P. aeruginosa* numbering) (Fülöp *et al.*, 1995), with two of the water molecules establishing H-bonds with the E haem A propionate group (Figure 5B).

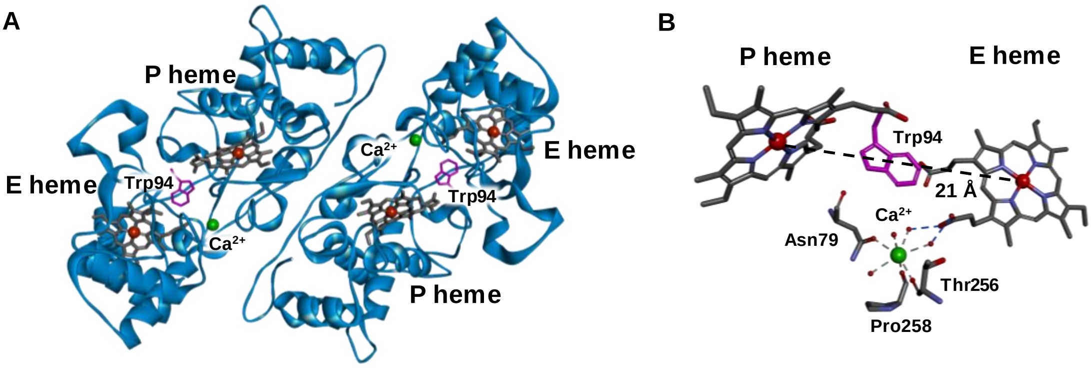


Figure 5 – (A) Structure of *P. aeruginosa* BCCP in the mixed-valence form. The asymmetric unit is a homodimer. Each monomer has two haems, E haem and P haem, a calcium atom (green sphere) between the two haem domains and the conserved tryptophan (purple). (B) The calcium binding site is coordinated by four water molecules and the oxygens from carboxyl group from Thr256 and Pro258, and from the side chain of Asn79. Two of the waters are coordinated by the E haem A propionate group. Figure prepared using PDB ID: 2VHD.

2.4.1.2 Spectroscopic and structural changes in the activation mechanism

Prior to the structural elucidation of the changes involved in the reductive activation, the formation of the high-spin P haem during reductive activation was thoroughly characterized using different spectroscopic techniques, besides the previously mentioned MCD and UV-visible, such as ^{1}H NMR (Gilmour *et al.*, 1993; Prazeres *et al.*, 1993), Mössbauer (Prazeres *et al.*, 1995), resonance Raman (Pauleta *et al.*, 2001; 2008; Rönnberg *et al.*, 1980) and EPR (Arciero and Hooper, 1994; Echalier *et al.*, 2008; Foote *et al.*, 1984; Timóteo *et al.*, 2003).

A property of this high-spin P haem was that it became low-spin at cryogenic temperatures. However, this low-spin species was not the same as the low-spin P haem signal in the oxidized form even though the EPR spectroscopic properties were interpreted considering a bis-His ligation (Foote *et al.*, 1985; Gilmour *et al.*, 1993). In the EPR spectra, the low-spin signal from the P haem in the mixed-valence form was also sensitive to the presence of calcium ions. For instance, in *P. aeruginosa* BCCP, in the absence of calcium ions the g values were 2.96, 2.26 and 1.49, while in the presence of calcium ions the g values changed to 2.86, 2.36 and 1.53 (Echalier *et al.*, 2008). The first set of g values corresponded to the bis-His coordination and the second to a different sixth distal ligand. Similar pairs of g values are found in the EPR spectra of BCCPs (Arciero and Hooper, 1994; Gilmour *et al.*, 1993; Greenwood *et al.*, 1988; Nóbrega *et al.*, 2017; Pulcu *et al.*, 2012; Seidel *et al.*, 2012; Timóteo *et al.*, 2003).

The nature of this P haem ligand had been proposed to be a water-derived molecule from the early nIR-MCD spectroscopic characterization of the *P. aeruginosa* BCCP (Foote *et al.*, 1985; Greenwood *et al.*, 1988), which was later summarized by Fülöp, Watmough & Ferguson (Fülöp *et al.*, 2001). The observation of a temperature dependent high-spin haem in the EPR and visible spectra, and of a nIR-MCD band at 1150 nm at room temperature for the mixed-valence BCCP is consistent with P haem having a His/OH^- axial coordination in this oxidation state (Fülöp *et al.*, 2001; Nobrega *et al.*, 2018; Smulevich *et al.*, 2016).

Later, when the first mixed-valence BCCP crystallographic structure was solved, for *P. pantotrophus* BCCP, it was observed an oxygen atom as distal axial ligand of P haem (Echalier *et al.*, 2006). This observation was interpreted as P haem being distantly coordinated by a water molecule. Without taking into account the earlier interpretation, the authors explained the previous spectroscopic data considering that although water is typically a weak ligand, it acted as a strong ligand at cryogenic temperatures in the case of BCCPs (Echalier *et al.*, 2006). Nevertheless, the presence of an OH^- can also be consistent with the crystallographic data, as protons are not observed in the electron density maps.

In summary, a His/OH^- coordination exhibits a thermal equilibrium of spin-states, and has an absorption band at 610 nm in the visible spectrum of its ferric form, while a His/H_2O coordinated haem would be high-spin regardless of the temperature, as observed for myoglobin, exhibiting a 630-640 nm absorption band in the visible spectrum of its ferric form (Fülöp *et al.*, 2001; Smulevich *et al.*, 2016). Moreover, since the EPR spectra of P haem in the mixed-valence BCCP is modulated by calcium ions, and for some classical BCCPs a high-spin form of this haem is still observed at cryogenic temperatures (Seidel *et al.*, 2012; Timóteo *et al.*, 2003), it is proposed that calcium ions can modulate the p*Ka* of this axial ligand (Nobrega *et al.*, 2018). In the absence of calcium ions, there would be an increase in the p*Ka* shifting the equilibrium towards a His-H_2O coordinated P haem, with an absorption band observed at 630 - 640 nm and being high-spin still at cryogenic temperatures. Therefore, the spectroscopic data of mixed-valence classical BCCPs need to be re-interpreted at the light of the observations presented here.

The reductive activation involves a series of conformational changes that were elucidated by comparison between the first BCCP crystallographic structures obtained for the oxidized form (De Smet *et al.*, 2006; Fülöp *et al.*, 1995), with the ones of the mixed-valence form (Echalier *et al.*, 2008; Echalier *et al.*, 2006; Hoffmann *et al.*, 2009; Schütz *et al.*, 2011; Seidel *et al.*, 2012) (Figure 6). These structures clarified the identity of the haem ligands in the different redox forms of the enzyme and were crucial to understand how reduction of E haem triggers the removal of the P haem distal axial ligand, since these two haems are in distinct protein domains, and the role played by the calcium ions in this mechanism.

The activation mechanism has been described as follows (Echalier *et al.*, 2008; Echalier *et al.*, 2006) (Figure 6): (i) when E haem receives an electron, there is a redox-linked protonation of E haem D propionate group that triggers the movement of loop regions, mainly a loop between the two haem domains, described in *P. aeruginosa* BCCP as the region 91-118 containing Arg97 that interacts with P haem D propionate; (ii) this loop moves to the dimer interface leading to the loss of P haem distal axial ligand (located in this loop); (iii) at the dimer interface, the loop carrying P haem histidine ligand (His71), stabilizes the dimer by a π-stacking interaction between the tryptophan side chain and the peptide bond of a glycine in the opposite monomer (Gly72-Trp73 residues in *P. aeruginosa* BCCP(Echalier *et al.*, 2008)). This tryptophan residue is conserved in most of the BCCPs characterized to up-to-date, with the exception of the enzymes from *N. gonorrhoeae* and *M. capsulatus* (Nóbrega *et al.*, 2017).

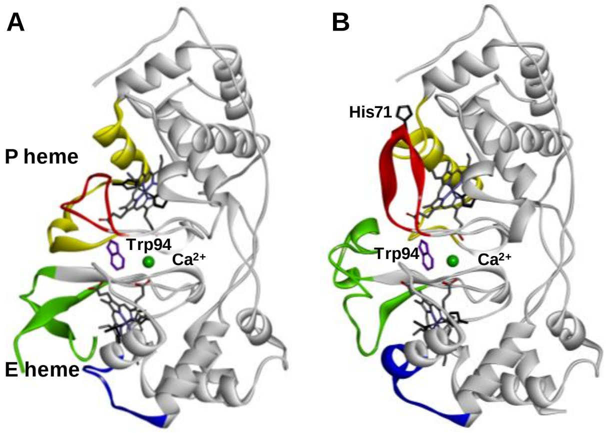


Figure 6 – Structure of *P. aeruginosa* BCCP in the oxidised (A) and mixed-valence form (B), evidencing the structural changes that occur during the reductive activation. For simplicity, only the monomer is presented. Red: Pro66-Arg78 (loop containing the P haem distal axial ligand His71), Yellow: Ala91-Thr118 (containing Arg97 that interacts with P haem D propionate; this loop establishes interdomain interactions via the calcium ion in the oxidized form), Green: Tyr211-Tyr243 (establishes contacts between the two domains via the calcium ion in the mixed-valence form), Blue: Gly276-pro286. E and P haem, the axial ligands and Trp94 are represented as sticks and the calcium atom as a green sphere. Figure prepared using PDB ID: 1EB7 and 2VHD.

The stabilization of this loop at the dimer interface explains why dimerization was considered essential for activity. However, a recent study with a *S. oneidensis* BCCP mutant unable to form a dimer, proved that in the monomeric mixed-valence form this enzyme was active, but the lifetime of the activated form decreased compared to the wild-type (Ellis *et al.*, 2012). Therefore, dimerization could have a role in stabilizing the conformation of the active form, mainly of the loop carrying the P-haem histidine ligand, reason why all described BCCPs form homodimers in the mixed-valence form, even the ones that are membrane anchored (Nóbrega *et al.*, 2017).

Moreover, as mentioned calcium ion is indirectly coordinated by the E haem A propionate group, which has been proposed to modulate its p*Ka* so that it is never protonated. Thus, when E haem is reduced the only group that can undergo a redox-linked Bohr effect is the D propionate, an event that triggers the conformational changes described above and required for activation of BCCP.

In addition, the structural reorganization that occurs in P haem cavity during the activation not just leaves P haem with a vacant position for substrate binding, but it also reorients two residues that have been proposed to be important for catalysis, Gln104 and Glu114 (see Section 2.4.2).

2.4.2 Catalytic mechanism

BCCPs catalyse the reduction of hydrogen peroxide to water. To reduce one molecule of hydrogen peroxide at the P haem, it requires two consecutive electrons to be delivered to E haem by electron shuttle proteins (Equation 10), which also return the enzyme to the initial active form (Equation 11) releasing a total of two water molecules, as follows (iron atom in the equations represents P haem):

$$H_2O_2 + e^- + Fe^{3+} \rightarrow H_2O + Fe^{4+}{=}O \qquad (10)$$

$$Fe^{4+}{=}O + e^- + 2\,H^+ \rightarrow H_2O + Fe^{3+} \qquad (11)$$

In Figure 7 is presented a general model of the catalytic mechanism of BCCP, considering the initial non-active fully oxidized form, the reductive activation and the proposed intermediate species. To reduce H_2O_2 two electrons are needed, one electron from the ferrous E haem and one from P haem, which becomes Fe^{4+} releasing a water molecule (Figure 7B to 7C). The remaining oxygen from the H_2O_2 forms an intermediate Fe^{4+}-oxo species (oxoferryl, Figure 7C). This intermediate species (Compound I) was first observed using rapid mixing and freezing visible absorption, MCD and EPR spectroscopies in *P. aeruginosa* BCCP (Ellfolk *et al.*, 1983; Greenwood *et al.*, 1988; Rönnberg *et al.*, 1984; Rönnberg *et al.*, 1985). The spectra showed features of the equivalent oxoferryl species in eukaryotic monohemic peroxidases (horseradish and yeast peroxidase), but no evidences for the presence of a π-cation radical. The reduction of the oxoferryl species by one electron (donated by the small electron shuttle proteins) is coupled to proton transfer: the first forms a Fe^{3+}-OH species (Figure 7D) and the second releases the second water molecule (Figure 7E). This second intermediate species was also observed in the initial spectroscopic studies (Greenwood *et al.*, 1988; Rönnberg *et al.*, 1985), to be in an equivalent oxidation state as the inactive as-isolated protein, but P haem is still penta-coordinated, allowing a rapid turnover.

As long as there is sufficient reducing power, BCCP does not decay back to the as-isolated form since this is a slow process (Pettigrew *et al.*, 2006). This inactivation was proposed as a defence mechanism, not allowing hydrogen peroxide to bind when there are insufficient electrons to complete the catalytic cycle and reduce it to water, which also avoids radical damage to the active site (Pettigrew *et al.*, 2006).

The proton donors in the catalytic cycle, are proposed to involve two conserved residues in P haem catalytic cavity, Gln104 and Glu114 (other conserved residues are Phe93, Pro108 and Met 115, *P. aeruginosa* BCCP numbering). In fact, mutation of the conserved glutamine, glutamate and methionine residues in *R. capsulatus* BCCP results in an inactive enzyme (De Smet *et al.*, 2006), and in *S. oneidensis* BCCP a mutation of the phenylalanine residue also inactivates the enzyme (Frato *et al.*, 2016).

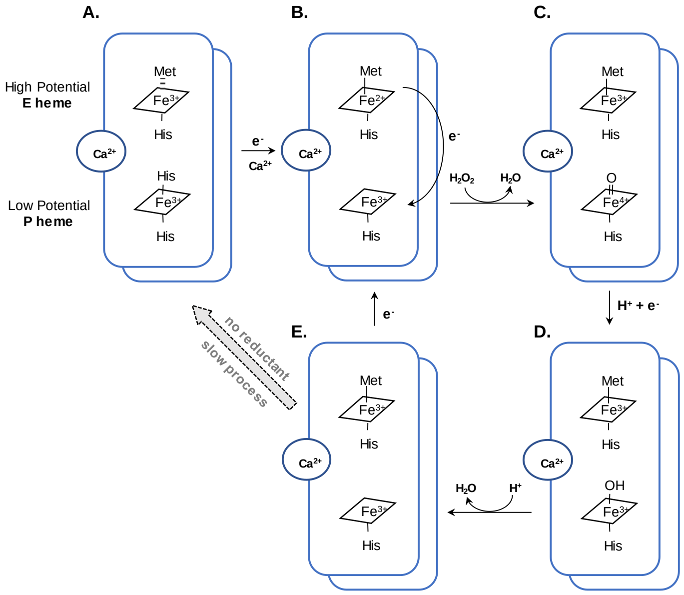


Figure 7 – Schematic representation of the catalytic cycle of classical BCCPs. (A) The as-isolated BCCP is fully oxidized and inactive. (B) The electrons are delivered by a small redox protein to E haem, which becomes reduced leading to conformational changes that remove the distal histidine ligand of P haem. (C) E haem transfers one electron to P haem, the active site, which reduces hydrogen peroxide, releasing a water molecule and one oxygen remains bound, forming a Fe^{4+}-oxo intermediate species (Compound I). (D) One electron from the electron donor and one proton forms the Fe^{3+}-OH (Compound II) and (E) addition of another proton releases a second water molecule. When there is enough reducing power, the cycle continues to (B), otherwise it slowly reverts to (A). The dashed methionine bond in the as-isolated form is representative of the E haem high/low-spin equilibrium at room temperature.

To further infer of the nature of the residues involved in these protonation steps, one can analyse the pH dependence of the catalytic activity, considering these to be part of the rate-limiting step. Usually, BCCP peroxidatic activity has a bell-shaped curve *versus* pH, with one p*Ka* value around pH 6 and another between 8-10 (Table 1), with the optimum pH being around 7.5. In *P. aeruginosa* BCCP, a lower p*Ka*, with a value of 4.4, was associated to an acid group involved in the formation of Compound I (Rönnberg *et al.*, 1984), but this seems to be a unique trade of this enzyme.

The lower p*Ka* can be attributed to the conserved glutamate in P haem cavity (Glu114 in *P. aeruginosa* BCCP). This residue needs to be deprotonated to help the heterolytic cleavage of the peroxide O-O bond that leads to the formation of the oxoferryl intermediate species (Shimizu *et al.*, 2001), similarly to what was proposed for chloroperoxidases. In other eukaryotic peroxidases, this role is played by a distal histidine ligand (Casadei *et al.*, 2014), but in the mixed-valence classical BCCPs, such a histidine residue is too far from the P haem cavity to play this a role.

The higher p*Ka* value, that was observed in the catalytic activity of classical BCCPs, could be associated with the proton transfer required for the release of the second water molecule in its catalytic cycle. The conserved glutamine residue in the active site (Gln104 in *P. aeruginosa* BCCP) might mediated this proton transfer, as no activity is observed when the equivalent residue is mutated (De Smet *et al.*, 2006). In fact, the side chain of this residue forms a hydrogen bond with the water-derived molecule coordinating P haem in the active form of the enzyme (Echalier *et al.*, 2006), and at higher pH values the proposed proton transfer might be hindered leading to a decrease in activity.

The catalytic mechanism of the classical BCCP does not foresee the formation of a cation radical, such as the one proposed to occur in eukaryotic peroxidases, involving a nearby tryptophan residue (yeast cytochrome *c* peroxidase) or porphyrin π-cation radical (horseradish peroxidase) (Erman and Vitello, 2002; Erman *et al.*, 1989). Instead, the second electron required for the formation of Compound I comes from the reduced E haem, that becomes oxidized. The presence of this reducing entity renders the presence of an oxidizing radical species to be very short-lived, with rapid formation of Compound I, which is formed with a rate constant closed to the diffusion limit (Ronnberg *et al.*, 1981a).

Nevertheless, the formation of this oxoferryl species might involve a transient protein radical species. Visible and EPR spectroscopic studies of *P. aeruginosa* BCCP H71G mutant, that lacked the P haem distal histidine ligand, showed the formation of a protein radical when the oxidized form of this mutant was mixed with excess of hydrogen peroxide (Hsiao *et al.*, 2007; Y. Lee *et al.*, 2007). A similar shift in the Soret band was observed in the visible spectra of horseradish peroxidase and interpreted to be the formation of a tryptophan radical species. The presence of this radical species was also confirmed by its EPR spectra (Morimoto *et al.*, 1998). Brittain and collaborators proposed a “charge hopping” mechanism for the intra-molecular electron transfer in classical BCCPs and that a possible candidate for this short-lived radical was the tryptophan residue between the two haem domains (Hsiao *et al.*, 2007; Y. Lee *et al.*, 2007) (see Section 2.4.3.1).

Although, for classical BCCPs that required reductive activation this radical species might be so short-lived that there are no experimental evidences for its formation in the wild-type enzyme, an intermediate radical species has been proposed in the catalytic cycle of *N. europaea* BCCP. This will be discussed in the following section.

2.4.2.1 *Nitrosomonas europaea* BCCP – a different catalytic mechanism?

N. europaea BCCP was the first bacterial peroxidase to be reported not to require reductive activation for maximum activity (Arciero and Hooper, 1994). This trait has only been found in one other classical BCCP isolated from *M. capsulatus* Bath (Zahn *et al.*, 1997). The crystallographic structure of *N. europaea* BCCP was the first available structure of an active form of classical BCCPs, in which P haem was penta-coordinated (Shimizu *et al.*, 2001). Although the protein was in the fully oxidized form, later it was clear that its structure was in fact identical to the mixed-valence structure of other classical BCCPs. In this structure all the typical features identified in the other classical BCCPs were present: the calcium binding site, E and P haems, a tryptophan between the two haems and other structural resemblances. This would suggest that the catalytic mechanism of *N. europaea* BCCP would be similar to the one of the classical BCCPs (Figure 7).

However, an apparently different intermediate Fe^{4+}-oxo species was detected when adding hydrogen peroxide to the oxidized *N. europaea* BCCP. Rapid changes in the Soret region of the visible spectra were consistent with the formation of a Fe^{4+}-oxo radical species (Fe^{4+}=O R$^{+\cdot}$) (Arciero and Hooper, 1994), which was proposed to be a porphyrin π-cation radical (Bradley *et al.*, 2004). Catalytic protein film voltammetry estimated a high value for the catalytic potential, E_{cat} of + 540 mV, a unique feature of *N. europaea* BCCP. This catalytic potential was assigned to an active species at the P haem that is pH dependent and represents a one electron proton-coupled process, such as the one proposed in Figure 7 between (C) and (D) (redox couple Fe^{4+}=O/Fe^{3+}-OH) (Elliott *et al.*, 2007). The major difference in *N. europaea* BCCP, according to Elliot *et al.*, is that the E haem does not store the electron for the catalytic reaction in the P haem, as hydrogen peroxide binds to the fully oxidized form of the enzyme, forming a Fe^{4+}=O R^{+}, not requiring a reduced E haem (Bradley *et al.*, 2004; Elliott *et al.*, 2007). The formation of a similar intermediate species has also been proposed by protein film voltammetry in *S. oneidensis* BCCP, as it was also observed under turnover condition the formation of a catalytic wave centred at approximately at + 450 mV, which shifts to more positive potentials in the presence of H_2O_2, and has a similar pH dependence as *N. europaea* BCCP (Frato *et al.*, 2016). An important mention is that in the case of *S. oneidensis* BCCP the enzyme was adsorbed in the mixed-valence state contrary to *N. europaea* BCCP, which was examined in the fully oxidized form. However, this result might just be due to the special environment of P haem cavity in *S. oneidensis* BCCP, as similar experiments using other classical BCCP did not produce similar observations (Paes de Sousa *et al.*, 2011a). Nevertheless, these data corroborate the previous hypothesis of Brittain and collaborators that there might be formation of a protein radical in a residue close to P haem, but short life-time of this intermediate species has preclude it to be properly characterized.

It is also important to point out that although *N. europaea* BCCP does not need reductive activation *in vitro*, it does not seem plausible to consider that *in vivo* E haem would not be reduced prior to H_2O_2 binding or that *in vivo* the electrons would be delivered directly to P haem. Therefore, there are no clear evidences that the catalytic mechanism of *N. europaea* BCCP is different from the one presented for the classical BCCPs (Figure 7).

To sum up, so far, there are no structural evidences for any of the intermediate species in the catalytic cycle of classical BCCPs, which would be useful to define the catalytic cycle in detail and to infer the plausible electron and proton transfer pathways. Such data could be obtained using neutron cryo-crystallography, which for the yeast cytochrome *c* peroxidase showed the formation of the oxoferryl species and the protonation state of the distal histidine and of other important residues in the haem *b* cavity (Casadei *et al.*, 2014).

2.4.3 Intra and intermolecular electron transfer pathways

2.4.3.1 Intramolecular electron transfer pathway

For the proposed catalytic cycle to occur two electrons need to be delivered consecutively from the E haem to the P haem, for the reduction of one H_2O_2 molecule. However, E and P haems are distant from each other (with Fe-Fe distance of 21 Å), and thus without an electron tunnelling or a charge hopping mechanism this transfer could not occur (Gray and Winkler, 2005). The electron tunnelling refers to the electron transfer between two redox centres with the protein matrix facilitating this process but not forming any chemical intermediate, while in the charge hopping mechanism the electron is transferred in a series of steps (hops) and the protein matrix intervenes in the process through the formation of transient radical centres (Gray and Winkler, 2015). Although the first mechanism can occur over long distance in very short time scales, the second enables electron transfer through even longer distances (Gray and Winkler, 2015). This later mechanism has been proposed by Gray & Winkler to occur through Tyr/Trp side chain and might protect enzymes form oxidative damage (Gray and Winkler, 2015).

In the case of BCCPs, which has not yet been analysed by these authors, such a charge hopping mechanism might be in place, as there is a conserved tryptophan reside in between E and P haems. The indole ring of this tryptophan (Trp94 in *P. aeruginosa* BCCP) is positioned between the propionate groups of two haem groups (a shorter distance of ≈10 Å) and can act as a bridge between the two redox centres (Fülöp *et al.*, 1995).

Point mutations in this conserved tryptophan in *P. aeruginosa* and *R. capsulatus* BCCPs abolished their catalytic activity (De Smet *et al.*, 2006; Y. Lee *et al.*, 2007), corroborating its role in electron transfer. Moreover, the charge hopping mechanism is supported by the formation of a protein radical intermediate species as the ones observed in *P. aeruginosa* BCCP mutants (Hsiao *et al.*, 2007; Y. Lee *et al.*, 2007).

Another evidence for the involvement of Trp94 in the intramolecular electron transfer between E and P haems came from the analysis of the X-ray structure of *M. hydrocarbonoclasticus* BCCP at a non-physiological pH (pH 4.0), in which the calcium binding site was empty (Dias *et al.*, 2004). The crystallized enzyme was reduced after exposure to the synchrotron radiation, but both haems remained hexa-coordinated and the position of the conserved Trp94 was moved from its usual position. On the other hand, when the enzyme was crystallized at pH 5.3, its calcium site was filled, and Trp94 occupied its usual position (Dias *et al.*, 2004). Although, the analysis of these structures showed the importance of the Trp94 and calcium site for BCCP electron transfer process, the conformational changes observed between the structures at the two pH values are not relevant as they were both acquired in non-physiological conditions.

2.4.3.2 Intermolecular electron transfer pathway

When considering the intermolecular electron transfer process between two proteins there are three steps that need to occur (Pettigrew *et al.*, 2006):

(i) formation of a competent donor-acceptor complex,

(ii) electron tunnelling between the two proteins, and

(ii) dissociation of the donor-acceptor pair.

Little data is usually available for the electron tunnelling steps, as this is a very fast step and the whole process will be dominated by the dynamics of the first and second steps, to search for a productive position for the electron transfer to occur and then dissociation of the complex. Moreover, there are very few data on the exact structural geometries of these steps (i and iii), due to the transient nature of these complexes. Nevertheless, structural information on the nature of the protein-protein interactions and protein complex interface can be obtained by NMR titrations, microalorimetry and kinetic assays, and a structural model of the complex using molecular docking simulations (Almeida *et al.*, 2016). These experiments have a down-side since they are usually performed with both proteins in the same oxidation state, for the results not be masked by the electron transfer process.

The formation of these electron transfer complexes are usually entropy driven by excluding water molecules from the interface (Cameron *et al.*, 2010; Pauleta *et al.*, 2004a; Pettigrew *et al.*, 2006), though some water molecules are proposed to remain and mediate intramolecular electron transfer. Moreover, a small interface, weak and fast interactions, are essential to achieve the high catalytic turnovers.

In the case of classical BCCPs little is known about the surface residues involved in the electron transfer, due to the absence of structural and mutagenesis data. The small redox proteins that donate electrons to the BCCPs are either monohaem *c*-type cytochromes or type-1 copper proteins, such as azurin and pseudoazurin (Nóbrega and Pauleta, 2018; Pauleta *et al.*, 2004b; Pettigrew *et al.*, 1999; Ronnberg *et al.*, 1981b). In some cases, mitochondrial cytochrome *c* and small compounds have also been used as an artificial electron donors in binding assays (Pauleta *et al.*, 2004b; Pettigrew *et al.*, 2003a), as well as in kinetic assays (Ellis *et al.*, 2011; Hoffmann *et al.*, 2009; Nóbrega *et al.*, 2017; Seidel *et al.*, 2012).

The steady-state kinetics of classical BCCPs showed that the maximum catalytic activity of the mixed-valence form can be achieved with concentrations of hydrogen peroxide in the micromolar range, with affinity constants, K_M, lower than 40 µM H_2O_2 (Table 1) and high turnover numbers, as the value estimated for *R. capsulatus* BCCP/cytochrome c_2 redox pair, 1060 s^{-1} (De Smet *et al.*, 2001) (Table 1). The lowest K_M value reported up-to-date is for the tag-free *S. oneidensis* BCCP/cytochrome c_5 physiological redox pair, in unsaturated conditions, with a K_M of 0.3 µM H_2O_2 (Pulcu *et al.*, 2012). Heteronuclear NMR and docking studies with *P. pantotrophus* BCCP and cytochrome c_{550} or pseudoazurin as electron donors showed that these two small redox proteins interact at the same site, near the solvent exposed E haem edge of *P. pantotrophus* BCCP, which has a hydrophobic patch surrounded by negatively charged residues (Pauleta *et al.*, 2004a; Pettigrew *et al.*, 1999). A similar binding site can be proposed for other BCCPs, though no experimental is available.

These electron transfer complexes can be electrostatic as described for *P. pantotrophus* (Pauleta *et al.*, 2004a) and *R. capsulatus* cytochrome c_2 (Koh *et al.*, 2003). The cytochrome c_2 has an interaction surface with charged lysines that when is lost, disrupts the interaction with BCCP (Koh *et al.*, 2003). A ring of lysines residues surrounding a hydrophobic patch containing the ET site (histidine residue that coordinates the type-1 copper centre) has also been described in *P. pantotrophus* pseudoazurin. On the other hand, the ET complex can have a hydrophobic character, as for *M. hydrocarbonoclasticus* BCCP/cytochrome c_{552} (Alves *et al.*, 1999), and *N. gonorrhoeae* BCCP/Laz (Lipid-modified azurin) (Nóbrega and Pauleta, 2018), for which the activity remains unchanged throughout a wide range of ionic strengths.

The stoichiometry and dissociation constant have only been determined for the complexes involving *P. pantotrophus* BCCP and *G. sulfurreducens* MacA. These enzymes form with their physiological electron donors a complex with a 1:1 stoichiometry (Dantas *et al.*, 2017; Pauleta *et al.*, 2004a; Pettigrew *et al.*, 2003b), while the complex formed between *P. pantotrophus* BCCP and the mitochondrial cytochrome *c* has a stoichiometry of 1:2 (Pauleta *et al.*, 2004a; Pettigrew *et al.*, 2003b). The *P. pantotrophus* BCCP complexes have also been characterized using microcalorimetry and analytical ultracentrifugation, showing that its dissociation constant are in the micromolar range (10-

20 µM) (Pauleta *et al.*, 2004a; Pauleta *et al.*, 2004b; Pettigrew *et al.*, 2003a), which explains the fast dissociation and thus the high turnover numbers that this enzyme can attain.

2.5 Non-classical three-haem bacterial peroxidases

The gene coding for trihaem BCCPs is found in known pathogenic bacteria, but only a few of these enzymes have been isolated and biochemically characterized to date. Reports for homologues from *A. actinomycetemcomitans* (Abe *et al.*, 2017; Takashima and Konishi, 2008; Takashima *et al.*, 2010; Yamada *et al.*, 2007), *Z. mobilis* (Balodite *et al.*, 2014), and *E. coli* (Khademian and Imlay, 2017; Nobrega *et al.*, 2018), are the only found in the literature.

These enzymes have been produced heterologously, mainly without its transmembrane region (Figure 3D) as soluble proteins (Nóbrega and Pauleta, 2018; Takashima *et al.*, 2010). The isolated *E. coli* BCCP (YhjA) has three *c*-type haems covalently bound to the polypeptide chain, behaving as a monomer in solution. Contrary to classical BCCPs this enzyme does not dimerize in the presence of calcium ions nor upon reduction of its haems by sodium ascorbate (Nóbrega and Pauleta, 2018), which was demonstrated by size-exclusion chromatography and differential scanning calorimetry (DSC). The thermogram of *E. coli* BCCP comprises two transitions, which was interpreted as the unfolding of two independent domains: N-terminal domain and C-terminal domain homologous to classical BCCP. The redox potential of these haems was estimated at pH 7.5 to be 140±10 mV, 220±10 mV and -190±10 mV, for the N-terminal, E and P haems, respectively (Nóbrega and Pauleta, 2018).

E. coli BCCP was spectroscopically characterized in the oxidized formby visible and EPR spectroscopies, showing features of two low-spin and one high-spin haems. The N-terminal domain is Met/His hexacoordinated, with the distal methionine being proposed to be the conserved Met125. The homologous E is also Met/His hexacoordinated and is in a high-low spin thermal equilibrium in the fully oxidized form, as in the classical BCCPs. The homologous P haem is high-spin OH^-/His coordinated in the oxidized form. The spin state of this haem is temperature dependent and modulated by the presence of calcium ions as observed in the mixed-valence classical BCCPs (Nóbrega and Pauleta, 2018). P haem is available to bind the substrate in the oxidized form forming an oxo-ferryl radical species that was observed by visible spectroscopy (Nóbrega and Pauleta, 2018), that might involve formation of a protein radical species at the Trp250 (the homologous tryptophan that is found between the two haem domains in classical BCCPs), though spectroscopic evidences for the formation of a radical are lacking.

The catalytic properties of *E. coli* BCCP was studied using different electron donors, with the enzyme being a quinol peroxidase. This enzyme, like *A. actinomycetemcomitans* BCCP (QPO), does not require reductive activation, but contrary to QPO needs calcium ions for maximum activity (Nóbrega and Pauleta, 2018; Yamada *et al.*, 2007). *E. coli* BCCP is a poor peroxidase with a K_M of 0.6 mM for H_2O_2 and a p*Ka* of 8.4 when hydroquinone was used as electron donor (Nóbrega and Pauleta, 2018). This *in vitro* K_M value is much higher than the one estimated *in vivo* for this enzyme, 5 µM, a discrepancy that can be simply because the enzyme in the *in vitro* studies lacks the regions of quinol binding and because the native electron donor was not the one used in the assays. In fact, in *E. coli*, BCCP is proposed to receive electron from menaquinol (Khademian and Imlay, 2017).

In the case of *Z. mobilis* (PerC) and *A. actinomycetemcomitans* (QPO) trihemic BCCPs, a quinol peroxidase activity has also been observed. Furthermore, in *A. actinomycetemcomitans* a null QPO mutant showed higher sensitivity towards H_2O_2 and a lower growth rate under aerobic conditions (Takashima and Konishi, 2008). In the case of *Z. mobilis*, it was initially suggested that PerC was involved in an incomplete aerobic respiratory pathway, as it lacked a terminal oxidase, but later its role as a quinol peroxidase was recognized (Balodite *et al.*, 2014).

The isolated *A. actinomycetemcomitans* BCCP was spectroscopically characterized and the reduction potentials of its three haems were shown to be more positive, + 67 (P), + 156 (NT) and + 290 mV (E), at pH 7.5 (Takashima *et al.*, 2010), than the ones determined for *E. coli* BCCP. The major difference lies in the reduction potential of P haem, which has a positive value, a feature that has never been observed for a P haem in bacterial peroxidases (Pettigrew *et al.*, 2006). The catalytic mechanism of this enzyme is characterized by a Ping Pong Bi Bi system using ubiquinol-1 as electron donor, with a K_M value of 107±8 µM and it was observed that the enzyme was damaged at high concentrations of H_2O_2 (Abe *et al.*, 2017). The pH dependence has a bell-shaped profile centred at pH 7.5 but no p*Ka* were estimated (Yamada *et al.*, 2007).

To gain further insights on non-classical trihaem BCCPs its three-dimensional structure should be determined and site-directed mutagenesis could be employed to elucidate the role of the extra N-terminal haem in the electron transfer pathway from the quinol pool (Figure 1). The high sequence homology between YhjA from *E. coli* and homologous enzymes from *Salmonella typhimurium* and *Yersinia pestis* pin points the importance in understanding the role of this group of bacterial peroxidases, as well as its catalytic mechanism, as they can play important roles as virulence trait or be therapeutic targets. In fact, it was suggested that FNR-dependent regulation allows intestinal pathogens, from the *Salmonella* and *Shigella* genus (that also encode a *yhjA* homologous gene in their genome), to infect the epithelial cell layer as the mucosa can present a slightly higher oxygen concentrations as explained in Section 2.3, inducing key virulence factors (Marteyn *et al.*, 2010). In an infection setting, YhjA, from a pathogenic *E. coli* strain, can also play a key role in preparation for cell invasion, providing the ability to respire exogenous H_2O_2 present in these environments.

3. Final Remarks

Bacterial peroxidases reduced hydrogen peroxide to water in a reaction that requires two electrons other donated by small redox proteins or by the quinol pool. These enzymes can be divided into classical and non-classical BCCPs depending on the presence of an additional N-terminal haem.

Most of the spectroscopic, kinetic, mechanistic and structural studies have been focused on the classical BCCP, which led to the identification of the reductive activation mechanism required for activity and the role played by calcium ions, which presence in the structure is not just merely structural. Future work would focus on defining the intermolecular electron transfer pathway using site directed mutagenesis and on understanding the origin of the two protons required for catalysis (either from a residue of a nearby solvent molecule).

In the case of the non-classical BCCPs the field would benefit by having its crystal structure which would help understand the role of the additional haem domain in modulating activity and electron transfer from the quinol pool, as well as the quinol binding site. Moreover, enzymatic assays under more physiological conditions (using a menaquinol analogue) and/or with the enzyme embedded in the membrane will clarify the different affinity for H_2O_2 reported for the BCCP between *in vitro* and *in vivo* assays.

The physiological function of bacterial peroxidase is now clearer, and the proposed involvement in ROS detoxification is abandoned. In fact, considering that H_2O_2 equilibration across the outer membrane is fast, the enzyme would not be able to lower its periplasmic concentrations below the environmental level that the bacteria would be experiencing. The fact that BCCP confers the ability to respire H_2O_2 in anoxic environments resolves the paradox of the gene encoding this enzyme being under the regulation of FNR but the enzyme would be dealing with an oxidative stress. Moreover, this might be a very ancient trait that was not lost during evolution, when the atmosphere was still anoxic but reactive oxygen species would be abundant due to chemical reactions and photolysis (Imlay, 2018).

Another open question is the role played by bacterial peroxidase in virulence as homologues of *ccp* gene are present in many pathogenic bacteria.

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Author contributions

SRP has planned and wrote the manuscript with contributions from CSN.

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Table 1 – Summary of the biochemical properties of classic BCCPs studied up-to-date.

Species name	**Electron donor ([ED])** [a]	**K_M H_2O_2 (µM)**	**K_M ED** [b] **(µM)**	**k_{cat} (s^{-1})**	**p*Ka*** [c]	**Reduction potential (mV *vs.* SHE)**		**Ref.**
						E heme	P heme	
Geobacter sulfurreducens CcpA	ABTS^{2-} (3 mM) [d]	6.2	-	15.5	6.8; 9.1	-	-	(Ellis *et al.*, 2011; Hoffmann *et al.*, 2009)
Geobacter sulfurreducens MacA	ABTS^{2-} (3 mM) [d]	38.5	-	0.46	-	-	-	(Seidel *et al.*, 2012)
Marinobacter hydrocarbonoclasticus	cytochrome c_{552} (38 µM)	-	122	460	-	-	-	(Alves *et al.*, 1999)
Methylococcus capsulatus	cytochrome c_{555} (9 µM)	0.5	-	7.1	-	+ 432	- 254	(Zahn *et al.*, 1997)
Neisseria gonohrroeae	ABTS^{2-} (3 mM) [d]	4	-	79	5.9; 8.4	+ 310	-190/-300	(Nóbrega and Pauleta, 2018; Nóbrega *et al.*, 2017)
	lipid modified azurin (10 µM)	0.4	-	39	5.1; 8.5			
Nitrosomonas europaea	horse heart cytochrome *c* [d]	-	-	3	6.5; 8.4	+ 450	- 260	(Arciero and Hooper, 1994; Bradley *et al.*, 2004; Elliott *et al.*, 2007)
Paracoccus pantotrophus	cytochrome c_{550} (23 µM)	-	13	1417	7.0	+ 226	- 200	(Gilmour *et al.*, 1993; Gilmour *et al.*, 1994; Paes de Sousa *et al.*, 2007; Pauleta *et al.*, 2004b)
	pseudoazurin (20 µM)	-	> 50	-				
Pseudomonas aeruginosa	cytochrome c_{551} (13 µM)	6	88	143	4.4; 7.0; 10.0	+ 320	-330	(Becker *et al.*, 2009; Ellfolk *et al.*, 1983; Foote *et al.*, 1992; Rönnberg and Ellfolk, 1975; Rönnberg *et al.*, 1984; Soininen and Ellfolk, 1972)
	azurin (40 µM)	11	120	196				
Pseudomonas stutzeri	cytochrome c_{551} (7 µM)	1.8	> 50	88.3	-	-	-	(Timóteo *et al.*, 2003)
Rhodobacter capsulatus	cytochrome c_2 (6 µM)	33	60	1060	-	+ 270	- 190/-310	(De Smet *et al.*, 2001; Hu *et al.*, 1998)
Shewanella oneidensis	cytochrome c_5 (4 µM)	0.3	-	73	6.4; 8.8 [e]	-	-	(Bolton, 2002; Frato *et al.*, 2016; Pulcu *et al.*, 2012; Stohs and Bagchi, 1995)

[a] Concentrations in parenthesis are the ones used to calculate the kinetic parameters presented (K_M and k_{cat}) nad p*Ka*.

[b] K_M values for the electron donor are an estimative, as the concentrations used are below this value, except for cytochrome c_{550} from *P.pantotrophus*.

[c] p*Ka* values determined by steady-state kinetics or protein-film voltammetry.

[d] In the absence of a physiological electron donor, the synthetic electron donor ABTS^{2-} or the reduced horse heart cytochrome *c* were used.

[e] These values were determined for a H81G BCCP mutant.