UNIVERSIDADE DE LISBOA
FACULDADE DE MEDICINA VETERINÁRIA

PROTEIN DISULFIDE ISOMERASE AS A TARGET FOR BESNOITIOSIS THERAPY. MOLECULAR CHARACTERIZATION AND STUDIES OF ITS ROLE IN INFECTION AND HOST IMMUNE RESPONSE.

EDUARDO MIGUEL BAPTISTA FERREIRA MARCELINO

Orientador(es): Doutor José Alexandre da Costa Perdigão e Cameira Leitão

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Tese especialmente elaborada para obtenção do grau de Doutor em Ciências Veterinárias na Especialidade de na especialidade de Sanidade Animal.

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Abstract

Besnoitia besnoiti is an apicomplexan parasite responsible for bovine besnoitiosis, a disease with a high prevalence in tropical and subtropical regions and re-emerging in Europe. Despite the great economical losses associated with besnoitiosis, this disease has been underestimated and poorly studied, and neither an effective therapy nor a vaccine to be used in Europe is available. Protein disulfide isomerase (PDI) is an essential enzyme for the acquisition of the correct three-dimensional structure of proteins. Current evidence suggests that in *Neospora caninum* and *Toxoplasma gondii*, which are closely related to *B. besnoiti*, PDI plays an important role in host cell invasion, is a relevant target for the host immune response, and represents a promising drug target and/or vaccine candidate. In this work, we presented the nucleotide sequence of the *B. besnoiti* PDI gene and a 3D theoretical model was built by comparative homology using Swiss-Model server. *B. besnoiti* expresses a PDI with 471 amino acids, structurally similar to human and yeast PDIs, with four thioredoxin-like domains **a**, **b**, **b'**, **a'** and a C-terminal extension **c**. The **a** and **a'** domains present the characteristic active site pattern CxxC, in this case CGHC and CGYC, respectively. Analysis of the phylogenetic tree for PDI within the phylum Apicomplexa reinforced the close relationship among *B. besnoiti*, *N. caninum* and *T. gondii*. Recombinant *B. besnoiti* PDI (recBbPDI) and truncated versions corresponding to domains **a**, **b**, **b'** and **a'c** were expressed in a heterologous system. Mice were immunized with recBbPDI for the production of monoclonal antibodies (mAbs) by hybridoma technology and four mAbs were produced and characterized. RecBbPDI and domain **a'c** (recBb-a'c) were functionally active and exhibited a dose dependent cross-linking activity of insulin. In the presence of bacitracin, tocinoic acid, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 4-chloromercuribenzoic acid (pCMBA) activity of both enzymes was inhibited, in a dose dependent manner. The same happened with recBbPDI in the presence of mAbs (with the exception of T8a), but not with recBb-a'c, whose activity was not sensitive to the presence of mAbs. *In vitro* proliferation of *B. besnoiti* tachyzoites was diminished in the presence of PDI inhibitors and anti-PDI mAbs, indicating that this enzyme seems to intervene in the process of host cell adhesion/invasion. In this way, considering the inhibitions obtained, both in the host cell invasion ability and in the enzyme catalytic activity, PDI can represent a potential target for addressing the treatment and/or prevention of besnoitiosis. The panel of monoclonal antibodies here developed represents an important tool for future studies.

Keywords: *Besnoitia besnoiti*, protein disulfide isomerase, PDI, monoclonal antibody, host cell invasion.

Título: A enzima isomerase de dissulfureto como alvo terapêutico contra a besnoitiose bovina. Caracterização molecular e estudo do seu papel na infeção e na resposta imunitária.

Resumo

Besnoitia besnoiti, o agente etiológico da besnoitiose bovina, pertence ao filo Apicomplexa, família Sarcocystidae, subfamília Toxoplasmatinae, estando filogeneticamente próximo dos géneros *Neospora* e *Toxoplasma.* A besnoitiose bovina é uma doença severa mas geralmente não fatal, endémica em vastas áreas tropicais e subtropicais de África e responsável por elevadas perdas económicas. Na Europa, após as primeiras descrições há mais de um século, em França e Portugal, a doença recebeu pouca atenção até finais do século XX, altura em que a sua incidência começou a aumentar com relatos em Portugal, Espanha, Itália e França. Mais recentemente o avanço geográfico da doença é relatado com casos na Grécia, Suíça, Alemanha, Hungria, Croácia e Irlanda. A fase aguda da doença é marcada por alterações respiratórias, febre, anasarca, diarreia e, por vezes, aborto. Na fase crónica, o animal apresenta grande espessamento da pele (elefantíase) com soluções de continuidade, muitas vezes complicadas por infecções oportunistas, má condição corporal e, nos machos, orquite necrosante e aspermia. Apesar de ser conhecida há mais de um século, assim como a sua etiologia parasitária, a besnoitiose bovina mantém muitos aspectos da sua epidemiologia desconhecidos, incluindo o ciclo de vida do seu agente etiológico. Outras espécies do género *Besnoitia* têm um ciclo heteroxeno mantido por uma relação presa-predador entre pequenos mamíferos e o gato. Acredita-se que o ciclo de vida de *B. besnoiti* seja semelhante, mas até à data ainda não foi identificado um hospedeiro definitivo. A transmissão homoxena de bradizoítos e taquizoítos de *B. besnoiti* foi comprovada experimentalmente, em bovinos e em diversos animais de laboratório, e uma maior incidência da doença nos meses mais quentes do ano, quando existe maior actividade de insetos hematófagos, sugere um papel destes na transmissão de *B besnoiti*. No entanto, o verdadeiro modo de transmissão deste parasita na natureza mantém-se desconhecido. A doença pode atingir uma elevada taxa de morbilidade, o que, associado à gravidade das manifestações clínicas e à inexistência de vacina segura ou tratamento eficaz, justifica a necessidade de aprofundar o conhecimento sobre a biologia deste parasita, tentando desenvolver novas abordagens à terapêutica e à formulação de vacinas.

A enzima isomerase de dissulfureto (PDI, do inglês protein disulfide isomerase), uma das mais abundantes do retículo endoplasmático, catalisa a formação de pontes dissulfureto e o rearranjo de emparelhamentos dissulfureto incorrectos, permitindo que as proteínas adquiram a sua correta conformação tridimensional. As suas funções (ou disfunções) têm

sido implicadas em doenças neurodegenerativas como Alzheimer ou Parkinson ou na capacidade proliferativa de células neoplásicas. Sabe-se hoje que, para além da sua localização maioritariamente reticular, a PDI está presente à superfície das células, onde participa em diversas funções biológicas, como a ativação e agregação plaquetária, a adesão leucocitária ou a internalização de agentes patogénicos como alguns vírus e clamídias. Em diversos parasitas do filo Apicomplexa, incluindo *Neospora caninum* e *Toxoplasma gondii*, foram já identificadas PDIs na superfície dos taquizoítos e foi demonstrado que a inibição desta proteína, através de drogas ou anticorpos específicos, tem um efeito negativo sobre a capacidade de proliferação dos parasitas, evidenciando o papel que esta parece desempenhar no processo de adesão/invasão da célula hospedeira. Por outro lado, a PDI parece ter um papel relevante na resposta imunitária do hospedeiro, uma vez que foi identificada como uma proteína imunodominante e que foi demonstrada a presença de anticorpos anti-*T. gondii*-PDI e anti-*N. caninum*-PDI no repertório inato do homem e da vaca, respetivamente. Em conjunto, estas evidências sublinham a importância da PDI no processo de invasão celular e na resposta imunitária do hospedeiro.

Neste trabalho determinámos a sequência completa do gene da PDI em *B. besnoiti*, clonámos o respectivo cDNA e expressámos a proteína recombinante, assim como versões truncadas da mesma, num sistema heterólogo. A PDI de *B. besnoiti* (BbPDI) pertence à superfamília das tiorredoxinas (cluster 00388), estando incluída na família PDI_a (cluster defined cd02961) e subfamília PDI_a_PDI_a'_c (cd02995). Construímos um modelo tridimensional da BbPDI por homologia de comparação usando o software disponível no servidor Swiss-Model e tendo como modelo a estrutura cristalográfica da Tapasina-ERp57 (código do PDB: 3F8U chain C). Verificámos que *B. besnoiti* expressa uma PDI composta por 471 aminoácidos, com a organização típica descrita para a PDI humana e de levedura, ou seja os 4 domínios estruturalmente semelhantes à tiorredoxina, domínio **a**, **b**, **b'**, **a'**, com uma extensão C-terminal **c.** Os domínios activos **a** e **a'** apresentam os característicos centros ativos CxxC, no caso de *B. besnoiti* com a sequência aminoacídica CGHC e CGYC, respectivamente. A análise filogenética para a PDI colocou a *B. besnoiti* no mesmo ramo que a *N. caninum* e *T. gondii*. Com a PDI recombinante de *B. besnoiti* (recBbPDI), imunizámos murganhos com o objetivo final de produzir anticorpos monoclonais (mAbs) anti-PDI, pela tecnologia de hibridomas. Obtivemos 4 mAbs, que foram caracterizados no reconhecimento das versões truncadas de PDI, correspondentes aos domínios **a**, **b**, **b'** e **a'c** e na capacidade de reconhecerem cruzadamente a PDI de *N. caninum* e *T. gondii.* Todos os anticorpos produzidos, T8a, S4a, R60b e S16p, reconhecem a proteína recombinante e a natural de *B. besnoiti*, tanto em condições desnaturantes como em condições nativas. Dois mAbs (T8a e S4a) reconhecem o domínio **a'c**, um anticorpo reconhece o domínio **b'** (R60b) e o outro (S16p) não reconhece nenhuma versão truncada da proteína. O anticorpo T8a é

específico para a PDI de *B. besnoiti,* enquanto os restantes reagem cruzadamente com a PDI de *N. caninum* e *T. gondii*, quando testados por western blot e ELISA. Foi avaliada a actividade cinética da proteína recombinante pelo método da agregação da insulina. Este consiste na avaliação turbidimétrica da precipitação da insulina como resultado da formação de pontes dissulfureto, ação que é catalisada pela PDI. Foram avaliadas as versões truncadas dos domínios ativos (**a** e **a'c**) e a proteína integral, tendo-se verificado ausência de atividade para a versão truncada correspondente ao domínio **a**. Contudo, a versão truncada **a'c** (recBb-a'c) e recBbPDI mostraram-se funcionalmente ativas. Na presença de bacitracina, ácido tocinóico, ácido ditionitrobenzoico (DTNB) e ácido p-cloromercurobenzóico (pCMBA) a atividade destas enzimas foi, de uma forma geral, inibida de um modo dose-dependente. O mesmo aconteceu com a atividade catalítica da recBbPDI quando testada na presença dos mAbs produzidos (excepto mAb T8a), mas não na actividade de recBb-a'c, que não foi inibida por nenhum dos mAbs. Verificou-se a presença de PDI no produto de secreção/excreção de taquizoítos e, para avaliar o envolvimento desta no processo de invasão da célula hospedeira, foram colocados taquizoítos de *B. besnoiti* sobre um tapete confluente de células Vero na presença das drogas e dos mAbs acima mencionados. Após uma hora, removeram-se os inibidores e os taquizoítos em suspensão e as culturas foram incubadas por mais onze horas para permitir a fácil visualização dos vacúolos parasitóforos, por imunofluorescência indireta. A presença de inibidores da PDI e de mAbs anti-PDI permitiram diminuir a taxa de invasão de *B. besnoiti*, demonstrando a acção desta enzima no processo de adesão/invasão da célula hospedeira. Tendo em conta os resultados de inibição obtidos, quer na capacidade de invasão de célula hospedeira, quer na atividade cinética da enzima, a PDI pode de facto representar um alvo para o desenvolvimento de estratégias terapêuticas e/ou profilácticas contra a besnoitiose bovina. O painel de anticorpos monoclonais aqui desenvolvidos representa uma ferramenta importante para estudos futuros.

Palavras chave: *Besnoitia besnoiti*, enzima isomerase de dissulfureto, PDI, anticorpos monoclonais, invasão célula hospedeira.

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Abbreviations

ABS	Absorvance
BbPDI	*Besnoitia besnoiti* protein disulfide isomerase
BCR	B-cell receptor
BPTI	Bovine pancreatic trypsin inhibitor
cDNA	Complementary DNA
DAPI	4',6-diamidino-2-phenylindole
DMEM	Dulbeco's Minimal Essential Medium
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
DsbA	Disulfide bond formation protein A (thiol-disulfide oxidoreductase enzyme)
DTNB	5,5′-dithiobis(2-nitrobenzoic acid)
DTT	1,4-Dithiothreitol
EFSA	European Food Safety Authority
ELISA	Enzyme-linked immunosorbent assay
EM	Electron microscopy
ER	Endoplasmic Reticulum
FBS	Fetal bovine serum
FITC	Fluorescein isothiocyanate
GR	Glutathione reductase
GSH	Reduced glutathione
GSSG	Oxidized glutathione
GST	Glutathione S-transferase
HAT	Hypoxanthine, Aminopterin, Thymidine
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HGPRT	Hypoxanthine guanine phosphoribosyl transferase
hPDI	Human protein disulfide isomerase
ICW	Inner cyst wall
IFAT	Immunofluorescence antibody test
Ig	Immunoglobulin(s)
IgA	Immunoglobulin, isotype A
IgG	Immunoglobulin, isotype G
IgM	Immunoglobulin, isotype M
IMC	Inner membrane complex
ITS1	Internal transcribed spacer 1

mAb	Monoclonal antibody
NADPH	Nicotinamide adenine dinucleotide phosphate
NTZ	Nitazoxanide
OCW	Outer cyst wall
ORF	Open reading frame
pCMBA	4-chloromercuribenzoic acid, p-chloromercuribenzoic acid
pCMBS	p-chloromercuribenzenesulfonic acid
PCR	Polymerase chain reaction
PDB	Protein data base
PDI	Protein disulfide isomerase
PEG	Polyethylene glycol
PMDB	Protein model database
PMDs	Protein misfolding disorders
p-NPP	p- Nitrophenylphosphate
PV	Parasitophorous vacuole
PVDF	Polyvinylidene difluoride
rDNA	Ribossomal DNA
recBbPDI	Recombinant *B. besnoiti* protein disulfide isomerase
RMS	Root mean square
RNA	Ribonucleic acid
RNase	Ribonuclease
RT-PCR	Reverse transcription polymerase chain reaction
SDS-PAGE	Sodium dodecyl sulphate-polyacrylamide gel electrophoresis
SSU	Small (ribosomal) subunit
TEM	Transmission electron microscopy
TRITC	Tetramethylrhodamine
TRX	Thioredoxin
UV	Ultra violet
WB	Western blot
yPDI	Yeast protein disulfide isomerase

Chapter 1 |
Introduction

1.1 Bovine besnoitiosis

Besnoitiosis is a protozoal disease of cattle caused by the cyst-forming apicomplexan parasite *Besnoitia besnoiti*. It is a severe but usually non-fatal disease that leads to important economic losses due to significant reduction in productivity (Agosti, Belloli, Morini & Vacirca, 1994; Basso et al., 2013; Bigalke, 1968; Cortes et al., 2003; European Food Safety Authority [EFSA], 2010; Juste, Cuervo, Marco & Oregui, 1990; Olias et al., 2011; Pols, 1960). The first mention related to bovine besnoitiosis was published in 1884, when Cadéac described a skin disease in cattle from southern France, that he then, having no knowledge of the etiology, named bovine elephantiasis and anasarca («l'elephantiasis et de l'anasarque du boeuf») (Cadéac, 1884). The history of bovine besnoitiosis is, in fact, plenty of confusion and uncertainty concerning the identity of the etiological agent and the name of the disease, which conduced to different synonym based on the clinical aspects of the disease or the succeeding denominations for the parasite. With the investigations of Besnoit and Robin (1912), that revealed the presence of a large number of thick-walled cysts harboring numerous spores in the skin and subcutaneous tissues of affected cattle, the parasitic etiology of the disease was established. They realized that it was a new parasite, and suggested that it might be a S*arcocystis* sp., designating the disease as (cutaneous) sarcosporidiosis. In the same year, Marotel (1912) pointed out that nothing similar had been found previously in cattle and, despite noting morphological characteristics that clearly differentiate this parasite from the known *Sarcocystis* spp. at the time, he proposed the species name of *Sarcocystis besnoiti*. The following year, Henry (1913), considering this morphological distinction, argued that the protozoa of Besnoit and Robin could not be placed in the genus *Sarcocystis*. He proposed the creation of the genus *Besnoitia* and that the parasite be referred as *Besnoitia besnoiti*, keeping the authorship of the species to Marotel. Meanwhile, in Portugal, Franco and Borges (1915, 1916) conducted a detailed description of the disease, in 67 animals they encountered in Lisbon abattoir from 1885 to 1914. In agreement with Henry (1913), they noticed the distinct characteristics that differentiated this parasite from the genus *Sarcocystis* and proposed also the name of *Besnoitia besnoiti.* However, in 1920 (Nöller), in a revision of the relevant literature up to the year of 1918 about the generic nomenclature of the genera *Globidium*, *Gastrocystis*, *Ileocystis*, *Lymphocystis*, *Haplogastrocystis* and *Besnoitia*, regarded that the parasites with unknown complete life cycle should be included in the genus *Globidium*, for the sake of simplicity. He then suggested the name *Globidium besnoiti* for the etiological agent of the Besnoit and Robin's sarcosporidiosis. This suggestion was followed by other authors in subsequent publications (Pols, 1960), and lead to another designation of the disease: bovine globidiosis. In

subsequent years, the debate on how to classify this protozoan continued, but the consensus about the *Besnoitia* genus came in the mid twentieth century.

1.1.1 The genus *Besnoitia* and *B. besnoiti*

1.1.1.1 Life cycle and biological features

Besides *B. besnoiti* (type species), the genus *Besnoitia* comprises other three named species infecting large ungulates (*Besnoitia caprae*, *Besnoitia bennetti* and *Besnoitia tarandi*) and six infecting small mammals and lizards (*Besnoitia akodoni*, *Besnoitia darlingi*, *Besnoitia jellisoni*, *Besnoitia neotomofelis*, *Besnoitia oryctofelisi* and *Besnoitia wallacei*) (Cortes, Leitao, Gottstein & Hemphill, 2014; Olias et al., 2011). Presently, there are still questions regarding the differentiation of some of these species and their taxonomic status because only four have their life cycle clarified and morphological and molecular differences among the remaining species are poorly defined (Dubey, van Wilpe, Blignaut, Schares & Williams, 2013; EFSA, 2010; Olias et al., 2011).

It has been suggested that *Besnoitia* species, like other cyst-forming coccidian, have a facultative heteroxenous life cycle with a predator as a definite host and a prey as intermediate host (Basso, Schares, Gollnick, Rutten & Deplazes, 2011; Olias et al., 2011). The life cycle is well characterized only for four *Besnoitia* species found in small mammals from the American continent (*B. darlingi*, *B*. *neotomofelis*, *B. oryctofelisi* and *B. wallacei*), for which the cat was found to act as the definite host (Dubey & Lindsay, 2003; Dubey et al., 2002; Dubey et al., 2003b; Dubey & Yabsley, 2010; Frenkel, 1977; Smith & Frenkel, 1977, 1984; Wallace & Frenkel, 1975). Nevertheless, it is important to point that not all life forms (cysts/bradyzoites, tachyzoites or oocysts) of these parasites have been identified in nature. For *B. darlingi*, *B*. *neotomofelis* and *B. oryctofelisi*, the tissue cysts, containing bradyzoites, were identified in the intermediate host and the cat experimentally infected (Dubey & Lindsay, 2003; Dubey et al., 2002; Dubey et al., 2003b; Dubey & Yabsley, 2010; Smith & Frenkel, 1977, 1984), while for *B. wallacei*, the oocysts were isolated in the feces of one stray cat and susceptible intermediate hosts (*Rattus norvegicus*, *R. exulans*, and *Mus musculus*) experimentally infected (Frenkel, 1977; Wallace & Frenkel, 1975). In this way, while the life cycle of these species was experimentally maintained through a cat-prey-cat cycle, the natural intermediate host for *B*. *wallacei* remains unknown, as well as the cat real role in nature for the remaining species. No definite host for *B. jellisoni* and *B. akodoni* has been identified so far.

In regard to the *Besnoitia* species infecting large ungulates, although they have been studied for more than a century now (namely *B. besnoiti*, (Besnoit & Robin, 1912)), the definite host in the putative heteroxenous life cycle is still to be found (Alvarez-Garcia, Frey, Mora & Schares, 2013; Cortes et al., 2014; Olias et al., 2011). Homoxenous, direct transmission of bradyzoites and tachyzoites between intermediate hosts has been established experimentally in ungulates and several laboratory animals (Basso et al., 2011; Bigalke, 1968; Ng'ang'a & Kasigazi, 1994; Pols, 1960). Whether sexual reproduction really exists for all species of this genus is unknown, but since the closely related *T. gondii*, *N. caninum* and *Hammondia* spp. use terrestrial carnivores to fulfill this process and the cat has been identified has definite host in four *Besnoitia* species, it is very suggestive that large ungulate *Besnoitia* species also have a two-host life cycle (Olias et al., 2011). There have been several attempts to identify a definite host for these *Besnoitia* species, but only Peteshev, back in 1974, stated that both domestic cats and a wild cat (*Felis lybica*), shed oocysts after ingestion of *B. besnoiti* cyst-containing tissues (Peteshev, Galuzo & Polomoshov, 1974). This observation, however, was never sustained by other authors. Diesing et al. (1988), fed domestic cats (*Felis domestica*), a dog (*Canis familiaris*), jungle cats (*Felis chaus*), caracals (*Caracal caracal*), small spotted genets (*Genetta genetta*), a lion (*Panthera leo*), leopards (*Panthera pardus*), cheetahs (*Acinonys jubatus*), banded mongooses (*Mungos mungo*), black-backed jackals (*Canis mesomelas*), a cape fox (*Vulpes chama*), six species of snakes and white-backed vultures (*Gyps africanus*), with *B. besnoiti* cystic material from chronically infected cattle and concluded that none of these species represents the definitive host of *B. besnoiti*, as no oocysts were found in their feces. Recently, these results were confirmed in experimental infections in domestic cats and beagle dogs (Basso et al., 2011). In the same way, experimental infections with *B. tarandi* failed in studies with domestic cats (Ayroud, Leighton & Tessaro, 1995), domestic cats and dogs (Dubey et al., 2004) and raccoon (*Procyon lotor*), domestic cat and arctic fox (*Alopex lagopus*) (Glover, Swendrowski & Cawthorn, 1990), as well as experimental infections with *B. caprae* in cats (Ng'ang'a & Kasigazi, 1994). Evidences of a definitive host also fail in a serological survey of *B. besnoiti* in free-living carnivores in Spain (Millan et al., 2012)

Apart from the search for the missing final host it should be considered that ungulates may in fact represent accidental hosts, playing a role as a reservoir of the parasite in a sylvatic life cycle (Basso et al., 2011; Diesing et al., 1988; EFSA, 2010; Kiehl et al., 2010; Olias et al., 2011). To the present date, there is no evidence of parasites of the genus *Besnoitia* being infective for humans (Cortes et al., 2014; Olias et al., 2011).

1.1.1.2 Taxonomy and phylogeny

The obligate intracellular protozoan parasites of the genus *Besnoitia* are classified in the sub-family Toxoplasmatinae of the family Sarcocystidae, within the phylum Apicomplexa (Ellis et al., 2000; Olias et al., 2011; Tenter et al., 2002). Phylogenetic analysis based on 18S rDNA sequences of *B. besnoiti*, *B. jellisoni*, *B. caprae*, *Hammondia hammondi*, *Isospora* spp., *Frenkelia* spp., *Neospora caninum*, *Sarcocystis* spp. and *Toxoplasma gondii* showed that *Besnoitia* species comprise a monophyletic and sister group to a clade containing other medically and veterinary important parasitic protozoa, such as *Toxoplasma gondii*, and *Neospora caninum* (Figure 1) (Ellis et al., 2000; Olias et al., 2011). These results were supported by Dubey et al. (2004) with analysis of both large and small subunit rDNA sequences.

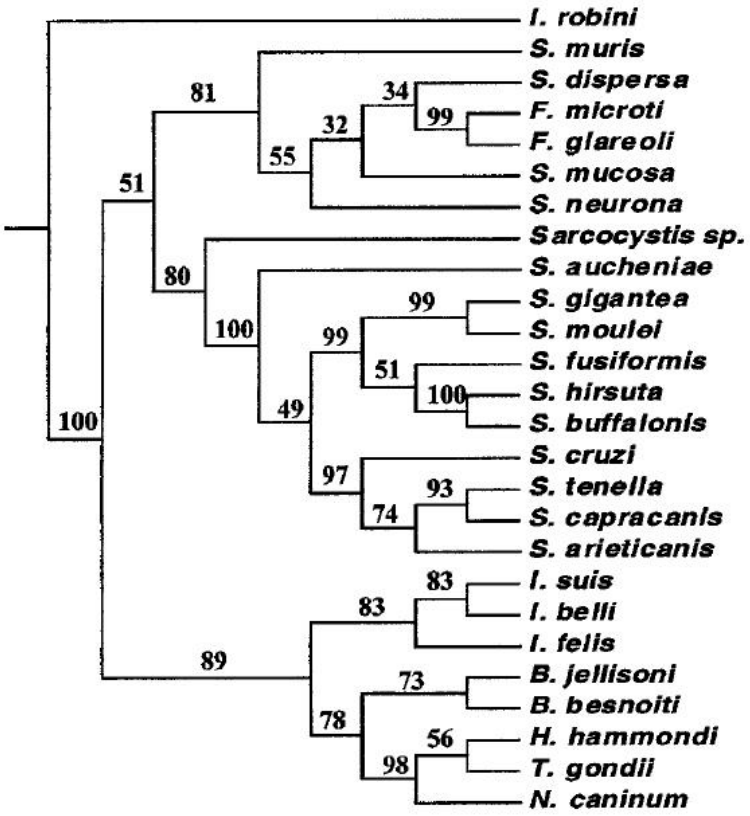

Figure 1. Phylogenetic tree based on 18S rDNA sequences.
Tree constructed from the parsimony analysis of the structure alignment of the SSU rDNA, representing the relationships amongst the cyst-forming coccidia sequences. *Isospora robini* was used as the outgroup. The numbers at the nodes represent the bootstrap values (% out of 500) (reprinted from Ellis et al., 2000, with permission from Elsevier)

Evolutionary relationships among *Besnoitia* species have been only scarcely investigated and are hindered by the deficit of gene sequences published for the different species and from different geographic regions. A striking example, is the fact that for *B. wallacei*, despite being known for forty years now (Wallace & Frenkel, 1975), there is no publically available sequence [GenBank search (http://www.ncbi.nlm.nih.gov/genbank/) for the term "*Besnoitia wallacei*"; accessed on 18 February 2016]. The published phylogenetic analyses on the genus *Besnoitia* have been based on the availability of ITS1 gene sequences. Although sequence information is not entirely complete for all species, it was possible to identify a *Besnoitia* genus-specific cluster within the ITS1 region, which, by multiple alignments, was demonstrated to be independent from other apicomplexans (Cortes et al., 2014). Phylogeny suggests that the *Besnoitia* genus is comprised of two distinct groups. One group includes the *Besnoitia* isolates from large ungulates (*B. besnoiti, B. bennetti, B. caprae and B. tarandi*), while the other contains *Besnoitia* isolates from small mammals from the American continent (*B. akodoni*, *B. darlingi*, *B. jellisoni*, *B. oryctofelisi* and *B. neotomofelis*) (Dubey & Yabsley, 2010; Kiehl et al., 2010; Olias et al., 2011). According to the Olias et al. (2011) analysis of available ITS1 sequences, that did not include the recently described *B. neotomofelis* (Dubey & Yabsley, 2010), small mammal *Besnoitia* spp group show a genetic divergence to large ungulate species group of 23.1% to 25.5%. Within the small mammal *Besnoitia* species there is a higher degree of ITS1 sequence variation, between 1.6 to 5.9%, while within large mammal *Besnoitia* species the sequences showed no variation (Figure 2). These differences might result from the contribution of the diploid phase, during the parasites differentiation and life cycle, where there is a bigger chance of recombination to occur. As previously discussed, in the case of *B. besnoiti*, the oral route of infection through oocysts shed by a definitive host has not been identified to date. However, it was demonstrated that transmission can occur by blood feeding insects such as horse flies and the stable fly, *Stomoxys calcitrans* (Bigalke, 1968), therefore simply bypassing the sexual cycle and promoting the parasite clonality (Cortes et al., 2014). This scenario is supported by comparison of the ITS1 sequences of *B. besnoiti* from different geographic regions, that show a lower degree of sequence variation for mechanically transmitted *Besnoitia* species. Olias et al. (2011) compared *B. besnoiti* ITS1 sequences from Israel, Portugal and South Africa and encountered no variation, while Kiehl et al. (2010) analyzed sequences from isolates of Israel, Portugal, Spain and Germany and found nearly identical sequences, with only a single nucleotide different in two of them.

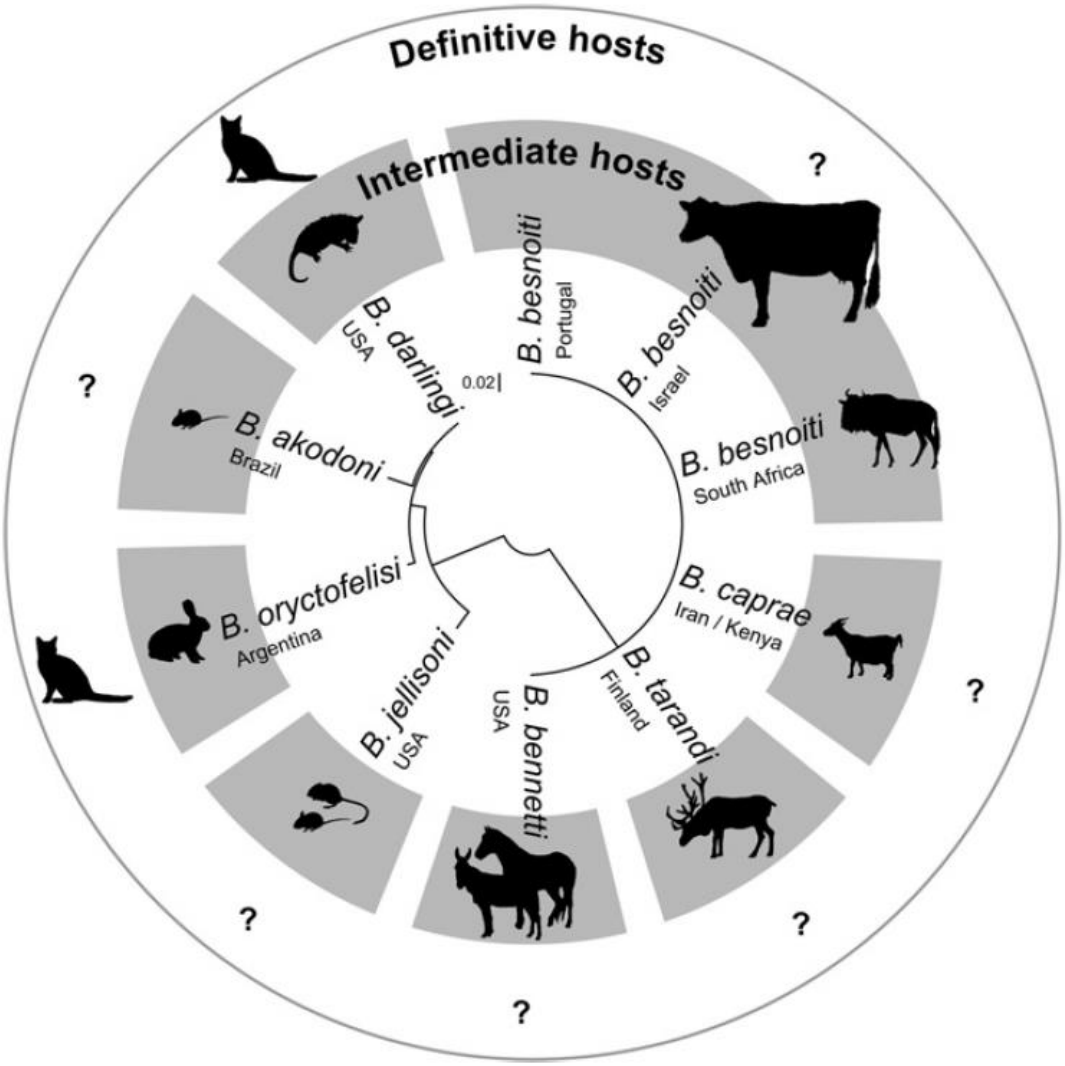

Figure 2. Phylogenetic tree of the ITS1 region of parasite isolates in the genus *Besnoitia*. Tree constructed by unrooted neighbor-joining method. Eight out of 10 named *Besnoitia* species shown, as well as their known natural intermediate host (in gray). No natural definitive hosts are known for these species and the cat here depicted was established experimentally. Scale bar indicates genetic distance (reprinted from Olias, Schade & Mehlhorn, 2011, with permission from Elsevier).

Phylogenetic analysis of the ITS1 sequence of *B. neotomofelis*, the latest identified *Besnoitia* specie, placed it in a clade with *B. jellisoni*, which is a sister clade to one containing *B. akodoni*, *B. darlingi*, and *B. oryctofelisi* within the small mammal clade (Dubey & Yabsley, 2010) of the genus *Besnoitia* (Figure 3).

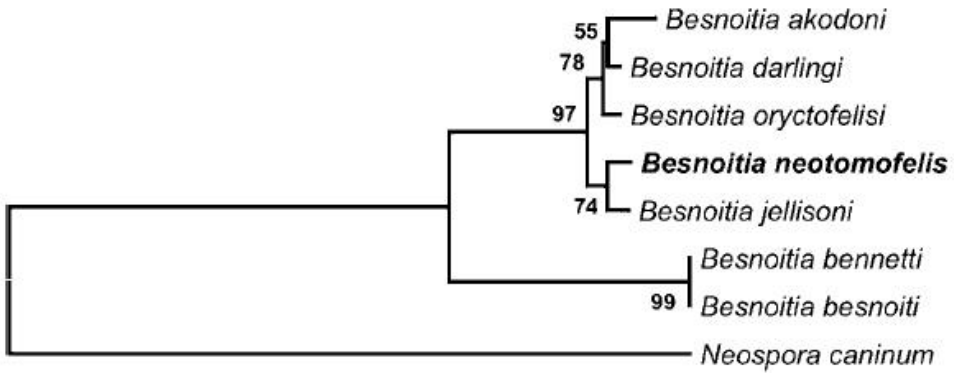

Figure 3. Phylogenetic tree based on ITS1 region sequences of *Besnoitia neotomofelis* and related organisms.
Percentages of 1000 bootstrap samplings that supported clades are shown on branches for Neighbor-joining analysis (reprinted from Dubey & Yabsley, 2010, with permission from Cambridge University Press).

Molecular analysis set in motion the debate about species differentiation that, especially in the case of coccidian, was traditionally based on phenotypic characters that include the morphology/ultrastructure of the available parasite stage and the host specificity. One problem with this approach is that in most cases only the oocyst stage and the 'host' by which it was shed were known when a new species was named. Such incomplete species descriptions are confounded by the fact that the 'host' described for the new species may not be its true natural host, because many animals may passage oocysts through their intestine without being a host for these parasites (Tenter et al., 2002). This may be further complicated in the case of heteroxenous or polyxenous parasites, where an incomplete understanding of the parasite life cycle can lead to different named species based on phenotypic characters such as the host specificity. An example of this situation is the well-known *T. gondii*, that, before the discovery of the oocyst and it's heteroxenous life cycle (more than 60 years later from the description of the first stage of the parasite in the gondi (*Ctenodactylus gundi*)), several other species of *Toxoplasma* were named in accordance to the host species in which they were detected (Levine, 1977; Tenter et al., 2002). *Besnoitia* spp. may or may not be writing the same history than *Toxoplasma* and only further research, both at the molecular level and biological/epidemiological level, can elucidate this matter. Presently, we should deduce the status of a species from the analysis of molecular, morphological, biochemical, and/or ecological data all together (Kiehl et al., 2010). In the case of *Besnoitia* species, looking into the ITS1 sequences of large ungulates, we might be

drawn to conclude that presently there is no sufficient genetic evidence to consider *B. caprae*, *B. tarandi*, *B. bennetti* and *B. besnoiti* separated species, since all published sequences to date are identical or different with only one or two base insertions when compared to *B. besnoiti* (Ellis et al., 2000; Olias et al., 2011). Nevertheless, some points should be considered in this matter: first, as previously mentioned, genetic data available for these parasites from different isolates is limited and for sure represents a biased sampling of the biological diversity of *Besnoitia* spp. (Tenter et al., 2002); second, phylogenic definition of a species is almost impossible based in molecular data derived only from one gene, especially when there are no hints in which degree of diversity is needed to define a specie (Kiehl et al., 2010); Nevertheless, the size and sequence content of the ITS1 is recognized as a well-characterized species-specific marker amongst the coccidian such that parasite populations sharing the same, or highly similar, ITS1 sequences are likely to be derived from the same specie (Ellis et al., 2000); third, biological and ultrastructural details indicate substantial differences that, as stated, have to be weighed along with molecular data. Still, the *Toxoplasma* example shows us that we can have virulent and avirulent populations of T. gondii, which differ in their biological (e.g. spectrum of disease in the mouse) and genetic (e.g. SAG2 and RAPD PCR profiles) properties, possess identical ITS1 sequences (Ellis et al., 2000). When comparing *B. besnoiti* and *B. caprae*, we can found significant ultrastructural differences (Njenga, Bwangamoi, Kangethe, Mugera & Mutiga, 1995) and differences in host susceptibility: *B. besnoiti* is infectious for rabbits, mice, guinea pigs, hamsters, rats, sheep and cattle but *B. caprae* is not (Ng'ang'a & Kasigazi, 1994; Njenga, Bwangamoi, Mutiga, Kangethe & Mugera, 1993). Similarly, we can find differences in the ultrastructure and host susceptibility between *B. besnoiti* and *B. tarandi* (Dubey, Shkap, Pipano, Fish & Fritz, 2003a; Dubey et al., 2004), and between *B. besnoiti* and *B. bennetti* (Bigalke, 1970; Dubey et al., 2005; Pols, 1960). In this way, what we can presently sustain is that either *B. caprae, B. tarandi* and *B. bennetti* are different species with the same ITS1 sequence than *B. besnoiti* or that they represent distinct populations/strains of *B. besnoiti*. Clearly, additional information is required to resolve this controversy.

1.1.1.3 Morphological features

The rapidly dividing *B. besnoiti* tachyzoites represent the parasite proliferative stage responsible for the acute phase of the disease. With the onset of the host immune response and the presence of other physiological factors, tachyzoites differentiate into the slowly replicating encysted bradyzoites and a persistent tissue cyst infection is established along with the chronic phase of the disease (Alvarez-Garcia, Garcia-Lunar, Gutierrez-Exposito, Shkap & Ortega-Mora, 2014b; Buxton, McAllister & Dubey, 2002; Cortes et al., 2014;

Jacquiet, Lienard & Franc, 2010; Lyons, McLeod & Roberts, 2002). Tachyzoites and tissue cysts (containing bradyzoites) are the two tissue stages of *B. besnoiti* (and other *Besnoitia* species) found in intermediate hosts (Alvarez-Garcia et al., 2014b; Dubey et al., 2013).

B. besnoiti tachyzoites are typically crescent-shaped with a slightly pointed anterior compared with the rounded posterior end (Langenmayer et al., 2015; Shkap, Yakobson & Pipano, 1988), measuring about 6-7.5 x 2.5-3.9 μm in cell culture (Reis et al., 2006). Similar to *T. gondii* and *N. caninum*, these tachyzoites can invade a wide range of host cells *in vitro*, with an asexual multiplication by endodyogeny (Cortes et al., 2014; Göbel, Widauer, Reimann & Munz, 1985), that takes place in the parasitophorus vacuole, a cytoplasmic compartment formed upon entry of the parasite into the host cell. By light microscopy tachyzoites of *B. besnoiti*, *N. caninum* or *T. gondii* cannot be distinguished. *In vitro* replication rates depend largely on the cell line used, going from 0.14 (on Vero cells) to 20 (on BHK_{21} cells) tachyzoites per hour per tachyzoite inoculated (Schares et al., 2009). Bradyzoites however, show a replication rate more than 100 times slower than that of cell cultures inoculated with tachyzoites (Schares et al., 2009). Morphologically they are very similar to tachyzoites, but reported measures fluctuate accordingly the author (Dubey et al., 2003a; Pols, 1960; Rostaher, Mueller, Majzoub, Schares & Gollnick, 2010; Sannusi, 1991), differing from 4.6 x 1.8 μm (Cortes et al., 2003) to 7-9 x 2.0 μm (Mehlhorn et al., 2009).

As with other apicomplexans, *B. besnoiti* bradyzoites persist for extended periods of time in the chronically infected animal, harbored in cysts in long living tissues, mainly the skin and aponevrosis (Cortes et al., 2014; Walker et al., 2014). The typical morphology of *B. besnoiti* tissue cysts is a strikingly large host cell containing a parasitophorous vacuole (PV) with large numbers of bradyzoites surrounded by a thick wall. They can grow up to 0.6 mm diameter (Pols, 1960) and are morphologically unusual because the host cell nuclei is incorporated inside the cyst and is hypertrophied (Dubey & Lindsay, 2003; Dubey et al., 2003b; Dubey et al., 2003c; Dubey et al., 2013; Dubey & Yabsley, 2010; Ernst, Chobotar, Oaksec & Hammond, 1968; Paperna & Lainson, 2001; Wobeser, 1976). This originates the characteristic double layer cyst-wall, that clearly distinguishes *B. besnoiti* cysts from its related apicomplexans (Cortes et al., 2014). Accurate morphological description of *Besnoitia* cysts is complicated by observed variation in cysts size and the thickness of the cyst wall, features that depend largely on the host affinity (natural or experimental host), tissue affinity and duration of infection (Dubey et al., 2003a). For *B. besnoiti*, although the existence of several studies that describe the cysts structure in naturally infected cattle, the nomenclature regarding the composition of the cysts varies significantly between authors. For example, Dubey et al. (2003a) and Cortes et al. (2006c) describe the cyst structures in a similar way (from in to out): intracellular cyst wall delineated by the parasitophorous vacuole membrane, host cell cytoplasm and membrane and outer cell cyst wall; Mehlhorn et al. (2009) talks of a

primary cyst wall and a secondary cyst wall; Later, Dubey et al. (2013) described *B. besnoiti* tissue cysts has having 3 layers or compartments: the outer layer with connective-like tissue, the middle layer incorporating host nuclei, and the cyst proper containing the bradyzoites. Recently, Langenmayer et al. (2014), in a study that followed cyst development on cattle through an 84-day trial post natural infection, proposed a new nomenclature for describing *B. besnoiti* tissue cysts: 1) structures within the host cell should be called by their respective name (bradyzoites, parasitophorous vacuole, membrane of the parasitophorous vacuole, host cell cytoplasm, nuclei, host cell membrane, etc.). 2) For the outermost acellular layer, they proposed the term outer cyst wall (OCW). The OCW is composed of interwoven collagenous material, is about 10-12 µm in thickness (Cortes et al., 2006c; Dubey et al., 2003a) and seems to be the result of the host physiological response (Cortes et al., 2014). 3) For the cyst wall of undeveloped cysts, which in developed cysts lies between the OCW and the membrane of the host cell, they proposed the name of inner cyst wall (ICW). This is a thick layer of up to 1 µm, also acellular, consisting of proteoglycan particles and fine filaments. Both OCW and the ICW seem to be the result of the host physiological response (Cortes et al., 2014). 4) The whole hypertrophied host cell, including the ICW and, if present, the OCW, is called a tissue cyst.

The parasitophorous vacuole is limited by a single cell membrane, frequently lined on the luminal side by a granular layer of approximately 0.2 µm thick (Dubey et al., 2003a; Langenmayer et al., 2015; Mehlhorn et al., 2009). No septation exists inside *Besnoitia* cysts as it is characteristic for *Sarcocystis* species (Mehlhorn et al., 2009). The host cell cytoplasm usually contains multiple large nuclei with occasional invaginations of the nuclear membrane. The organelles consisted mostly of dilated tubules of the ER, normal or swollen mitochondria and lysosomes. They are arranged in a circular manner around the PV, which occupies most of the host cell cytoplasm (Langenmayer et al., 2015; Mehlhorn et al., 2009).

1.1.1.4 Bradyzoite and tachyzoites ultrastructure features

The phylum Apicomplexa consists of intracellular parasites that have a characteristically polarized cell structure and a complex cytoskeletal and organellar arrangement at their apical end (Black & Boothroyd, 2000). Because the Apicomplexa are obligate intracellular parasites, survival depends on their ability to invade host cells (evading the host humoral immune response), avoid degradation by the host cell machinery (by blocking/modulating fusion of the vacuole with lysosomes, blocking vacuolar acidification and delaying premature cell death until parasite development is complete), and propagate intracellularly (recruiting cell resources and optimizing nutrient acquisition) (Binder & Kim, 2004; Nyalwidhe, Maier & Lingelbach, 2003; Sibley, 2011; Sinai & Joiner, 1997). The defining feature of Apicomplexa is

a complex assemblage of unique structural and secretory elements at the apical end of the parasite invasive life-cycle stages, named apical complex, that is intimately associated with these functions. (Binder & Kim, 2004; Katris et al., 2014; Kreier & Baker, 1987; Tenter et al., 2002).

The apical complex plays a key role in the mechanism of parasite motility, adhesion, invasion and formation of the parasitophorous vacuole, by providing both a semi-rigid framework to the parasite cell and a focal point for secretory organelles that release numerous invasion factors that mediate these functions. The apical complex is organized around an apical polar ring that serves as a microtubule organizing center that nucleates an array of subpellicular microtubules that descend toward the posterior of the cell (Katris et al., 2014; Morrissette & Sibley, 2002). These microtubules subtend flattened membrane sacs, or alveoli, that line most of the plasma membrane. A fibrous proteinaceous membrane skeleton supports the alveolar sacs against the microtubules. The alveoli and the proteinaceous skeleton form a structure called the inner membrane complex (IMC), which, together with the subpellicular microtubules, provides the shape and stability of the cell (Black & Boothroyd, 2000; Katris et al., 2014; Morrissette & Sibley, 2002). The plasma membrane is closely apposed over the IMC, forming together a structure called pellicle (Morrissette & Sibley, 2002). The apical polar ring marks the apical extremity of the IMC. A mobile conoid, consisting of tightly bent tubulin filaments fused to form a tapered hollow barrel, sits within the apical polar ring (Katris et al., 2014). The conoid can either be recessed in the cell, so that its tip is even with the apical polar ring, or, during invasion, be extruded from the apical polar ring to form an extended point to the cell. At the tip of the conoid are two preconoidal rings, and a pair of short microtubules sit eccentrically within the conoid. These preconoidal rings and interconoidal microtubules move together with the conoid during extrusion. The structural elements of the apical complex provide orientation to the cell, and the focal point for arrays of secretory organelles, micronemes and rhoptries and dense granules, that are organized towards the base of the conoid in readiness for a staged sequence of release (Katris et al., 2014). The electron-opaque dense granules are evenly distributed throughout the parasite cytoplasm, while the large club-shaped rhoptries and the small rod shaped micronemes are strictly confined to the anterior third of the cell (Carruthers, 1999). Several studies in other apicomplexans, especially *T. gondii*, have shown that microneme contents are secreted first, prior to invasion, and coat the parasite with proteins that facilitate host cell adhesion, gliding motility, and contribute to formation of an annular moving junction with the host plasma membrane through which the parasite enters the cell. During invasion rhoptries secrete further elements of the moving junction, as well as proteins that establish the properties of the parasitophorous vacuole in which the parasite will reside intracellularly (Katris et al., 2014; Sibley, 2011; Straub, Cheng, Sohn & Bradley, 2009). This junction can

be thought of as a “ring of contact” between the invading tachyzoite and the host cell membrane and is used by the parasite to forcibly enter the host cell. Once the parasite is completely inside the host cell, the moving junction disappears as the parasitophorous vacuole is sealed (Dubremetz, 2007; Walker et al., 2014). Finally, as the complete parasitophorous vacuole is detaching from the plasma membrane, dense granules penetrate the IMC within the apical region and liberate their content, by exocytosis, to the vacuole. Dense granule secretion coincides with the formation of a network of membrane tubules in the vacuole (named vacuolar network) and continues during the intracellular residence of the parasite (Figure 4) (Dubremetz, Achbarou, Bermudes & Joiner, 1993; Ngô et al., 2000).

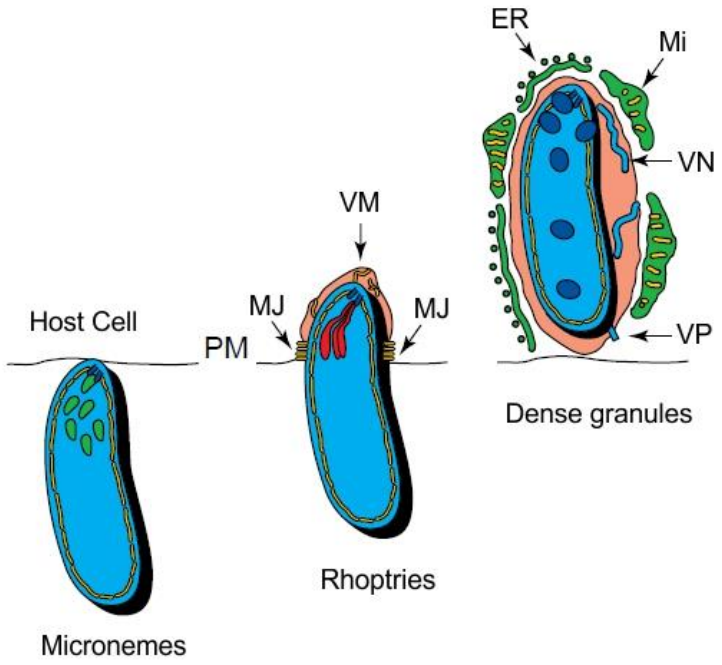

Figure 4. Schematic representation of host cell invasion.
There is a sequential exocytosis of micronemes (green), rhoptries (red) and dense granules (blue) from different cellular locations. ER, host endoplasmic reticulum; Mi, host cell mitochondria; MJ, Moving junction PM, host cell plasma membrane; VM, vacuole membrane; VN, vacuole network; VP, vacuole pore. (adapted from Ngô, Hoppe & Joiner, 2000, with permission from Elsevier)

The elements of the apical complex are highly conserved throughout Apicomplexa (Katris et al., 2014), as it is the presence of the apicoplast, a plastid without photosynthetic ability (Arisue & Hashimoto, 2015). There are however some variations within the taxon and some species lack one or other feature of the apical complex (conoid in piroplasms, for example) or even lack the apicoplast (e.g. cryptosporidea) (Adl et al., 2012). *B. besnoiti,* being a coccidian, features the complete apical complex and the apicoplast and its ultrastructure is schematically presented in Figure 5. Here we can see the previously described elements, as well as other organelles and structures, including the nucleus, endoplasmic reticulum, Golgi complex, mitochondria, micropore, amylopectin granules and inclusion bodies (Dubey et al., 2013; Langenmayer et al., 2015; Shkap et al., 1988), similar to what is described for *T. gondii* (Dubey, Lindsay & Speer, 1998). The nucleus is usually situated in the middle or posterior third of the parasite (Langenmayer et al., 2015). In *B. besnoiti* zoites twenty two subpellicular microtubules radiate from the polar ring and extend far more than the 2/3 of the parasite length, sometimes reaching 4/5 of the cell body (Reis et al., 2006), as was defined for *B. jellisoni* (D'Haese, Mehlhorn & Peters, 1977).

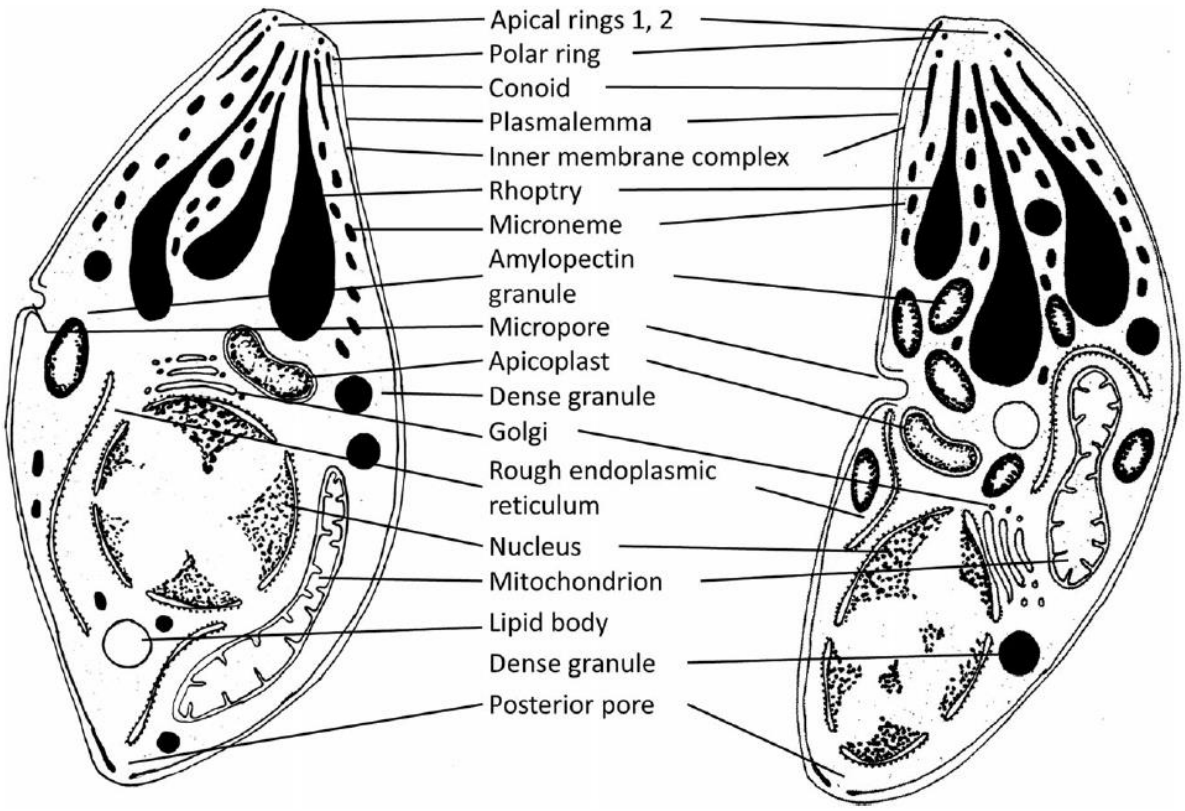

Figure 5. Schematic representation of the ultrastructure of *Besnoitia besnoiti.*
Bradyzoites (left) and Tachyzoites (right) (reprinted from Langenmayer et al., 2015, with permission of Springer)

1.1.2 Epidemiology

First reported cases of bovine besnoitiosis occurred more than a century ago in Europe, first in France (Besnoit & Robin, 1912; Cadéac, 1884) and after in Portugal (Franco & Borges, 1915, 1916). For years, besnoitiosis was considered an endemic sub-Saharan disease, because of the succeeding cases reported here and the lack of new cases reported in Europe. In Africa, the occurrence of bovine besnoitiosis was first discovered in 1945, by the South African state veterinary Christian Hofmeyr (Hofmeyr, 1945), with several other cases reported both in South Africa (Pols, 1960) and other countries, like Swaziland, Botswana, Namibia, Zimbabwe, Angola, Congo, Kenya, Tanzania, Uganda, Sudan, Cameroon and Nigeria (EFSA, 2010). In Asia the disease was reported in Israel (Goldman & Pipano, 1983; Neuman, 1972; Shkap, Reske, Pipano, Fish & Baszler, 2002; Shkap, Pipano & Greenblatt, 1987b), South Korea (Lee, Bak, Moon & Shin, 1970) and Russia (Krasov, Omarov Zh, Uvaliev & Khvan, 1975; Peteshev et al., 1974).

Bovine besnoitiosis remained silent in Europe until the last decade of the twentieth century, when new cases were described in northern Spain (Juste et al., 1990) and in Alentejo (Malta & Silva, 1991). But was until the beginnings of the twenty first century that the disease started to be a concern, having spread from southern France over western and central France (Alzieu, Dorchies, Schelcher & Gottstein, 2007; Bourdeau et al., 2004) and propagated within Portugal (Cortes et al., 2006c; Cortes et al., 2003; Cortes et al., 2005) and Spain (Castillo, Marcén, Ortega-Mora & Álvarez-García, 2009; Fernandez-Garcia et al., 2010; Fernandez-Garcia et al., 2009a; Fernandez-Garcia et al., 2009b; Irigoien et al., 2000). *B. besnoiti* is considered endemic in large areas in Spain, Portugal and France, and recently this status was also proposed for Italy (Gazzonis et al., 2014; Gentile et al., 2012a). Isolated outbreaks, described as resulting from the introduction of infected cattle from endemic areas, have been reported in Germany (Mehlhorn et al., 2009), Switzerland (Basso et al., 2013; Lesser et al., 2012), Italy (Agosti et al., 1994; Gentile et al., 2012b; Gollnick, Gentile & Schares, 2010; Mangili et al., 2012; Manuali et al., 2011; Mutinelli et al., 2011), Greece (Papadopoulos et al., 2014), Hungary (Hornok, Fedak, Baska, Hofmann-Lehmann & Basso, 2014), Croatia (Beck, Štoković & Pleadin, 2013) and Ireland (Ryan et al., 2016). The growing numbers of animals affected and the geographic expansion lead the European Food Safety Authority (EFSA) to consider bovine besnoitiosis as an emerging disease in Europe (EFSA, 2010).

There are still many aspects of the epidemiology of bovine besnoitiosis that are poorly understood, including prevalence and incidence data in endemic areas, routes of transmission and risk-factors associated to infection and disease. The complete life cycle of *B. besnoiti* is not known and the putative definite host(s), that sheds oocysts after ingestion

of infected tissues, has not been identified yet, despite several studies with that purpose (Basso et al., 2011; Diesing et al., 1988; Millan et al., 2012). Hitherto, the only experimentally confirmed modes of transmission among cattle are mechanically, either through hematophagous insects, or iatrogenically through hypodermic needles (Bigalke, 1968; Pols, 1960). However, until now, no natural transmission of *Besnoitia* species has been proven and it is very likely that livestock trade of infected animals serves as a very important vehicle for long distance horizontal transmission of *B. besnoiti* (Frey et al., 2013; Olias et al., 2011), as it is discussed for recent outbreaks in France, Germany, Italy, Switzerland and Hungary (Agosti et al., 1994; Basso et al., 2013; Hornok et al., 2014; Mehlhorn et al., 2009; Mutinelli et al., 2011). After long-distance transport of single infected animals, the parasite might find adequate conditions to be transmitted locally to contact cattle, presumably by insect vectors and/or by direct contact between animals (Bigalke, 1968). Bigalke (1968), experimentally infected cattle orally with tachyzoites (bradyzoites were non-infective) and through the naso-pharyngeal route, with the inoculation of bradyzoites in the nostrils, but in nature, a feasible mechanism for the release of viable parasites (in infective quantities) from a chronically infected animal would have to be found. Skin cysts that might occasionally be exposed by trauma or spontaneously rupture to the surface are most unlikely to form a sufficiently reliable source of infection (Bigalke, 1968). The same should hold true when considering the venereal transmission of *B. besnoiti*, even though it has been suggested as possible (Frey et al., 2013; Gentile et al., 2012a; Gollnick, Scharr, Schares & Langenmayer, 2015). Consequently, the epidemiological significance of these modes of transmission is still unknown and further investigation is required. Moreover, it is not known if there are differences in the host specificity among *B. besnoiti* isolates from different parts of the world, or if other intermediate hosts than bovids are involved in the epidemiology of bovine besnoitiosis (Basso et al., 2011). In Europe, *B. besnoiti* natural infections were only confirmed in cattle, but in South Africa it was also described in wild ungulates (kudu (*Tragelus strepsiceros*), blue wildebeest (*Connochaetes taurinus*) and an impala (*Aepyceros melanpus*) (McCully, Basson, Van Niekerk & Bigalke, 1966). For this reason, and also because it can experimentally infect small mammals, the EFSA (2010) stated that wild ruminants and rodents should not be disregarded as reservoirs of the parasite. A serological survey in wild ungulates in Spain detected specific antibodies against *Besnoitia* spp. in a red deer (*Cervus elaphus*) and a roe deer (*Capreolus capreolus*) from the endemic region of the Pyrenees, suggesting a link between the sylvatic and domestic life cycles of *Besnoitia* spp. similar to what is observed in the lifecycles of other closely related apicomplexan parasites (Gutierrez-Exposito et al., 2013). In the same way, the relationship between *B. besnoiti* and other *Besnoitia* spp. isolated from ungulates (*B. tarandi* and *B. caprae*) should be investigated to assess the risk of infection for domesticated ruminants (EFSA, 2010).

Moreover, a sylvatic cycle involving birds as intermediate hosts should be considered, since some endemic regions of besnoitiosis (e.g., Pyrenees, southern Portugal, Israel, South Africa, Nigeria, Kenya) correspond to important staging spots and funnels of migratory birds in the African Eurasian flyway. Whether birds can become infected by *Besnoitia* species as final or intermediate hosts and play a role as long distance vectors similar to *T. gondii* remains to be shown and warrants further investigation (Olias et al., 2011)

In endemic areas, infection is widespread within cattle populations, but the proportion of infected cattle that develop the disease is low (Pols, 1960; Bigalke, 1968; Legrand, 2003; Bourdeau et al., 2004; Cortes et al., 2006b; Alzieu et al., 2007). The prevalence of clinical cases varies from 1 to 10% and the incidence between 2% and 5% (Jacquiet et al., 2010) Three clinical scenarios can be considered after the initial contamination of a herd with *B. besnoiti*: (1) a small proportion of animals with typical clinical signs; (2) a larger subset of seropositive individuals with sclero-conjunctival cysts; (3) the largest subset of asymptomatic, seropositive individuals (Jacquiet et al., 2010; Lienard et al., 2011). In areas where the disease is emerging, up to 20% of newly contaminated cattle show typical signs (Jacquiet et al., 2010). In endemic areas most clinical cases are reported in two to four-year old cattle, even though younger animals (from six months upwards) may present clinical signs (Alzieu et al., 2007; Jacquiet et al., 2010). It was found a positive correlation between the prevalence of the disease and the age of the animal (Ashmawy & Abu-Akkada, 2014; Fernandez-Garcia et al., 2010; Gutierrez-Exposito et al., 2014; Waap, Nunes, Cortes, Leitão & Vaz, 2014). All cattle breeds seem to be susceptible to besnoitiosis (Alvarez-Garcia et al., 2014b; EFSA, 2010; Jacquiet et al., 2010; Lienard et al., 2011), and no gender predisposition was established (Alvarez-Garcia et al., 2014a; Goldman & Pipano, 1983; Gutierrez-Exposito et al., 2014; Waap et al., 2014), despite Jacquiet et al. (2010) has suggested that males are more susceptible than females, showing more acute clinical manifestations with a higher mortality rate. Bovine besnoitiosis occurs preferentially from spring to autumn with maximum clinical expression in summer (Ferrié, 1984; Legrand, 2003). This is coincident with the period of higher activity of hematophagous insects and some authors use this observation to rationalize the occurrence of mechanically transmission of *B. besnoiti* in nature (Alvarez-Garcia et al., 2013; Alvarez-Garcia et al., 2014b; Cortes et al., 2014). While agreeing that it might play a role in the epidemics of besnoitiosis, one has to consider that infection might be constant throughout the year, and that it is the climatic/environmental factors, like higher UV exposition and higher temperatures for example, that determine the severity of clinical signs in this time of year (Bigalke, 1968). In a recent longitudinal study of besnoitiosis seroprevalence that combined entomological survey of hematophagous flies in an endemic area, no clear relationship between peaks of positive seroconversions or antibody levels and stable fly activity measured by Vavoua traps was found (Lienard et al., 2011).

The first serological studies looking into the prevalence of *B. besnoiti* infection were done in Israel and South Africa and reported seroprevalences of around 50 to 60% for the first (Frank, Pipano & Rosenberg, 1977; Goldman & Pipano, 1983; Neuman, 1972) and around 50% for the latter (Janitschke, De Vos & Bigalke, 1984). Recently in Egypt animal prevalences of 13.7% were found (Ashmawy & Abu-Akkada, 2014), while in Jordan values of 6.0% individual animal prevalence and 26.7% herd prevalence were reported (Talafha, Al-Majali, Ababneh & Abutarbush, 2015). In Europe, cross-sectional studies investigating the prevalence and incidence of the disease are still sparse. Recent studies in endemic regions of Spain, determined individual animal prevalences of 16% in Navarra (Alvarez-Garcia et al., 2014a) and herd prevalences of 87.3%, with within-herd prevalences ranging from 15.1% to 95.7%, with a mean of approximately 50 % in the province of Huesca, Aragon (Gutierrez-Exposito et al., 2014). Similarly, another report from the Basilicata region of southern Italy (recently considered an endemic region) found a herd prevalence of 83.0% and an individual animal prevalence of 44.1% (Rinaldi et al., 2013). In Northern Greece, considered a non-endemic region, a prevalence of 22% of infected animals was found (Papadopoulos et al., 2014). In a wide survey in Portugal, covering the all continental country (i.e. extending far beyond the endemic region of Alentejo), Waap et al. (2014), determined a herd prevalence of 5.1 % in Portugal, while the prevalence of seropositive animals within infected herds ranged from less than 10.3% up to 72.4 %, with a mean of 33.0%. The lower intra-herd values may be explained by the recent introduction of infection in the herd or, considering an older introduction of infection, by a slower transmission of disease in herds through unknown epidemiological constrains. The high within-herd prevalence could be the result of a more successful transmission cycle or a long-year cohabitation with infection. Nevertheless, after the initial herd infection with *B. besnoiti*, the individual animal prevalence can rise from initial values of around 30-40% up to 70-90% in a matter of few months (Cortes et al., 2006b; Fernandez-Garcia et al., 2010; Lienard et al., 2011).

1.1.3 Clinical signs and pathogenesis

Bovine besnoitiosis is clinically characterized has having an acute stage, that starts with an initial febrile syndrome and usually evolves to the characteristic generalized edema or anasarca, and a chronic stage, that is characterized by the formation of numerous tissue cysts containing bradyzoites, leading to the typical scleroderma (Alvarez-Garcia et al., 2014b; Bigalke & Prozesky, 2004; Jacquiet et al., 2010; Lienard et al., 2011; Radostits, Gay, Hinchcliff & Constable, 2006). Jacquiet et al. (2010) and Pols (1960) refer an incubation period of 6 to 10 days, but experimental infections revealed an incubation period from 1 to 13

days, depending on the method of parasite inoculation (Basson, McCully & Bigalke, 1970; Bigalke, 1968; Bigalke & Prozesky, 2004). The mean incubation period of the experimental mechanical transmission of *B. besnoiti* by hematophagous flies accomplished by Bigalke (1968), was 13 days. In a recent study about the chronology of disease progression in naturally infected cattle (Gollnick et al., 2015), the authors proposed the assignment of specific clinical features to differentiate bovine besnoitiosis stages. Animals in the acute stage had to show fever (body temperature > 39.0 °C) or at least two of the following clinical signs/diagnoses: depression, conjunctivitis, subcutaneous edema, lymphadenitis or lameness. Cattle were classified as chronically infected when first parasitic cysts were visible in the scleral conjunctivae or mucous membranes. The time period between the acute and chronic stage is referred to as subacute stage of disease.

Hyperthermia is the first clinical sign and, as previously stated, marks the beginning of the acute phase. It may last for 3 to 10 days, can reach 42 °C (Alvarez-Garcia et al., 2014b; Jacquiet et al., 2010) and is accompanied by progressive inappetence, culminating in complete anorexia in severe cases, with consequent loss of weight, declining body condition and diminished milk production (Bigalke & Prozesky, 2004; Cortes et al., 2014). Affected animals are usually apathetic and prefer to lie down; they can show photophobia, serous nasal and ocular discharges, increased heart and respiratory rates, hyperemia of unpigmented skin and mucous membranes and swelling of superficial lymph nodes (Alvarez-Garcia et al., 2014b; Cortes et al., 2014; Gollnick et al., 2015; Pols, 1960). In this phase, the fast proliferating tachyzoites start invading macrophages, fibroblasts and endothelial cells within blood vessels, causing necrotic vascular lesions, vasculitis, and thrombosis, specially of capillaries and small veins in the dermis, subcutis fascia, testicles and upper respiratory mucosa (Alvarez-Garcia et al., 2014b; Cortes et al., 2014). They multiply by endodyogeny in the host cell cytoplasm and the released tachyzoites disseminate trough the blood stream (Pols, 1960) to infect new cells and repeat the parasite lytic cycle. Proliferative organisms may be detected in blood from the 3rd up to 12th day after the beginning of the febrile reaction (Basson et al., 1970; Bigalke, 1968; Pols, 1960). These cumulative lesions, together with a possible toxic effect of the parasite, cause an increased vascular permeability, resulting in edema and sometimes small hemorrhages and foci of necrosis in the afore-mentioned sites (Bigalke & Prozesky, 2004). The evolving anasarca may vary from a slight swelling of the face and thickening of the skin fold of the neck, back or chest regions, to a generalized subcutaneous edema covering the entire body. The anasarca often goes unnoticed and its presence is only revealed when fluid accumulates along the ventral part of the body, such as brisket, sternum, legs, udder, prepuce and scrotum. Animals may recover by the third week of the reaction, with the disappearance of the mentioned signs, or progress sequentially to the scleroderma stage (Bigalke & Prozesky, 2004). The edemas in joints cause painful

movements that may lead to permanent posterior lameness. In severe cases, edemas in alveolar and interstitial tissues in the lungs may also cause severe respiratory disorders and be accompanied by pneumonitis and emphysema (Alvarez-Garcia et al., 2014b). In males, an acute orchitis develops, with painful testes and transient or definitive infertility (Cortes et al., 2005; Dubey et al., 2013). In pregnant animals, this pathophysiological condition can lead to abortion (Alvarez-Garcia et al., 2014b; Cortes et al., 2014). Death may occur sometimes in the acute stage, usually by secondary complications like pneumonia or septicemia (Bigalke & Prozesky, 2004; Pols, 1960). In that matter, Dubey et al. (2013), reported the death of a bull in the acute stage of the disease due to a protein-losing nephrotic syndrome and posed the question of the role of protein-losing glomerulophaty in the pathogenesis of acute besnoitiosis anasarca.

The subsequent chronic stage is characterized by the formation of myriads of tissue cysts containing the slow replicating bradyzoites. These cyst can grow up to 0.6 mm (Pols, 1960) and are formed in the same tissues where the initial proliferation occurred, namely dermis, subcutis, upper respiratory tract, scleral conjunctiva, vestibulum vaginae and vagina, testis, connective tissues, muscles and sometimes spleen, liver, lung and heart (Basson et al., 1970; Olias et al., 2011). Recently, Frey et al. (2013) assessed the intra-organ parasite distribution in naturally infected cattle, in a subclinical stage (i.e., seropositive animals, that were clinically symptomatic in the acute stage, that had visible tissue cysts in the past that cleared by the time of slaughter and did not show evident chronic clinical signs). They showed that, in the six cows studied, the most frequent cyst locations were the upper respiratory tract (rhinarium, larynx/pharynx), followed by the distal genital tract (vulva/vagina) and the skin of the neck. Cyst formation occurs almost synchronously throughout different tissues (Bigalke, 1968) with the first ones being noticed in skin biopsies around the second week after the beginning of the acute stage (Dubey et al., 2013). At 4 to 5 weeks post-infection, tissue cysts measuring more than 100 µm can be found, and cyst development apparently reaches completion at 10 weeks post-infection (Basson et al., 1970). The small cysts visible by the naked eye in the scleral conjunctiva, vulvar region and nasal mucous tissues may be detected upon close visual inspection as early as 8 weeks post-infection (Alvarez-Garcia et al., 2014b; Cortes et al., 2014). The multifocal whitish pinpoint tissue cysts in the scleral conjunctiva are considered pathognomonic, representing a feature with considerable value in clinical diagnosis (Jacquiet et al., 2010; Rostaher et al., 2010; Sannusi, 1991). It is not certain if new tissue cysts are formed during the chronic stage and how long the cysts persist in cattle (Bigalke and Prozesky, 2004), but it is accepted that they last for years, probably the all lifespan of the animal (Cortes et al., 2014) Although some tissue cysts do degenerate and cause inflammation, there is no evidence that besnoitiosis can be reactivated from the chronic to acute stage (Bigalke and Prozesky, 2004).

The skin lesions that arouse in the chronic stage may be explained by the presence of numerous tissue cysts in the dermal and stratum papillare, accompanied by a granulomatous reaction and fibrosis surrounding the cysts. These events, together with vascular lesions that interfere with the skin blood supply, are responsible for alopecia (Bigalke, 1968). The most characteristic clinical sign is the scleroderma, with the progressive thickening, hardening and prominent folding and wrinkling of the skin, which may be widespread or more localized. Progressive alopecia, accompanied by dermatitis, and hyperkeratosis, hyperpigmentation and dermatitis crustosa also occurs (Bigalke & Prozesky, 2004; Cortes et al., 2014; Pols, 1960). Deep, raw fissures sometimes develop between folds of the skin in sites such as leg joints and bonny prominences. The superficial lymph nodes are swollen and usually a low-grade intermittent fever is present, causing further anorexia and weight loss (Bigalke & Prozesky, 2004).

In the male reproductive tract, the presence of tissue cysts in different locations may lead to permanent sterility. Dubey et al. (2013), reported a diffuse interstitial lymphoplasmocytic orchitis with an absence of spermatogenesis and the presence of cysts in the epididymis, in the testicles (scattered between the seminiferous tubules), and in bigger amounts in the pampiniform plexus. Back in 1981, a study with severely and chronically affected bulls in Nigeria reported severe orchitis in 64% of the animals (Kumi-Diaka, Wilson, Sanusi, Njoku & Osori, 1981). The authors found: several cysts of varying sizes in the scrotum displacing normal tissues; the tunicas (dartos, vaginalis and albuginea) were infiltrated by besnoitia cysts, fibrosis and some inflammatory cells; also, numerous cysts of varying sizes were observed in the testes and epididymis (interstitial tissues and seminiferous tubules) with accompanying inflammatory cellular infiltration of the interstitium; the testes and the testicular germinal epithelium were displaced and degenerated in many cases, with partial or complete arrest of spermatogenic activity. Cortes et al. (2005) also described severe anatomic and histological alteration on the teste of a chronically infected bull. The severity of lesions observed in testes and the consequent oligospermia/aspermia are the result of a combination of events: (1) reduced blood supply to the testes as the result of the development of cysts in the pampiniform plexus; (2) displacement and direct pressure on the germinal epithelial cells originated by the *Besnoitia* cysts that develop in the interstitial spaces and under the epithelial cells of the seminiferous tubules; this interferes with normal spermatogenesis and causes atrophy and necrosis of epididymal and/or testicular tissues; (3) poor heat exchange through the thickened acanthotic scrotum or partially devitalized scrotum (Alvarez-Garcia et al., 2014b; Kumi-Diaka et al., 1981).

Concerning the female reproductive tract, despite cysts have been found in the genital tract (Basso et al., 2013; Frey et al., 2013; Nobel et al., 1981), it is assumed that they do not interfere with normal functioning (Nobel et al., 1981). In fact, chronically infected cows may

become pregnant and successfully give birth to healthy calves that develop normally (Shkap, Pipano, Marcus & Krigel, 1994).

Death may also occur in the chronic stage of the disease regardless of the gender, and animals may be culled due to poor body condition (Pols, 1960). Some affected animals, however, seem to recover from these clinical manifestations, at least partially (Jacquiet et al., 2010), but they retain the large number of cysts (Cortes et al., 2014).

1.1.4 Diagnosis

Diagnosis of bovine besnoitiosis can be easily made when the animal presents the typical skin lesions of the chronic phase by demonstrating the presence of the parasite. The characteristic tissue cysts, may be observed macroscopically and can be confirmed by direct tests such as microscopy smears (scleral conjunctival cysts scraping) to identify bradyzoites (Sannusi, 1991), by histological/histopathological examination of skin biopsies to identify the typical cysts (Bigalke, 1968; Dubey et al., 2013; Pols, 1960; Sannusi, 1991) or by molecular techniques (PCR) to demonstrate the parasite DNA in tissue samples (Cortes et al., 2007b; Schares et al., 2011b). However, typical clinical signs are not always present neither are skins cysts. At the acute, short lasting stage, clinical signs are very unspecific and can be easily confused with other diseases, such as blue tongue or malignant catarrhal fever (EFSA, 2010; Jacquiet et al., 2010); there are no detectable parasite stages in tissues and anti-*B. besnoiti* antibodies will only be present around 2 weeks after the onset of clinical signs (Jacquiet et al., 2010; Schares et al., 2011b). Passing the acute stage, not all animal evolve to the typical chronic stage, but maintain a sub-clinical infection (Bigalke, 1968; Pols, 1960). These animals may play an important role in the epidemiology of the disease, as previously discussed, and represent the bigger challenge for diagnostic. For sub-clinically infected animals, histopathology is not suitable, and more sensitive methods such as PCR are required (Cortes et al., 2014). Nevertheless, sub-clinically infected animals with a low number of cysts in the skin may still not present *B. besnoiti* DNA in the sample and might be PCR negative (Schares et al., 2011b). Despite this limitation, PCR was shown to be appropriate for the diagnosis of bovine besnoitiosis in the acute stage of the disease, during which no anti-*B. besnoiti* antibodies are found yet (Cortes et al., 2014; Schares et al., 2013).

Assessment of antibodies against *B. besnoiti* in sera of infected animals is an appropriate approach for the diagnosis of clinical and subclinical disease. A number of tests have been developed so far, including indirect fluorescence antibody test (IFAT) (Cortes et al., 2006a; Goldman & Pipano, 1983; Janitschke et al., 1984; Schares et al., 2010; Shkap et al., 2002), commercial enzyme linked immunosorbent assay (ELISA) (ID Screen® Besnoitia, ID.vet,

France; PrioCHECK® Besnoitia Ab, Prionics, Switzerland), in-house ELISAs (Cortes et al., 2006a; Fernandez-Garcia et al., 2010; Janitschke et al., 1984; Shkap, Ungar-Waron, Pipano & Greenblatt, 1984), western blot (Cortes et al., 2006a; Fernandez-Garcia et al., 2009a; Schares et al., 2010) and modified agglutination test (MAT) (Waap et al., 2011). Although all tests available perform well during the chronic phase of the disease, they show low sensitivity during the acute stage, since detectable antibody levels have not yet developed. Additionally serological tests may yield a low percentage of chronically infected animals undiagnosed as a result of a low level of circulating antibodies and the possible presence of seronegative animals with tissue cysts (Fernandez-Garcia et al., 2010; Garcia-Lunar et al., 2013a; Schares et al., 2010). Furthermore, specificity of this tests, especially ELISA, have been questioned when used routinely in diagnostics with field samples. Recently in Italy, Gazzonis et al. (2014), detected 712 animals (22.7%) with a positive reaction in ELISA, but only 10 of them (0.3%) were confirmed by WB, while in Australia, testing with the PrioCHECK® initially revealed 160 (18,4%) positive animals, with none being confirmed by immunoblot and IFAT (Nasir, Lanyon, Schares, Anderson & Reichel, 2012).

The IFAT, when performed with the appropriate expertise, shows no cross-reaction with *N. caninum* or *T. gondii* antibodies. The cut-off dilution for *Besnoitia* IFAT ranges from 1/200 to 1/256. Western blotting is clearly more expensive and time consuming compared with IFAT. It may show cross-reactions with related protozoans, especially *N. caninum*, but at a lower extent than ELISA and is usually recommended as a confirmation test in combination with other methods (Cortes et al., 2006a; Garcia-Lunar et al., 2013a; Millan et al., 2012; Schares et al., 2010; Schares et al., 2011a). The ELISA is more suitable for analyzing larger numbers of samples, but, for the serological diagnosis of individual cases at least one other confirmation test, IFAT or western blot, should be used (Garcia-Lunar et al., 2013a; Jacquiet et al., 2010). Recently, Schares et al. (2013) described an ELISA (using immune affinity chromatography purified *B. besnoiti* antigens), with 100% sensitivity for sera of chronically infected animals, and 99.8% specificity when tested with positive *N. caninum* sera. False positive results can thus be avoided and the time consuming and expensive confirmatory tests are no longer necessary, but the added usefulness of this test, compared to existing ones, still has to be proven with routine field samples. Nevertheless, the author was able to identify acute cases of bovine besnoitiosis by the detection of low avidity IgG in this ELISA. The direct agglutination test, based on the complex formation of formalin-treated parasites in the presence specific IgG antibodies, is also very suitable for the screening of large amounts of samples and is relatively inexpensive. It offers the major advantage of avoiding the use of secondary specie-specific antibodies, which turns this technique particularly attractive for epidemiological purposes, by allowing testing a large variety of species that may be implicated in the life cycle of this protozoan parasite (Waap et al., 2011).

1.1.5 Treatment, vaccines and control measures

Presently there is no specific treatment for bovine besnoitiosis (Alvarez-Garcia et al., 2013; Cortes et al., 2014; EFSA, 2010; Jacquiet et al., 2010). No suitable drug is available, despite the testing of several compounds in experimentally infected rabbits, gerbils and cattle (Pols, 1960; Shkap, De Waal & Potgieter, 1985; Shkap, Pipano & Ungar-Waron, 1987a). Affected animals should receive supportive therapy and be treated symptomatically (Bigalke & Prozesky, 2004; Radostits et al., 2006).

A live vaccine against bovine besnoitiosis was developed in the Onderstepoort Veterinary Institute, South Africa (Bigalke, Basson, McCully, Bosman & Schoeman, 1974a; Bigalke, Schoeman & McCully, 1974b), and has been in use there since 1974. It is administered subcutaneously to weaners or older animals and each dose contains approximately 10^6 organisms of a blue wildebeest strain of *B. besnoiti* grown in cell culture. A single dose was shown to protect cattle from the clinical form of the disease for observation periods of up to 4 years, but did not entirely prevent subclinical infection from taking place. Annual re-vaccination for 2 to 3 years is recommended (Bigalke, 1981). Israel also uses a live vaccine derived from attenuated cell cultured parasites (Pipano, 1997), but no information regarding its safety or efficacy is found in scientific literature. However, the use of any of these vaccines is geographically limited, because live attenuated vaccines pose a risk of introducing the parasite into uninfected herds and of inducing carriers among the vaccinated animals. This is of particular concern when, as in the case of *B. besnoiti*, the knowledge about the biology, transmission and life cycle of the parasite is scarce (Cortes et al., 2014).

In this way, with the lack of treatment and effective vaccine, this Europe's reemerging disease can only be battled, at least for now, with the implementation of sanitary control measures, summarized by Jacquiet et al. (2010). First, to prevent introduction of *B. besnoiti* to non-endemic areas, all purchased/imported cattle from endemic areas should be tested serologically before or at purchase and ideally, the analysis should be repeated after 3 to 4 weeks to discard false negative results due to very recent infections (Basso et al., 2013). Second, in the case of an outbreak in a previously *Besnoitia*-free herd, infected animals should be separated from non-infected ones, and skin and stable insecticides and/or traps should be used to minimize the fly population around the herd (Mehlhorn et al., 2009); a rapid and systematic evaluation of individual infection status should be done in order to cull all seropositive animals as soon as possible. Third, in endemic areas, herds should adopt regular programs to control fly population (for example with regular application of pyrethroid insecticides) and also evaluate individual infection status to be able to systematically eliminate positive ones. With several years of systematic elimination of chronically infected cattle, Bigalke (1968) noted a reduction of new infections. Finally, an intensive information

campaign is necessary for veterinarians and for cattle breeders, especially in the regions of recent emergence.

In this way, the gap of knowledge around *B. besnoiti*, not only in some fundamental aspects of its biology, but also in the possible means to address the disease it causes prompted us to focus our studies in this parasite. In particular, considering recent reports on other Apicomplexa that refer protein disulfide isomerase (PDI) as a potential target for drug or vaccine formulation, we concentrated our studies in this protein, hoping to understand its role in the biology of *B. besnoiti* and if it can be addressed for besnoitiosis therapy.

1.2 Protein disulfide isomerase

Protein disulfide isomerase (PDI) is a multifunctional protein that acts as a thiol–disulfide oxidoreductase, being able to catalyze disulfide bond formation, breakage and rearrangement in all non-native protein and peptide substrates reported to date, depending on the redox state of its cysteine containing active sites (Hatahet & Ruddock, 2009; Wang et al., 2015). Independently of its redox activity, PDI can also act as a chaperone, catalyzing the correct folding of newly synthesized or denatured proteins into their native conformations (Kozlov, Määttänen, Thomas & Gehring, 2010). The sulfhydryl (-SH) side group (thiol) of cysteine is unique among amino acids, because when two cysteines are within close proximity, a disulfide bond can form. Disulfide bond is a covalent linkage that requires the deprotonation of one cysteine thiol and donation of two electrons to an acceptor such as oxygen. Disulfide bond formation is oxidation, breaking of a disulfide bond is reduction, and rearrangement (simultaneous reduction and oxidation) of a bond within a protein is isomerization; these processes are dictated by the local redox conditions (Sevier & Kaiser, 2002) (Figure 6). Disulfide bonds serve important functions during protein folding and in stabilizing protein structures (both intra or intermolecular) and have the capacity to work as regulatory switches in redox signaling (Appenzeller-Herzog & Ellgaard, 2008).

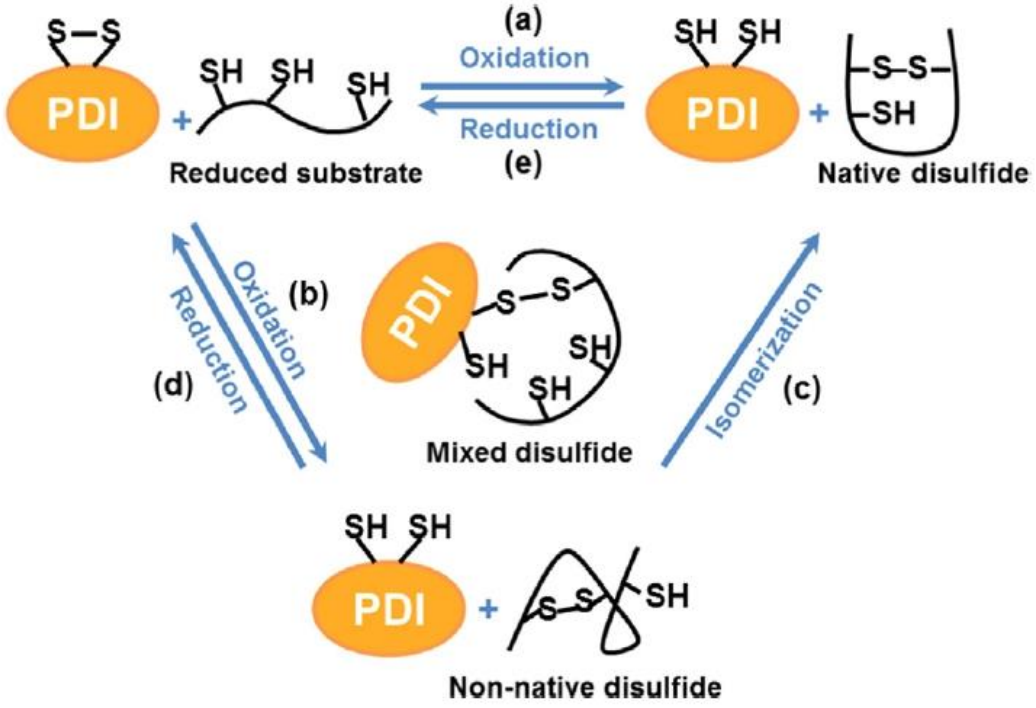

Figure 6. Schematic representation of thiol–disulfide interchange reactions catalyzed by PDI. PDI-catalyzed disulfide formation occurs when oxidizing equivalents are transferred from the active site of oxidized PDI to reduced substrate, resulting in either a native disulfide (a) or a nonnative disulfide (b) in the substrate and reduced PDI. The mispaired disulfide can be isomerized by reduced PDI through direct intramolecular disulfide rearrangement (c). Alternatively, the nonnative disulfide can be converted to the native one via the pathway of reduction (d) and reoxidation (a). In some cases, the disulfide inactive substrate can be reduced by reduced PDI (e) to facilitate substrate unfolding. Note that in all the thiol–disulfide interchange reactions, a mixed disulfide is formed between the N-terminal cysteine in the PDI active site and a cysteine in the substrate. Only one catalytic domain of PDI is shown for simplicity. (reprinted from Wang, Wang & Wang, 2015, with permission from Elsevier)

The first description of PDI-like activity was simultaneously published by two independent groups in the early sixties of the twentieth century, one working with pigeon and chicken pancreatic extracts (Venetianer & Straub, 1963b) and the other with rat and bovine liver microssomes (Goldberger, Epstein & Anfinsen, 1963). It was the first folding catalyst to be reported half a century ago, back then in studies with the reactivation of reduced enzymes, mainly ribonuclease (RNase) and lysozyme (Givol, Goldberger & Anfinsen, 1964; Goldberger et al., 1963; Goldberger, Epstein & Anfinsen, 1964; Venetianer & Straub, 1963a, 1963b). PDI was first purified in 1966 from bovine liver microssomes, and described as “an enzyme which catalyzes sulfhydryl-disulfide interchange in proteins” (De Lorenzo, Goldberger, Steers, Givol & Anfinsen, 1966). It was given the enzyme classification number EC 5.3.4.1 in 1972, along with its subsequent officially accepted name – protein disulfide isomerase, a name that was mentioned for the first time in a publication of Hawkins and Freedman in 1975. However,

despite the concurrent identification of other substrates of PDI *in vitro*, including soybean trypsin inhibitor (Steiner, De Lorenzo & Anfinsen, 1965), immunoglobulins (De Lorenzo et al., 1966; Murkofsky & Lamm, 1979; Roth & Koshland, 1981), vasopressin and oxytocin (Varandani, Nafz & Chandler, 1975), insulin (Varandani & Nafz, 1970; Varandani et al., 1975), bovine serum albumin (Teale & Benjamin, 1976), cholera toxin (Moss, Stanley, Morin & Dixon, 1980), bovine pancreatic trypsin inhibitor (Creighton, 1992), ricin (Barbieri, Battelli & Stirpe, 1982), and procollagen (Forster & Freedman, 1984; Wilson, Lees & Bulleid, 1998), the importance of this enzyme only started to be recognized about 20 years later, in the mid-eighties, with the publication of the first highly cited review specifically on PDI (Freedman, 1984) and the sequence of rat PDI (Edman, Ellis, Blacher, Roth & Rutter, 1985). In addition, the subcellular localization of PDI to the ER was confirmed (Lambert & Freedman, 1985) and its detailed action in catalyzing a specific folding pathway was defined (Creighton, Hillson & Freedman, 1980). However, the accurate identification of the domain boundaries took almost another 20 years. The domain structure of PDI has been determined by theoretical (Ferrari & Soling, 1999) and experimental (Alanen et al., 2003; Denisov et al., 2009; Dong, Wearsch, Peaper, Cresswell & Reinisch, 2009; Freedman et al., 1998; Kemmink, Darby, Dijkstra, Nilges & Creighton, 1997; Kemmink, Darby, Dijkstra, Scheek & Creighton, 1995; Kemmink, Darby, Dijkstra, Nilges & Creighton, 1996; Kemmink et al., 1999; Kozlov et al., 2006; Nguyen et al., 2008; Tian, Xiang, Noiva, Lennarz & Schindelin, 2006) procedures and it is now known to comprise four thioredoxin-like domains in the order of **a**, **b**, **b'**, and **a'**, with a C-terminal acidic extension **c** and an **x**-linker between domains **b'** and **a'** (Alanen et al., 2003; Hatahet & Ruddock, 2009; Pirneskoski et al., 2004; Wang et al., 2015) (Figure 7). The thioredoxin-like domains can be catalytic or noncatalytic and are identified as either **a** or **b**-type domains based on the presence or absence of a catalytic motif, with use of the prime symbol to indicate their position in the protein. The **a**-type domains functionally resemble thioredoxin and usually contain two cysteines in a CXXC tetrapeptide active-site motif with an intermediate GH sequence being the most common in the PDIs. The **b**-type domains, although structurally similar to thioredoxin, do not contain catalytically active cysteines and therefore are not redox active (Appenzeller-Herzog & Ellgaard, 2008; Kozlov et al., 2010). Instead, the **b**-type domains appear to act as spacers, and are likely responsible for substrate binding and specificity (Byrne et al., 2009; Denisov et al., 2009; Klappa, Ruddock, Darby & Freedman, 1998a; Pirneskoski et al., 2004). Generally, the sequence identity is much higher for catalytic a-type domains than for non-catalytic **b**-type domains since the latter are missing a number of residues important for catalysis that are conserved among the **a** domains (Appenzeller-Herzog & Ellgaard, 2008; Hatahet & Ruddock, 2009).

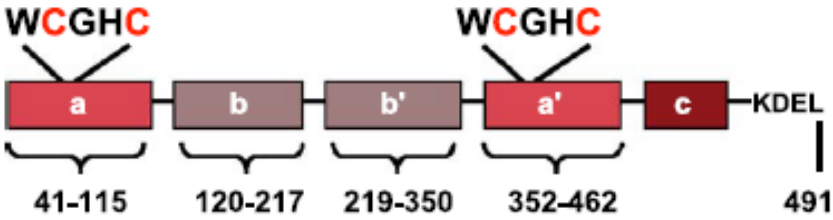

Figure 7. Domain organization of rat PDI.
Domain boundaries are shown in brackets below each domain. Endoplasmic reticulum retention signal KDEL is identified as well as the active sites (CGHC) in the a-type domains. (reprinted from Wilkinson & Gilbert, 2004, with permission from Elsevier)

PDI is found in all multicellular organisms, including higher plants, and is expressed in yeast but not bacteria. Instead, bacteria have homologous Dsb proteins to facilitate oxidative protein folding and isomerization in the periplasmic space (Arts et al., 2013; Inaba, 2009). It is regarded that all eukaryotes possess a species-dependent PDI family of enzymes (Pirneskoski et al., 2004), that share a common structural feature of having at least one thioredoxin-like domain (Kozlov et al., 2010; Wang et al., 2015). For example, in *Saccharomyces cerevisiae* there are five PDI family members (Pdi1p, Mpd1p, Mpd2p, Eug1p, and Eps1p) (Xiao et al., 2004), while in humans more than 20 members have been characterized so far (Appenzeller-Herzog & Ellgaard, 2008; Benham, 2012; Kozlov et al., 2010). It is the modular combinations of thioredoxin-like catalytic and noncatalytic domains that give rise to structural and functional differences of PDI family members (Benham, 2012). A thioredoxin-like domain has been loosely defined as anything with a predicted thioredoxin-like structure. However, we could define it more specifically as a domain capable of reacting with cysteines. This definition appears to make the most sense, as members of the disulfide isomerase family should be capable of reacting with cysteine side chains. Nevertheless, the founding member of this family, PDI, also exhibits non-specific polypeptide-binding chaperone activity, and thus, a more inclusive definition of PDI family members would comprise proteins that contain non-thiol-reactive thioredoxin-like domains with chaperone-like activities for ER folding and secretion of proteins (Kozlov et al., 2010).

Thioredoxin is a small, ubiquitous protein of approximately 12 kDa involved in several cellular activities (Holmgren, 1985, 1989). In addition to being the founding member of the thioredoxin superfamily of proteins, the structure of thioredoxin forms the foundation of a protein fold known as the thioredoxin fold, that is found in a wide range of proteins including thioredoxin, DsbA, PDI, glutathione peroxidase, glutathione S-transferase, arsenate reductase, calsequestrin, and circadian oscillation regulator, many of which are involved in

thiol metabolism or use thiol-based chemistry (Hatahet & Ruddock, 2009). The thioredoxin fold is characterized by the following sequence of α-helices and β-strands: β1 – α1 – β2 – α2 – β3 – β4 –α3. It forms a three-layer α – β – α structure with two α-helices packing onto one side of a four stranded β-sheet, of which strand three is antiparallel to the others, and with an additional α-helix packing onto the other side of the β-sheet (Ferrari & Soling, 1999; Hatahet & Ruddock, 2009). Nevertheless, although the thioredoxin fold is defined by these three α -helices and four β-strands core, this is not the structure of thioredoxins, which have an additional β – α before the thioredoxin fold and thus a β0 – α0 – β1 – α1 – β2 – α2 – β3 – β4 – α3 structure (Figure 8) (Forman-Kay, Clore, Wingfield & Gronenborn, 1991; Holmgren, Soderberg, Eklund & Branden, 1975; Katti, LeMaster & Eklund, 1990).

Crystallization studies of full-length *S. cerevisiae* PDI provided the first direct evidence of the long suspected PDI flexibility, since in yeast PDI, crystals grown at different temperatures exhibits two opposite forms: (1) at 4 °C, the four thioredoxin domains are arranged in the shape of a twisted ‘‘U’’, with the active sites facing each other (a-type domains) across the long sides of the ‘‘U’’, the inside surface of the ‘‘U’’ (b-type domains) acts as a rigid base and is enriched in hydrophobic residues, that facilitate interactions with misfolded proteins (Tian et al., 2006); (2) at 22 °C, yeast PDI shows a “boat-shaped” form, as the result of the drastic rotation and translation of the **a** domain, preventing the two active sites from facing each other (Tian et al., 2008). This extended conformation was already previously suggested by Solovyov and Gilbert (2004) for the rat PDI. PDI must be a highly flexible enzyme in order to accommodate its structurally diverse substrates, of varied sizes and conformations (e.g. procollagen and insulin), in a way that permits the access of the active sites to the critical substrate thiols (Laurindo, Pescatore & Fernandes, 2012). An analysis of the inter-domain contacts in the two crystal structures suggests that PDI is a molecule with two flexible arms, represented by the a-type domains, that are connected to a relatively rigid base formed by the b-type domains module (Tian et al., 2008; Tian et al., 2006). Tian et al. (2008) showed that restricting the flexibility of the **a** domain results in a 60% decrease in enzymatic activity, whereas restricting the flexibility of the **a’** domain results in only 24% reduction. They identified the junction between domains **a** and **b** as a major site of flexibility and suggested that the loops connecting the **a** and **a’** domains to the **b**-**b’** base enable the yeast PDI to facilitate these conformational changes. Nevertheless, a recent work about the plasticity of human PDI, indicated that, in contrast to the situation reported in yeast PDI, the C-terminal half (**b’** and **a’** domains and x-linker region) is more flexible than the N-terminal half (**a** and **b** domains) of the protein (Wang et al., 2010a). The x-linker region, allows the human PDI to partially swing into and out of the binding site on the **b’** domain, originating capped and uncapped conformers of PDI, which respectively restrict or allow substrate accessibility and binding (Nguyen et al., 2008; Wang et al., 2010a).

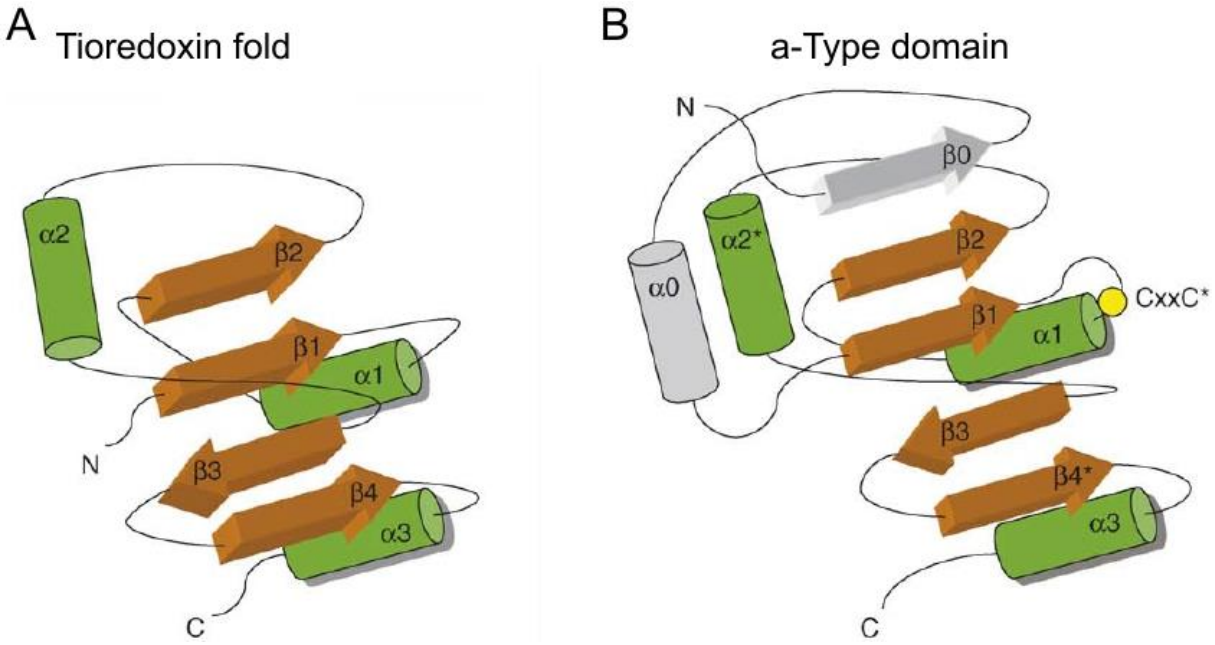

Figure 8. Schematic representation of the organization of the thioredoxin fold and PDI domains. (A) The thioredoxin fold incorporates an N-terminal βαβ motif, β1-α1-β2, that is connected to a C-terminal ββα motif, β3-β4-α3, by a loop containing a third α-helix, α2. (B) Thioredoxin structure is represented by a thioredoxin-fold organization that includes an extra N-terminal βα motif, β0-α0 (gray); A a-type domain is represented here with the position of the active site motif CxxC indicated by the yellow sphere. α Helices are shown in green and β sheets in orange. (adapted from Gruber, Cemazar, Heras, Martin & Craik, 2006, with permission from Elsevier)

PDI is ubiquitously expressed in most tissues and cell types of mammals, especially in tissues active in the synthesis and secretion of disulfide-bonded proteins, namely liver and pancreas (Hillson, Lambert & Freedman, 1984). It is retained in the ER by a C-terminal ER retention signal, usually the KDEL sequence in mammalian cell or the HDEL sequence in *S. cerevisiae*, but other closely related sequences can occur (Alanen, Raykhel, Luukas, Salo & Ruddock, 2011; Pelham, 1990). In this way, PDI is primarily located in the ER lumen where its concentration can approach the millimolar range, although it has also been found in other intracellular compartments, such as mitochondria, nucleus, cytosol, and even at the cell surface and extracellular space (Noiva, 1999; Turano, Coppari, Altieri & Ferraro, 2002). There are increasingly evidences of the importance of chaperones and protein folding catalysts in cell signaling (Ellis, 2006; Henderson & Pockley, 2010; Lee, Park, Kang & Ahn, 2009). Although it is unclear how PDI escapes the ER, the role of PDI in cell surface has been documented in several biological functions (Jordan & Gibbins, 2006; Turano et al., 2002) such as platelet activation and aggregation (Essex, 2009; Essex & Li, 2003), thrombus formation (Cho, Furie, Coughlin & Furie, 2008; Jasuja, Furie & Furie, 2010; Popescu, Lupu &

Lupu, 2010; Reinhardt et al., 2008), thyroid hormone synthesis (Delom, Mallet, Carayon & Lejeune, 2001), leukocytes adhesion (Bennett, Edwards, Sklar & Rogelj, 2000) and lymphocyte migration (Bi, Hong, Lee & Baum, 2011). In addition, PDI and PDI family members are directly and indirectly involved with the immune system function. Indirectly, because they are responsible by the ER quality control of proteins that are involved in immune defense, like the major histocompatibility complex (MHC) molecule (Kang, Park, Oh, Cho & Ahn, 2009; Kim et al., 2009; Lee et al., 2009; Peaper & Cresswell, 2008; Raghavan, Del Cid, Rizvi & Peters, 2008) or immunoglobulins (Ig) (Anelli et al., 2002; Anelli et al., 2007). Directly, since PDI has been ascribed a role in internalization of pathogens such as viruses, as was documented for the *in vitro*, cell cultured, infection of polyomavirus (Gilbert, Ou, Silver & Benjamin, 2006), Newcastle disease virus (Jain, McGinnes & Morrison, 2007, 2008, 2009), Dengue Virus (Diwaker, Mishra, Ganju & Singh, 2015) and HIV (Bi et al., 2011; Gallina et al., 2002; Markovic et al., 2004; Ryser, Levy, Mandel & DiSciullo, 1994), or chlamydia, where native PDI on the cell surface was proven to be required for the effective attachment of this obligate intracellular bacterial pathogen of eukaryotic cells (Abromaitis & Stephens, 2009; Conant & Stephens, 2007; Davis, Raulston & Wyrick, 2002). Moreover, PDI family enzymes are emerging as important players in other processes that require changes in cell adhesion or migration, such as cancer (Benham, 2012). PDI has been implicated in the survival and progression of some cancer cells, such as malignant glioma (Goplen et al., 2006), breast cancer (Lee, Lim, Cheong, Singh & Gam, 2012), ovarian cancer (Xu et al., 2012; Zhao et al., 2015) and prostate cancer (Welsh et al., 2001) by compensating for endoplasmic reticulum stress with the upregulation of the protein-folding capacity (Yu et al., 2014). It has been suggested as a potential drug target for chemotherapy in several tumors (Xu, Sankar & Neamati, 2014), including melanomas (Lovat et al., 2008), lung adenocarcinomas (Tufo et al., 2014) and breast cancer (Chen, Chang, Wu, Kung & Wu, 2015).

Also, PDI family enzymes are being implicated in the pathogenesis of many neurodegenerative diseases that are collectively classified as protein misfolding disorders (PMDs), including Parkinson's and Alzheimer's disease, Huntington's disease, prion related disorders and amyotrophic lateral sclerosis (Andreu, Woehlbier, Torres & Hetz, 2012; Benham, 2012; Conway & Harris, 2015). PMDs are diseases where at least one protein or peptide has been shown to misfold, aggregate, and accumulate in tissues where the disease-specific damage occurs (Mukherjee, Morales-Scheihing, Butler & Soto, 2015; Soto, 2003), and, besides the neurodegenerative disorders, include other systemic ones such as the highly prevalent type II diabetes (Mukherjee et al., 2015). These diseases are generally related with aging and a progressive decline and failure of cellular protein folding and quality control systems (Andreu et al., 2012; Cuanalo-Contreras, Mukherjee & Soto, 2013). Misfolded proteins can lose their function and accumulate as large, insoluble aggregates that

interfere with cell function, result in inherent cytotoxicity and may originate tissue inflammation (Soto, 2003; Wilkinson & Gilbert, 2004).

1.2.1 Protein disulfide isomerase in apicomplexan parasites

Although much as been learned about PDIs in higher eukaryotes, limited information is available regarding PDIs in protozoa parasites. Nevertheless, growing interest in this family of proteins has led to significant advances recently. Hitherto, several PDIs and PDI-like enzymes were identified in protozoan parasites, specifically in *N. caninum* (Lee et al., 2003; Liao et al., 2006; Liao et al., 2005; Naguleswaran et al., 2005), *T. gondii* (Meek, Back, Klaren, Speijer & Peek, 2002a; Meek, Back, Klaren, Speijer & Peek, 2002b), *P. falciparum* (Florenta et al., 2000; Mahajan et al., 2006; Mouray et al., 2007), *P. chabaudi* (Novo, Martins, Prata, Lopes & Armada, 2009), *P. vivax*, *P. knowlesi* and *P. berghei* (Mahajan et al., 2006), *P. yoelli* (Carlton et al., 2002), *L. donovani* (Padilla et al., 2003), *L. major* (Achour, Chenik, Louzir & Dellagi, 2002), *L. amazonensis* (Hong & Soong, 2008), *Theileria parva* (Ebel, Bender, Bocskor, Binder & Lipp, 2002), *Cryptosporidium parvum* (Blunt, Montelone, Upton & Khramtsov, 1996), *Eimeria tenella* (Han et al., 2014), *Giardia lamblia* (Gillin et al., 1990; Knodler et al., 1999), *Trypanosoma brucei* (Rubotham, Woods, Garcia-Salcedo, Pays & Nolan, 2005) and *Babesia caballi* (Ikadai et al., 2005). The functional importance of PDI should be the same for these parasites as previously described for other eukaryotes, as well as the primary ER localization. Nevertheless, it was shown that PDI is also present at the surface of *N. caninum* (Naguleswaran et al., 2005), *T. gondii* (Meek et al., 2002a), *P. falciparum* (Florenta et al., 2000), *L. donovani* (Padilla et al., 2003) and *T. parva* (Ebel et al., 2002) and that it is present in the excretory-secretory compartment of *N. caninum* (Liao et al., 2006), *T. gondii* (Zhou et al., 2005) and *P. berghei* (Lal et al., 2009). In apicomplexans the secretory products have been implicated in parasite host cell entry, intracellular survival, and interaction with the host (Carruthers, 1999, 2002; Zhou et al., 2005) and several studies demonstrate that inhibition of PDI has a negative correlation with parasite *in vitro* proliferation. This was shown for *N. caninum* by Naguleswaran et al. (2005) and Liao et al. (2006), as the preincubation of *N. caninum* tachyzoites with PDI inhibitors (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), p-chloromercuribenzoic acid (pCMBA), tocinoic acid and bacitracin) and anti-PDI antibodies resulted in decreased adhesion of tachyzoites to the host cell, with consequence inhibitory effect on the growth of the parasite. In *L. amazonensis* promastigotes, incubation of parasites with bacitracin and pCMBA lead to a decreased number of viable parasites, where pCMBA concentrations above 0.5 mM were able to kill all promastigotes in the first 24 hours (Hong & Soong, 2008). Similar result were obtained with

L. major (Achour et al., 2002). Also, the action of the broad spectrum anti-parasitic (and anti-viral) drug nitazoxanide (NTZ), and some derivatives lacking the nitro group, was linked to PDI inhibition, since affinity chromatography with thiazolide-coupled-resin binds *N. caninum* PDI and these compounds also inhibited the enzymatic activity of recombinant *N. caninum* PDI (Muller, Naguleswaran, Muller & Hemphill, 2008). NTZ and some non-nitro-thiazolides compounds efficiently inhibit the *in vitro* propagation of *N. caninum* (Esposito et al., 2005), *B. besnoiti* (Cortes et al., 2007a), *T. gondii* (Galvan-Ramirez Mde et al., 2013), *G. lamblia* (Adagu, Nolder, Warhurst & Rossignol, 2002; Muller, Ruhle, Muller, Rossignol & Hemphill, 2006) and *Cryptosporidium parvum* (Gargala et al., 2000; Giacometti, Cirioni, Barchiesi, Ancarani & Scalise, 2000; Theodos, Griffiths, D'Onfro, Fairfield & Tzipori, 1998). Similarly, affinity chromatography showed that the antiplasmodial compound DS61 (1,4-bis(3-[N-(cyclohexylmethyl) amino]propyl)piperazine) targets *P. falciparum* PDI (Florenta et al., 2000).

The relevance of PDI was further strengthened with the description of *T. gondii* PDI as a major target for naturally occurring human IgA antibodies secreted at mucosal sites (Meek et al., 2002a). Secretory IgA (sIgA) antibodies, represent the humoral component of the mucosal immune system (MIS) and serve as the first line of defense against pathogens, by preventing adhesion and entry into the epithelium, and facilitating their clearance by peristaltic or mucociliary movements (Corthesy, 2009; Mantis & Forbes, 2010), in a process called immune exclusion. By recognizing polyvalent antigens on the surfaces of pathogens, sIgA are able to agglutinate them and retard or abolish the ability of the pathogen to adhere to and/or penetrate the epithelium (Strugnell & Wijburg, 2010). Meek, Klaren, van Haeringen, Kijlstra and Peek (2000), showed that there is an anti-*T. gondii* IgA response in human tears against this ubiquitous parasite, with 81 % of the tear samples tested recognizing *T. gondii* antigens. The authors found no relation between the presence of anti-*T. gondii* IgA in tears and chronic infection, since only 23% of individuals had evidence of systemic immunity against the parasite and suggested that these antibodies might belong to the natural antibody repertoire. In subsequent studies, Meek et al. (2002a) identified the major 49 kDa antigen as PDI and determined that the IgA epitopes recognize conserved regions of this enzyme and are able to cross react with other apicomplexan PDIs, namely *P. falciparum* (Meek et al., 2002b). Also, they demonstrated the presence of anti-*T. gondii*-PDI sIgA in human milk and in tears of, very likely, *T. gondii* naïve infants, facts that further support the notion that anti-PDI sIgA form part of the natural immune memory (Meek et al., 2002b). Likewise, *N. caninum*-PDI is also targeted by sIgA in bovine tears, suggesting that PDI-specific antibodies constitute part of the mucosal antibody repertoire possibly involved in the defense against protozoan parasites (Liao et al., 2006). In addition, works on the analysis of *N. caninum* and *T. gondii* antigenic proteins recognized several immunodominant antigens associated with the processes of invasion, proliferation and egress of apicomplexan

parasites, being PDI one of the constantly identified antigens (Ma et al., 2009; Shin et al., 2004; Shin et al., 2005; Zhou et al., 2014). A recent Portuguese study in patients with acute malaria (*P. falciparum*), also detected PDI as one of the major immunogenic proteins (Costa, Nogueira, de Sousa, Vitorino & Silva, 2013). In *Leishmania* species, PDI is suspected to have an important role in the virulence of these parasites (Achour et al., 2002; Amit et al., 2014). Furthermore, recent works on neosporosis, applying the acute disease mouse model, determined 90% protection against disease and a significant reduction of cerebral parasite burden and numbers of viable tachyzoites in brain tissue, when mice were intranasally vaccinated with recombinant *N. caninum* PDI (Debache, Guionaud, Alaeddine & Hemphill, 2010). Interestingly, intraperitoneal vaccination was ineffective (Debache et al., 2010; Debache et al., 2011), suggesting that the profile and/or homing of the immune response elicited are critical for this protective effect (Hemphill et al., 2013).

In conclusion, all these evidences together further support the importance of PDI in apicomplexan parasites and in the host immune response against them. First, because of PDI fundamental role in the ER quality control by assuring the correct folding of proteins; Misfolded protein can lose their function, form aggregates and interfere with cell function. The obligate intracellular apicomplexan parasites depend on their ability to interact with the host cell for survival and this interaction is in a major part achieved by the sequentially discharge of their secretory organelles, which contain a vast set of proteins that are, obviously, essential for this process. One point that differentiates apicomplexan parasites from other intracellular pathogens, is that invasion of the host cell is a parasite driven and not a host cell driven process, fact that puts more pressure on the parasite for quality control of essential proteins and renders it more vulnerable for aiming these proteins as drug targets. Second, because of PDI explicit engagement in the host cell adhesion/invasion process, either directly, through an undescribed direct interaction with the host cell, or indirectly, by guarantee the adequate redox state of extracellular environment and correct folding of secreted proteins. Third, because PDI seems to be an immunodominant antigen that plays an important role in host immune response

In this way, and since the disruption of PDI function (either through drug inhibition or anti-PDI antibodies) was shown to negatively affect parasite growth, this protein has been proposed as a suitable target for anti-protozoan drugs and for protozoa vaccine development (Achour et al., 2002; Amit et al., 2014; Florenta et al., 2000; Han et al., 2014; Hemphill et al., 2013; Hong & Soong, 2008; Liao et al., 2006; Mouray et al., 2007; Naguleswaran et al., 2005; Novo et al., 2009).

1.3 Aim of the work

Currently, it is generally accepted that PDI is present at the surface of apicomplexan parasites, where it participates in the host cell adhesion/invasion process. In addition, PDI has been identified as a major immunogenic protein in several parasitic diseases and also as a target of the natural antibody repertoire. Moreover it was shown that interference with PDI function has a negative impact in the growth of several apicomplexan parasites, especially in the closely related *N. caninum* and *T. gondii*. In this way, we hypothesize that *B. besnoiti* PDI plays a crucial role on host cell invasion and in bovine response to infection and that it can be, in agreement with what was proposed by several authors, a promissory drug target. The main purpose of this work was to characterize the enzymatic activity of *B. besnoiti* PDI and to evaluate its role in parasite-host cell interaction and in the bovine immune response. For this we identify the following objectives:

- Isolation and sequencing of *B. besnoiti* PDI cDNA.
- Cloning and heterologous expression of recombinant *B. besnoiti* PDI (recBbPDI) and truncated versions of PDI.
- Development of specific monoclonal anti-recBbPDI antibodies by means of cellular fusion (hybridoma technology).
- Characterization of the enzymatic activity of recBbPDI and its sensitivity to drug inhibitors and specific antibodies.
- Immunolocalization of PDI in *B. besnoiti* bradyzoites and tachyzoites and evaluation of the role of PDI in host cell invasion.
- Evaluation of bovine immune response to PDI.

Chapter 2 |

Besnoitia besnoiti protein disulfide isomerase (BbPDI): Molecular characterization, expression and *in silico* modelling.

2.1 Introduction

Besnoitia besnoiti is a cyst-forming apicomplexan parasite of cattle with high veterinary relevance in many tropical and subtropical regions, with particular emphasis for sub-Saharan Africa (Bigalke & Prozesky, 2004). In Europe, besnoitiosis has been recently reported in Portugal (Cortes et al., 2006c; Cortes et al., 2005), Spain (Castillo et al., 2009; Fernandez-Garcia et al., 2010; Fernandez-Garcia et al., 2009a; Fernandez-Garcia et al., 2009b; Irigoien et al., 2000)., France (Alzieu et al., 2007; Bourdeau et al., 2004; Jacquiet et al., 2010), Italy (Agosti et al., 1994; Gentile et al., 2012b; Gollnick et al., 2010; Mangili et al., 2012; Manuali et al., 2011; Mutinelli et al., 2011), Greece (Papadopoulos et al., 2014), Hungary (Hornok et al., 2014), Croatia (Beck et al., 2013), Switzerland (Basso et al., 2013; Lesser et al., 2012) and Germany (Mehlhorn et al., 2009; Schares et al., 2009). There is no treatment against this infection despite the efforts to identify and develop effective agents (Cortes et al., 2007a; Shkap et al., 1987a). Live-attenuated vaccines have been used in Israel and South Africa (Basson et al., 1970; Bigalke et al., 1974a) but they do not prevent subclinical infection and their use is geographically limited. Therefore, there is an urgent need for studying potential drug targets and vaccine candidates. Protein disulfide isomerase (PDI) is a key enzyme that enables proteins to acquire their correct three dimensional structure (Appenzeller-Herzog & Ellgaard, 2008; Hatahet & Ruddock, 2009; Wilkinson & Gilbert, 2004). This enzyme, one of the most abundant in the endoplasmic reticulum (ER) and classically described as an ER-resident protein (Lambert & Freedman, 1985), is a member of the thioredoxin superfamily (Ferrari & Soling, 1999) and consists of two homologous catalytic domains, **a** and **a'** , comprising each an active site with a CxxC motif, separated by two homologous non-catalytic domains, **b** and **b'** , and a highly acidic C-terminal extension **c** (Byrne et al., 2009; Edman et al., 1985; Kemmink et al., 1999; Kozlov et al., 2010). PDI catalyzes disulfide formation and the rearrangement of incorrect disulfide pairing during the oxidative protein folding process (Schwaller, Wilkinson & Gilbert, 2003; Wilkinson & Gilbert, 2004). Misfolded proteins can lose their functionality and accumulate as large, insoluble aggregates that might interfere with cell viability (Wilkinson & Gilbert, 2004). Current evidence suggests that PDIs are also involved in a wide range of other biological functions in many cellular compartments including the cell surface, where they may participate in the reducing exterior environment and/or may be involved in cell adhesion (Turano et al., 2002). Parasite PDI-like enzymes were identified in *Neospora caninum* (Naguleswaran et al., 2005), *Toxoplasma gondii* (Meek et al., 2002a; Meek et al., 2002b), *Theileria parva* (Ebel et al., 2002), different *Leishmania* species (Achour et al., 2002; Hong & Soong, 2008; Padilla et al., 2003), *Cryptosporidium parvum* (Blunt et al., 1996) and several species of *Plasmodium* (Florenta et al., 2000;

Mahajan et al., 2006; Mouray et al., 2007; Novo et al., 2009). *T. gondii* and *N. caninum*, both closely related to *B. besnoiti* (Ellis et al., 2000), have been shown to express highly homologous molecules, which could indicate that PDI plays a key role in the biology of neosporosis and toxoplasmosis (Ma et al., 2009; Meek et al., 2002b; Naguleswaran et al., 2005). In this way, the important role of PDI in these two species and presumably in other apicomplexan parasites as well, might not allow for the accumulation of a large number of mutations in the protein sequence, rendering this molecule an interesting drug target. In fact, bacitracin and anti-PDI antibodies are effective at inhibiting the enzymatic activity of PDI in CHO cells (Mandel, Ryser, Ghani, Wu & Peak, 1993), and allowed the demonstration of the fundamental role of PDI in *N. caninum* host cell invasion (Naguleswaran et al., 2005). Furthermore, *T. gondii* PDI is targeted by mucosal IgA in humans (Meek et al., 2002b) and *N. caninum* PDI by IgA in cattle (Liao et al., 2006), suggesting that PDI specific antibodies may be involved in the host defense against these parasites. In addition, the analysis of *N. caninum* antigenic proteins identified PDI as one of the immunodominant proteins associated with the processes of invasion, proliferation and egress of apicomplexan parasites (Shin et al., 2004; Shin et al., 2005). The inhibitory effect of *N. caninum* PDI antiserum on the growth of this parasite was also demonstrated (Liao et al., 2006). Recently, intranasal vaccination of mice with recombinant *N. caninum* PDI conferred 90% protection against disease and significantly reduced the cerebral parasite burden and the numbers of viable tachyzoites in brain tissue (Debache et al., 2010). All the evidence obtained so far points towards the importance of PDI in the host immune response and cell invasion process, and prompted us to characterize this molecule in *B. besnoiti*.

2.2 Materials and methods

2.2.1 Tissue culture and parasite purification

The *B. besnoiti* Bb1Evora03 strain (Cortes et al., 2006c) was maintained by serial passages in confluent monolayers of Vero cells grown in DMEM with Glutamax (Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum and 20mM HEPES, at pH 7.2–7.5 as previously described (Cortes et al., 2006c; Reis et al., 2006). For parasite purification, freshly egressed tachyzoites were collected from the cell cultures supernatants, centrifuged at 800 g for 10 min, passed three times through a 25 G needle, washed three times with PBS, and passed through PD10 columns according to the manufacturer's instructions (GE Healthcare). Bradyzoites were freshly obtained from mechanical disruption of cysts, collected from fresh skin biopsies of a chronically infected cow. Biopsies were made on an aseptically prepared site, with 8 mm biopsy punch, and immediately collected to PBS solution. Liberated bradyzoites were washed and purified as above.

2.2.2 *B. besnoiti* PDI-gene sequence

B. besnoiti tachyzoites were purified from in vitro cultures, and genomic DNA was isolated using DNeasy Blood and Tissue Kit (Qiagen). Primers for the initial PCR amplifications were designed based on conserved regions of the aligned PDI nucleotide sequences from *N. caninum* (AY751081) and *T. gondii* (AJ312317). PCR amplification was carried out in a 20 μl PCR mixture (1x reaction buffer, 1 U of Taq DNA polymerase (Fermentas), 10 pmol of each primer, and 0.2 mM dNTP's), using *B. besnoiti* genomic DNA, primers PDIBesF2 and PDIBesR2 (Table 1), and running 30 cycles (90 °C for 30 s, 55 °C for 1 min, 72 °C for 1 min 15 s). PCR products were cloned in the pTZ57R/T vector (Fermentas), and sequenced in both directions by dideoxy-chain termination method (Sanger, Nicklen & Coulson, 1977). Primers for genome walking were designed based on the sequence obtained.

Genome walking libraries were generated and amplifications were carried out according to the manufacturer's instructions (Clontech, USA). 2.5 μg of genomic DNA was digested separately with AluI, HaeIII, HpaI or SmaI. Subsequently, the GenomeWalker adaptors were ligated to the DNA. PCR amplification of DNA fragments from genomic libraries was carried out using Taq DNA polymerase (Fermentas) and gene-specific primers PDIBesGWF1, PDIBesGWF2, PDIBesGWR1 and PDIBesGWR2 (Table 1). After the first genome walking, the products were cloned and sequenced and a second round of amplification was done with

primers PDIBesGWR3 and PDI BesGWR4. All clones were again sequenced in both directions, as above.

Table 1. List of primers used for PCR amplification in this chapter.
In lowercase letters is the sequence for the restriction enzyme.

Primers' name	Sequence (5'- 3')
PDIBesF2	GAGTGGATCGAGAAGATG
PDIBesR2	GTGCCGTCCATCTTGGCAACA
PDIBesGWF1	AGGAGGCTGTCAAGGTTGTCGTCGGCAAGA
PDIBesGWF2	GATCTACGCTCCGTGGTGCGGCTACTGCAA
PDIBesGWR1	AAGTTCTCGGCGTTGATGGGGCCAAGCAGA
PDIBesGWR2	GCCACTGAAGTCGTCAGTGCCCTCCTCGTA
PDIBesGWR3	CGAGACATGCTGCTTTTGGAGGCCGACCAA
PDI BesGWR4	GAAGCGACGCTGTCTGGAGACGTTCTGCAA
PDIBesEX1	gaatccATGCGGGCTGGGGTCTGCTACCT
PDIBesEX2	gctcgagTTACAATTCCTCGCCCTTGTCGTCCT

2.2.3 Production of recombinant *B. besnoiti* PDI (recBbPDI)

2.2.3.1. Cloning and sequencing of B. besnoiti PDI cDNA

For cDNA synthesis, *B. besnoiti* tachyzoites were purified from in vitro cultures and total RNA was extracted (High Pure RNA Isolation Kit – Roche). RNA obtained from 10^8 tachyzoites was reverse transcribed by a two-step RT-PCR procedure (First Strand cDNA Synthesis Kit – Fermentas) for the production of cDNA. The PDI ORF was PCR-amplified using the forward primer PDIBesEX1 and the reverse primer PDIBesEX2 (Table 1), that contain EcoRI and XhoI sites respectively, and cDNA as template. Amplification was carried out using the conditions described above and performing 30 cycles (90 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min 15 s). PCR products were cloned into the pTZ57R/T vector (Fermentas), and sequenced.

2.2.3.2 Plasmid construction and expression

Full-length *B. besnoiti* PDI cDNA was released from pTZ57R-PDI by restriction-reactions with EcoRI and XhoI, subcloned into pGEX-6P-1 expression vector hydrolyzed with the same enzymes, and sequenced for confirmation. Production and purification of recombinant glutathione S-transferase (GST) fusion protein was conducted according to the manufacturer's recommendation (GE Healthcare). Briefly, the pGEX-6P-PDI construct was transformed into *E. coli* BL21 cells, cultures were grown in liquid broth and induced for 2 h with 1mM IPTG when absorbance at 600 nm was 0.8. The cell pellet was collected and lysis performed by sonication. The recombinant *B. besnoiti* PDI (recBbPDI) was purified from the lysate using Glutathione Sepharose 4B and following the brand indications with the exception that 0.1% Triton X-100 was used in binding and washing steps. Protein quantification was performed using a NanoDrop 2000 spectrophotometer assuming an extinction coefficient of 93000 M^{-1} cm^{-1} and a molecular weight of 81 kDa. The extinction coefficient and molecular weight were calculated based on the amino acid sequence, using the ProtParam tool of the ExPASy Bioinformatics Resources Portal from the Swiss Institute of Bioinformatics (http://web.expasy.org/protparam/) (Artimo et al., 2012; Gasteiger et al., 2005)

2.2.4 Determination of the reductase activity of recBbPDI and its inhibition by bacitracin

RecBbPDI activity was measured by a turbidimetric assay based on the polymerization of reduced insulin (Lundstrom & Holmgren, 1990) as adapted by Smith et al. (2004). Commercial bovine PDI (Sigma) was used as a control. The assay was performed in 96-well microtiter plates and all solutions were made with assay buffer: 100mM potassium phosphate, 2mM EDTA, pH 7. Bacitracin, at concentrations ranging from 0.5mM to 4 mM, or assay buffer (5 µL) were added to respective wells; the reaction solution was made by mixing 70 µL assay buffer with 10 µL of insulin (1.6 mM) and the enzymes or buffer (blank); 75 µL of reaction solution were added to each well and the reaction was started with the addition of 20 µL of 5mM DTT. Absorbance at 650 nm was read in a SECTRAmax® 340 pc microplate spectrophotometer over 60 min every 5 min. Enzyme activity was calculated from the increase of turbidity over time accordingly to the formula:

$$(\mathrm{ABS}_{650}\ t_x - \mathrm{ABS}_{650}\ t_0)_{Enzyme} - (\mathrm{ABS}_{650}\ t_x - \mathrm{ABS}_{650}\ t_0)_{Blank}$$

2.2.5 Antiserum production

One mouse was intraperitoneally immunized and one rabbit subcutaneously immunized, both three times in intervals of three weeks, with 10 µg and 500 µg, respectively of recBbPDI emulsified in Freund's incomplete adjuvant (MP Biomedicals, Inc.). The animals had free access to food and water and were housed and managed according to the applicable National and EU regulations. Four days after the last injection they were bled and the specific anti-recBbPDI antibody titre determined by enzyme-linked immunosorbent assay (ELISA). Briefly, coating was performed with 0.1 µg/well of recBbPDI and alkaline phosphatase-conjugated secondary antibodies were used with p-nitrophenylphosphate (Sigma) (1 mg mL^{-1}) in 100mM glycine containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$, pH 10.4 as enzyme substrate. The plate was incubated 30 min in the dark at room temperature and the reaction stopped by the addition of 50 µL 3 N NaOH. Absorbance was read at 405 nm in a TIM 200 Inter Med plate reader.

2.2.6 Western blotting

Protein samples were prepared from purified tachyzoites or bradyzoites. The cellular pellet (10^8 parasites) was resuspended in Laemmli sample buffer (BioRad) and heated for 5 min at 95 °C. Proteins were separated by SDS–PAGE (12%) under reducing conditions and blotted onto nitrocellulose (Millipore) at 100 Volts for 1 h in Tris–glycine buffer with 20% (v/v) methanol. The membranes were blocked overnight at 4 °C with 1% fish gelatin (Sigma) and probed with mouse anti-recBbPDI followed by incubation with alkaline phosphatase-conjugated goat anti-mouse-IgG secondary antibody (Sigma) diluted 1:5000. The final enzyme reaction was developed using the BCIP/NBT kit (Bio-Rad).

2.2.7 Immunofluorescence

Purified tachyzoites or bradyzoites were allowed to adhere to poly-L-lysine-coated microscopy coverslips for 30 min, and were fixed for 10 min with cold methanol (-20 °C). Parasites were then probed with mouse anti-recBbPDI serum and FITC-conjugated anti-mouse antibodies (Invitrogen). To visualize the entire cells, parasites were simultaneously probed with rabbit anti-*B. besnoiti* (Cortes et al., 2006b) and TRITC-conjugated anti-rabbit antibodies (Invitrogen). All these sera were used at 1:500 dilutions. DNA was stained with DAPI (1 µg mL^{-1}; Sigma) for 2 min and the slides were mounted with Mowiol (Calbiochem) and examined under a UV light microscope.

2.2.8 Database search and biocomputing approaches

Similarity searches on the Protein databank were done using the Blast search program (Altschul et al., 1990). Alignment between two protein amino acid sequences was done using SIM (http://expasy.org/tools/sim-prot.html) (Huang & Miller, 1991). Multiple sequence alignment of PDI amino acid sequences was performed using MEGA 4 and ClustalW (Kumar, Nei, Dudley & Tamura, 2008). Search for BbPDI neighbors and phylogenic tree construction were performed using the web server phylogeny.fr (http://www.phy- logeny.fr) (Dereeper et al., 2008). Pattern search was performed using ScanProsite (http://expasy.org/prosite/) (Gattiker, Gasteiger & Bairoch, 2002) and Superfamily 1.69 version (http://supfam.org/SUPER- FAMILY/) (Gough, Karplus, Hughey & Chothia, 2001).

2.2.9 Comparative modelling and model validation

A theoretical three-dimensional model for PDI from *B. besnoiti* (BbPDI) was constructed by comparative modelling by the Swiss-Model server v.3.7 (Arnold, Bordoli, Kopp & Schwede, 2006; Kiefer, Arnold, Kunzli, Bordoli & Schwede, 2009; Peitsch, 1996) (http://swissmodel.expasy.org/) using as template the heterodimer Tapasin-ERp57 crystallographic deduced model (Dong et al., 2009) (PDB code: 3F8U chain C). Superimposition of models and calculation of the root mean square values (RMS) between the model and modelling template using the Carbon alpha fitting, were made by Swiss-Pdb Viewer v. 3.7 (Guex & Peitsch, 1997). RasMol v. 2.6 (Sayle & Milner-White, 1995) and Swiss- Pdb Viewer v. 3.7 were used for visualization of models. Search for structurally similar proteins was done using Dali server v. 2.0 (http://www.embl-ebi.ac.uk/dali/) (Holm & Sander, 1993). Model structure validation was performed by Procheck programs (Laskowski, MacArthur, Moss & Thornton, 1993; Morris, MacArthur, Hutchinson & Thornton, 1992). The substrate pocket was predicted using the WHAT IF program (http://swift.cmbi.ru. nl/servers/html/index.html) (Vriend, 1990). The model was deposited in the PMDB (https://bioinformatics.cineca.it/PMDB/) (Castrignano, De Meo, Cozzetto, Talamo & Tramontano, 2006) under the identification number PM0075875. In this manuscript the BbPDI amino acid sequence numbering is based on ABF48402 entry.

2.3 Results and discussion

2.3.1 Molecular characterization of the gene coding for BbPDI

The gene sequence of *B. besnoiti* PDI was determined and deposited at GenBank under the accession number DQ490130. The sequence analysis showed an ORF of 1416 nucleotides, comprising three exons, the first one spanning 164 bp, the second one 199 bp and a third one 1053 bp, which are separated by two introns of 343 bp and 97 bp respectively. The deduced amino acid sequence revealed a protein with 471 residues, presenting the characteristic catalytic thioredoxin motifs CxxC, and showing high amino acid conservation within the catalytic sites regions motif with other PDI entries in databases (Figure 9). A comparison of the BbPDI amino acid sequence with available PDI sequences from other species of the phylum Apicomplexa revealed identity levels as follows (Table 2): 88.5% for *N. caninum* (BAD67151) (NcPDI) and 87.7% for *T. gondii* (CAC28361) (TgPDI); between 48.1% and 48.2% for *Plasmodium* species, *P. chabaudi* (AAV36000) (PcPDI) *P. yoelli* (EAA17481) (PyPDI), *P. berghei* (CAH95379) (PbPDI), *P. falciparum* (CAC15387) (PfPDI), and *P. vivax* (ABB72222) (PvPDI); 37.1% for *C. parvum* (AAB40710) (CpPDI); between 35.8% and 34.3% for *Babesia* and *Theileria* species, *B. bovis* (EDO05830) (BboPDI), *B. caballi* (BAE54313) (BcPDI), *T. annulata* (CAI75629) (TaPDI) and *T. parva* (EAN31157) (TpPDI).

Table 2. Identity and similarity levels of PDI from *B. besnoiti, N. caninum*, *T. gondii*, *P. falciparum* and *B. Taurus.*
Light gray highlights the high identity and similarity between *B. besnoiti*, *N. caninum* and *T. gondii*. Results are given in percentage based on the amino acidic sequence of *B. besnoiti* PDI compared to the other organisms and using PAM250 as the scoring matrix.

		Identity				
		B. besnoiti	*N. caninum*	*T. gondii*	*P. falciparum*	*B. taurus*
Similarity	*B. besnoiti*	—	88,5	87,7	48,1	30,7
	N. caninum	96,2	—	98,5	42,9	31,1
	T. gondii	96,0	94,3	——	44,0	30,9
	P. falciparum	69,3	68,7	68,7	—	27,5
	B. taurus	52,4	53,2	53,4	51,5	—

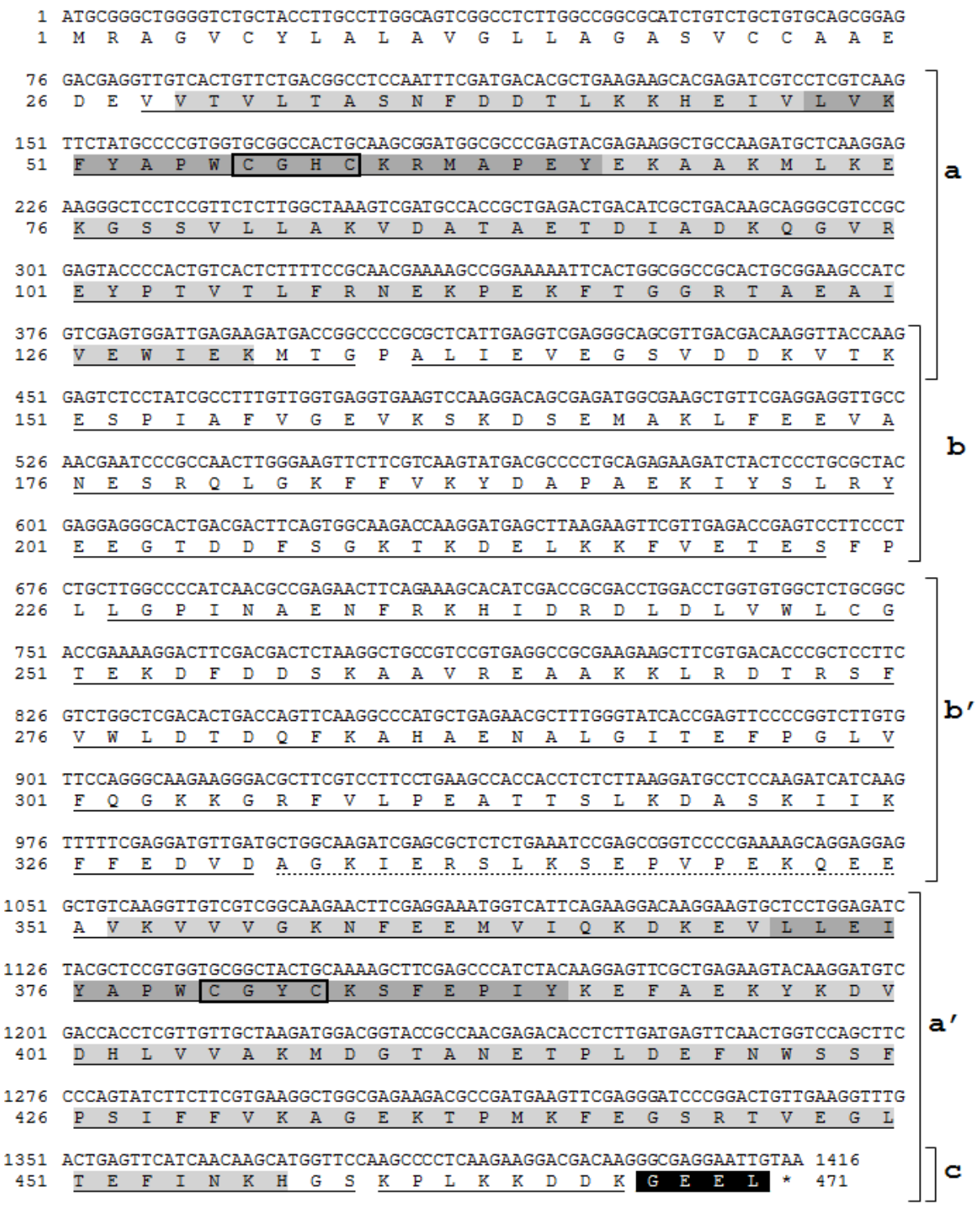

Figure 9. The cDNA encoding BbPDI (GenBank accession number. DQ490130.1).
Nucleotide sequence (above) and deduced amino acid sequence (below) are shown. Light gray highlights hits to the Pfam thioredoxin model (PF00085) and medium gray denotes the thioredoxin family active site Prosite pattern (PS00194). The characteristic catalytic thioredoxin motifs CxxC are boxed. The C-terminal putative ER retention signal is displayed on a black background. The stop codon is indicated by *. Based on the alignment with chain C of Tapasin Erp57 (PDB code 3F8U) the proposed delimitation of **a**, **b**, **b'** and **a' and c** domains is showed by brackets and underlined; x-linker is dotted-underline.

As expected for members of the thioredoxin superfamily, two thioredoxin domains (PS51352) were identified in BbPDI. These are localized between amino acids 28–134 (**a** domain) and 351– 459 (**a'** domain), and contain the thioredoxin family active site pattern (PS00194) [LIVMF]-[LIVMSTA]-x-[LIVMFYC]-[FYWSTHE]- x(2)-[FYWGTN]-C-[GATPLVE]-[PHYWSTA]-C-x(6)-[LIVMFYWT] between amino acids 48–66 (a domain) and amino acids 372–390 (**a'** domain). A putative thioltransferase domain is present between amino acids 223–335. The BbPDI **a** domain presents the characteristic CGHC eukaryotic active site motif, but there is a substitution of the histidine by a tyrosine in the **a'** domain active site motif (CGYC), similar to what was described for *N. caninum* (Naguleswaran et al., 2005) and *T. gondii* (Meek et al., 2002a). The putative ER retention motif GEEL is present at C terminus of the amino acid sequence, diverging from the classical KDEL sequence found in eukaryotic ER-resident proteins (PS00014), but again in agreement with *N. caninum* (Naguleswaran et al., 2005) and *T. gondii* (Meek et al., 2002a).

2.3.2 *B. besnoiti* PDI and its relationship to other members of the thioredoxin-like superfamily

BbPDI belongs to the thioredoxin like superfamily (cluster 00388) and is included in the PDI_a family (cluster defined cd02961) and the PDI_a_PDI_a'_c subfamily (cd02995). This sub-family includes ER-resident eukaryotic proteins involved in the oxidative protein folding (Marchler-Bauer et al., 2009). Structurally, PDI consists of four thioredoxin-like domains, **a**, **b**, **b'** and **a'**, plus a highly acidic C-terminal extension **c**. Two domains contain a redox-active catalytic site (**a** and **a'**), and the other (**b** and **b'**) do not (Kemmink et al., 1995; Kozlov et al., 2010; Marchler-Bauer et al., 2009). Catalytic domains usually contain two cysteines in a CxxC active-site motif (Appenzeller-Herzog & Ellgaard, 2008).

The search for protein neighbors through the web server phylogeny.fr on non-redundant databases, identified orthologues in the Muridae family (*Rattus norvegicus*, *Mus musculus*), Hominidae family (*Homo sapiens*, *Pan troglodytes*), Bovidae family (*Bos taurus*), Nematoda phylum (*Caenorhabitis elegans*), in the Phaniasidae family (*Gallus gallus*), the Amphibia (*Xenopus laevis*) and Insecta class (*Culex quinqiuefasciatus*), in the Branchistomidae family (*Branchiostoma floridae*); Viridiplantae (*Arabidopsis thaliana*, *Medicago sativa*), in the phyla Apicomplexa (*P. falciparum*), Platyhelminthes (*Schistosoma mansoni*), Dinoflagellata (*Perkinsus marinus*) and in the Tetraodontidae family (*Tetraodon nigroviridis*). No orthologue was detected in bacteria.

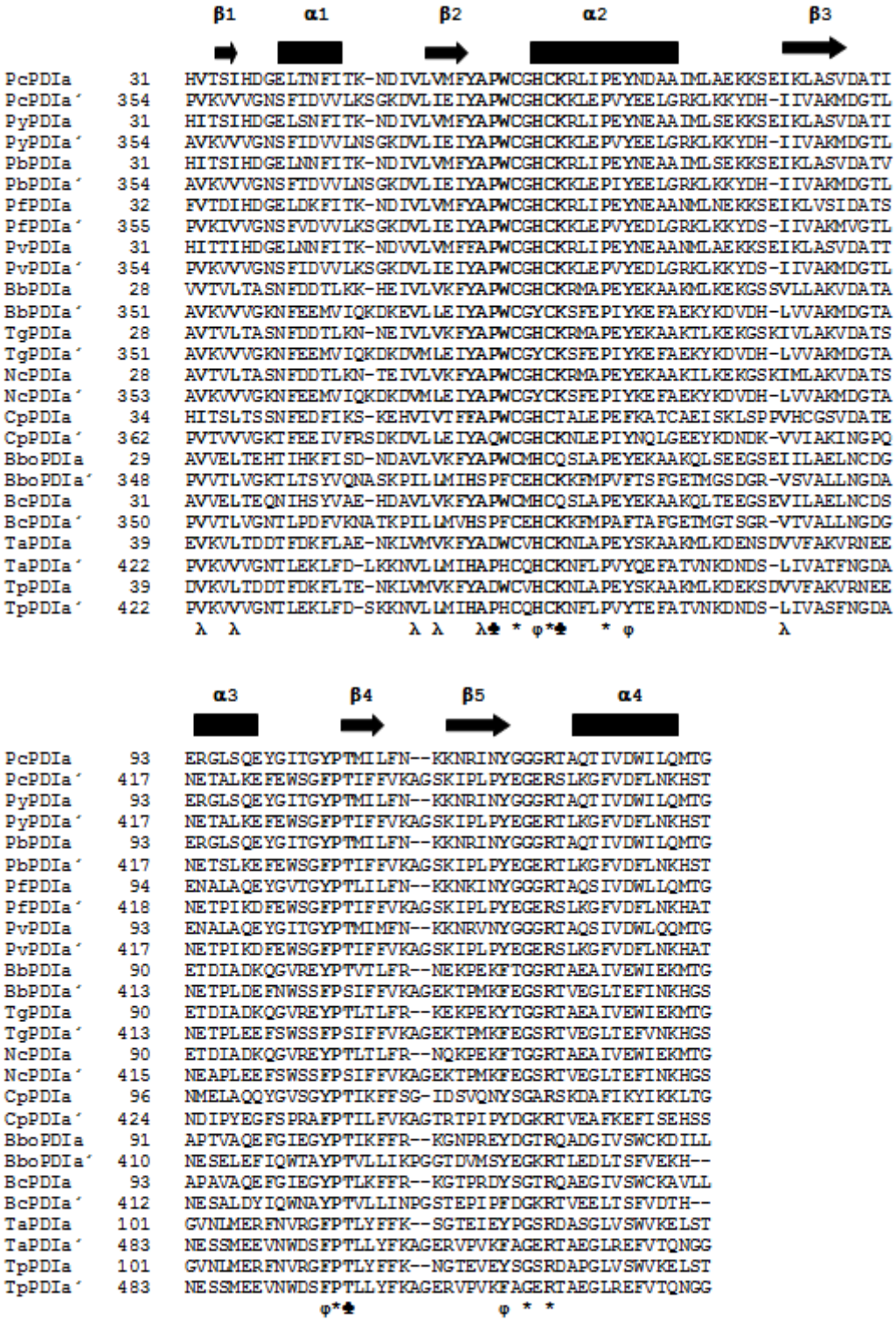

Figure 10. Multiple sequence alignment between the catalytic **a** and **a'** domains of Apicomplexa PDI family and predicted Secondary Structure Elements for the BbPDI model.
Conserved residues in all sequences are in bold and marked (*****). Residues with >90% identity are in bold and marked (♣). Residues with conserved properties are in bold and marked: hydrophobic (λ) and aromatic (φ). Localization of alpha and beta Secondary Structure Elements are indicated: α-helices (▬), (▬), β-strand (➡). Residues numbering according entries in database were used except for PyPDI (starting at mature enzyme). *P. chabaudi* (Pc), *P. yoelli* (Py), *P. berghei* (Pb), *P. falciparum* (Pf), *B. besnoiti* (Bb), *T. gondii* (Tg), *N. caninum* (Nc), *C. parvum* (Cp), *B. bovis* (Bbo), *B. caballi* (Bc), *T. annulata* (Ta), *T. parva* (Tp)

The multiple alignment between the **a** and **a'** domains of the PDI amino acid sequences from apicomplexan parasites (Figure 10) showed that from the 20 residues around the active site motif, VlVkfYAPWCgHCKrmaPeY (*B. besnoiti* sequence) 12 are identical or their chemical properties are conserved. Also the P103-T104 and R120 residues (a domain) and P426 and R445 (a' domain) (*B. besnoiti* numbering) are conserved. The aligned residues to T104 in the a0 domain are also conserved in all the apicomplexan PDIs except for the Sarcocystidae in which the Threonine residue was changed to a Serine residue (similar chemical properties), S427 (*B. besnoiti, T. gondii*) and S429 for N. caninum. This residue conservation is similar to the one described for the human PDI family (Lappi et al., 2004) and indicates similar functional activities as reported for the equivalent residues in the human PDI family.

Analysis of the phylogenetic tree (Figure 11) showed that at the first divergent node the Apicomplexa are divided into two branches, the first one comprising the *Plasmodium* (orthologue) and the *Toxoplasma*, *Neospora*, *Besnoitia*, *Babesia* and *Theileria* species homologues and the second one comprising the Cryptosporidiidae members. The first branch diverges again into one containing *Toxoplasma*, *Besnoitia* and *Neospora* PDI homologues and one containing the *Babesia* and *Theileria* homologues as expected. Orthologues from *P. marinus* are phylogenically closer to the Cryptosporidiidae than to *B. besnoiti*. Phylogenetically, *B. besnoiti* PDI is relatively divergent from *B. taurus* PDI, the target host for *B. besnoiti* infection, with an amino acid sequence identity of only 30.7% in 485 amino acids

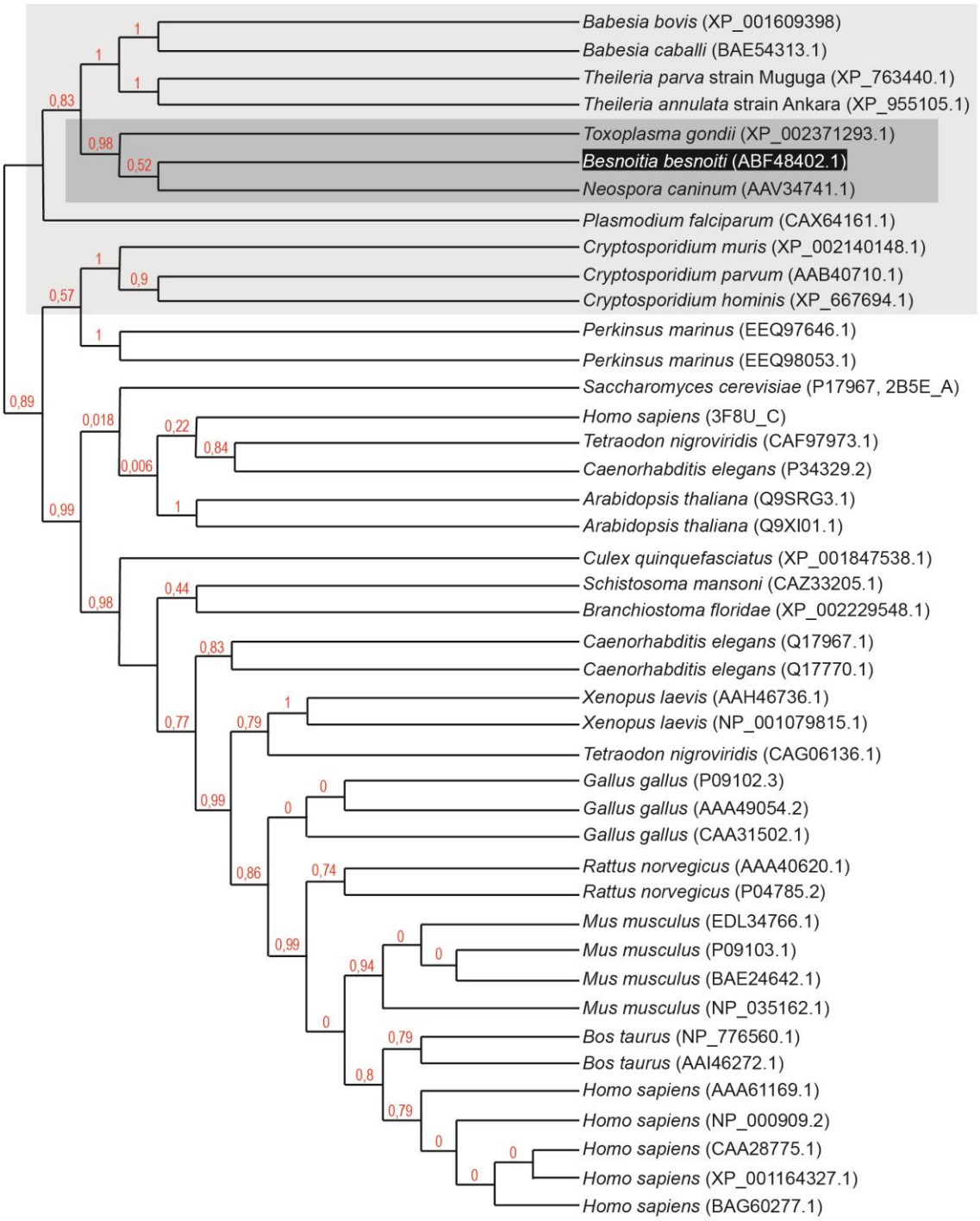

Figure 11. Mid-point rooting cladogram tree for PDI amino acid sequence.
Branch support values are presented. Accession numbers and parasite source were provided for PDI from Apicomplexa from the *Plasmodiidae*, *Theileriidae*, *Babesiidae* and *Sarcoscystidae* families. The polypeptide sequences from the crystallographic deduced model used as a template for *in silico* modelling of BbPDI were also introduced (*Homo sapiens*, 3FU8_C). Orthologues from different species are shown. Some orthologues from *M. musculus, P. troglodytes, P. marinus, C. quinquefasciatus, S. mansoni, B. floridae* and *X. laevis* were omitted due to their similarities to other entries already included. Light gray highlights phylum Apicomplexa and medium gray denotes subfamily Toxoplasmatinae. *B. besnoiti* is highlighted in black background.

2.3.3 Analysis of an *in silico* model of *B. besnoiti* PDI

The catalytic sites exhibit a structure that is reminiscent for thioredoxin domains. Both **a** and **a'** domains have a double-wound alternating α/β fold, consisting of a 5-stranded β-sheet core, enclosed by 4 α-helices. The active site disulfide is located in a short segment at the N-terminus of helix 2, as described (Kemmink et al., 1996), after a kink caused by the conserved Proline residue P64, (**a** domain) and P388 (**a'** domain). The view of the model (Figure 12A) shows the four thioredoxin domains. Domains **a**, **a'**, **b** and **b'** are, as expected, presenting the five β-strands characteristic of the fold.

The stereochemical quality of the model, checked by Procheck program, showed that 95.6% of residues are in the core plus allowed regions of the Ramachandran plot. The model presents 99.3% of M/c bond lengths, 95.1% of M/c bond angles and 88.1% of planar groups within the expected limits and an overall G factor of -0.04. The structural alignment obtained from the superimposition of the model with the 3F8U_C template model showed a RMS of 0.33 Å on 407 Ca atoms and a RMS of 1.15 Å on 119 Ca atoms from the superimposition with the *S. cerevisiae* PDI crystallographic deduced model 2B5E. Search for structurally similar proteins using Dali reveals a very high structural similarity with the used template 3F8U chain C (Z score 42.6) against a Z score 27.1 for 2B5E chain A. The phylogenetical distance of the H. sapiens Tapasin-ERp57 (PDB code 3F8U_C) and the S. cerevisiae PDI (PDB code 2B5E_A) to *B. besnoiti* PDI is similar for both cases. As the comparative modelling is highly dependent on the degree of amino acid sequence identity, the discrepancies observed on the structural alignment could be due essentially to the low amino acid sequence identity, 28.7% in 456 amino acid overlap for 2B5E protein and 33.6% in 446 amino acid overlap for 3F8U_C sequence.

2.3.4 Analysis of catalytic domains

In each catalytic site, Cys residues are placed on **a** and **a'** domains face to face. On domain **a**, Cys residues are in a reduced state, in contrast to the oxidized state on **a'** domain, as occurs in *Humicola insolens* (Serve et al., 2010). It was shown (Lappi et al., 2004) that the movement of the side chain of the Arg residues in human PDI, equivalent to *B. besnoiti* R120 and R445 residues, are used by the enzyme to maintain a high pKa value for the Cys residue, while allowing the reaction which requires a thiolate to proceed at an appropriate rate. Similar to what was described for human PDI, the side chains of R120 and R445 residues in BbPDI are also distant from the Cys residues of the active site suggesting an identical movement of the Arg side-chains during the catalytic mechanism.

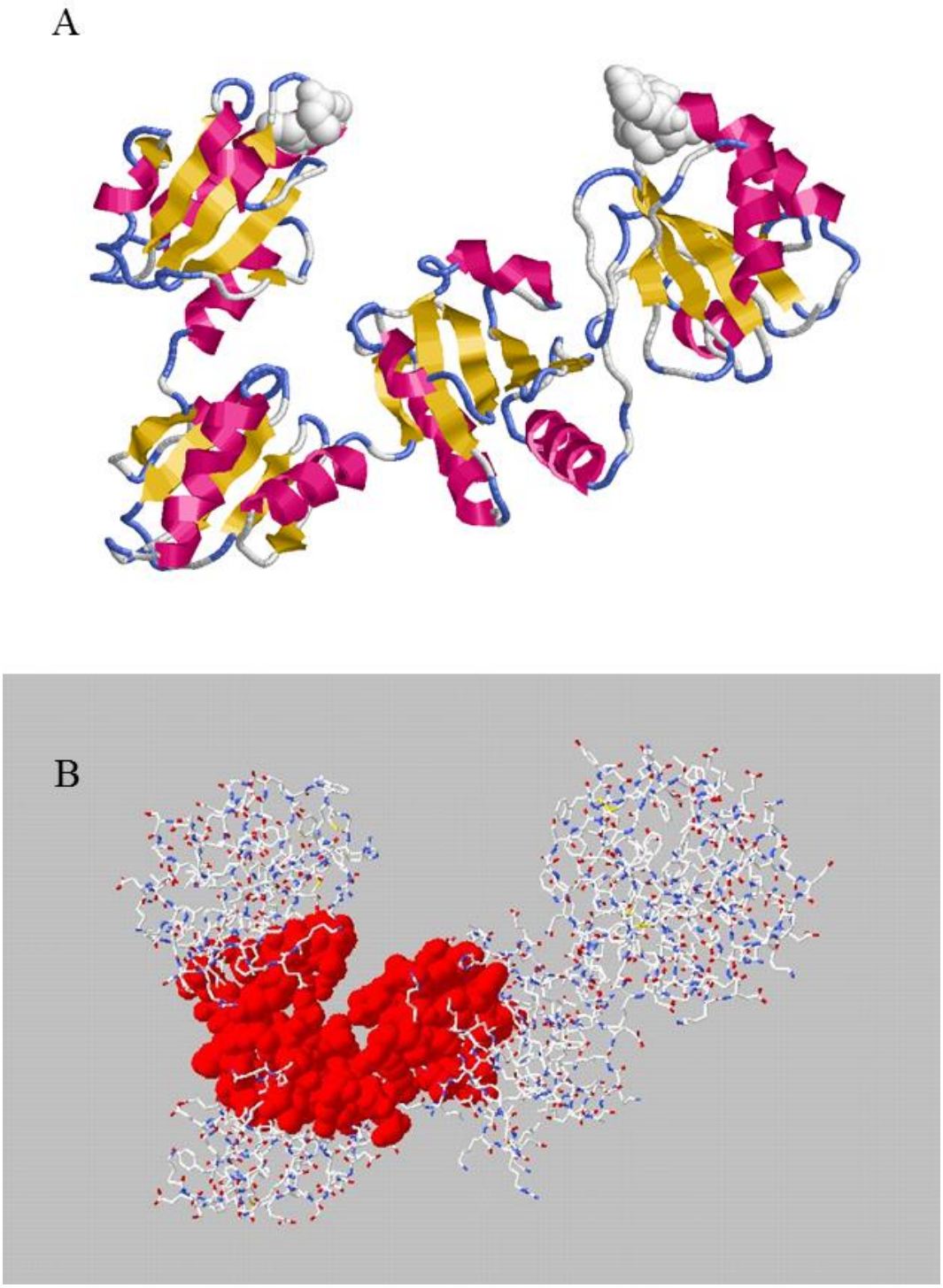

Figure 12. *B. besnoiti* PDI model and binding pocket.
(A) View of the *B. besnoiti* PDI model. Cartoon representation generated by Rasmol: α-helices (red), β-strand (yellow), turns (blue) and aperiodic structures (grey). Residues of the active center (CxxC) are spacefill displayed in grey in both domains **a** and **a'**. The β sheet, characteristic of each domain (in yellow), is visible from left to right, respectively domains **a, b, b'** and **a'**; (B) View of the binding pocket of *B. besnoiti* PDI. Representation generated by Swiss-Pdb Viewer v 3.7 by superimposition of the BbPDI model and the residues belonging to the pocket predicted by WHAT IF docking program. Residues belonging to the pocket are displayed in red. The pocket residues belonging to the domains **a, b** and **b'** are depicted from left to right.

Several studies showed that all domains of PDI act synergistically during catalysis. The experimental results from disulfide- bond isomerase (DsbC) from *E. coli* (Banaszak, Mechin, Frost & Rypniewski, 2004; McCarthy et al., 2000) and *Haemophilus influenza* (Zhang, Monzingo, Segatori, Georgiou & Robertus, 2004) crystal structures, suggest hinge-flexibility within the proteins, which allows movements of the active sites, in order to accommodate the substrate in the cleft between the catalytic domains, and also differences in the orientation of the catalytic domains (Banaszak et al., 2004; McCarthy et al., 2000). This flexibility, essentially due to the x-linker region between **b'** and **a'** domains, agrees with the experimental data (Nguyen et al., 2008; Wang et al., 2010b) and is fundamental for interaction with folding substrates.

In the BbPDI model the distance of Cys residues of **a** and **a'** catalytic domains is approximately 35 Å. In addition to the predicted movement of the R120 and R445 residues, these facts agree with the hypothesis that substrate binding induces domain-movements, leading to a different orientation and distance between **a** and **a'** catalytic domains Cys residues.

Substrate pocket prediction on BbPDI by WHAT IF program (Figure 12B), detected one pocket comprising residues belonging to **a**, **b** and **b'** domains. The inclusion in the pocket of residues from **b** and **b'** domains is in agreement with the described participation of several domains in the binding activities of PDI suggesting that **b** and **b'** domains act to fix substrates for further catalytic chain action of the **a** and **a'** domains.

2.3.5 RecBbPDI catalytic activity and bacitracin inhibition

The activity of recombinant GST-tagged *B. besnoiti* PDI expressed in E. coli, was tested by following the aggregation of insulin at 650 nm, as a consequence of disulfide bond reduction (catalyzed by PDI). RecBbPDI is functionally active and shows a dose dependent cross-linking activity in the range of 0.05 mM to 1.75 mM (Figure 13A). The profile of activity is similar to that observed for bovine PDI (bPDI) (Figure 13B), although at lower enzyme concentrations recBbPDI exhibited higher activity levels and at higher concentrations (0.88 mM and 1.75 mM) recBbPDI activity levels are lower compared to bPDI.

Recombinant BbPDI activities were sensitive to bacitracin, a classic PDI inhibitor, in a dose-dependent manner at enzyme concentrations ranging from 0.05 mM to 0.22 mM (Figure 13C, D and E). At higher enzyme concentrations, however, PDI activity was not inhibited in the range of bacitracin concentrations tested (0.5–4 mM) (data not shown). Bovine PDI used as control showed a dose dependent inhibition by bacitracin in the above-mentioned range (Figure 13F).

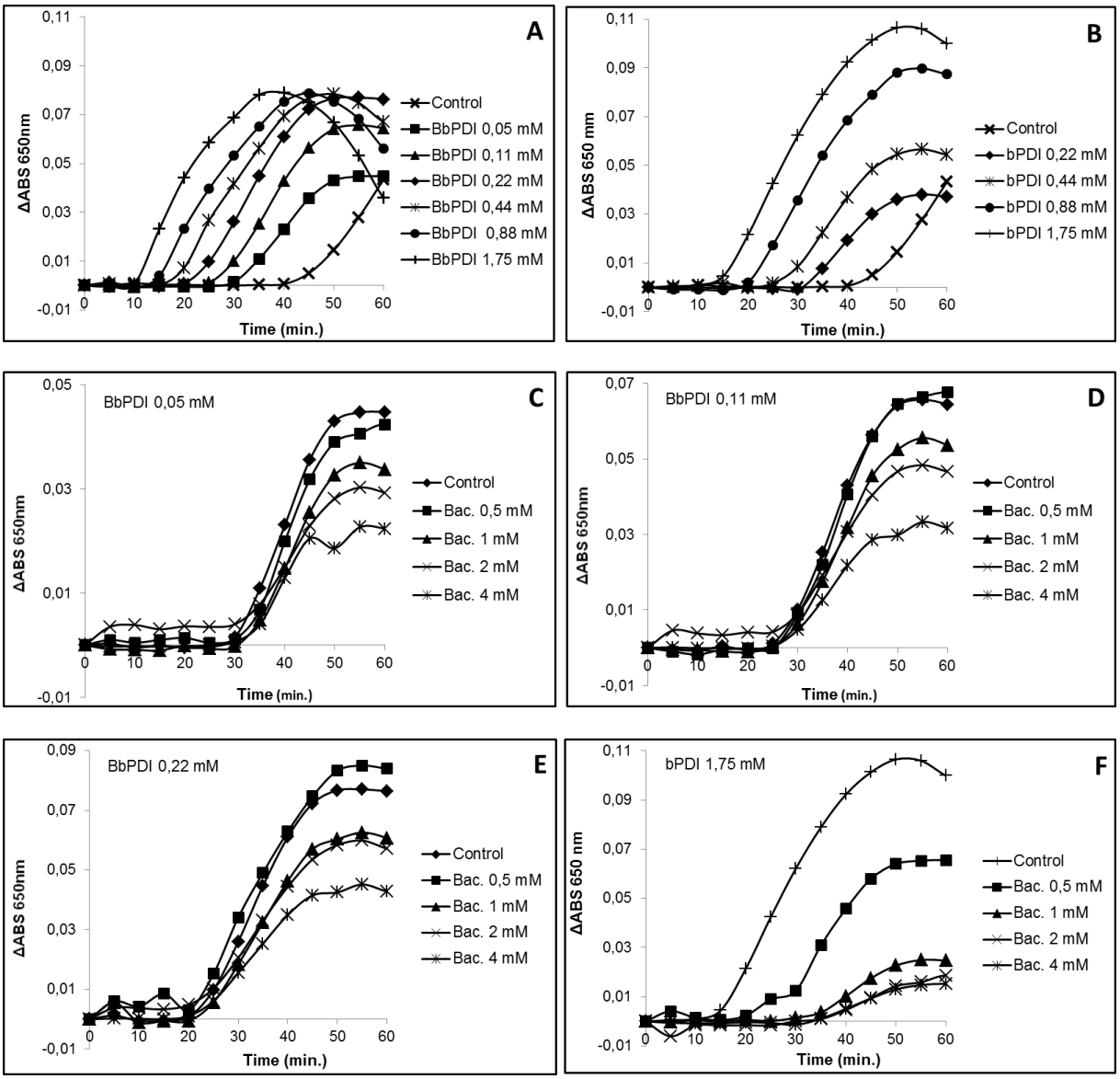

Figure 13. *B. besnoiti* PDI (BbPDI) enzymatic activity.
Reductase activity was tested by measuring the aggregation of insulin. (A) A dose dependent activity of BbPDI was observed in the range of 0.05 mM to 1.75 mM. (B) Bovine PDI (bPDI), used as control, shows also a dose dependent activity in the range of 0.22 mM to 1.75 mM. Decrease of activity observed when bacitracin, tested at concentrations ranging from 0.5 mM to 4 mM, was used with a concentration of BbPDI of 0.05 mM (C), 0.11 mM (D) and 0.22 mM (E) and bovine PDI (bPDI) at 1.75 mM (F).

In an attempt to understand differences in sensitivity of recBbPDI and bPDI to bacitracin *in silico* docking studies were performed. The data suggests that bacitracin makes hydrogen bonds within the substrate pocket residues Glu123 (**a** domain), Glu151 and Ser 152 (**b** domain) in the BbPDI model, and with Glu123, Glu153 and Ser154 in a *B. taurus* PDI model constructed by threading (data not shown). The comparative analysis suggests that

bacitracin, due to a different orientation in the substrate pocket, interacts more extensively with the **b'** domain in bPDI than in BbPDI

2.3.6 Western blotting analysis and immunolocalization of PDI

In Western blots of *B. besnoiti* tachyzoites and bradyzoites extracts, the anti-recBbPDI serum revealed a band of 53 kDa, as expected (Figure 14A and B). The antiserum also reacted with a band of similar molecular mass in *N. caninum* and *T. gondii* tachyzoite extracts (Figure 14C and D). This is not surprising, considering the high amino acid sequence homology of PDIs in the three species. Western blots of recBbPDI labeled with anti-recBbPDI antiserum revealed two bands, one corresponding to the GST-PDI-fusion protein (~81 kDa) and the other corresponding to free PDI devoid of the GST tag (~53 kDa) (Figure 14E).

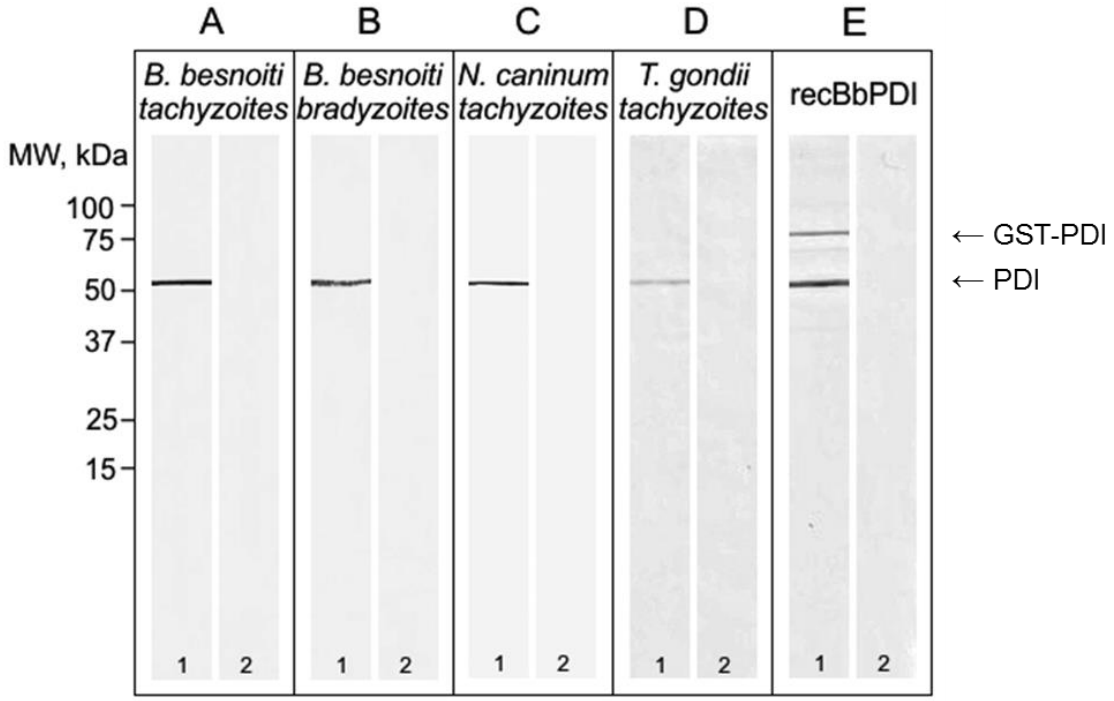

Figure 14. Western blot analysis using mouse serum**.**
(1) anti-recBbPDI hyper immune serum (diluted 1:1000); (2) pre-immune serum (diluted 1:1000); (A) *B. besnoiti* tachyzoite total protein extract, (B) *B. besnoiti* bradyzoite total protein extract, (C) *N. caninum* tachyzoite total protein extract, (D) *T. gondii* tachyzoite total protein extract and (E) GST-recBbPDI.

The anti-recBbPDI serum, tested by immunofluorescence on *B. besnoiti* tachyzoites and bradyzoites smears, showed a scattered and uneven intracellular staining, with higher intensity in the perinuclear area, a pattern that is compatible with ER labeling (Figure 15). *B. besnoiti* PDI staining is similar in both parasite stages, but tachyzoites seems to show a more scattered distribution whereas bradyzoites a more spotted and condensed one with a clear empty intracellular PDI staining area, as shown in the merge image.

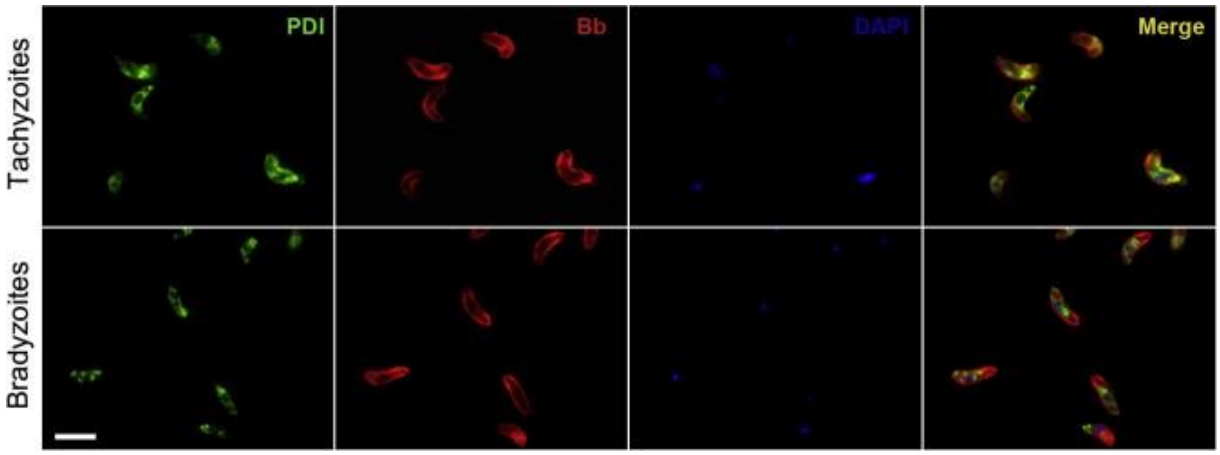

Figure 15. Indirect immunolocalization of *B. besnoiti* PDI
IFAT in purified tachyzoites (top panel) and bradyzoites (bottom panel), using mouse anti-recBbPDI serum and FITC conjugated secondary antibodies (PDI-green), rabbit anti-*B. besnoiti* and TRICT conjugated secondary antibodies (Bb-red) and DAPI to label the cell nuclei (blue). Merged image is presented on the right panel. Scale Bar: 6 µm

2.4 Conclusion

In conclusion, we showed that *B. besnoiti* expresses a PDI with 471 amino acid residues, presenting the two thioredoxin domains and the catalytic motif CxxC as expected for the members of the thioredoxin superfamily. Within the region of the catalytic sites there is a high amino acid sequence identity with PDIs from other apicomplexans, most notably with *N. caninum* and *T. gondii*. Phylogenetic analysis confirms that BbPDI belongs to a cluster comprising the PDIs from these two species. Using the SwissModel server it was possible to construct an *in silico* model by comparative modelling covering the four domains **a**, **b**, **b'** and **a'**. The results obtained by Procheck programs analysis, gave us confidence to use this model as a working hypothesis and the model presents the expected fold for all domains. As described in PDIs from other sources, the distance between catalytic domains and between Cys residues from each domain suggest the presence of the x region linking **b'** and **a'**

domain, and agree with the hypothesis that substrate binding induces domain movements, leading to different orientations and distances between **a** and **a'** catalytic domains and Cys residues during the catalytic reaction. This confirms what has been found earlier for other PDI models.

Recombinant BbPDI shows a dose dependent catalytic activity and is inhibited by bacitracin. Western blotting results confirm the specificity of produced antiserum against PDI in *B. besnoiti* tachyzoite and bradyzoite extracts, and cross-reactive activity with PDIs from other apicomplexans confirms the close phylogenetic relationship between these species. Immunofluorescence-based localization studies show that this protein is largely associated with the ER in both tachyzoites and bradyzoites. These studies form the basis for future investigations directed towards the exploitation of BbPDI as a potential drug target or vaccine candidate.

Chapter 3 |

Monoclonal antibody production and characterization

3.1 Introduction

One important function of the immune system is the production of soluble proteins that exhibit properties that contribute specifically to immunity and protection against foreign material and potentially damaging entities, such as bacteria, virus or parasites. These soluble proteins are known as antibodies or immunoglobulins and share a common structure that allows them to bind to a nearly unlimited number of specific antigens, including proteins, carbohydrates, glycoproteins, polysaccharides, nucleic acids and lipids (Abbas et al., 2014; Actor, 2014). Immunoglobulins can be secreted or membrane-bound. Secreted antibodies are produced by a terminally differentiated B-lymphocyte (or B-cell), called plasma cell, and circulate freely in extracellular fluids. Membrane-bound antibodies are present on the surface of B-cells where they serve as antigen-specific receptors and participate in the formation of the B-cell receptor (BCR) (Coico & Sunshine, 2015a). All immunoglobulin molecules consist of a basic unit (monomer) with a molecular weight of approximately 150 kDa, that comprises a symmetrical core structure composed of two identical light chains (25 kDa) and two identical heavy chains (55 kDa), held together by one or more interchain disulfide bonds (Nunez-Prado et al., 2015) (Figure 16). Both the light chains and heavy chains contain a series of repeated homologous units, each about 110 amino acid residues in length, that fold independently in a globular motif that is called an Ig domain. Also, all chains contain an amino-terminal variable region (V) and a carboxyl-terminal constant region (C). In the heavy chains, the V region is composed of one Ig domain, and the C region is composed of three or four Ig domains. Each light chain is composed of one V region Ig domain and one C region Ig domain. Like the name implies, variable regions vary in their amino acid sequences among antibodies made by different B cell clones. The V region of one heavy chain (V_H) and the adjacent V region of one light chain (V_L) form the antigen-binding site (see Figure 16). The C region Ig domains are distant from the antigen-binding site and do not participate in antigen recognition. The heavy chain C regions (C_H) are responsible for most of the biologic functions of antibodies, since this is where the interaction with other effector molecules and cells of the immune system takes place (Abbas et al., 2014; Lipman, Jackson, Trudel & Weis-Garcia, 2005).

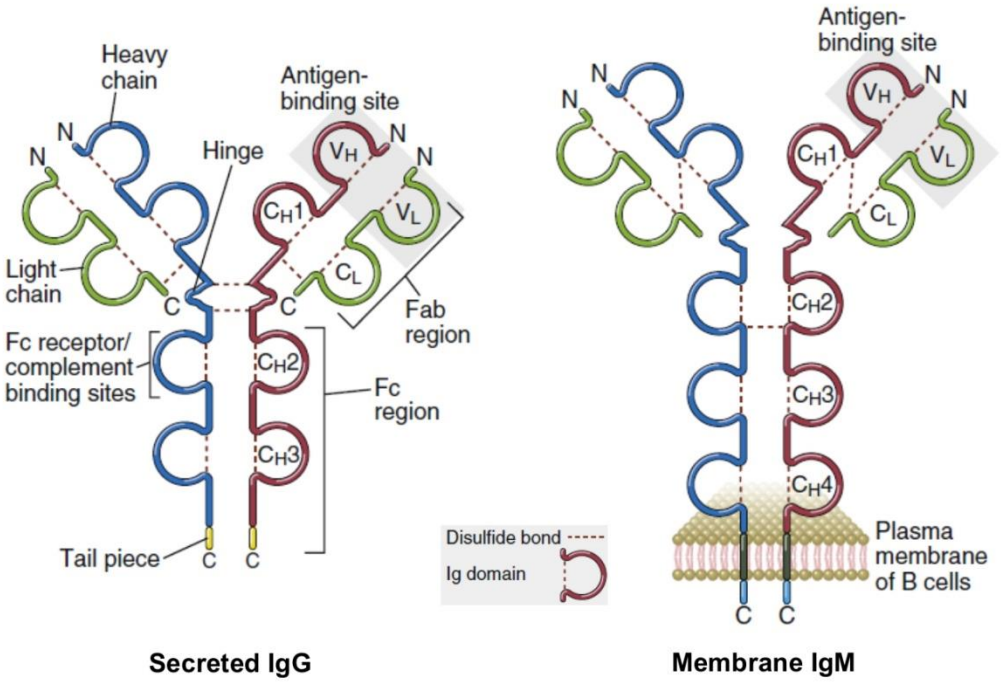

Figure 16. Structure of an antibody molecule.
Schematic diagram of a secreted IgG molecule (on the left) and a membrane-bound IgM molecule (adapted from Abbas, Lichtman & Pillai, 2014, with permission from Elsevier)

The analysis of the structural characteristics and biological activity of immunoglobulins really began in 1959 with the distinct works of Edelman (1959) and Porter (1959). They decided to address this issue by splitting the immunoglobulins and, despite previous attempts (Porter, 1950), for the first time they were able to separated them into analyzable parts suitable for further study. Edelman showed that reduction of the disulfide bonds of antibodies in the presence of denaturizing agents led to dissociation of the molecule into four fragments: two identical chains with a molecular weight of about 22 kDa each and two others of about 53 kDa each. They were named light chain and heavy chain and it was first proposed that IgG molecule consisted of two heavy and two light chains linked by disulfide bonds and noncovalent interactions (Edelman, 1959). Porter's (1959) experiments focused on a specific IgG of rabbit serum that he cleaved with papain. This enzyme hydrolyzed peptide bonds and produced three fragments of similar molecular weight (50kDa). Two of these fragments were found to retain ability to bind antigen specifically, although, unlike the intact molecule, they could no longer precipitate the antigen from solution. These two fragments are referred to as Fab (fragment, antigen-binding), are identical to each other and consist of the complete light chain (V_L and C_L) associated with a V_H-C_H1 fragment of the heavy chain. The third fragment is composed of two identical disulfide-linked peptides, that

correspond to the heavy chain C_H2 and C_H3 domains and is called Fc (fragment, crystallizable), because it has a propensity to aggregate and to crystalize into lattice. The crystals of the Fc fragments coming from antibodies with different specificities were practically homogeneous. It should be noted that if pepsin is used instead of papain to cleave rabbit IgG, proteolysis occurs C-terminally to the disulfide bridge between the heavy chains at the hinge region, generating a divalent fragment of IgG, referred as F(ab')2 (Figure 17). In this situation the inter-chain disulfide bonds are kept intact and only two fragments are originated from the proteolysis (Abbas et al., 2014; Coico & Sunshine, 2015a).

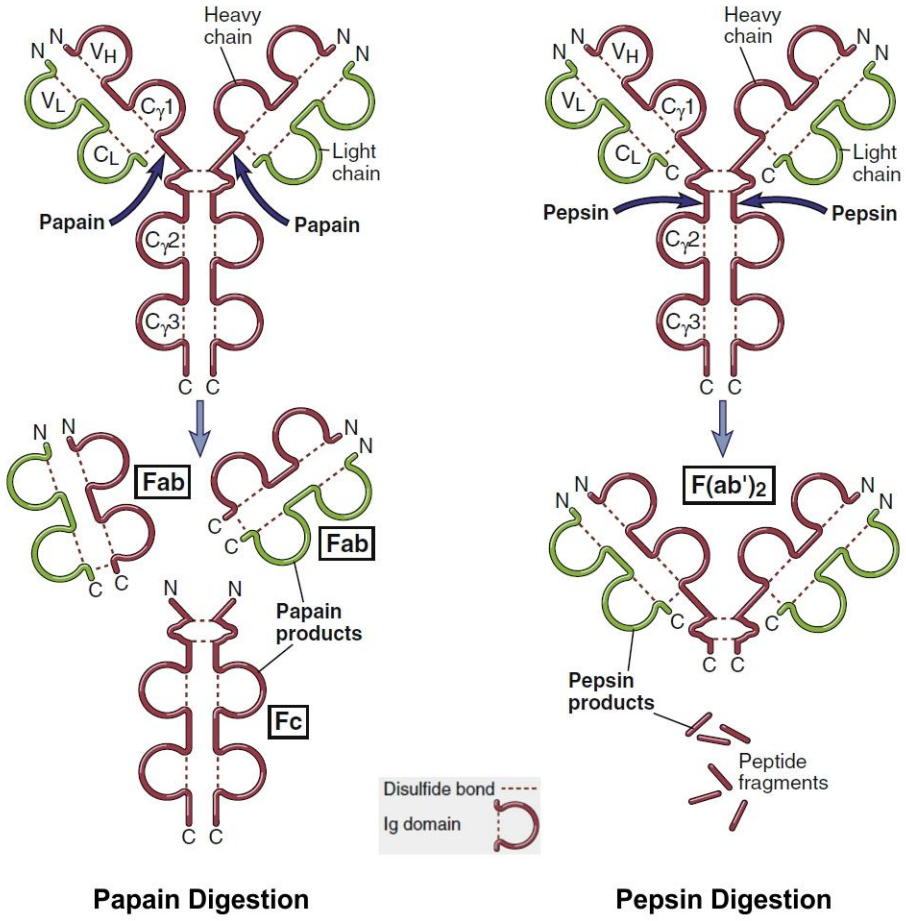

Figure 17. Proteolytic digestion of IgG by papain (left) and pepsin (right). Cleavage occurs at the sites indicated by arrows. Papain digestion allows separation of two antigen-binding regions (Fab fragments) from the portion of the IgG molecule that binds to complement and Fc receptors (Fc fragment). Pepsin generates a single bivalent antigen-binding fragment, the F(ab')2. (adapted from Abbas et al., 2014, with permission from Elsevier)

Each clone of a B-cell produces an antigen receptor (immunoglobulin) with a unique antigen-binding structure, that is determined during the process of lymphocyte maturation, through complex process of gene rearrangement and genetic recombination. Briefly, the genes that encode diverse antigen receptors are generated by the rearrangement of different variable (V) region gene segments with diversity (D) and joining (J) gene segments. This process, named V(D)J recombination, involves selection of one V gene, one D segment (when present), and one J segment in each lymphocyte and rearrangement of these segments to form a single V(D)J exon that will code for the carboxyl-terminal ends of an immunoglobulin variable region. It is this random use of different V, D and J segments that contributes to the diversity of the antigen receptor repertoire. Early in the maturation of lymphocytes, low-affinity antibodies are produced and it is the process of affinity maturation, where somatic mutation of Ig V genes and selection of B-cells with high-affinity antigen receptors occurs, that will latter generate antibodies with high affinity for antigen (Abbas et al., 2014).

Quite apart from the structure and the function in the immune response, antibodies have long been an important tool for investigators, who capitalize on their specificity to identify or label particular molecules or cells and to separate them from a mixture. The physiological response to a specific antigen, i.e. the antibody production, is highly heterogeneous. There is a huge diversity of B lymphocytes, the precursors of plasma cells, in the spleen of a mouse or a man. All are derived from a common stem cell, but each line develops, as briefly mentioned, an independent capacity to make an antibody that recognizes a different antigenic determinant. When an animal is injected with an immunizing agent, it responds by making diverse antibodies directed against different antigen molecules on the injected substance and/or different determinants on a single antigen, and even different antibodies that recognize, more or less well, a single determinant. So conventional antiserums contain mixtures of antibodies, that vary from animal to animal, making it very difficult or even impossible to make reproducible reagents (sera) against a specific antigen (Milstein, 1980). Each antibody is made, however, by a different line of lymphocytes and their derived plasma cells. If we could harvest one such cell (making a single specific antibody) and grow it in culture, this single cell's progeny, or clone, would be a source of large amounts of identical antibody against a single antigenic determinant: a monoclonal antibody (mAb). Unfortunately antibody-secreting cells cannot be maintained in *in vitro* culture. There are however malignant tumors of the immune system called myelomas that are immortal and able to secrete large amounts of immunoglobulins. They can be cultured *in vitro*, but it is impossible to determine to what antigen is the secreted antibody directed or to induce myelomas that produce antibodies to a specific antigen (Coico & Sunshine, 2015b; Goding, 1996). A major

breakthrough was achieved in 1975, when Georges Köhler and César Milstein described for the first time a method for immortalizing individual antibody-secreting cells from an animal immunized with a known antigen by fusing them with a myeloma cell line and growing them in a way that unfused normal and tumor cells cannot survive (Köhler & Milstein, 1975). The resultant fused cells that grow out are called hybridomas and each hybridoma makes only one antibody, derived from one B-cell from the immunized animal. The antibodies secreted by many hybridoma clones are screened for binding to the antigen of interest, and this single clone with the desired specificity is selected and expanded. The products of these individual clones are monoclonal antibodies, each specific for a single epitope on the antigen used to immunize the animal and to identify the immortalized antibody-secreting clones (Goding, 1996). With this technique, antibodies could be transformed in a well-defined chemical reagent that can be reproduced at will, making it a valuable tool for scientific research and clinical medicine. Monoclonal antibodies have not only fueled breakthrough discoveries in basic research, but have also been developed as clinical diagnostics, reagents for high throughput drug screening, and more importantly, life-saving medicines (Abbas et al., 2014; An, 2010).

In apicomplexan parasites, references to the use and development of monoclonal antibodies started early in the 1980's, especially in *Plasmodium* spp. and *Toxoplasma*. Many of these early studies were already directed to different purposes like diagnostics (Araujo, Handman & Remington, 1980; Erlich, Rodgers, Vaillancourt, Araujo & Remington, 1983) or treatment (Cox, 1980; Freeman, Trejdosiewicz & Cross, 1980; Johnson, McDonald & Neoh, 1983b), but most of them were descriptive of a specific mAb developed and it's characterization, in terms of antigen recognition and/or the effect in experimental parasite growth (for example: Handman, Goding & Remington, 1980; Handman & Remington, 1980; Johnson, Haynes, Leppard, McDonald & Neoh, 1983a; Perrin, Ramirez, Er-Hsiang & Lambert, 1980; Pinder & Hewett, 1980; Potocnjak, Yoshida, Nussenzweig & Nussenzweig, 1980; Sethi, Endo & Brandis, 1980; Yoshida, Nussenzweig, Potocnjak, Nussenzweig & Aikawa, 1980). Consequently, the first studies started to report the inhibitory effect of specific antibodies on the invasion of apicomplexan parasites and their intracellular replication, especially in *Toxoplasma gondii* (Hauser & Remington, 1981; Johnson et al., 1983b; Schwartzman, 1986; Sethi, Endo & Brandis, 1981), *Plasmodium falciparum* and *P. vivax* (Hollingdale, Nardin, Tharavanij, Schwartz & Nussenzweig, 1984), *Cryptosporidium parvum* (Perryman, Riggs, Mason & Fayer, 1990) and *Eimeria bovis* (Whitmire, Kyle, Speer & Burgess, 1988). In these early times however, hyper-immunization of mice was most frequent accomplished by using mixed antigenic molecules, like the parasite itself or parasite protein extracts whose contents were far from defined, originating a blind monoclonal antibody production, that was after tested for effect or to probe major antigenic proteins. This

was in fact the case for the only two published studies concerning mAb development for *B. besnoiti.* One study fused spleen cells of mice immunized with total protein extracts of purified tachyzoites (Shkap, Pipano & Zwernemann, 1995) whereas the other of mice immunized with purified live tachyzoites (Njagi, Entzeroth, Nyaga & Musoke, 2004). Shkap et al. (1995), reported one mAb with a strong immunofluorescence labelling of the polar organelle complex, that recognized a 70 kDa polypeptide on immunoblots, while Njagi et al. (2004) reported three mAb recognizing antigens of >200 kDa and one with 75 kDa, with different fluorescence patterns against *B. besnoiti* tachyzoites. However, both studies could not ascribe the specific location of the reactive epitope to a particular organelle or specifically identify the target proteins. Nowadays, in this post-genomic era, the means to address a more specific production of mAb are present, not only because we can immunize mice with purified and specific peptides but also because screening of epitope recognition is much more sensitive. In this way, since PDI has been recognized as an important player in host cell invasion process and host immune response, we proceeded with the production of anti-PDI mAb, to acquire a powerful tool for further studies addressing these issues.

3.2 Materials and methods

3.2.1 Production of recombinant *B. besnoiti* PDI (recBbPDI) and truncated versions.

For cDNA synthesis, *B. besnoiti* tachyzoites (10^8) were purified from in vitro cultures, RNA was extracted (High Pure RNA Isolation Kit – Roche) and reverse transcribed by a two-step RT-PCR procedure (First Strand cDNA Synthesis Kit – Fermentas). For the full length *B. besnoiti* PDI the cDNA was PCR-amplified using the forward primer PDI_F_Nde and the reverse primer PDI_R_Xho, that contain Nde and XhoI recognition sites respectively (primer sequences are shown in Table 3). Truncated versions of BbPDI were produced corresponding to the domains **a**, **b**, **b'** and **a'c** using the primers presented in Table 3 containing the mentioned recognition sites, as follows: domain **a**, forward primer PDI_F_Nde and reverse primer PDI_a_R_Xho; domain **b**, forward primer PDI_b_F_Nde and reverse primer PDI_b_R_Xho; domain **b'**, forward primer PDI_b'_F_Nde and reverse primer PDI_b'_R_Xho; and domain **a'c**, forward primer PDI_a'c_F_Nde and reverse primer PDI_R_Xho. Schematic representation of the truncated version is depicted in Figure 18**.** PCR amplification was carried out in a 25 µl PCR mixture (1x reaction buffer, 1 U of Taq DNA polymerase (Fermentas), 10 pmol of each primer, and 0.2mM dNTP's) and performing 30

cycles (90 °C for 30 s, 60 °C for 1 min, 72 °C for 1 min 15 s). PCR products were cloned into the pGEM-T Easy Vector, its nucleotide sequence was confirmed and then subcloned in the pET28a vector using the Xho I and Nde I restriction sites of the polylinker. The strain Rosetta(DE3)pLysS was used for expression purposes; Protein expression was induced by the addition of isopropyl-D-thiogalactopyranoside to a final concentration of 1 mM when bacterial cultures attained an OD of 0.6 to 0.9 (measured at 600 nm wavelength), followed by incubation for 2 hours. The domains were purified by affinity chromatography using Ni Sepharose 6 Fast Flow (GE Healthcare) according to the manufacturer instructions.

Table 3. List of primers used for PCR amplification of the full length recBbPDI and the truncated versions **a**, **b**, **b'** and **a'c**.
Underlined is the sequence for the restriction enzyme together with the necessary added nucleotides for efficient cleavage in lowercase letters.

Primers' name	Sequence (5'- 3')
PDI_F_Nde	ggaattccatatgCGGGCTGGGGTCTGCTACCTT
PDI_R_Xho	ccgctcgagttaCAATTCCTCGCCCTTGTCGT
PDI_a_R_Xho	ccgctcgagttaCAACGCTGCCCTCGACCTC
PDI_b_F_Nde	ggaattccatatgGACGACAAGGTTACCAAGGAG
PDI_b_R_Xho	ccgctcgagttaTAGAGTCGTCGAAGTCCTTTTC
PDI_b'_F_Nde	ggaattccatatgACCGAAAAGGACTTCGACGAC
PDI_b'_R_Xho	ccgctcgagttaCAACCTTGACAGCCTCCTCC
PDI_a'c_F_Nde	ggaattccatatgCAGGAGGAGGCTGTCAAGGTTGT

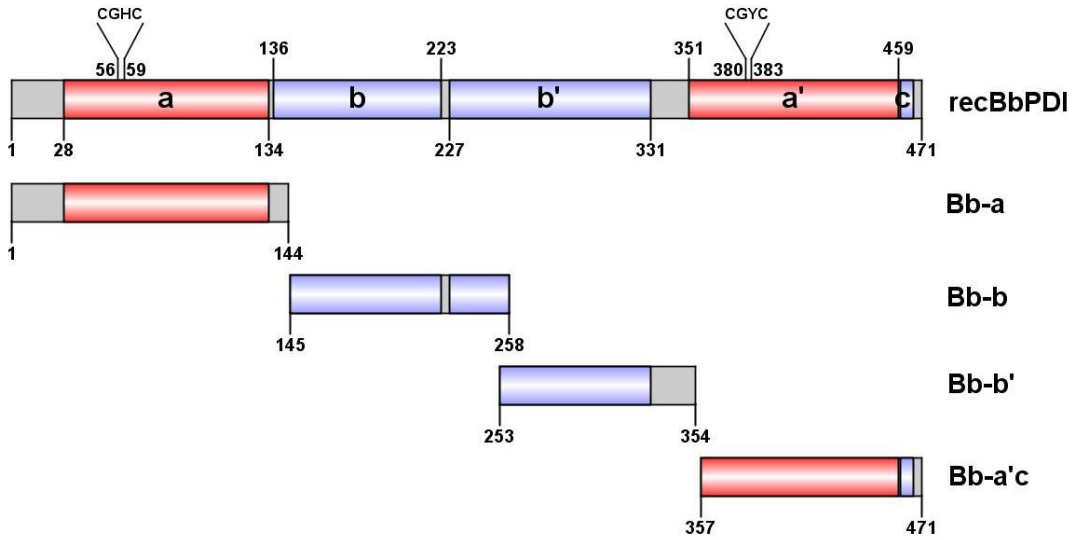

Figure 18. Schematic representation of the truncated version of recombinant BbPDI produced. Recombinant BbPDI is shown on the top. Domain boundaries are illustrated and labeled, with the a-type domains in red and the b-type and **c** domain in blue. Location of the active sites CGHC and CGYC is shown. Truncates version are shown under recBbPDI.

As domain **a** and domain **b** were present in the insoluble fraction of the bacteria lysate they were purified in denaturing conditions, by the addition of 8M urea. All fractions were analyzed by SDS-PAGE and elution fractions were pooled and concentrated using a ultrafiltration centrifugal device (Amicon, Millipore) with the appropriate nominal molecular weight limit, accordingly with the manufacture's recommendations and instructions. Protein quantification was performed using a NanoDrop 2000 spectrophotometer assuming the extinction coefficient and the molecular weights presented in Table 4. The extinction coefficients and molecular weights were calculated based on the amino acid sequence, using the ProtParam tool of the ExPASy Bioinformatics Resources Portal from the Swiss Institute of Bioinformatics (http://web.expasy.org/protparam/) (Artimo et al., 2012; Gasteiger et al., 2005).

Table 4. Recombinant proteins produced in this chapter.
Expected molecular weight (MW) and extinction coefficient (ε) was calculated by ProtParam tool based on the amino acidic sequence of the protein and includes the initiating N-terminal methionine residue and the histidine tag from pET-28a. Molecular weights assuming that cysteines from active sites form disulfide bonds

	Number of amino acids	MW (Da)	Theoretical pI	ε ($M^{-1}cm^{-1}$)
RecBbPDI	491	55039.7	5.57	49890
Domain a	164	17804.4	6.5	17210
Domain b	135	15402.1	5.44	9970
Domain b'	125	13965.7	7.11	5500
Domain a'c	145	16526.7	5.98	17085

3.2.2 Production of monoclonal antibodies against *B. besnoiti* PDI

3.2.2.1 Mice immunization

Four weeks old, female BALB/c mice were intraperitoneally immunized three times, every 2 weeks, with 10 μg of recBbPDI emulsified in incomplete Freund's adjuvant (Sigma). After the third injection the mouse was bled and the anti-PDI antibody titre determined by enzyme linked immunosorbent assay (ELISA). Three days after the last immunization the mice were sacrificed and the spleen aseptically collected to harvest cells for cell fusion.

3.2.2.2 Anti-recBbPDI antibody titre determination

A blood sample was collected and serum titre determined by ELISA using as antigen to sensitize the plates recBbPDI, at 0.2 μg/well in carbonate buffer 0.5 M (pH 9.5), overnight at 4ºC. As secondary antibody a goat anti-mouse alkaline phosphatase-conjugated IgG (heavy and light chain) (Sigma) diluted at 1:5000. Incubations with antibodies were carried out at 37 ºC in the dark. p-Nitrophenylphosphate (Sigma) (1 mg ml^{-1}) in 100mM glycine containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$, pH 10.4 was used as enzyme substrate and the plate incubated 30 minutes in the dark at room temperature. The absorbance was read at 405nm in a TIM 200 Inter Med plate reader.

3.2.2.3 Cell fusion and HAT selection

Myeloma cells Sp2/0 Ag14 were maintained *in vitro* in exponential growth in Dulbeco's Minimal Essential Medium (DMEM) supplemented with 10% fetal calf serum at 37 °C, 5% CO_2 and 95% of relative humidity. They were collected by centrifugation (300 g for 5 min.), washed twice in serum-free medium and counted by trypan blue exclusion in a Neubauer chamber.

In a laminar flow hood, the spleen was transferred to a petri dish and then repeatedly injected with serum-free DMEM using a 25 gauge needle to drive the spleen cells free into the medium. The cells are harvested by centrifugation (300 g for 5 min.), washed twice in medium and counted as above.

A third and final wash in serum-free DMEM was made while combining splenocytes (10^8) and myeloma cells (5×10^7) in the same tube and centrifuging at 300g for 5 min. The supernatant was discarded and care was taken to assure that the cell pellet is dry. Cell were fused in the presence of 50% polyethylene glycol (PEG, Hybri-Max™ Sigma): while gently stirring, 0.8 mL PEG was gradually added over a period of 1 min; then, one milliliter of serum-free DMEM was added slowly with gentle agitation over one minute, and the PEG further diluted with new addition of 20 mL of DMEM over a 4 minute period with gentle agitation. The cells were centrifuged and resuspended in 50 mL of selection medium (DMEM supplemented with 1×10^{-4}M Hypoxanthine, 4×10^{-7} M Aminopterin, 1.6×10^{-5} M Thymidine (HAT, Sigma) and with 10% (v/v) inactivated fetal calf serum), 100 µL distributed through 8 microculture plates wells and incubated at 37 °C, 5% CO_2 and 95% of relative humidity. On day one 100 µL of selection medium was added to each well and, every 2 to 3 days after, half the medium of each well was replaced with fresh medium. When hybrids start to grow and be visible under the inverted microscope, the supernatants were tested by ELISA for antibody presence.

3.2.2.4 Cell expansion and cloning

Positive wells for anti-recBbPDI antibody presence were expanded gradually to 24 wells plates, T25 and T75 culture flasks. When semi-confluent monolayers were achieved cells were frozen in liquid nitrogen and the supernatant stored for testing antibody presence. For cloning, selected flasks were scrapped, the hybrid cells centrifuged at 300 g for 10 minutes and then resuspended in culture medium (DMEM supplemented with 10% heat-inactivated fetal bovine serum, 20 mM HEPES, 250 µg/mL amphotericin B, 100 U/mL penicillin and 100 µg/mL streptomycin). The number of hybrid cells was determined by counting with trypan blue exclusion in a Neubauer chamber, and the concentration of cells adjusted to 5 cells/mL. From this solution, 100 µL (i.e. 0.5 cells per well) were distributed into each well of 5

microculture plates and incubated as previously described. On day one 100 μL of culture medium was added to each well and, every 2 to 3 days after, half the medium of each well was replaced with fresh medium. When cells started to grow and be visible under the inverted microscope, the supernatants were again tested by ELISA for antibody presence. Positive wells were gradually expanded to 24, 12 and 6 wells plates. At this stage supernatants were also tested by western blot (WB), over recBbPDI and its truncated versions, and indirect immunofluorescence (IFAT), over fixed *B. besnoiti* tachyzoites, in order to distinguish different antibodies. Desired flasks were selected to proceed once more with cloning as just described. Again, positive wells for antibody production were expanded and the cell clones supernatants were tested by ELISA, WB and IFAT.

3.2.2.5 Enzyme-linked immunosorbent assay (ELISA)

Soluble protein extracts were prepared from purified tachyzoites of *B. besnoiti*, *N. caninum* and *T. gondii* by sonication. RecBbPDI (0.2 μg/well) or soluble protein extracts (2 μg/well) coated plates were incubated with mAb (1μg/mL), for 1 hour at 37 °C. As secondary antibodies, AP-conjugated anti-mouse polyvalent immunoglobulins (G,A,M) (Sigma) were used in a 1:10.000 dilution. p- Nitrophenylphosphate (Sigma) (1 mg ml^{-1}) in 100mM glycine containing 1 mM $MgCl_2$ and 1 mM $ZnCl_2$, pH 10.4 was used as enzyme substrate. The plate was incubated 30 minutes in the dark at room temperature and the absorbance was read at 405 nm in a TIM 200 Inter Med plate reader.

3.2.2.6 Western blot (WB)

Protein samples prepared from purified tachyzoites of *B. besnoiti*, *N. caninum* and *T. gondii*, and purified recBbPDI and domains **a**, **b**, **b'** and **a'c** were separated by SDS–PAGE (12%) and blotted onto nitrocellulose membrane. For ease of operations, a Prep/2D comb was used, so that small strips from the nitrocellulose membrane could be cut for batch analysis. Also, considering the different molecular weights, recBbPDI, domain **a** and domain **b'** were pooled together in the same membrane. Membranes were blocked with 1% fish gelatin solution, probed with the mAb (hybrid cell culture supernatant or 2 μg/mL of purified mAb) and then incubated with an AP-conjugated secondary antibody (Sigma). Enzyme reaction was developed with the BCIP/NBT kit (Bio-Rad).

3.2.2.7 Immunofluorescence

Purified parasite tachyzoites were allowed to adhere to poly-L-lysine-coated microscopy coverslips for 30 min, and were fixed for 10 min with cold (-20°C) methanol. Parasites were then probed with mAb supernatant and FITC-conjugated anti-mouse antibodies (Invitrogen). To visualize the entire cells, parasites were simultaneously probed with rabbit anti-*B. besnoiti* (Cortes et al., 2006b) and TRITC-conjugated anti-rabbit antibodies (Invitrogen). All these sera were used at 1:500 dilutions; supernatants were used undiluted. DNA was stained with DAPI (1 μg mL; Sigma) for 2 min and the slides were mounted with Mowiol (Calbiochem) and examined under a UV light microscope.

3.2.2.8 Monoclonal antibody purification and isotype determination.

The clones selected for purification were gradually adapted to serum free medium (EX-CELL®, Sigma) and expanded to 175cm^2 tissue culture flasks. Secreted antibodies from the cell culture medium were collected by centrifuging at 300g for 10 minutes to sediment cells and filtering through a 0.45 μm PVDF membrane. The antibody was purified by affinity chromatography using HiTrap Protein G HP columns (GE Healthcare), accordingly to the manufacturer instructions. All fractions were collected and analyzed by SDS-PAGE. Fractions of purified mAb were pooled and concentrated using an Amicon Ultra 15 with a nominal molecular weight cut off of 50 kDa. Antibody concentration was determined using a NanoDrop 2000 spectrophotometer with the pre-configured IgG reference (extinction coefficient of 13.7 $M^{-1}cm^{-1}$ at 280 nm for a 1% (10 mg/mL) IgG solution)

The isotype of the antibodies obtained was determined by ELISA, using a commercial kit accordingly to the manufacturer's instructions (Mouse Mono Ab-ID Kit, Zymed).

3.2.2.9 Storage of hybrid cells

Throughout the process of mAb production, hybrid cells were frequently frozen in liquid nitrogen. For that, cells were scraped from the tissue culture flasks, centrifuged at 300 g for 10 min, resuspended in plain DMEM and placed on ice. Cells were counted in a Neubauer chamber by trypan blue exclusion and the concentration adjusted to approximately 2x10^7 cells/mL. Ice cold frozen medium (DMEM with 40% FBS and 20% DMSO) was then slowly added to the cell suspension while gently stirring, yielding a final suspension of approximately 10^7 cells in DMEM with 20% FBS and 10% DMSO. Cell suspension was transferred to cryotubes, left on ice for 30 min and then frozen in liquid nitrogen.

Thawing of cells was done rapidly in a 37 °C water bath. The cells were transferred to a culture flask already with 37 °C heated culture medium and left in the incubator for 12 hours. Cells were then washed twice with PBS and fresh culture medium added.

3.2.3 Epitope prediction

Prediction of mAb epitope recognition in BbPDI was made combining *in silico* prevision based in the amino acidic sequence and the three-dimensional protein structure with the results obtained with WB and ELISA. Linear prediction of epitopes was done with BepiPred (http://www.cbs.dtu.dk/services/BepiPred/) (Larsen, Lund & Nielsen, 2006), based on the deduced amino acid sequence encoding BbPDI (GenBank accession No. DQ490130). The prediction based on the 3D structure was performed by ElliPro (http://tools.immuneepitope.org/ellipro/) (Ponomarenko et al., 2008) based on the theoretical three-dimensional model developed in Chapter 2, and deposited in the PMDB (https://bioinformatics.cineca.it/PMDB/, (Castrignano et al., 2006)) under the identification number PM0075875. Multisequence alignment was executed by Clustal Omega from the European Molecular Biology Institute (http://www.ebi.ac.uk/Tools/msa/clustalo/) (Li et al., 2015; Sievers et al., 2011), using the deduced amino acid sequence encoding *B. besnoiti* PDI (GenBank accession No. DQ490130), *N. caninum* PDI (AAV34741) and *T. gondi* PDI (AJ306291).

3.3 Results and discussion

For the successful production of novel monoclonal antibodies based on hybridoma technology there are three critical points. The first point is immunization to facilitate differentiation of B-lymphocytes into more matured forms. The second point is the fusion of targeted antigen-sensitized B-lymphocytes with myeloma cells and the *in vitro* selection of the hybridoma. The third point is the initial screening assay to access the desired mAb production (Shepherd & Dean, 2000; Tomita & Tsumoto, 2011). If these critical points are achieved, hybridoma technology allows generation of monoclonal antibodies with high affinity and specificity.

Mice immunization followed the standard protocol of three antigen inoculations, two weeks apart, with recBbPDI emulsified in incomplete Freund's adjuvant to slow down the otherwise rapid dispersion of the soluble immunogen and to elicit a strong cellular infiltrate of

neutrophils and macrophages at the site of injection, stimulating the proliferation of specific antibody-producing B cells and their precursors in the local lymph nodes and spleen. As mature antibody-secreting cells fuse poorly with myelomas and it is the committed precursors that are required for hybridoma production, the animals were given a final boost with antigen three days before the spleen was harvested, accordingly the protocol described by Shepherd and Dean (2000). With this protocol high antibody titers are usually obtained but these were confirmed by ELISA at the time of the third inoculation. In fact, all mice had titers higher that 1:50000 two weeks after the second inoculation, as assessed by ELISA using recBbPDI sensitized plates, and were used to proceed to the cell fusion process as scheduled.

The introduction of PEG for somatic cell fusion and its commercial (ready-to-use) availability resulted in standardization of the hybridoma technology (Davidson & Gerald, 1976, 1977; Lane, 1985; Maggio, Ahkong & Lucy, 1976). Nevertheless, the fusion process itself is random and poorly controlled and hybrid cells will result from the desired fusion of myeloma cells with splenocytes, but also from the unwanted fusion between two splenocytes or two myeloma cells. Thus, a key point in this technique is the selection of the desired long-term hybrid cell line (Goding, 1996), that was accomplished by using an enzyme-deficient myeloma cell line, unable to grow in hypoxanthine-aminopterin-thymidine (HAT) selective medium, a procedure first described by Littlefield (1964). The myeloma cell line Sp2/0 Ag14, was engineered to be deficient in the enzyme hypoxanthine-guanine phosphoribosyl transferase (HGPRT). When the main biosynthetic pathway for guanosine is blocked by the aminopterin added to the medium, HGPRT becomes essential in the salvage pathway for DNA synthesis, by converting the hypoxanthine to guanosine monophosphate. In this way, HGPRT-negative cells (i.e. immortal myeloma cells) will die when cultured in HAT medium, but they can survive if they are made HGPRT-positive my fusing with and HGPRT-positive cell such as a splenocyte. Every cell not immortalized by fusing with a myeloma cell will die within days of *in vitro* culture (Goding, 1996; Hope & Graves, 1978; Shepherd & Dean, 2000).

The initial screening of the hybridoma culture supernatants for specific antibody production also represents a key step for the success of the technique, since it is on the basis of these results that a hybridoma colony is picked, expanded and cloned. Initial testing was done by indirect ELISA and started when considerable cell growth was visible under the inverted microscope, usually around the 10^{th} to 15^{th} day post-fusion. At this time testing has to be quick and specific considering the large number of supernatants to test and so, it relied solely on the recognition of recBbPDI in ELISA. A polyvalent and polyclonal affinity purified secondary antibody was used in order to guarantee that we could detect all the expected classes of antibodies (IgG, IgM and IgA). Best performing positive wells were gradually expanded and samples frozen in liquid nitrogen as soon as possible. With higher volumes of

supernatants available initial screening was diversified in an attempt to perceive differences in the mAbs secreted. In this way, selection for native *B. besnoiti* PDI was accomplished by labeling *B. besnoiti* tachyzoites by IFAT and soluble *B. besnoiti* tachyzoites protein extracts by ELISA as well as total parasite protein extracts by WB. For differences in epitope recognition truncated versions of BbPDI were used for screening, both by ELISA and WB. The screening results for the fusions performed were always consensual in the way that no differences were observed when testing by ELISA (where conformational epitopes are recognized) or by WB (where linear epitopes are labeled), i.e., independently of the antigen used for labeling (soluble extracts, purified protein or domains) either we obtained positive supernatants in both techniques or negative ones for both. Regarding PDI domain recognition, we noticed that the vast majority of antibodies produced recognized domain **a'c**, since around 90% of positive supernatant hybridomas tested labeled this domain. No labeling, in either WB or ELISA, was achieved for domains **a** and **b** and the remaining 10% were divided in supernatants that recognized the domain **b'** and supernatants that labeled only the full length PDI and not any domain. Considering that mice were immunized with full length recBbPDI, that was functionally active, we can consider domain **a'c** as more immunogenic than the others domains.

After the screening process, 5 hybridoma cell lines were selected from the panel of hybrid cells produced. Differences in the labeling of recBbPDI and truncated versions produced by WB and ELISA were the basis for picking different hybridomas, since the IFAT never produced differences in the labelling of *B. besnoiti* tachyzoites among the antibodies tested. However, we could never achieve the labeling of domain **a** or domain **b** by ELISA or WB. The selected cells were cloned by the limiting dilution method, following standard protocols (Coller & Coller, 1983; Lefkovits & Waldmann, 1984; Waldmann & Lefkovits, 1984). After cloning, hybridomas were again tested for the production of mAb by the methods just described and expanded. It is not uncommon that hybridomas suffer chromosome lost and sometimes the ability to produce antibodies, which warrants the need for continuous screening along all processes of mAb production (Goding, 1996). In fact, this happened with one of the cell lines picked for cloning. Hybridoma selection was once more based on the screening results but care was taken to pick up a hybrid cell line derived from a single colony. Nevertheless, to be absolutely sure that we ended up with a true clone, a second cloning procedure was performed.

For purification, with the loss of mAb production ability of one of the cell lines, we ended up with 4 different hybrid cell lines, each producing a different mAb. At this time, a chemically defined serum free medium was used to expand cells, in order to guarantee that no interference from proteins in the FBS would hamper the purification method or the purity of obtained mAbs. Adaptation to serum free medium was previously tested, as well as the mAb production ability in the new medium. Initially, crescent amounts of serum free medium were gradually used, while reducing the normal medium. Later, we could assess that medium change could be made immediately without prejudice to cell viability or mAb production capability. Purification was performed using a peristaltic pump and HiTrap Protein G HP columns in aseptic conditions (Figure 19).

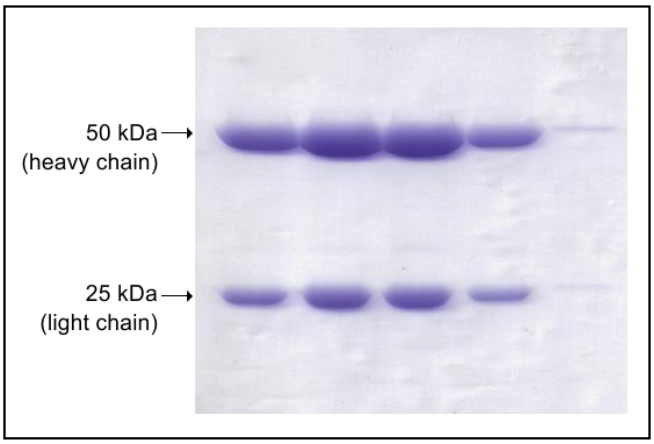

Figure 19. Coomassie stained SDS-PAGE (10%) of purified mAb (only elution fractions are shown). Under denaturing conditions the two light and heavy chains are separated.

Characterization of the purified mAbs was confirmed by defining the immunoglobulin isotype, using a commercial kit, and repeating the screening assays. Also cross reactivity with *N. caninum* and *T. gondii* was evaluated by ELISA and WB, since there is a high level of identity (88%) and similarity (96%) between PDI protein sequences of these apicomplexan. A resume of mAb characterization is presented in Table 5. All four mAb obtained are IgG1, with a *Kappa* light chain and can label both recBbPDI and *B. besnoiti* tachyzoite PDI by ELISA, WB and IFAT. Concerning domain recognition, mAb T8a and S4a label the domain **a'c**, while mAb R60b recognizes domain **b'**. Monoclonal antibody S16p was unable to recognize any of the produced truncated PDI fragments and is probably directed to a junction region of these fragments (Figure 20). Regarding PDI recognition of the above mentioned closely related

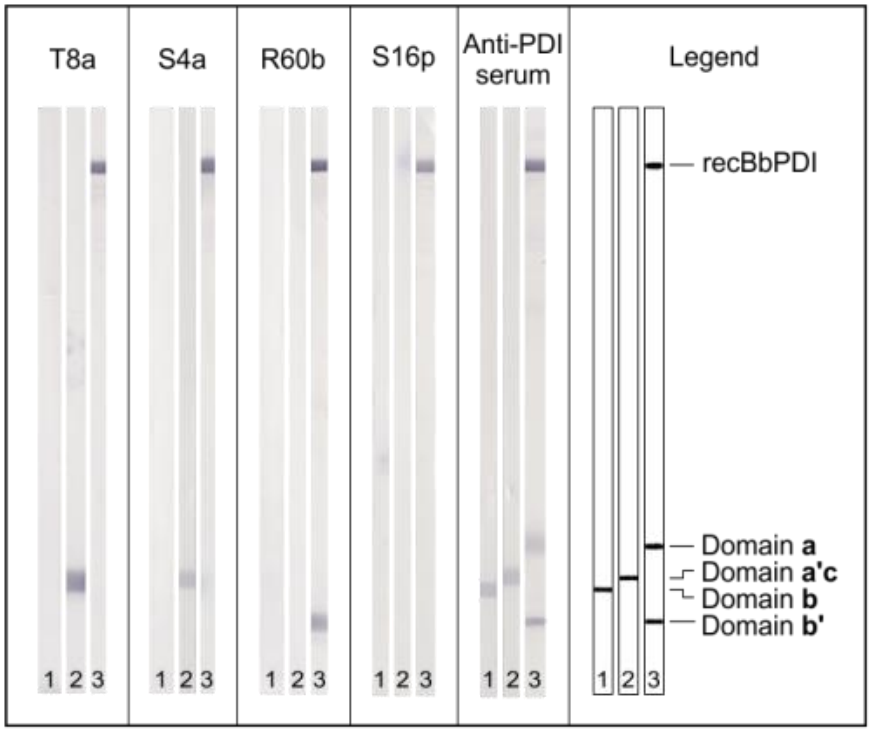

Figure 20. Western blot analysis of monoclonal antibodies T8a, S4a, S16p and R60b over recBbPDI and truncated version produced, corresponding to domains **a**, **a'c**, **b** and **b'**.
Schematic representation of probed nitrocellulose membrane is presented in the legend on the right. Considering the molecular weight of antigens, membrane had only domain **b** (1), only domain **a'c** (2) or recBbPDI with domain **a** and **b'** (3). Mouse Anti-PDI polyclonal serum was used as a positive control. All mAbs recognize the full length recBbPDI. Mab T8a and S4a labels domain **a'c.** Mab R60b labels domain **b'** and mAb S16p does not labels any isolated domain.

apicomplexan parasites, mAb T8a labels only *B. besnoiti* PDI; mAb S4a and S16p cross reacts with *N. caninum* PDI and labels it in both techniques; likewise, mAb R60b recognizes *N. caninum* PDI in both the ELISA and WB but is also able to recognize *T. gondii* PDI, but only by WB (Figure 21). In this way, mAb T8a is probably directed to a non-conserved region of domain **a'c**, since it only labels *B. besnoiti* PDI, while mAb S4a is probably directed to a conserved region of **a'c.** To better elucidate mAb epitope recognition and understand differences in specificity, the alignment of the amino-acidic PDI sequences of *B. besnoiti* (GenBank accession No. DQ490130), *N. caninum* (AAV34741) and *T. gondi* (AJ306291) was done, as well as the *in silico* prediction of epitopes, in an attempt to suggest probable sites for epitope recognition. Two *in silico* methods were used, one based on the amino acidic of PDI and the other based on the three-dimensional structure of the protein and the prediction output was compared with the results obtained in antigen recognition (Figure 22).

Table 5. Resume of monoclonal antibody characterization.
Labeling of antigens, either recBbPDI and truncated versions corresponding to domains **a**, **b**, **b'** and **a'c** or total protein extracts of *B. besnoiti*, *N. caninum* and *T. gondii*, is marked with **+**.

		Monoclonal antibody			
		T8a	**S4a**	**R60b**	**S16p**
Immunizing antigen		GST-PDI		His-PDI	
Isotype			IgG1, *Kappa* light chain		
Antigen recognition					
Full length recBbPDI		**+**	**+**	**+**	**+**
Domain a					
Domain b					
Domain b'				**+**	
Domain a'c		**+**	**+**		
Specificity					
B. besnoiti	ELISA	**+**	**+**	**+**	
	WB	**+**	**+**	**+**	**+**
N. caninum	ELISA		**+**	**+**	**+**
	WB		**+**	**+**	**+**
T. gondii	ELISA				
	WB			**+**	

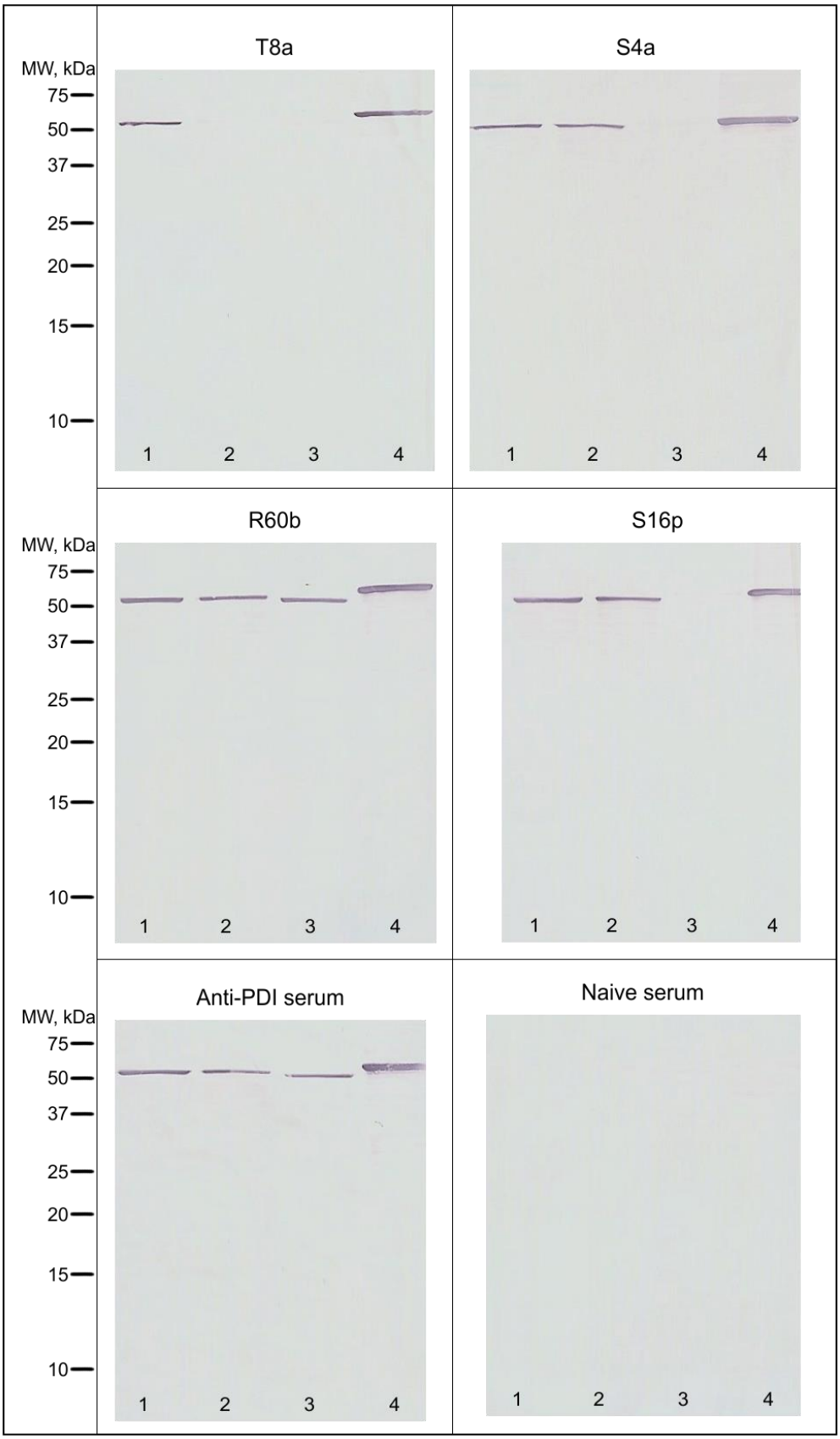

Figure 21. Evaluation of cross reactivity of monoclonal antibodies T8a, S4a, R60b and S16p with PDI of *N. caninum* and *T. gondii* tachyzoites.
Total protein extracts of *B. besnoiti* (1), *N. caninum* (2) and *T. gondi* (3) were separated by SDS-PAGE and blotted into nitrocellulose membrane, as well as recBbPDI (4). Polyclonal anti-PDI serum was used as positive control and a naïve serum as negative control.

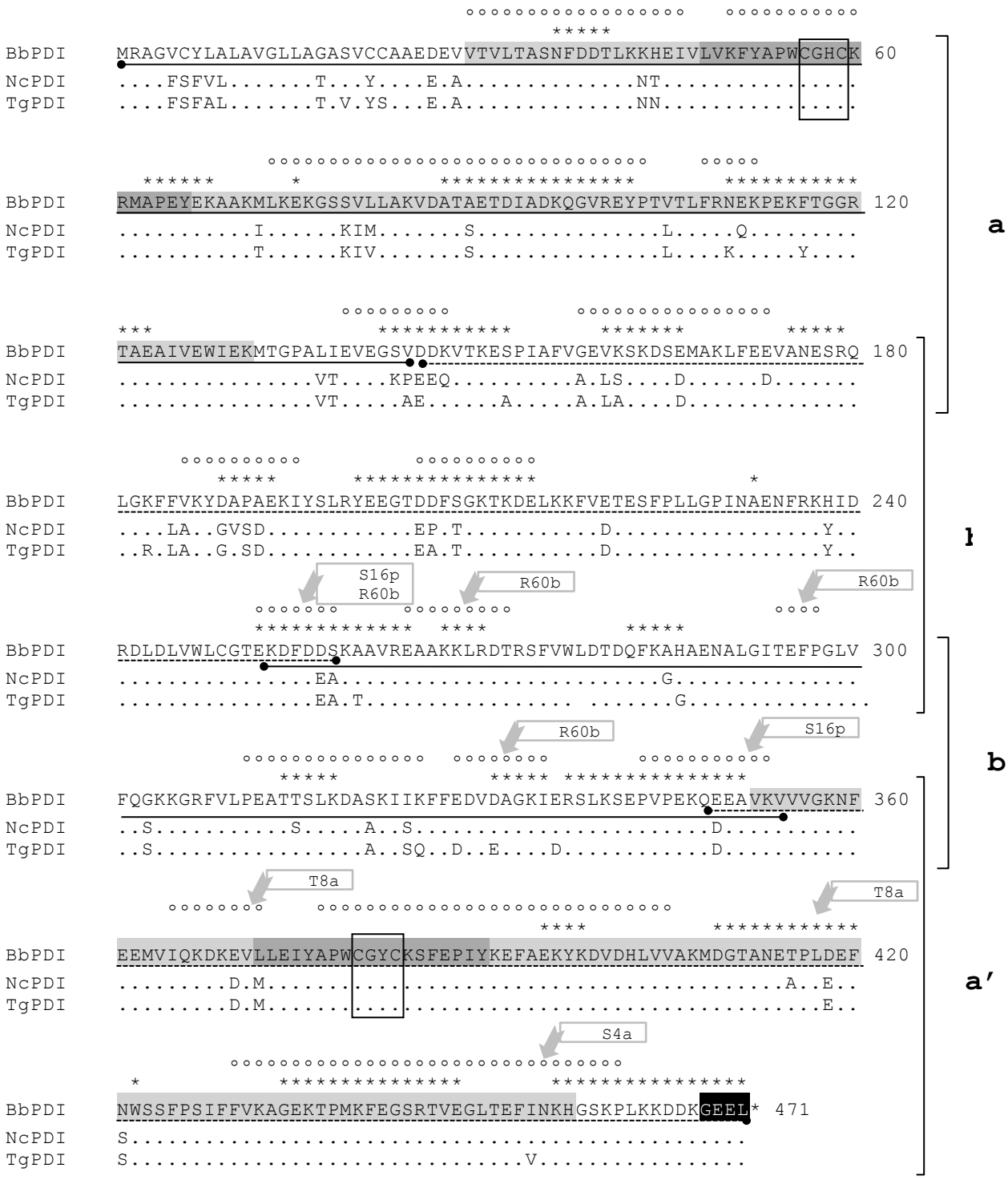

Figure 22. Epitope prediction of monoclonal antibodies produced in *B.besnoiti* PDI.
Alignment (Clustal Omega) of the deduced amino acid sequence encoding *B. besnoiti* (BbPDI; GenBank accession No. DQ490130), *N. caninum* PDI (NcPDI; AAV34741) and *T. gondi* PDI (TgPDI; AJ306291). Light gray highlights hits to the Pfam thioredoxin model (PF00085) and medium gray denotes the thioredoxin family active site Prosite pattern (PS00194). The characteristic catalytic thioredoxin motifs CxxC are boxed. The C-terminal putative ER retention signal is displayed on a black background. The stop codon is indicated by *. Delimitation of **a**, **b**, **b'** and **a'c** truncated versions of recBbPDI (as they were cloned and expressed) is showed by brackets and underlined; Epitope prediction by BepiPred (*) and ElliPro (º) for BbPDI is shown above its sequence. Arrows point hypothetical mAb epitope recognition considering results obtained for the labelling of total protein extracts of these parasites and truncated versions of PDI.

3.4 Conclusion

Through hybridoma technology we were able to obtain four monoclonal antibodies from mice immunized with recBbPDI. These were all able to recognize recBbPDI in WB and ELISA as well as natural *B. besnoiti* PDI in IFAT, WB and ELISA. Two mAbs (T8a and S4a) are directed to antigenic determinants in domain **a'c**, one (R60b) to antigenic determinants in domain **b'**, while the other is probably directed to a junction between the truncated versions produced. The ability to recognize PDI from the closely related parasites *N. caninum* and *T. gondii* was evaluated, and mAb T8a was found to be specific for BbPDI. These mAb may represent a valuable tool for research in the biology of this apicomplexan parasite and will allow for further studies about the role of PDI in *B. besnoiti* host cell invasion and host immune response.

The production of monoclonal antibodies through the hybridoma technology although relatively simple in the procedures is a very laborious and time consuming task. When expanding the hybrid cells, both after the fusion and cloning process, we are also increasing, almost exponentially, the amount of cell culture plates or flasks in the laboratory. These need to be taken care and frequently tested for mAb production and early on, as soon as possible, stored in liquid nitrogen. The mAbs here described and characterized are in fact the result of three distinct fusions of mice spleens with hybrid cells and may represent the tip of the iceberg of the potentially different antibodies that the obtained hybridomas can produce. In the future this bank of hybrid cells can be a valuable resource for obtaining other mAbs.

Chapter 4 |

Kinetic characterization of *B. besnoiti* PDI and its involvement in host cell interaction

4.1 Introduction

Protein disulfide isomerase (PDI) is one of the most abundant proteins of the eukaryotic cell, found primarily in the endoplasmic reticulum but also in other intracellular compartments and at cell surface (Hillson et al., 1984; Turano et al., 2002). It was the first folding catalyst to be reported half a century ago and the first enzyme known to possess disulfide isomerase activity (Givol et al., 1964; Goldberger et al., 1963; Goldberger et al., 1964; Venetianer & Straub, 1963a, 1963b). PDI is a member of the thioredoxin superfamily (Ferrari & Soling, 1999), that is characterized by one or more domains with sequence similarity to thioredoxin (a ubiquitous 12 kDa α-β protein), and an active site with two cysteines in a CxxC motif (Wilkinson & Gilbert, 2004). PDI has four structural thioredoxin-like domains, **a**, **b**, **b'**, and **a'**, a linker region **x** between the **b'** and **a'** domains, and a C-terminal acidic extension **c**. The enzyme catalyzes a variety of thiol-disulfide exchange reactions, including the formation and rearrangement of disulfide bonds during oxidative protein folding (Puig et al., 1997), and can also act as a chaperone, even for substrate proteins that do not form disulfide bonds (Cai, Wang & Tsou, 1994; Primm, Walker & Gilbert, 1996; Puig & Gilbert, 1994b; Wang & Tsou, 1993). The a-type domains contain the active site, and are sufficient alone to perform thiol–disulfide exchange reactions in simple substrates (Darby & Creighton, 1995; Darby, Penka & Vincentelli, 1998; Westphal, Darby & Winther, 1999). The b-type domains form a rigid base, providing a spacer for the attachment of more mobile active site domains that can access substrates from opposite sides simultaneously (Kozlov et al., 2010). They jointly contribute to the binding site, but the **b'** domain is the principal peptide and nonnative protein-binding site, and is required for isomerization reactions (Byrne et al., 2009; Darby et al., 1998; Denisov et al., 2009; Hatahet & Ruddock, 2009; Klappa, Ruddock, darby & Freedman, 1998b; Pirneskoski et al., 2004).

Several assays have been used to determine the activities of PDI and the reactions it catalyzes, which are all thiol-disulfide exchange reactions. We should distinguish at least three overall reactions that PDI can catalyze (Hatahet & Ruddock, 2009; Hawkins, Blackburn & Freedman, 1991): 1) Oxidation reactions, where, as the name suggest, the substrate attains a higher state of oxidation through the gain of electrons or a loss of oxygen. Thus, the protein or peptide substrate dithiol is oxidized to the disulfide state, with the simultaneous loss of a disulfide from the PDI active site, that must be reoxidized to complete the catalytic cycle. *In vitro*, oxidized glutathione (GSSG) is usually used as the terminal electron acceptor. This generates reduced glutathione (GSH) and results in a change in the reduction potential and redox-buffering capacity of the buffer. Care must be taken to ensure that sufficient GSSG is present to complete the reaction and that the change in reduction potential during

the reaction does not significantly affect the folding efficiency or yield. If no GSSG or equivalent electron acceptor is present, PDI is also able to reduce a disulfide bond in one nonnative protein molecule to form a disulfide in another nonnative protein. 2) Reduction reactions, where the substrate is driven to a lower oxidation state, through the gain of electrons or the loss of oxygen. Here, a protein or peptide substrate disulfide is reduced to the dithiol state, with the concomitant gain of a disulfide in the PDI active site. PDI must be reduced as well to complete the catalytic cycle. *In vitro*, GSH or DTT is often used as the electron donor. Again, this results in a change in the reduction potential and redox-buffering capacity of the buffer. 3) Isomerization reactions in which the disulfides and thiols in a protein or peptide substrate are rearranged to give a different disulfide-bonding pattern. In direct isomerization, no net change exists in the redox state of the active site of PDI, and so no other redox reagents are required.

The various *in vitro* assays hitherto described, despite the richness of variations between them, can be grouped in three major categories, according to Hatahet and Ruddock (2009): those based on the gain of activity of a substrate protein, widely used, in part because of their sensitivity and relative simplicity. They can detect activity after only a very small percentage of the refolding protein has reached the active state. Here the classic example is the use of ribonuclease A (RNase A), a one-domain protein that contains four disulfide bonds in the native state. The starting state of RNase can be: (a) reduced RNase (Creighton et al., 1980; Lyles & Gilbert, 1991; Xiao et al., 2004), to give an assay that looks primarily at oxidation; (b) ‘‘scrambled’’ RNase (Ibbetson & Freedman, 1976; Lambert & Freedman, 1983; Walker & Gilbert, 1997), where the protein has been reduced and allowed to oxidize under denaturing conditions, which produces a set of proteins in which the disulfide bonds are predominantly nonnative, to give an assay that looks primarily at isomerization, or (c) glutathionylated RNase T1 (Ruoppolo & Freedman, 1994, 1995), in which all of the cysteines in the protein are glutathionylated at the start, in an assay that looks at isomerization. One problem associated with these assays is that they do not directly measure disulfide-bond formation in the substrate protein and a gain of activity does not always directly correlate with disulfide-bond formation, since many folding intermediates may present biological activity. In addition, the biophysical conditions of the system are often varied, and care must be taken to assure that any changes observed relate to changes in the activity of PDI and not changes in activity or stability of the folded substrate, its folding intermediates or the reaction buffer. The second problem relates to the complexity of the substrate, since 764 different disulfide-bonded states are theoretically possible for RNase A. Certainly, only a small fraction of these may be experienced during refolding, but each intermediate is in fact a different substrate for PDI with different requirements for catalysis, and a global assay, such as the regain of activity, will miss many of the subtleties. This problem is exacerbated for ‘‘scrambled’’ RNase

in that the starting material itself is not homogeneous (and shows considerable batch-to-batch variation). The third problem is related to assaying the activity of RNase, where essentially two different assays are used: (a) the hydrolysis of RNA (Goldberger et al., 1964; Ibbetson & Freedman, 1976; Janiszewski et al., 2005; Laboissière, Sturley & Raines, 1995; Laboissière, Chivers & Raines, 1995), which is the natural substrate for this enzyme, but which requires very small changes in absorbance to be accurately measured with time, imposing the use of a good double-beam spectrophotometer. This method was first described by Anfinsen, Redfield, Choate, Page and Carroll (1954); or (b) the hydrolysis of cCMP (Lyles & Gilbert, 1991; Walker & Gilbert, 1997; Walker, Lyles & Gilbert, 1996; Xiao et al., 2004), an assay that, for detailed kinetics, requires the accurate measurement of very high absorbance values. In this way, although these methods can be used to measure the relative order of activity and compare different proteins, they are poor methods to determine PDI activity quantitatively, given the complexity of the system and the heterogeneity of the starting material in the ‘‘scrambled’’ RNase assay. Among other examples of assays that fall in this category we point out the use of denatured and reduced lysozyme (Goldberger et al., 1964; Puig, Lyles, Noiva & Gilbert, 1994) and the use of riboflavin-binding protein (Rancy & Thorpe, 2008). The first is based on the regain of lysozyme activity, that is measured by following the decrease in absorbance at 450 nm of a *Micrococcus lysodeicticus* cell wall suspension. The refolding of lysozyme has been widely studied and served as an important model for the understanding of protein folding mechanisms (Chaffotte, Guillou & Goldberg, 1992; Dobson, Evans & Radford, 1994; Eyles, Radford, Robinson & Dobson, 1994; Hooke, Radford & Dobson, 1994; Lin, Ruaan & Hsieh, 2007; Matagne, Radford & Dobson, 1997; Mizuguchi, Arai, Ke, Nitta & Kuwajima, 1998; Radford, Buck, Topping, Dobson & Evans, 1992; van den Berg, Chung, Robinson & Dobson, 1999; van den Berg, Chung, Robinson, Mateo & Dobson, 1999).

A second category of assays is based on changes in the biological/physical properties of a substrate. The insulin-reduction assay is the most used assay and was first described in works with thioredoxin by Holmgren (1979). It was latter adapted by Luthman and Holmgren (1982), Lundstrom and Holmgren (1990) and more recently by Smith et al. (2004), that suggested it as a good high-throughput turbidimetric assay for screening inhibitors of PDI activity. In this assay, PDI facilitates the reduction of insulin by GSH or DTT with the consequent aggregation of the β-chains of insulin. This aggregation results in turbidity and changes in light scattering, that are monitored by the increasing in absorbance at 650 nm. Again, this assay is very useful to determine the relative order of activity of different catalysts, but it is a poor method for quantification. Because the assay measures aggregation properties of the product, it is also very sensitive to changes in biophysical conditions. A more quantitative version of this assay is the coupled insulin-reduction assay, where the

reaction proceeds in glutathione redox system in conjunction with glutathione reductase (GR). In this way, the GSSG formed by the reduction of insulin by GSH is reduced by GR, with the concomitant oxidation of nicotinamide adenine dinucleotide phosphate (NADPH) and a decrease in absorbance at 340 nm (Maeda, Ado, Takeda & Taniguchi, 2007). This assay is quantitative, but only up to the time point at which turbidity starts to contribute to the apparent changes in absorbance at 340 nm due to light scattering (Hatahet & Ruddock, 2009). Other assays that fall into this category are peptide-based thiol-disulfide exchange assays in which an associated change in substrate fluorescence occurs (Christiansen, St Hilaire & Winther, 2004; Raturi, Vacratsis, Seslija, Lee & Mutus, 2005; Westphal, Spetzler, Meldal, Christensen & Winther, 1998).

A third grouping of assays is based on quenching dithiol-disulfide exchange reactions and then subsequently monitoring the disulfide bond state of the substrate. These quenching-based assays are considerably more time consuming and are always discontinuous, representing snapshots of the overall reaction at different time points. However, they have the potential to provide much more detailed kinetic analysis of even complex systems (Hatahet & Ruddock, 2009). Although various peptides and proteins have been used, the classic substrate in this type of assay is the bovine pancreatic trypsin inhibitor (BPTI) (Creighton et al., 1980).

PDI and PDI-like enzymes were identified in numerous apicomplexan parasites, as previously described (see Introduction, Chapter 1), but for only a few their corresponding enzymatic activity was demonstrated *in vitro*, namely *Giardia lamblia* (Knodler et al., 1999), *Leishmania major* (Achour et al., 2002), *L. donovani* (Amit et al., 2014; Padilla et al., 2003), *L. amazonensis* (Hong & Soong, 2008), *Neospora caninum* (Liao et al., 2006; Muller et al., 2008), *Plasmodium falciparum* (Mahajan et al., 2006; Mouray et al., 2007), and *Trypanosoma brucei* (Rubotham et al., 2005). There is a growing interest in this enzyme, since it has been described as a immunodominant antigen (Costa et al., 2013; Ma et al., 2009; Shin et al., 2004; Shin et al., 2005; Zhou et al., 2014), an important virulent factor (Achour et al., 2002; Amit et al., 2014) and a target for naturally occurring secretory IgA (Liao et al., 2006; Meek et al., 2002a; Meek et al., 2002b; Meek et al., 2000). Furthermore, PDI is believed to play an important role in host cell interaction and parasite survival, since several studies demonstrate that inhibition of PDI has a negative correlation with parasite *in vitro* proliferation. For *N. caninum*, preincubation of tachyzoites with PDI inhibitors, both drugs (5,5'-dithiobis(2-nitrobenzoic acid) (DTNB), p-chloromercuribenzoic acid (pCMBA), tocinoic acid and bacitracin) and anti-PDI antibodies resulted in decreased adhesion of tachyzoites to the host cell, with a consequent inhibitory effect on the growth of the parasite (Liao et al., 2006; Naguleswaran et al., 2005). Also, in *L. amazonensis* and *L. major* promastigotes, incubation of parasites with the same drug inhibitors lead to a decreased number of viable

parasites (Achour et al., 2002; Hong & Soong, 2008), with higher concentrations of bacitracin and pCMBA halting parasite growth. In addition, nitazoxanide (NTZ) and some non-nitro-thiazolides compounds efficiently inhibited the *in vitro* propagation of *N. caninum* (Esposito et al., 2005), *B. besnoiti* (Cortes et al., 2007a), *T. gondii* (Galvan-Ramirez Mde et al., 2013), *G. lamblia* (Adagu et al., 2002; Muller et al., 2006) and *Cryptosporidium parvum* (Gargala et al., 2000; Giacometti et al., 2000; Theodos et al., 1998). Thiazolides are a promising broad spectrum anti-parasitic and anti-viral family of drugs, that are also inhibitors of the enzymatic activity of PDI, with high affinity for this enzyme (Muller et al., 2008). Furthermore, recent works in neosporosis, applying the acute disease mouse model, determined 90% protection against disease and significantly reduction of cerebral parasite burden and numbers of viable tachyzoites in brain tissue, when mice were intranasal vaccinated with recombinant *N. caninum* PDI (Debache et al., 2010). Intraperitoneal vaccination was ineffective (Debache et al., 2010; Debache et al., 2011). However, this only shows that a protective effect of vaccination depends not only on the antigen but also on the route of antigen delivery (Hemphill et al., 2013). Altogether these are strong evidences that support the importance of PDI in apicomplexan parasites and host immune response, targeting this protein as a good candidate for anti-protozoan drugs and vaccine development, which warrants the need for further studies.

Here we intend to evaluate the kinetic activity of recombinant PDI and active site truncated versions and test the sensitivity to known PDI drug inhibitors (bacitracin, tocinoic acid, DTNB and pCMBA) and anti-PDI monoclonal antibodies developed in this work and presented in Chapter 3. The assay of choice for measuring PDI activity was the assay based in the insulin aggregation as described by Lundstrom and Holmgren (1990) and adapted by Smith et al. (2004), since it allows for further large scale screening of drug inhibitors. The influence of the PDI inhibitors in the *in vitro* growth of *B. besnoiti* tachyzoites was also evaluated.

4.2 Materials and methods

4.2.1 Catalytic activity of recBbPDI and truncated versions, corresponding to the active domains a and a'c

PDI activity was measured by a turbidimetric assay based on the polymerization of reduced insulin (Lundstrom & Holmgren, 1990) as adapted by Smith et al. (2004). The assay was performed in 96-well microtiter plates and all solutions were made freshly with assay buffer (AB): 100 mM potassium phosphate, 2 mM EDTA, pH 7. Reaction solution (RS) was made by diluting the enzyme in AB and adding 11 μL of Insulin (1.6 mM or 10 mg/mL; Sigma) to a total volume of 75 μL per well. For that, twice of the necessary volume of AB with enzyme was first prepared and then serially diluted 1:1 in separated tubes. To each tube the corresponding amount of insulin (i.e., 11 μL x no. of wells) was added and carefully agitated. Inhibitors or AB (10μL) were added to respective wells and then 70 μL of RS distributed through wells. The reaction was started by the addition of 20 μL of a 5 mM DTT solution. Absorvance at 650 nm was read at room temperature in a SPECTRAmax 340 pc microplate spectrophotometer, every 5 min, with a period of 30 sec agitation before each reading, for 60 to 90 min.

Enzyme activity was calculated from the increase in turbidity over time according to the formula:

$$(Enzyme_{ABS650}\ t - Enzyme_{ABS650}\ t_0) - (Blank_{ABS650}\ t - Blank_{ABS650}\ t_0)$$

Where "t" represents time and "blank" the reaction without enzyme. This formula allows the correction for the uncatalyzed rate of insulin aggregation observed in a parallel control, in the absence of PDI.

4.2.2 PDI in the excretory-secretory compartment of *B. besnoiti* tachyzoites

Freshly egressed *B. besnoiti* tachyzoites were collected and purified as previously described. For the preparation of excretory-secretory antigen (ESA) purified tachyzoites (10^8) were incubated in PBS and PBS with 1% ethanol at 37 °C for 15 minutes. Parasites were removed from supernatant by centrifuging at 800 g for 10 minutes at 4 °C. Supernatant was collected and a protease inhibitor cocktail (Roche) added. Pellet was resuspended in PBS (same volume as supernatant) and soluble protein extracts were prepared by sonication.

Samples were separated by SDS-PAGE (12%) and transferred to nitrocellulose membranes. These were blocked overnight at 4 °C with 1% fish gelatin (Sigma) and probed with mouse anti-recBbPDI followed by incubation with alkaline phosphatase-conjugated goat anti-mouse-IgG secondary antibody (Sigma) diluted 1:5000. The final enzyme reaction was developed using the BCIP/NBT kit (Bio-Rad).

4.2.3 *In vitro* invasion assay

Vero cells were grown to confluence in 12 mm glass coverslips. Freshly lysed out tachyzoites were collected and purified as previously described and $3.5x10^8$ tachyzoites/mL were incubated at room temperature for 10 min with drugs in microtubes. Drops of 300 µl containing 10^8 tachyzoites were placed over the coverslips and incubated for 1 hour at 37°C with 5% CO2 and relative humidity over 95%. Coverslips were then washed and transferred to a new cell culture plate with fresh medium and incubated for 11 hours. Finally, coverslips were collected, prepared for IFAT and the number of parasitophorous vacuoles were counted in 40 different fields under a UV light microscope.

4.2.4 Immunofluorescence

Coverslips were washed with PBS, fixed with 4% paraformaldehyde at room temperature for 30 min and then permeabilized with cold methanol (-20°C) for 10 min. Unspecific binding sites were blocked with 3% BSA in PBS, for 30 min at room temperature. Parasites were then probed with rabbit anti-*B. besnoiti* (Cortes et al., 2006b) and FITC-conjugated anti-mouse antibodies (Invitrogen). All sera were used at 1:500 dilutions. DNA was stained with DAPI (1 µg/mL; Sigma) for 2 min and the slides were mounted with Mowiol (Calbiochem) and examined under a UV light microscope.

4.3 Results and discussion

4.3.1 Catalytic activity of recBbPDI and active domains.

Initial activity assays were performed with a GST-tagged recBbPDI. While the recBbPDI produced in the Glutathione S-transferase (GST) Gene Fusion System was active and inhibited by bacitracin the activity levels were lower than the bovine PDI used as control (see Figure 13 on page 56). We were concerned that the relatively large size of the GST tag could difficult the PDI activity and since the purification of recBbPDI by cleavage of the GST tag was proven to be difficult and fastidious we changed the cloning strategy to produce a His-tagged recombinant protein. A new full length *B. besnoiti* PDI, as well as the truncated versions corresponding to the domains **a**, **b**, **b'** and **a'c**, were cloned, expressed and purified as described in chapter 3.

The activity of recombinant His-tagged proteins expressed in *E. coli*, was tested by following the aggregation of insulin at 650 nm, as described by Smith et al. (2004). This assay looks only at the reduction of the insulin disulfide catalyzed by PDI but it was chosen because of its relatively simplicity and the possibility of being used for rapid screening of PDI inhibitors, as validated in the above mentioned work. We tested the recBbPDI activity, as well as that of the truncated versions corresponding to the active domains **a** (recBb-a) and **a'c** (recBb-a'c) and observed that increasing concentrations of enzyme, either the full length recBbPDI (Figure 23A) or the truncated version recBb-a'c (Figure 23B), results in a faster aggregation of insulin, measured by solution turbidity. Using the truncated version recBb-a in these experiments no enzymatic activity was detected (data not shown).

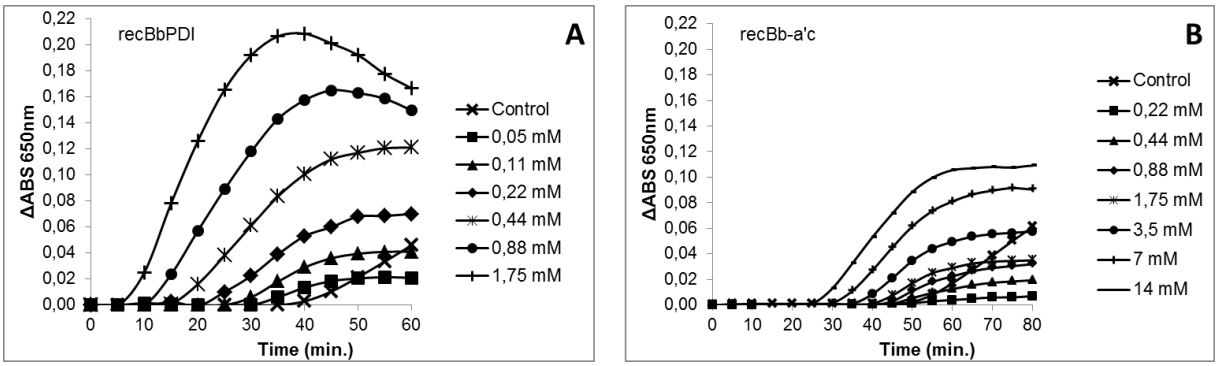

Figure 22. Reductase activity measured by the aggregation of insulin of (A) recombinant *B. besnoiti* PDI (recBbPDI) and (B) truncated version corresponding to domain **a'c** (recBb-a'c).
Results show that both recBbPDI (A) and recBb-a'c (B) are functionally active, in a dose dependent manner in the range of concentrations tested. The profile of activity is similar between the full length enzyme and the domain, but recBbPDI exhibited considerable higher activity levels, and a shorter lag phase than recBb-a'c. Control represents the non-catalyzed reduction of insulin.

The full length recombinant PDI and the domain **a'c** show a dose dependent cross-linking activity in the range of concentrations tested, from 0.05 mM to 1.75 mM and from 0.22 mM to 14 mM, respectively. The profile of activity is similar and both enzymes present a decrease in the lag time with the increase in enzyme concentration, similar to what Maeda et al. (2007) has observed. Nevertheless, full protein presents a shorter lag time in all concentrations, since it starts the cross-linking activity of insulin earlier than the truncated version. For example, at 1.75 mM turbidity starts to be noticed at around 5 min for recBbPDI and at about 40 min. for domain **a'c**. The higher concentration of **a'c** tested (14mM) starts to aggregate insulin and increase turbidity at the 25 min only. When comparing equimolar quantities, recBb-a'c exhibits considerable lower activity levels than the full length enzyme: only 10% of the recBbPDI activity at 0.22 mM and 0.44 mM and around 20 % at 0.88 and 1.75 mM. The multi-domain structure of PDI is known to be essential for high catalytic efficiency. Single a-type domains (or mixtures of these) have poor activity toward protein substrates, but they can still be able to catalyze oxidation, reduction, and isomerization. Nevertheless, for more complex isomerization reactions, the entire molecule is required for full activity (Darby & Creighton, 1995; Darby et al., 1998; Hatahet & Ruddock, 2009). In a functional study with multi-domain constructs, Darby et al. (1998) demonstrated that reconstruction of the PDI molecule from the isolated **a** and **a'** domains results in a progressive increase in catalytic efficiency as further domains are added. For more than 30% of expected activity, the minimal constructs required for the catalysis were either **abb'** or **b'a'c**. Constructs without the **b'** domain were less efficient catalysts in nearly all the reactions examined. The decrease to 10% to 20% in the reductase capabilities of domain **a'c** is in agreement with what was described by Sun, Dai, Liu, Chen and Wang (2000) for human **a'c** PDI domain. However, Puig et al. (1997) reported for a 21-kDa C-terminal fragment of rat PDI (corresponding to domains **a'c** with 37 amino acid from **b'** domain) an activity of 30% of that of PDI. Regarding the **a** domain, the lack of activity found agrees with what was described for yeast PDI, but not for human PDI. Katiyar, Till and Lennarz (2001), working with yeast PDI constructs also found no activity for a construct lacking 218 amino acids from the C-terminus (corresponding to the **a** domain with part of the **b** domain), but 30% activity for a construct lacking only 152 amino acids from the C-terminus. For human PDI, **a** domain is reported to retain 50% of the activity of normal PDI (Darby & Creighton, 1995; Darby, Kemmink & Creighton, 1996), while the construct of **ab** together preserves from 13% to 22%, depending on the assay used (Sun et al., 2000).

Comparing to previous results with GST-tagged recBbPDI, the His-tagged protein shows higher activity levels, attaining ABS values of 0.2 (at 1.75 mM) compared to the maximum of 0.079 of the GST-tagged enzyme. The His-tagged PDI also shows higher amplitude of activity across the enzyme concentrations tested, with a 9.7 fold increase in activity from the

lower enzyme concentration to the higher, while GST-recBbPDI only shows a 1.8 fold increase. Interestingly, in these assays, GST-recBbPDI seems to show a ceiling effect above the 0.44 mM concentration, meaning that higher enzyme concentrations are not able to attain higher turbidity or ABS values. Comparing to bovine PDI (see Figure 13 on page 56), His-recBbPDI shows about twice the activity levels, with a very similar profile of activity. Looking into equimolar quantities of enzyme, it is interesting to notice that the GST-recBbPDI shows higher aggregation of insulin at lower enzyme concentrations (0.05 to 0.22 mM) comparing to the His-recBbPDI, similar to what was described in chapter 2 for bovine-PDI used then as control. At enzyme concentration of 0.05 mM GST-recBbPDI shows twice the activity of His-recBbPDI, at 0.11 mM shows 1.6 fold, at 0.22 both show similar activity, and beyond this concentration the situation is inverted, with His-recBbPDI at 0.44 mM showing around 1.6 fold the activity of GST-recBbPDI, at 0.88 mM showing twice the activity and at 1.75 mM showing 2.6 fold more activity. Several studies showed that at substoichiometric concentrations PDI can have an anti-chaperone activity, facilitating the aggregation of the substrate protein (Puig & Gilbert, 1994a, 1994b; Puig et al., 1994; Puig et al., 1997; Song, Quan & Wang, 1997). However, an explanation for the different behavior of the GST-tagged and the His-Tagged enzyme is not clear for us.

4.3.2. Drug inhibition.

The activity of His-recBbPDI and recBb-a'c was tested in the presence of the known membrane impermeant PDI inhibitors bacitracin, tocinoic acid, 5,5′-dithiobis(2-nitrobenzoic acid) (DTNB) and 4-chloromercuribenzoic acid (pCMBA). Bacitracin is a potent metallopeptide antibiotic of narrow spectrum directed primarily against Gram positive cocci and bacilli that requires a divalent metal ion such as Zn^{2+} for its biological activity (Ming & Epperson, 2002). It is produced by *Bacillus subtilis* and *Bacillus licheniformis* as a mixture of closely related dodecapeptides, being bacitracin A the most abundant congener (Economou, Cocklin & Loll, 2013). Bacitracin was reported to be a PDI inhibitor in 1981 (Roth, 1981) and has been since widely used as a PDI specific inhibitor. More recently tough, this specificity was questioned by Karala and Ruddock (2010). Tocinoic acid, a cyclic hexapeptide (Cys-Tyr-Ile-Gln-Asn-Cys) with a disulfide bridge between the terminal cysteines, DTNB and pCMBA are not specific for PDI. They react nonspecifically with free thiols and covalently modify surface sulfhydryl to prevent disulfide bond formation (Davis et al., 2002; Mandel et al., 1993; Mou, Ni & Wilkins, 1998; Naguleswaran et al., 2005)

All drugs tested showed a dose dependent inhibition of enzyme activity, manifested in general by a longer lag phase and an attenuated increase in turbidity in the presence of inhibitor. Tested enzyme concentration ranged from 0.05 mM to 1.75 mM, for His-recBbPDI, and from 0.22mM to 7 mM, for recBb-a'c. Nevertheless, we noticed that for 0.22 mM of recBb-a'c we could not obtain consistency in the inhibition since the amplitude of the reaction was, by itself, very low and this concentration was disregarded.

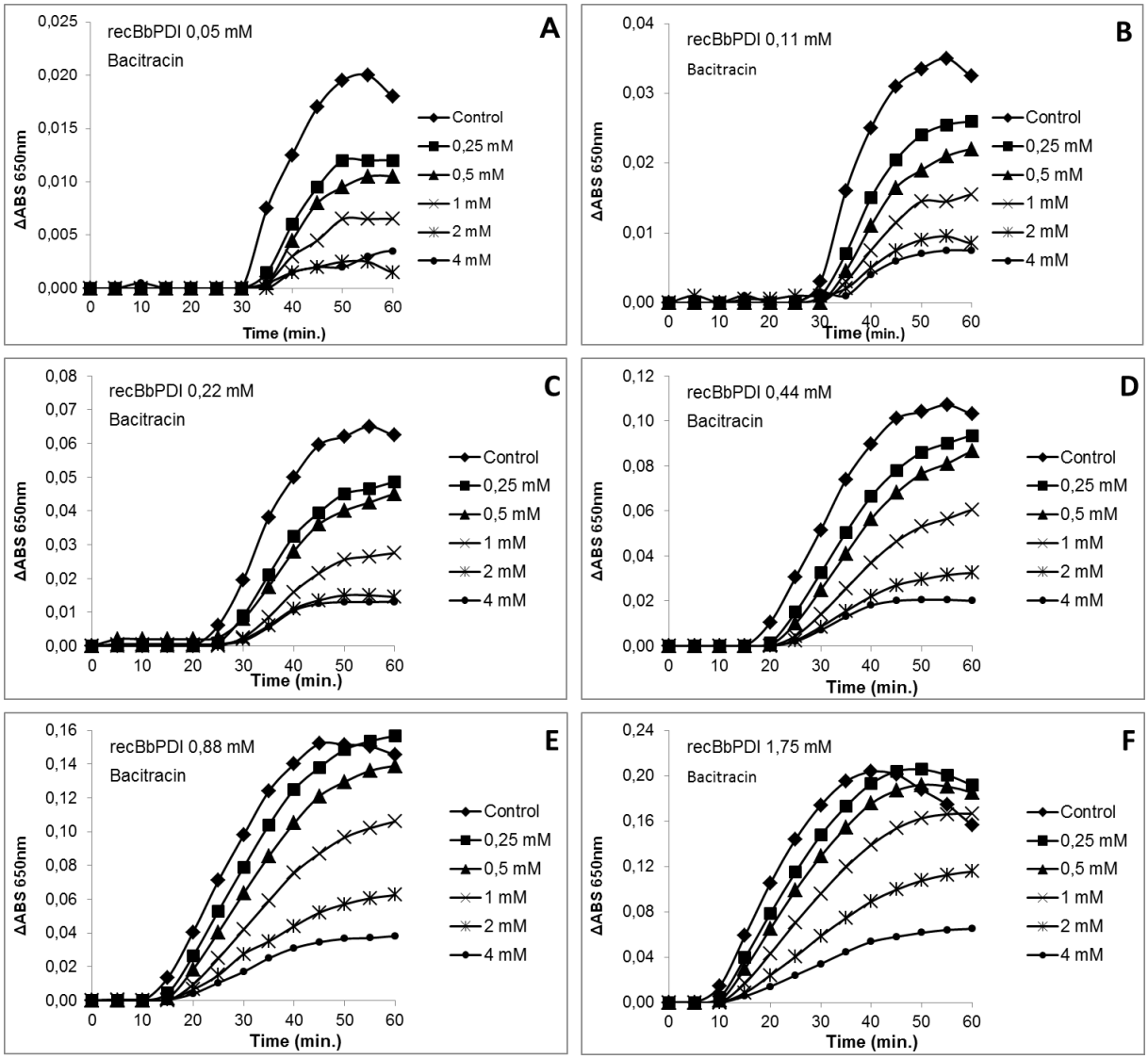

Figure 23. Reductase activity, measured by the aggregation of insulin, of recombinant *B. besnoiti* PDI (recBbPDI) in the presence of bacitracin.
Enzyme activity was tested in concentrations of 0.05 mM (A), 0.11 mM (B), 0.22 mM (C), 0.44 mM (D), 0.88 mM (E) and 1.75 mM (F) in the presence of 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM of drug. There is a dose dependent inhibition of reductase activity of recBbPDI in all enzyme concentrations tested. Control represents recBbPDI activity without the presence of inhibitor.

Enzyme sensitivity to bacitracin was tested at concentrations ranging from 0.25 mM to 4 mM of drug and a clear dose dependent inhibition was observed for both His-recBbPDI (Figure 24) and recBb-a'c (Figure 25). Inhibition varied from 5% to 80% in his-recBbPDI and from 1% to 50% in recBb-a'c depending on the molar ratio considered. The full length enzyme seems to be more sensitive than the domain a'c, since the higher drug concentration tested (4 mM) was able to inhibit enzyme activity in 80% for His-recBbPDI and only 50% in recBb-a'c in all enzyme concentrations tested. The mechanism of the inhibitory action of bacitracin on PDI is not clear yet. The only study addressing this issue hitherto published proposed the involvement of covalent binding of an open thiol form of bacitracin to free

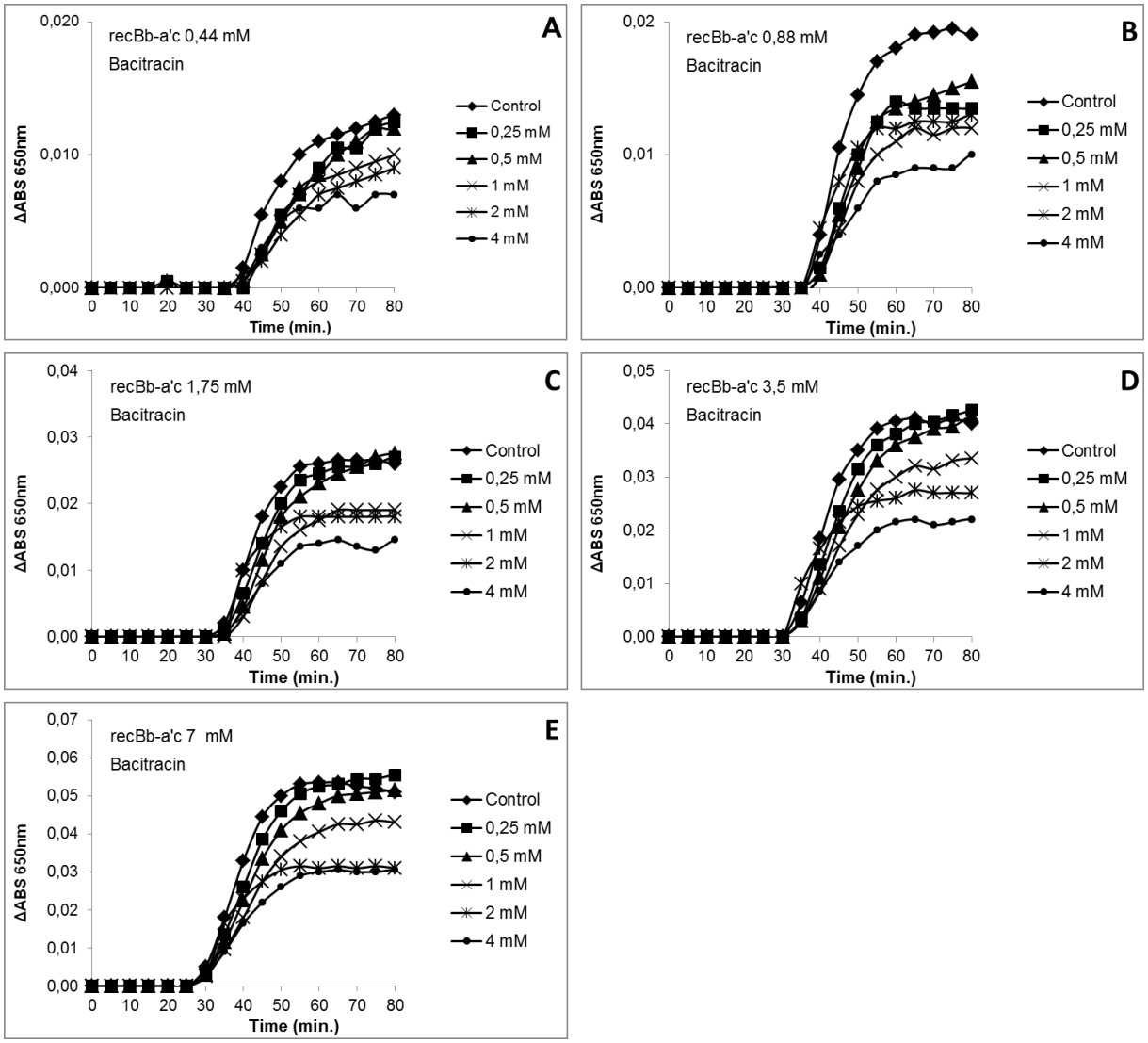

Figure 24. Reductase activity, measured by the aggregation of insulin, of truncated version corresponding to domain **a'c** (recBb-a'c) in the presence of bacitracin.
Enzyme activity was tested in concentrations of 0.44 mM (A), 0.88 mM (B), 1.75 mM (C), 3.5 mM (D) and 7 mM (E) in the presence of 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM of drug. There is a dose dependent inhibition of reductase activity of recBb-a'c in all enzyme concentrations tested. Control represents recBb-a'c activity without the presence of inhibitor.

cysteines in the substrate binding domain of PDI (Dickerhof, Kleffmann, Jack & McCormick, 2011). The authors proposed that the interaction of bacitracin with the hydrophobic surface of the substrate-binding site of PDI lead to the opening of the bacitracin ring with subsequent disulfide bond formation with free PDI cysteines. They showed by MALDI-TOF⁄ TOF MS disulfide bond formation between an open thiol form of bacitracin and Cys314 and Cys345 of PDI, that are present in the **b'**-domain and x-linker region respectively. However, BbPDI does not present these mentioned cysteines and has only one in the substrate binding pocket, located in the **b** domain (Cys249). Analysis of the *in silico* structural model of BbPDI developed in chapter 2 shows that Cys249 is free, but whether it participates or not in a

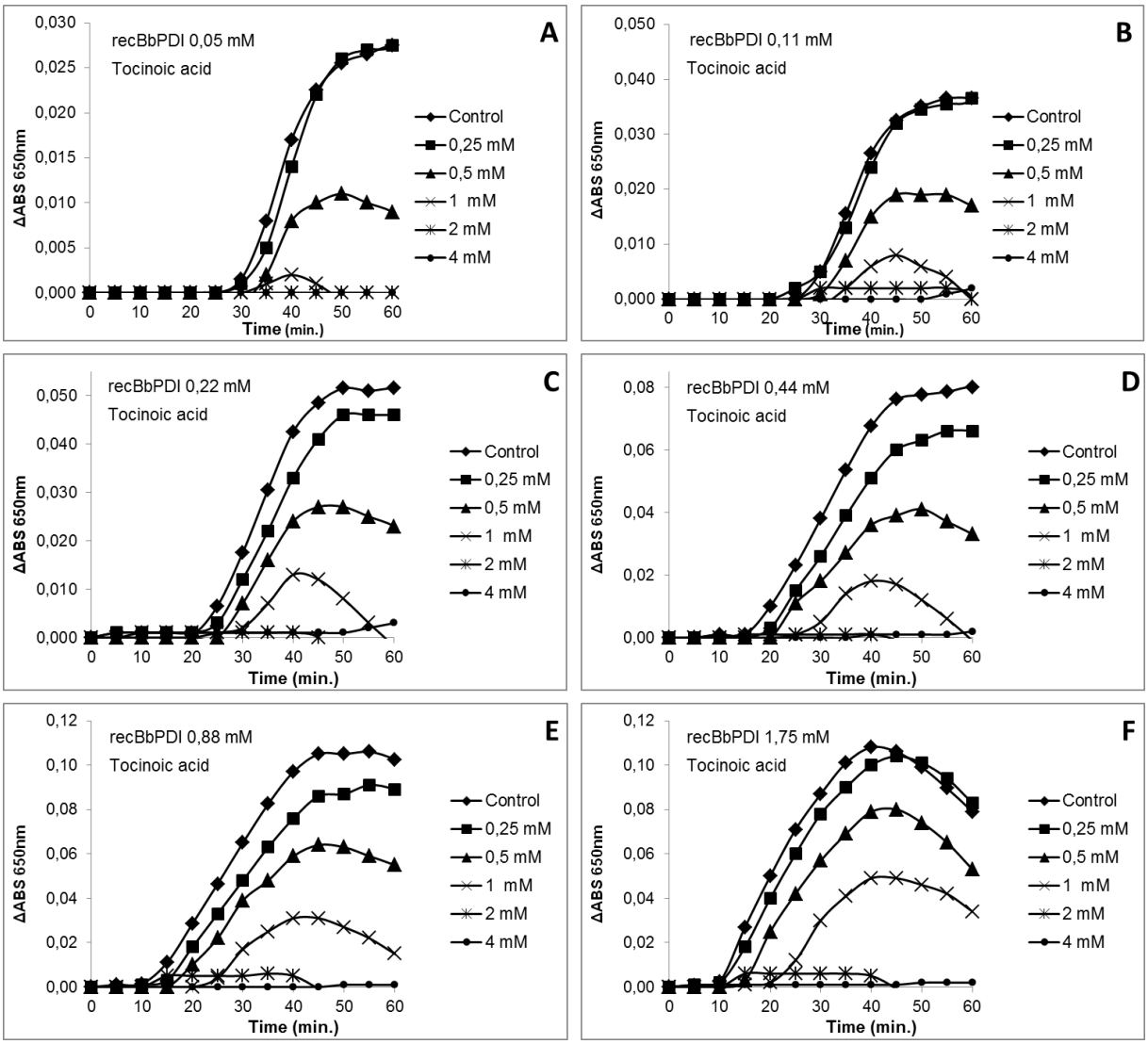

Figure 25. Reductase activity, measured by the aggregation of insulin, of recombinant *B. besnoiti* PDI (recBbPDI) in the presence of tocinoic acid.
Enzyme activity was tested in concentrations of 0.05 mM (A), 0.11 mM (B), 0.22 mM (C), 0.44 mM (D), 0.88 mM (E) and 1.75 mM (F) in the presence of 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM of drug. There is a dose dependent inhibition of reductase activity of recBbPDI, with drug concentrations abode 2 mM stopping enzyme function. Control represents recBbPDI activity without the presence of inhibitor.

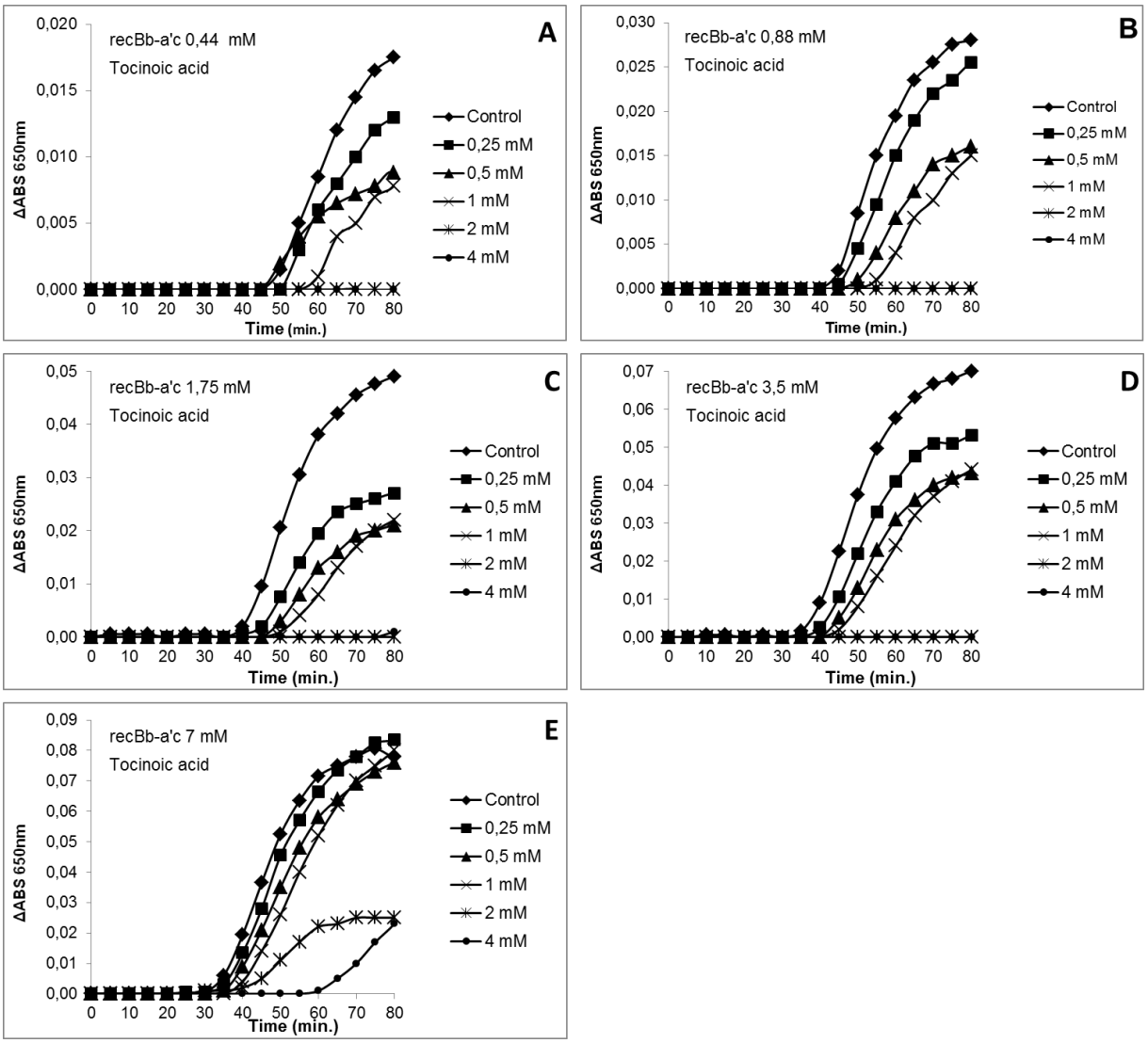

Figure 26. Reductase activity, measured by the aggregation of insulin, of truncated version corresponding to domain **a'c** (recBb-a'c) in the presence of tocinoic acid.
Enzyme activity was tested in concentrations of 0.44 mM (A), 0.88 mM (B), 1.75 mM (C), 3.5 mM (D) and 7 mM (E) in the presence of 0.25 mM, 0.5 mM, 1 mM, 2 mM and 4 mM of drug. There is a dose dependent inhibition of reductase activity of recBb-a'c. Drug concentrations above 2 mM can impair activity in all enzyme concentrations except 7 mM (G). Control represents recBb-a'c activity without the presence of inhibitor.

disulfide bond with bacitracin needs further assessment. Although to a lower extend than the full enzyme, bacitracin could also inhibit domain **a'c**, that presents only the two active site cysteines. Taking in account Dickerhof's et al (2011) observation, we can speculate that bacitracin may interact directly with PDI active sites with the differences in inhibition being justified by having only one active site to interact in recBb-a'c and two in the complete protein. Against this hypothesis, Karala and Ruddock (2010) state that the active site is probably not the target of inhibition of bacitracin, since they found no bacitracin inhibition with domain **a** and bacterial DsbA. Yet, as a measure of PDI reductase activity, the authors

considered only the lag phase, measuring merely the time before an apparent increase in absorbance of 0.1 was recorded. Despite the differences in calculating PDI activity, our results also show that, for recBb-a'c (but not recBbPDI), there is no increase in the lag phase with the addition of bacitracin to the reaction, but there is an attenuated increase in turbidity.

Tocinoic acid was tested in concentrations ranging from 0.25 to 4 mM and was also able to inhibit both enzymes but, unlike bacitracin, no significant differences in sensitivity were found between His-recBbPDI (Figure 26) and recBb-a'c (Figure 27). The lower tocinoic acid concentrations tested (from 0.25 mM to 1 mM) can reduce enzyme activity from 8% to 100%

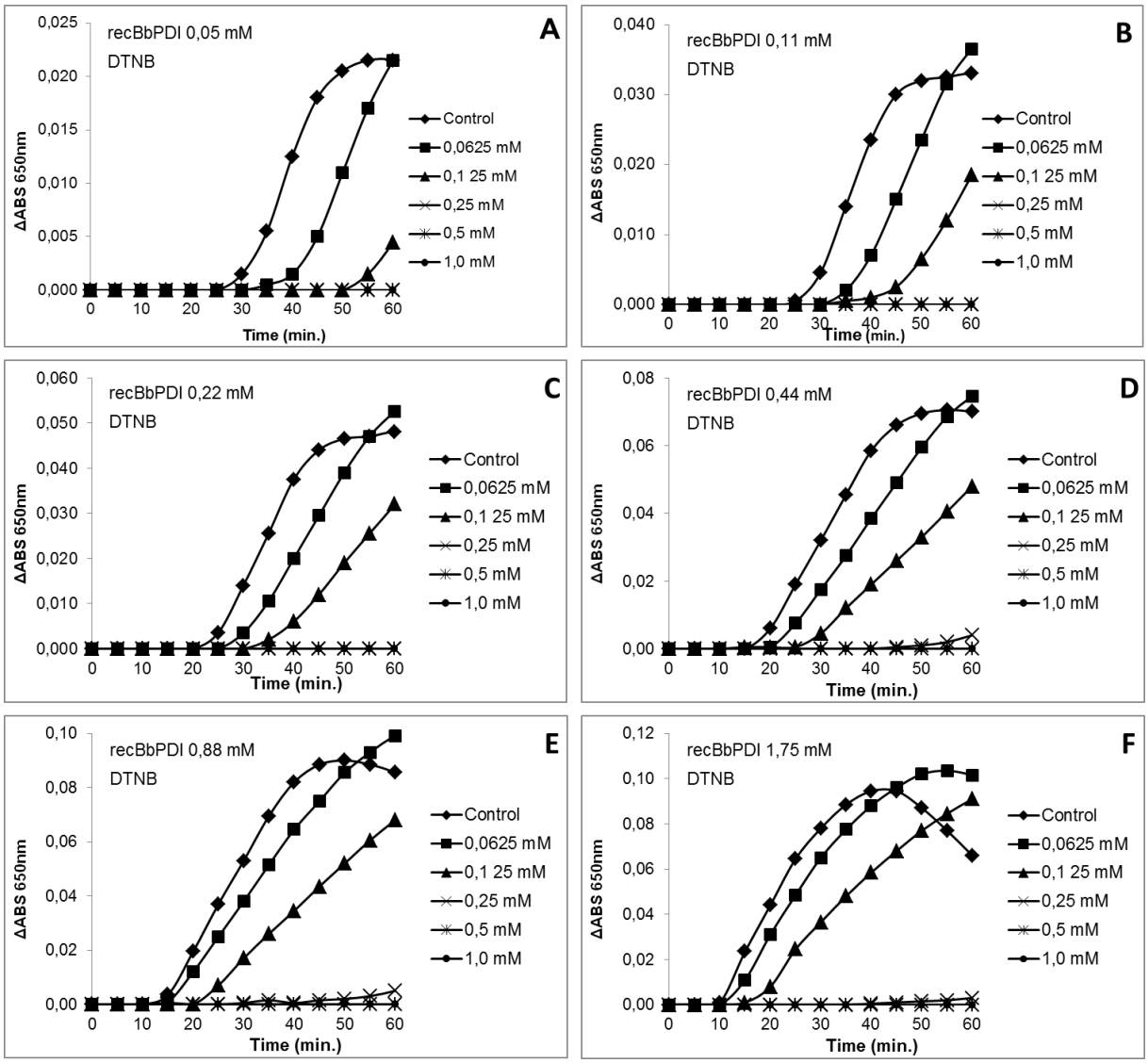

Figure 27. Reductase activity, measured by the aggregation of insulin, of recombinant *B. besnoiti* PDI (recBbPDI) in the presence of DTNB.
Enzyme activity was tested in concentrations of 0.05 mM (A), 0.11 mM (B), 0.22 mM (C), 0.44 mM (D), 0.88 mM (E) and 1.75 mM (F) in the presence of 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM and 1 mM of drug. There is a dose dependent inhibition of reductase activity of recBbPDI, with drug concentrations above 0.25 mM stopping enzyme function. Control represents recBbPDI activity without the presence of inhibitor.

depending on the molar ratio present. For example, at 0.05 mM His-recBbPDI, 1 mM of tocinoic acid completely impaired enzyme activity, while at 1.75 mM of enzyme, the same drug concentration only reduced activity by 65%. Higher concentrations of tocinoic acid (2 mM and 4 mM) were able to stop enzyme activity in all enzyme concentrations tested, with the exception of 7 mM of recBb-a'c that can still retain 30% and 20% of activity at tocinoic acid concentrations of 2 mM and 4 mM, respectively. In a similar way, DTNB showed the same behavior than tocinoic acid, since lower drug concentrations (0.0625 mM to 0.25 mM) were able to inhibit enzyme activity to a greater or lesser extent (considering a lower or

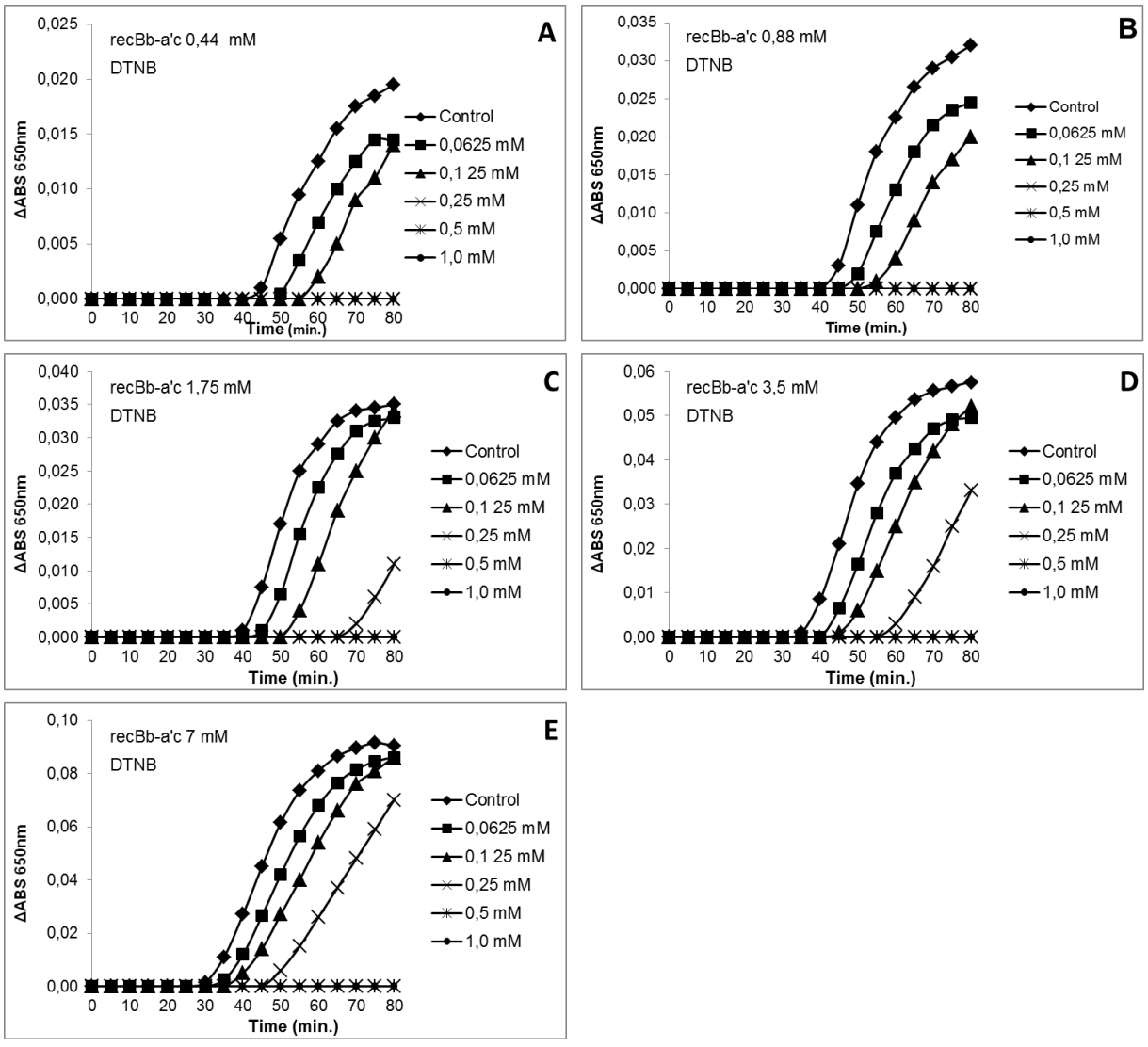

Figure 28. Reductase activity, measured by the aggregation of insulin, of truncated version corresponding to domain **a'c** (recBb-a'c) in the presence of DTNB.
Enzyme activity was tested in concentrations of 0.44 mM (A), 0.88 mM (B), 1.75 mM (C), 3.5 mM (D) and 7 mM (E) in the presence of 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM and 1 mM of drug. There is a dose dependent inhibition of reductase activity of recBb-a'c, with drug concentrations above 0.5 mM stopping enzyme activity. Control represents recBb-a'c activity without the presence of inhibitor.

higher enzyme concentration, respectively), while higher drug concentrations (0.5 mM and 1 mM) were able to halt the function of both enzymes (Figure 28 and Figure 29). The same was true for pCMBA, but only with recBb-a'c, for which higher drug concentrations (0.5 mM and 1 mM) could impair enzyme crosslinking of insulin, while lower drug concentrations could only reduce recBb-a'c activity. With the full length PDI, pCMBA showed a clear dose dependent reduction of enzyme activity, with the higher drug concentration (1 mM) allowing still 22% of enzyme activity (1.75 mM recBbPDI), but only around 5% at lower enzyme concentrations (0.05 mM and 0.11 mM recBbPDI) (Figure 30 and Figure 31).

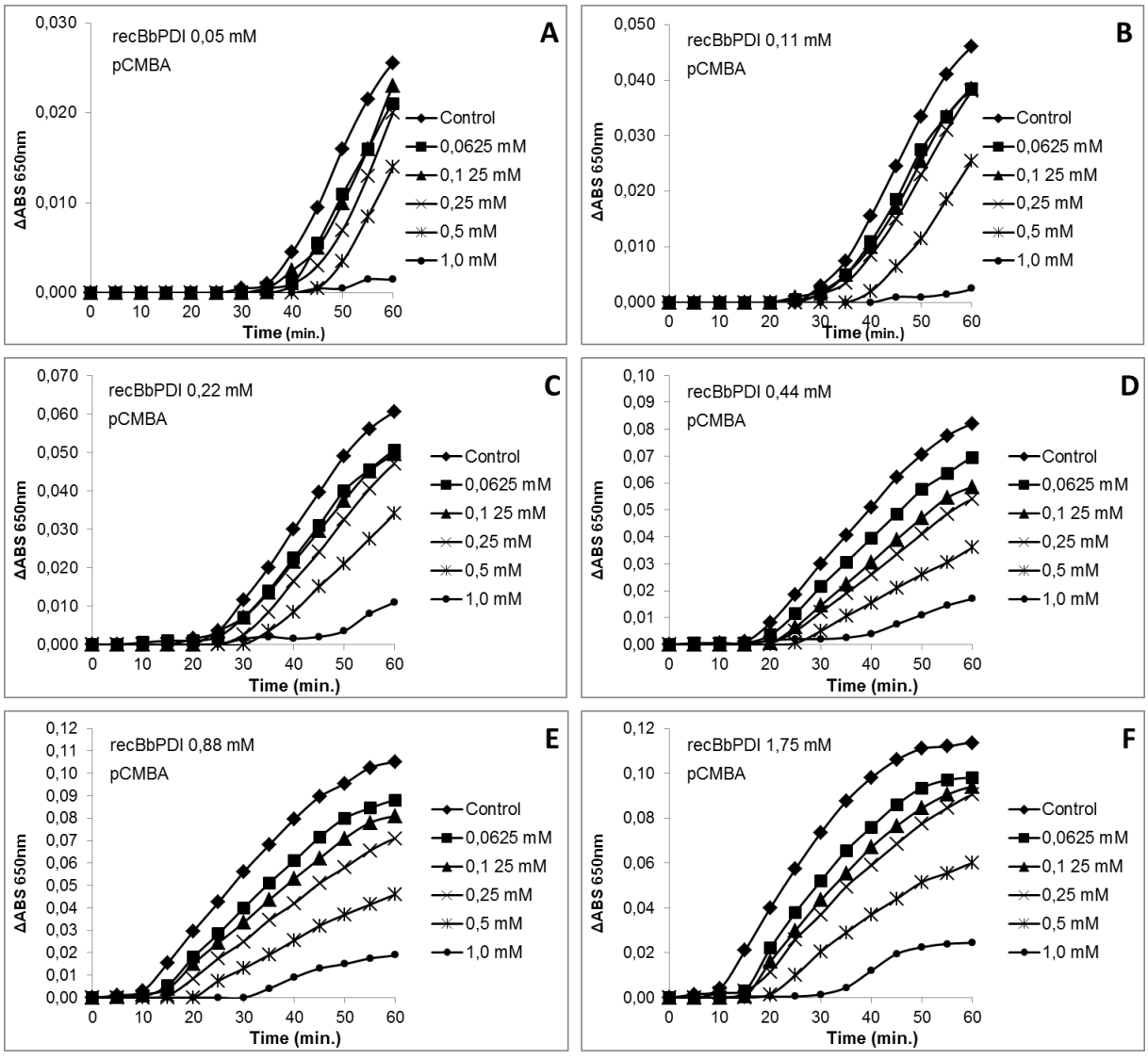

Figure 29. Reductase activity, measured by the aggregation of insulin, of recombinant *B. besnoiti* PDI (recBbPDI) in the presence of pCMBA.
Enzyme activity was tested in concentrations of 0.05 mM (A), 0.11 mM (B), 0.22 mM (C), 0.44 mM (D), 0.88 mM (E) and 1.75 mM (F) in the presence of 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM and 1 mM of drug. There is a dose dependent inhibition of reductase activity of recBbPDI. Control represents recBbPDI activity without the presence of inhibitor.

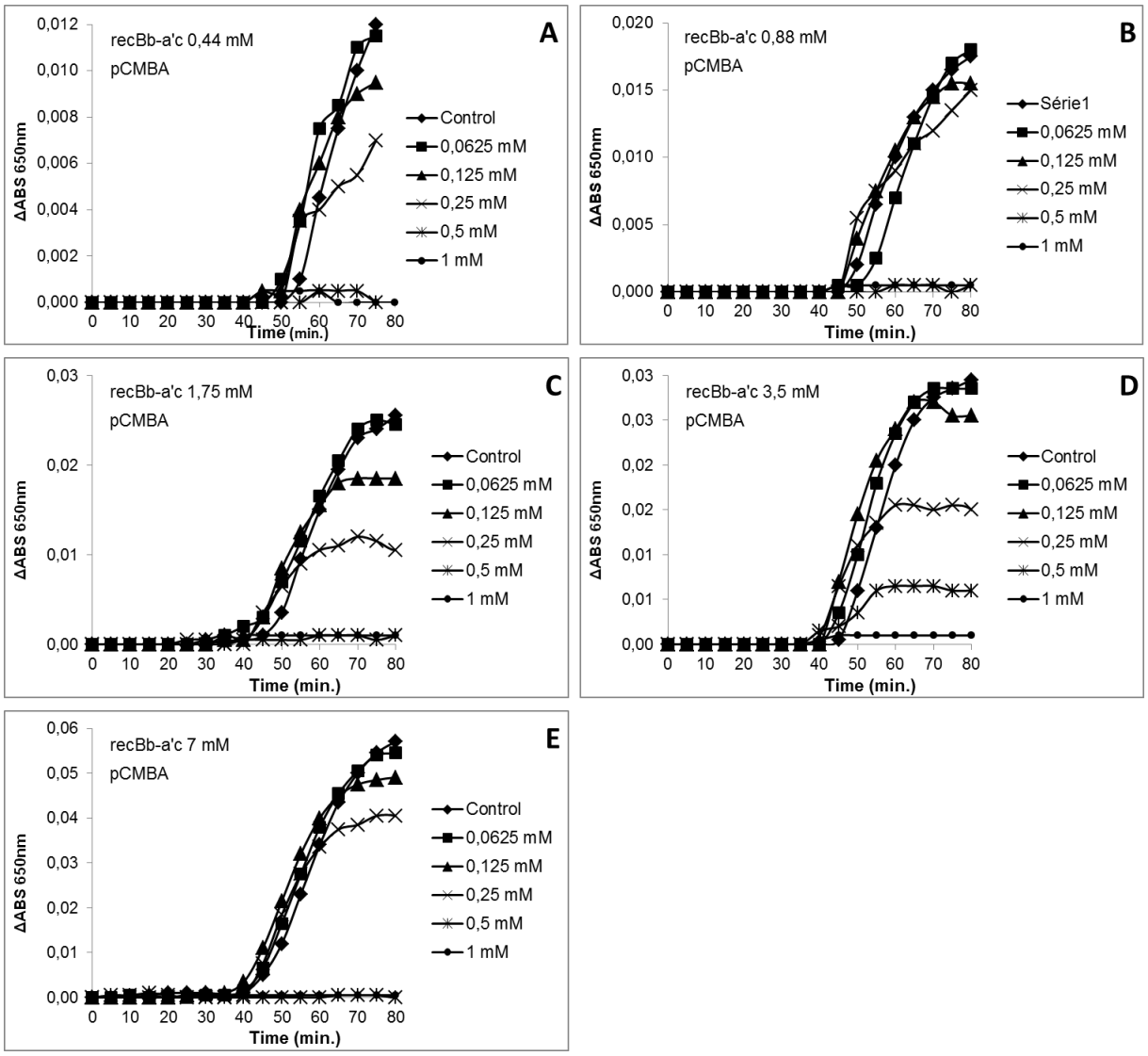

Figure 30. Reductase activity, measured by the aggregation of insulin, of truncated version corresponding to domain **a'c** (recBb-a'c) in the presence of pCMBA.
Enzyme activity was tested in concentrations of 0.44 mM (A), 0.88 mM (B), 1.75 mM (C), 3.5 mM (D) and 7 mM (E) in the presence of 0.0625 mM, 0.125 mM, 0.25 mM, 0.5 mM and 1 mM of drug. There is a dose dependent inhibition of reductase activity of recBb-a'c. Control represents recBb-a'c activity without the presence of inhibitor.

Overall, tocinoic acid, DTNB and pCMBA showed a very similar pattern of inhibition for both enzymes tested, with the two to three lower drug concentrations gradually decreasing enzyme activity and the tree to five higher drug concentration causing 100% enzyme inhibition. Concentrations above 2mM of tocinoic acid and 0.5 mM of DTNB and pCMBA can cause 100% inhibition in both enzymes, with the mentioned exception of His-recBbPDI and pCMBA, that seem to be less sensitive to this drug. Mandel et al. (1993), reported 100% inhibition for concentrations above 1 mM for DTNB and p-chloromercuribenzenesulfonic acid (pCMBS). Tocinoic acid, DTNB and pCMBA are thiol scavengers that covalently modify

surface sulfhydryl to prevent disulfide bond formation. Tocinoic acid presents a disulfide bond between its N-terminal and C-terminal cysteines, that might, similarly to what was described for bacitracin (Dickerhof et al., 2011), be involved in disulfide bond formation with PDI cysteines. DTNB, also known as Ellman's reagent, is composed of 2 molecules of 2-nitro-5-thiobenzoate linked by a disulfide bond that is easily reduced by thiols. As for pCMBA, an organic mercurial compound, it can react via mercury-sulfur affinity with free sulfhydryl groups. The fact that these drugs can inhibit the activity of domain **a'c**, that only possesses two sulfhydryl groups in the active site cysteines, leaves us to conclude that these molecules might interact directly with the active site. On the other hand, if that is the case, we should expect that full PDI expressed lower levels of inhibition with these molecules, since DTNB and pCMBA react stoichiometrically with thiols and the drug molar ratio to free cysteines is at least half of that of recBb-a'c (if we consider the two active sites in the complete protein). The result obtained however, show no significant differences in inhibition between recBbPDI and recBb-a'c.

4.3.3 Monoclonal antibody inhibition.

The activity of His-recBbPDI was tested in the presence of the four monoclonal antibodies described in chapter 3. Enzyme sensitivity was evaluated with mAb concentrations ranging from 0.05 mM to 0.4 mM. MAb T8a did not inhibited His-recBbPDI activity in any of the enzyme concentration tested (Figure 32). The remaining mAbs tested, S4a, S16p and R60b were all able to decrease in a dose dependent manner His-recBbPDI activity (Figure 33, Figure 34 and Figure 35). Characterization of antigen recognition, done in the previous chapter, showed that all mAbs can recognize the full length recBbPDI, that mAb T8a and S4a can also recognize the truncated version corresponding to domain **a'c** and that mAb R60b can also recognize the truncated version corresponding to domain **b'**. The mAb S16p is only able to label the full length PDI, but prediction of epitope recognition gave two possibilities, both in the non-catalytic b-type domains. PDIs are known for their flexibility and plasticity, necessary to accommodate diverse substrates, of different sizes and conformations (Laurindo et al., 2012; Tian et al., 2008; Tian et al., 2006). Tian et al. (2008) showed that restricting the flexibility of the **a** domain results in a 60% decrease in enzymatic activity, whereas restricting the flexibility of the **a'** domain results in only 24% reduction. They identified the junction between domains **a** and **b** as a major site of flexibility and suggested that the loops connecting the **a** and **a'** domains to the **b**-**b'** base enable the yeast PDI to facilitate these conformational changes. However, a recent work with human PDI, indicated that, in contrast to the situation reported in yeast PDI, the C-terminal half (**b'** and **a'** domains

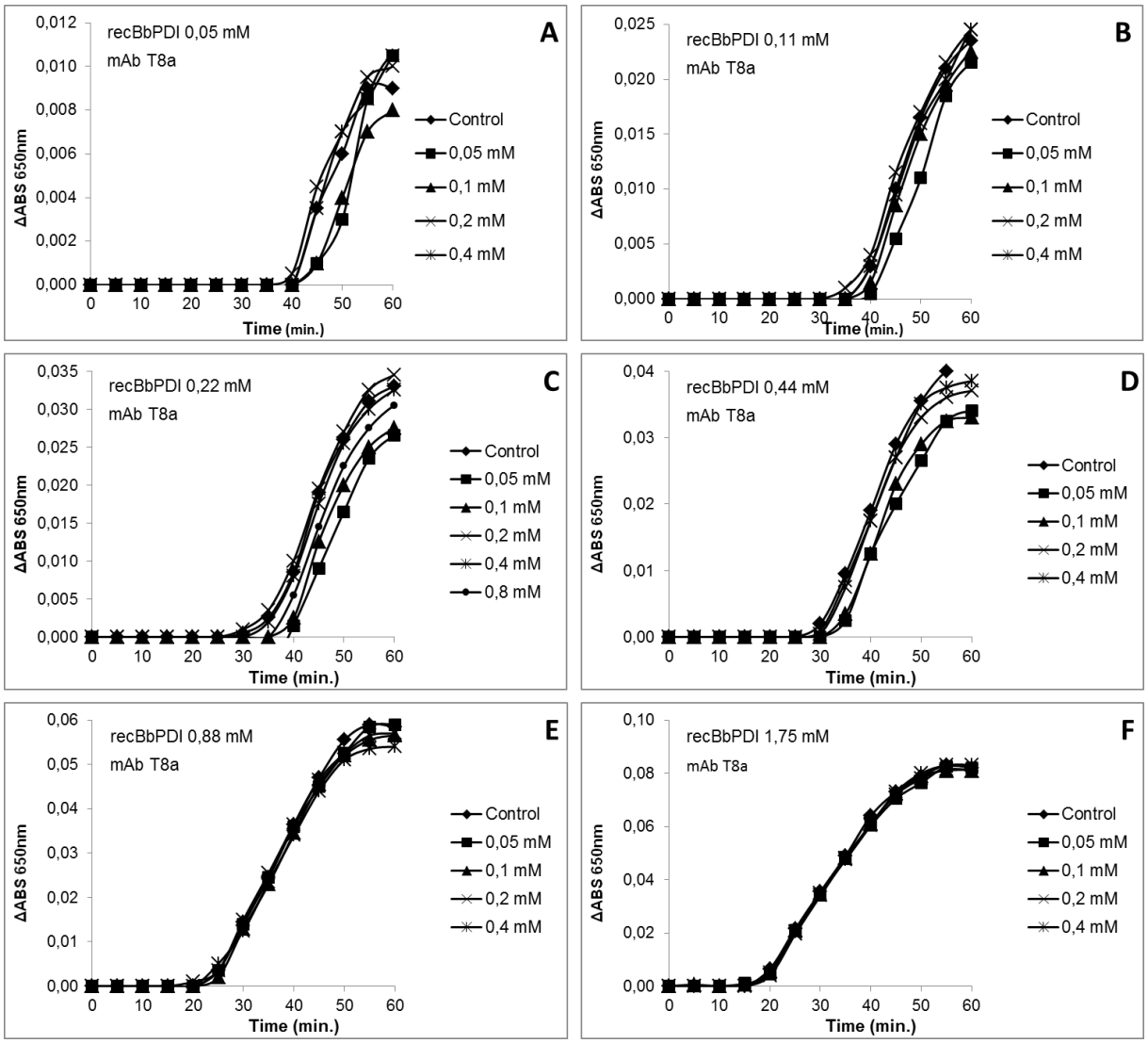

Figure 31. Reductase activity, measured by the aggregation of insulin, of recombinant *B. besnoiti* PDI (recBbPDI) in the presence of monoclonal antibody T8a (mAb T8a).
Enzyme activity was tested in concentrations of 0.05 mM (A), 0.11 mM (B), 0.22 mM (C), 0.44 mM (D), 0.88 mM (E) and 1.75 mM (F) in the presence of 0.05 mM, 0.1 mM, 0.2 mM and 0.4 mM of mAb T8a. No inhibition was obtained with T8a. Control represents recBbPDI activity without the presence of mAb.

and x-linker region) is more flexible than the N-terminal half (**a** and **b** domains) of the protein (Wang et al., 2010a). Fine tuning of epitope recognition will be needed in order to determine more precisely where these mAbs attach. Nonetheless, considering the relatively large size of an antibody molecule, the labeling of some part of one catalytic domain can probably constrain its mobility and the overall PDI plasticity or even block or difficult the access of substrate peptides to the binding site. This will also be true for the labeling of the non-catalytic domains, especially if epitope recognition lies within the substrate binding pocket.

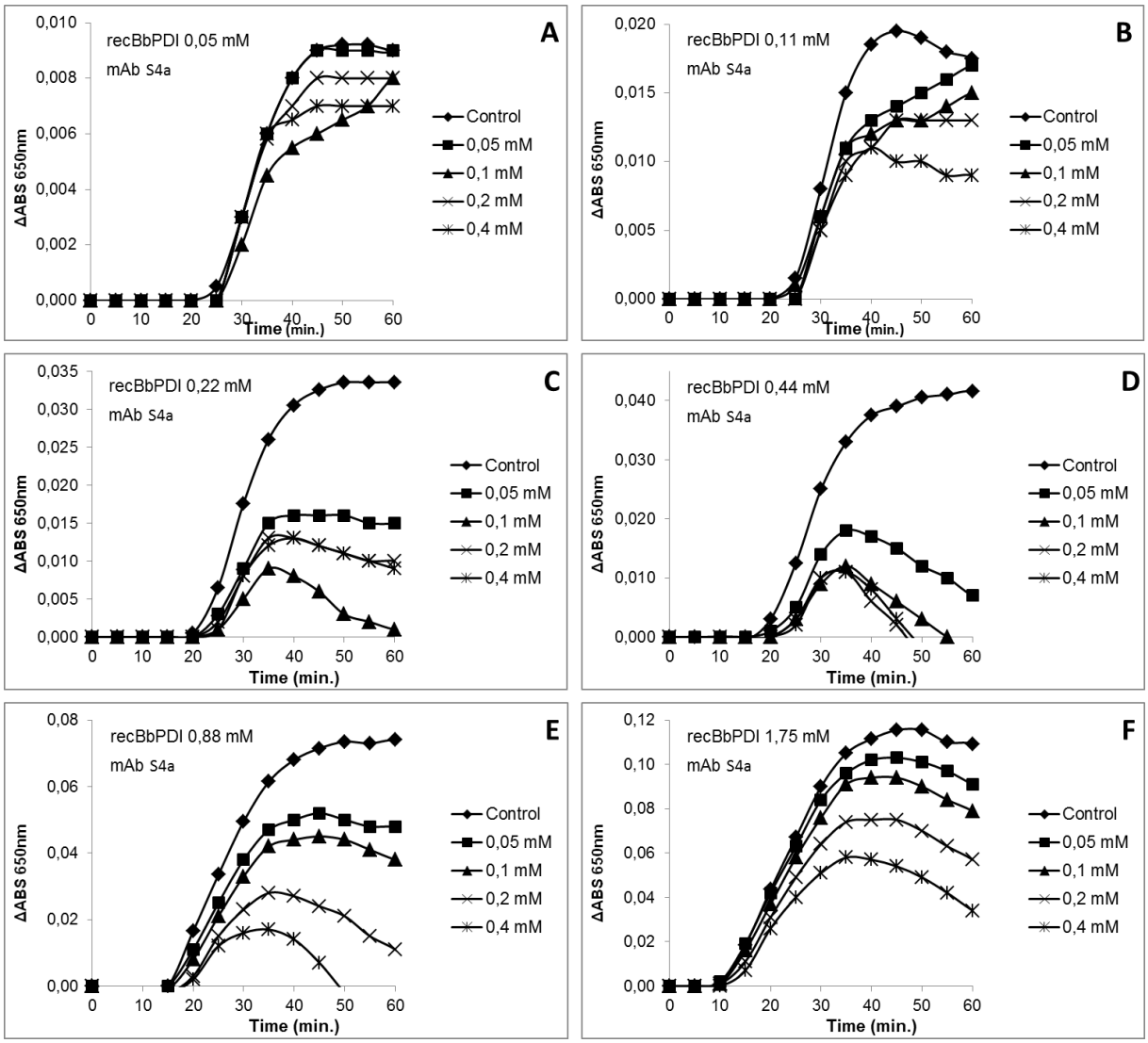

Figure 32. Reductase activity, measured by the aggregation of insulin, of recombinant *B. besnoiti* PDI (recBbPDI) in the presence of monoclonal antibody S4a (mAb S4a).
Enzyme activity was tested in concentrations of 0.05 mM (A), 0.11 mM (B), 0.22 mM (C), 0.44 mM (D), 0.88 mM (E) and 1.75 mM (F) in the presence of 0.05 mM, 0.1 mM, 0.2 mM and 0.4 mM of mAb S4a. There is a dose dependent inhibition of reductase activity of recBbPDI by mAb S4a. Control represents recBbPDI activity without the presence of mAb.

Despite recognizing domain **a'c**, mAb T8a and S4a are probably directed to distinct epitopes since the consequences on enzyme activity were quite different, with no noticeable interference in the former and a clear dose dependent inhibition in the latter. These results also agree with the differences predicted for epitope recognition on chapter 3.

In respect to the truncated version of BbPDI corresponding to the domain **a'c** (recBb-a'c) we tested its catalytic activity in the presence of mAb T8a, S4a and S16p. Enzyme concentration from 0.22 mM to 7 mM were used, and no inhibition was obtained with the presence of any of these mAbs (data not shown). Epitope recognition for these mAbs is probably away from the recBb-a'c active site, leaving it free to accomplish the insulin reduction.

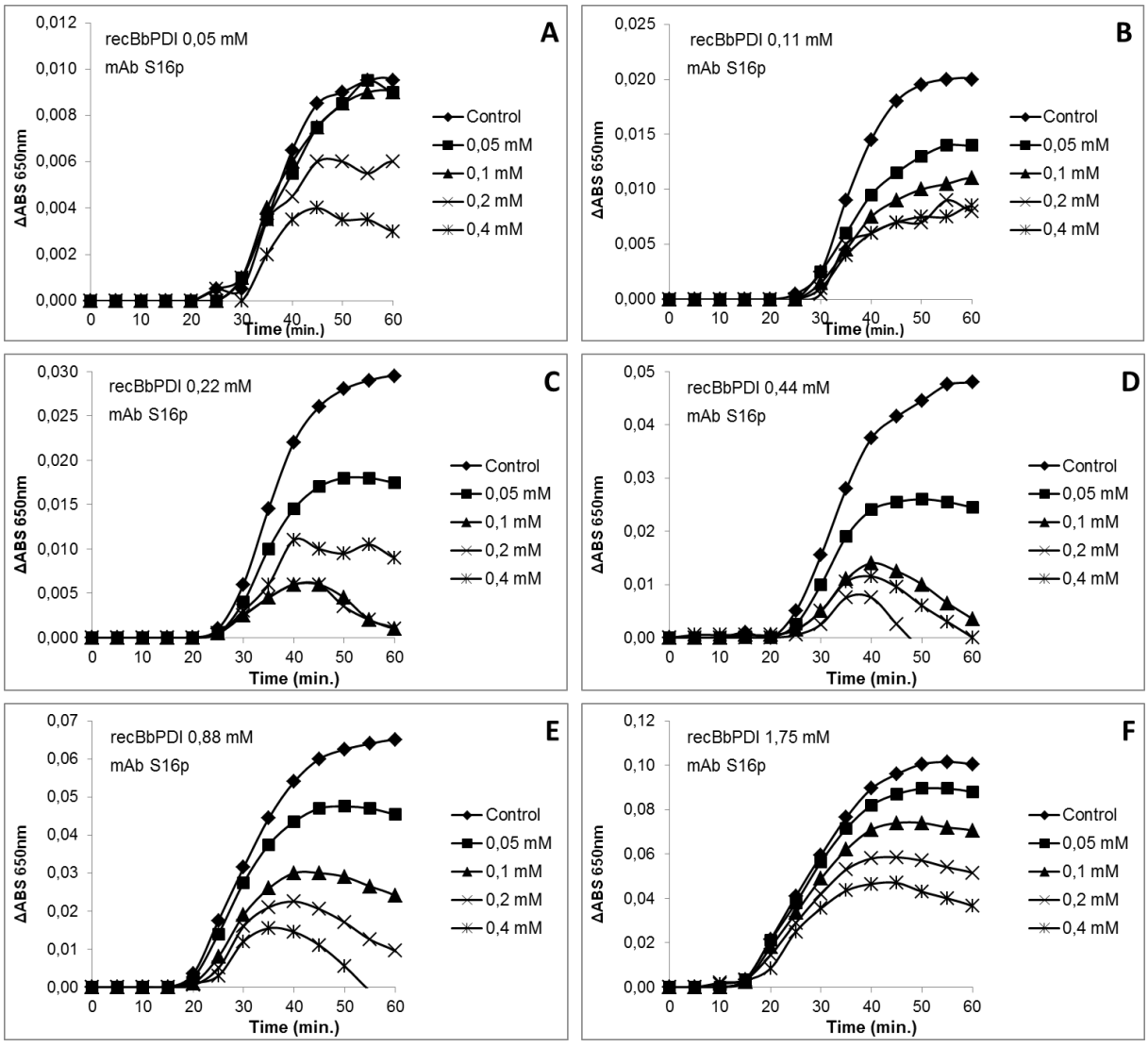

Figure 33. Reductase activity, measured by the aggregation of insulin, of recombinant *B. besnoiti* PDI (recBbPDI) in the presence of monoclonal antibody S16p (mAb S16p).
Enzyme activity was tested in concentrations of 0.05 mM (A), 0.11 mM (B), 0.22 mM (C), 0.44 mM (D), 0.88 mM (E) and 1.75 mM (F) in the presence of 0.05 mM, 0.1 mM, 0.2 mM and 0.4 mM of mAb S16p. There is a dose dependent inhibition of reductase activity of recBbPDI by mAb S16p. Control represents recBbPDI activity without the presence of mAb.

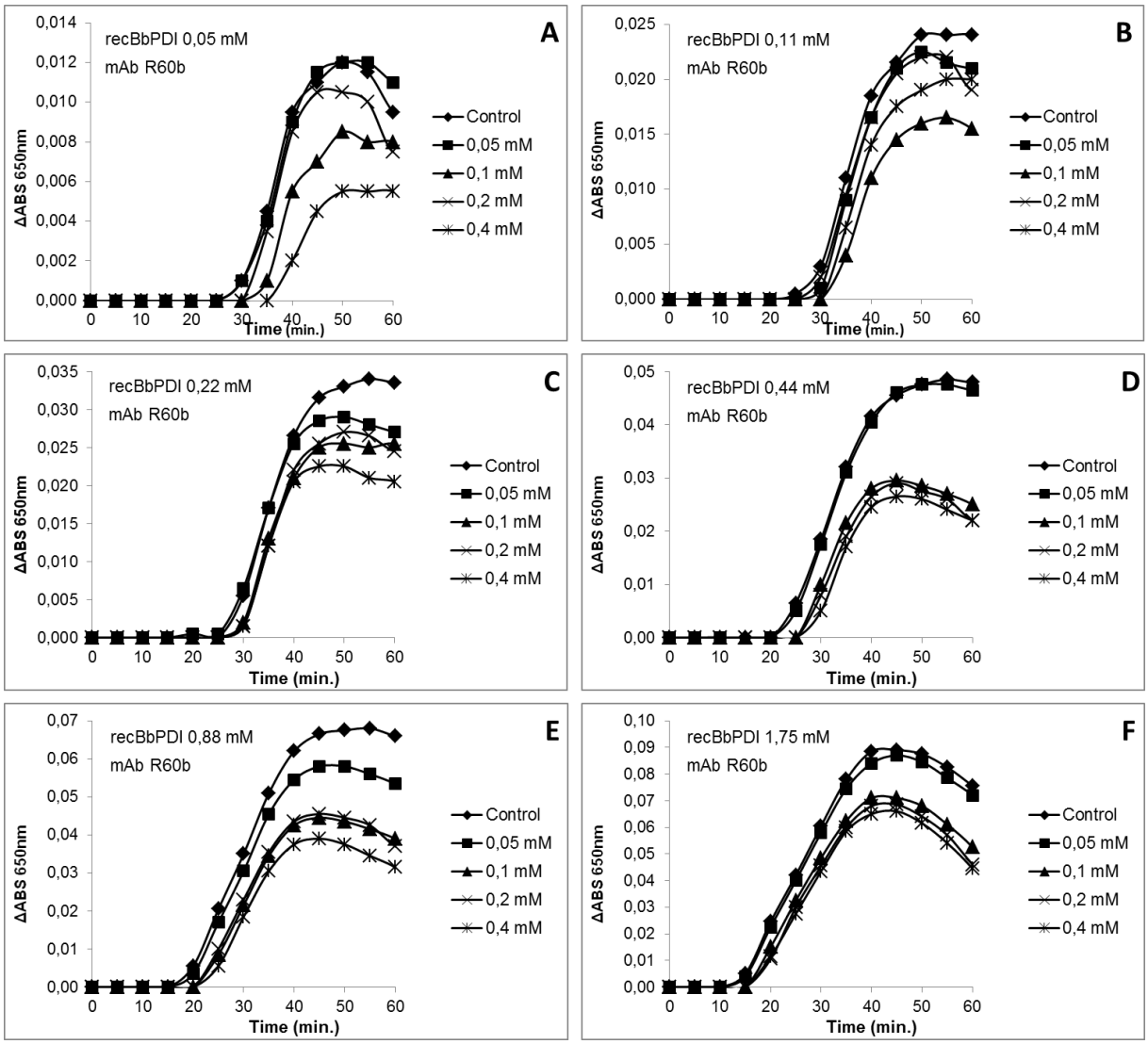

Figure 34. Reductase activity, measured by the aggregation of insulin, of recombinant *B. besnoiti* PDI (recBbPDI) in the presence of monoclonal antibody R60b (mAb R60b).
Enzyme activity was tested in concentrations of 0.05 mM (A), 0.11 mM (B), 0.22 mM (C), 0.44 mM (D), 0.88 mM (E) and 1.75 mM (F) in the presence of 0.05 mM, 0.1 mM, 0.2 mM and 0.4 mM of mAb R60b. Reductase activity of rec BbPDI is inhibited by mAb R60b. Control represents recBbPDI activity without the presence of mAb.

4.3.4 Involvement of BbPDI in host cell interaction.

B.besnoiti is an obligate intracellular parasite that must actively invade the host cell for survival and multiplication. Invasion is a dynamic and complex process in which the sequential release of proteins from micronemes, rhoptries and dense granules is fundamental (Binder & Kim, 2004; Katris et al., 2014; Sibley, 2011; Walker et al., 2014). There is a vast set of proteins involved in this process, playing different roles, but all essential for the successful invasion. Globally these proteins are regarded has good potential candidates for drug or immunogenic therapy (Darcy et al., 1988; Daryani, Hosseini & Dalimi, 2003). PDI was already identified in the excretory-secretory (ES) compartment of *N. caninum* (Liao et al., 2006), *T. gondii* (Zhou et al., 2005) and *P. berghei* (Lal et al., 2009) and at the cell surface of *N. caninum* (Naguleswaran et al., 2005), *T. gondii* (Meek et al., 2002a), *P. falciparum* (Florenta et al., 2000), *L. donovani* (Padilla et al., 2003) and *T. parva* (Ebel et al., 2002). To establish the presence of PDI in ES compartment purified tachyzoites were incubated in the absence and presence of ethanol. Ethanol specifically stimulates microneme discharge, but not rhoptry or dense granule release (Phelps, Sweeney & Blader, 2008).

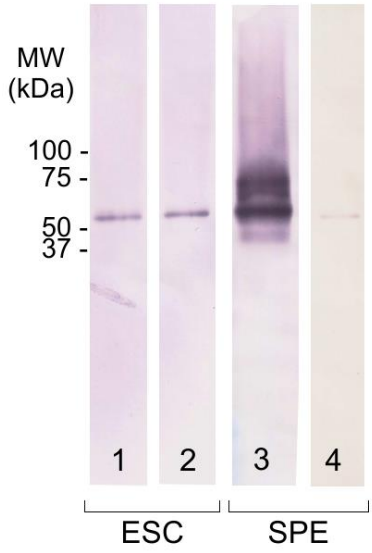

Figure 35. Detection of *B. besnoit*i PDI in the excretory-secretory compartment.
Western blot of supernatants (ESC) prepared after 15 min incubation of purified *B. besnoiti* tachyzoites and soluble protein extracts (SPE) of *B. besnoiti.* The excretory-secretory compartment (ESC) was evaluated in a PBS solution (1) and a 1% ethanol PBS solution (2). The soluble protein fraction was applied undiluted (3) and diluted 1:50 (4). Two fold serial dilutions were made until 1:1600 (not shown), but no labeling of PDI was achieved beyond 1:50 dilution. Membranes were probed with mouse anti-recBbPDI, revealing a band with the expected molecular weight of BbPDI.

When probed with anti-PDI serum we were able to label a band with the expected molecular weight of PDI, with a slight higher intensity for the ethanol induced ESA (Figure 36). For control, soluble protein extracts of *B. besnoiti* tachyzoites were used and diluted serially. This represents a strong evidence that in *B. besnoiti* PDI is also present in the ES compartment, highlighting the suspected importance of this protein in host cell invasion process.

In order to assess the importance of PDI in the process of cell invasion a simple assay was established, consisting in the pre-incubation of *Besnoitia besnoiti* tachyzoites with the inhibitors (drugs or mAbs), and the inoculation of host cells in the presence of inhibitors. For that, *B. besnoiti* tachyzoites were incubated for 10 min with the inhibitors and then transferred to a Vero cell monolayer where they were left to invade for 1 hour. To maximize the interaction between the Vero cells and tachyzoites and, in this way, the invasion process, only a small drop (300 μL) of medium containing the parasites was placed over the Vero cells. Vero cells were then washed to remove inhibitors and free tachyzoites and fresh medium (without inhibitors) was added. Intracellular tachyzoites were allowed to grow for a total of twelve hours. In this way, the effect of inhibitors was evaluated only during the invasion process, with minimal impact on host cell metabolism, that could subsequently influence parasite growth. Nevertheless, the viability of both tachyzoites and Vero cells in the presence of drug inhibitors was previously evaluated, by counting viable cells in a trypan blue exclusion test. A two hour incubation of Vero cells and tachyzoites did not increased cell death. Also, after this period of incubation, the viability of *B. besnoiti* tachyzoites was accessed by inoculating cell culture monolayers and performing routine serial passages. In the same way, viability of Vero cell was accessed in the subsequent days after the incubation with drugs. The two hour incubation period with the inhibitor drugs did not affect subsequent viability of tachyzoites or Vero cells.

The involvement of BbPDI in *B. besnoiti*-host cell interaction was in this way investigated by incubating tachyzoites with known PDI inhibitors, namely bacitracin, tocinoic acid, DTNB and pCMBA as well as with anti-PDI monoclonal antibodies. Results obtained showed a reduction on the host cell invasion rate of *B. besnoiti* tachyzoites for all drugs and mAbs tested (Figure 37 and Figure 38). On a molar basis, the most efficient inhibition was obtained with pCMBA, that with 5 μM can inhibit tachyzoite invasion in 20%, result that is only attained with 4 mM of Bacitracin (800 times pCMBA concentration) and 3 mM of tocinoic acid (600 times pCMBA concentration). Best overall result was obtained with DTNB, that can inhibit in 75% the parasite invasion rate, nevertheless, with a concentration of 2,5 mM. The concentration of drugs used for the invasion assay was adapted from the available literature. Comparable results were obtained in a similar study, but where *N. caninum* tachyzoites were only pre-incubated for 15 min. with inhibitors: 45% inhibition with 2.5 mM DTNB, 32% inhibition with 2 mM tocinoic acid and 42% with 10 μM pCMBA (Naguleswaran et al., 2005)

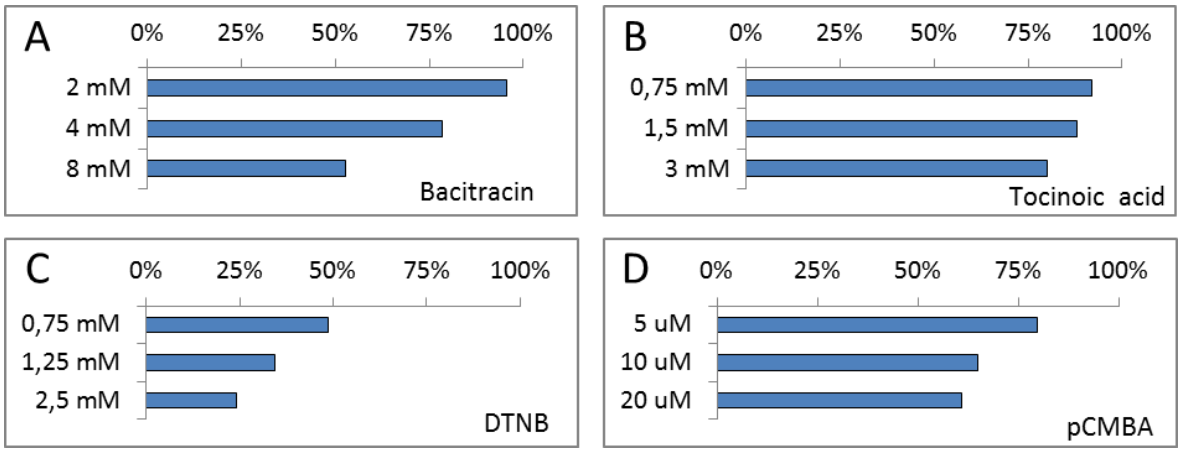

Figure 36. *In vitro* invasion of Vero cells by *B. besnoiti* tachyzoites in the presence of drug inhibitors. Different concentrations of bacitracin (A), tocinoic acid (B), DTNB (C) and pCMBA (D) result in a lower invasion rate of tachyzoites. Results shown as the percentage of invasion compared to control (100%)

Similarly, the presence of anti-PDI mAbs during the invasion process of *B. besnoiti* also had a negative impact in tachyzoite invasion rates with reduction ranging from 4% to 35%, depending on the mAb and the molar ratio considered. Curiously, mAb T8a, that could not inhibit *in vitro* reductase activity of recBbPDI, was one of the best performing mAb, along with S16p. In the invasion assay, conditions are very different from the ones established for measuring the catalytic activity of the recombinant protein. One key point that certainly changes, for example, is the enzyme substrate, that is not the same. The way mAb T8a binds to recBbPDI does not seem to interfere with activity of the recombinant enzyme over insulin, but appears to interfere with function of natural BbPDI, in a similar way as mAbs could not inhibit the catalytic activity of the truncated version recBb-a'c but could do so in the complete protein. When in the invasion process, *B. besnoiti* PDI function will probably go beyond the reductase activity performed over insulin, with the isomerase or chaperone functions being possibly the major tasks. It was shown for other PDIs, that the **b'** domain is essential for the isomerase function (Darby et al., 1998; Hatahet & Ruddock, 2009) since it represents the principal substrate binding site, and we can theorize that mAb T8a, despite recognizing an epitope on domain **a'c**, might interfere in a bigger extent with the binding of substrate peptides and in this way affect significantly the *in vivo* activity of BbPDI.

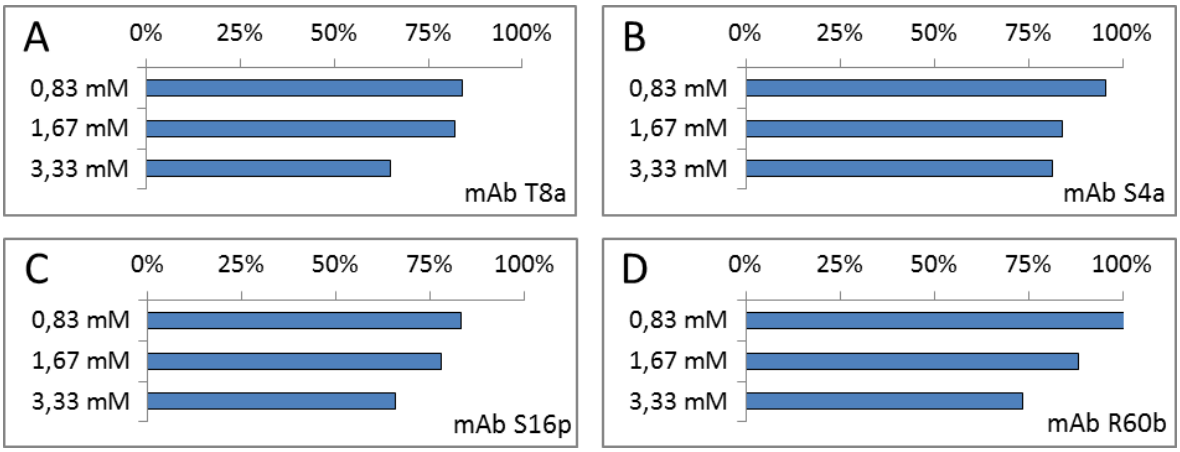

Figure 37. *In vitro* invasion of Vero cells by *B. besnoiti* tachyzoites in the presence of mAbs. Different concentrations of monoclonal antibody T8a (A), S4a (B), S16p (C) and R60b (D) result in a lower invasion rate. Results shown as the percentage of invasion compared to control (100%)

In general terms, these result agree with others that also show a negative impact of anti-PDI polyclonal sera and inhibitors in the growth of *N. caninum* (Liao et al., 2006; Naguleswaran et al., 2005), *L. amazonensis* (Hong & Soong, 2008) and *L. major* (Achour et al., 2002). In this assay, by using membrane impermeant PDI inhibitors, that can only act at the parasite surface, we show that interference with PDI function reduces *B. besnoiti* capacity to interact with the host cell, affecting ultimately its growth. Therefore, these results also point PDI as a potential target for drug therapy or vaccine development.

4.4 Conclusion

PDI is a ubiquitous multifunctional enzyme that is well characterized in mammals and yeast. We showed that recombinant BbPDI produced is functionally active, presenting the expected reductase activity over the reduced insulin. The truncated version corresponding to active site domain **a'c** is also active, but less than the full length protein, while active site domain **a** showed no reductase activity. In the presence of the drug inhibitors bacitracin, tocinoic acid, DTNB and pCMBA both recBbPDI and recBb-a'c were sensitive and showed a reduction in the catalytic activity. With mAbs, differences were observed between recBbPDI, that was sensitive to all mAbs except T8a, and recBb-a'c, whose reductase activity was not influenced by the presence of mAbs. The proposed method for high-throughput screening of PDI activity validated by Smith et al. (2004) was implemented for these initial assays and will allow for future screening of potential inhibitors.

We showed that *B. besnoiti* PDI is part of the excretory-secretory compartment in tachyzoites. Targeting surface associated PDI function with membrane impermeant inhibitors and anti-BbPDI mAbs in *B. besnoiti* tachyzoites invading Vero cells resulted in lower invasion rates which shows that this protein is probably involved in the host cell adhesion/invasion process. Although the exact role of this molecule at the parasite surface is not clarified, and needs to be further addressed, these results confirm the potential of PDI as a drug target.

Chapter 5 |

Concluding remarks and perspectives

Bovine besnoitiosis is a disease known for more than a century. It's mostly sub-Saharan location, with only a few pinpointed cases reported in Europe until the late 1990s, left it regarded as a tropical disease and fairly unnoticed for the veterinary and scientific community worldwide. Nevertheless, in between, other cases were reported in areas like Israel, Russia or South Korea. By the turn of the century the disease starts to be more noticed with a growing number of reports in Europe accompanied by a geographical expansion. This lead to the classification of besnoitiosis as an emergent disease in Europe by the EFSA. The genus *Besnoitia* comprises ten named species and has a worldwide distribution, but still with some questions regarding differentiation and taxonomic classification of these species. Only four *Besnoitia* species have their life cycle clarified, being similar to the closely related *T. gondii* and *N. caninum:* facultative heteroxenous with a predator as a definite host and a prey as intermediate host. For the remaining six species, where *B. besnoiti* is included, the life cycle is unknown. For these species no definitive host was identified and, although bovines were identified as intermediate hosts for *B. besnoiti*, the question whether they represent an accidental or the natural host remains to be answered. The disease in bovines can lead to important economic losses due to significant reduction in productivity, but is usually non-fatal, progressing to a debilitating chronic stage. There is no treatment and the use of the attenuated vaccines obtained in Israel and South Africa is geographically limited due to safety concerns. Therefore, control of disease is currently based solely on sanitary control methods. With so much to be known and clarified about this disease and parasite, arose the will to contribute to the building of knowledge on this subjects. And, for that matter, PDI had been recently pointed as an important protein, playing a key role in host cell invasion and in the host immune response, representing a promising drug target and/or vaccine candidate. PDI has been associated with adhesive functions and cell–cell and cell–pathogen interactions, and is now accepted that PDI is present at the surface of apicomplexan parasites, where it participates in host cell adhesion/invasion process. This process is crucial for the survival of these obligate intracellular parasites and we must consider that it is a mechanism driven solely by the parasite, where the host cell does not voluntarily participates, unlike phagocytosis for example. In this way, apicomplexan parasites need to carry with them all the tools to promote, first, adhesion, and then invasion. In this process they deploy a vast number of important proteins that exhibit an exceptional high number of disulfide bridges (Naguleswaran et al., 2005), that are responsible for their correct conformation and functionality. The pressure for quality control on the assembling of proteins is high, both at the endoplasmic reticulum and at the parasite surface. The exact mechanisms in which PDI is involved are unknown but certainly, and at least, it will be responsible for maintaining the structure and function of these crucial surface constituents (Jordan & Gibbins, 2006; Turano et al., 2002).

In this way, our main goal was to characterize the PDI of *B. besnoiti* and evaluate its role in host cell adhesion/invasion process and in the bovine immune response. We identified the complete coding sequence for PDI in *B. besnoiti*, originating the first publication of a complete gene sequence for a protein and the first protein to be identified and characterized in the genus *Besnoitia*. The enzymatic activity of the recombinant protein produced was evaluated by a turbidimetric assay based on the reduction of insulin, catalyzed by PDI. This assay, that can be used in the future for batch screening of PDI inhibitors, revealed a functionally active protein that was inhibited by drugs and monoclonal antibodies produced in this work. The advent of mAb technology has provided a powerful tool for the generation of limitless amounts of highly specific antibodies with multiple applications in research, diagnosis, and therapy. The mAbs produced were raised against recBbPDI, through the fusion of hyperimmunized mice spleens with a myeloma cell line. This hybridoma technology, although established more than 35 years ago, is still applied because of its simple protocol. We purified and characterized four mAbs, showing differences in epitope recognition of truncated versions of PDI and in the cross reactivity with *N. caninum* and *T. gondii.* These represent a valuable tool for future studies not only on the role of PDI, but also on fundamental biology of *B. besnoiti* and related Apicomplexa.

PDI is an immunodominant antigen as proteomics studies describe for other apicomplexan parasites and for *B. besnoiti,* where it is up-regulated in the tachyzoite stage (Garcia-Lunar, Regidor-Cerrillo, Gutierrez-Exposito, Ortega-Mora & Alvarez-Garcia, 2013b). Proteomic tools have allowed for the investigation of relevant biological processes as well as host immune responses associated with infections by other apicomplexan parasites of medical and veterinary importance. We addressed the diagnostic value of PDI, by performing ELISA over recBbPDI sensitized plates and also a competitive ELISA using mAbs. Despite initial results were encouraging, when starting to test a larger panel of sera, we soon realize that PDI is not a suitable antigen for this purpose due to lack of specificity (data not shown). Initially the panel tested included sera from chronically infected animals, recently infected animals, naïve animals, as well as sera from animals infected with the related *N. caninum* and *T. gondii.* Later we were able to add a well-defined batch of sera, from 4 animals followed during the course of disease, in a co-habitation trial (Gollnick et al., 2015; Schares et al., 2013). These animals were sampled at least from day 0 post seroconversion until approximately day 150. Since PDI is up-regulated in the tachyzoite phase, we would expect higher anti-PDI antibodies in the acute phase, that would decrease with the progression to the chronic stage. Nevertheless, although PDI was constantly identified in infected animals, no consistent variation was found in ELISAs throughout the course of disease (data not shown). Until the exact mechanism in which PDI intervenes is clarified we probably will not be able justify these observations.

When evaluating the role of PDI in host cell adhesion/invasion, we could place this protein in the excretory-secretory compartment of *B. besnoiti* tachyzoites, highlighting the hypothesized importance of PDI in this process. Furthermore, when incubating *B. besnoiti* tachyzoites with the PDI inhibitors tested in the kinetic assay (both drugs and mAbs), we could negatively affect the *in vitro* development of the parasite, by diminishing its invasion rate. This, not only confirms that PDI plays a role in the adhesion/invasion of the host cell, but also points to the potential use of PDI as a drug target or vaccine candidate. In *N. caninum*, PDI has been tested as a vaccine candidate in a mouse model, and different results were obtained depending on the antigen formulation and route of delivery. Intranasal vaccination with recombinant *N. caninum* PDI conferred approximately 90% protection against the disease. Considering the close relation to *B. besnoiti* and specially the high identity level between PDIs of these two parasites, these results indicate that in besnoitiosis PDI, despite not being a suitable antigen for diagnostics, can still be a good candidate to include in immunogenic formulations. The above mentioned results, obtained with PDI in the *N. caninum* mouse model, clearly point to the need of addressing this question together with the adjuvant formulation and route of administration in bovine.

In a therapeutic point of view, two complementary approaches can be used for further studies. One can be the *in silico* screening of new compounds to find suitable ligands for PDI, making use of the 3D model here developed. This can be a fertile field considering the large amount of compounds made available through the advent of combinatorial chemistry. The other approach can be the evaluation *in vitro* of the efficacy of selected compounds. These can first be tested in the insulin assay for their ability to inhibit PDI activity and then, best performing ones, can be used in invasion assays. Following these approaches we might be able to find good candidates for the treatment of *B. besnoiti* infections and possibly for the treatment of infections by other related Apicomplexa.

This work represents an initial approach to better understand the role of PDI in the biology of *B. besnoiti*. While demonstrating that PDI is an important enzyme for successfully accomplish host cell invasion, the exact mechanisms in which it participates are not clarified. Future works should both try to clarify these mechanisms and look for the potential of PDI as a drug target or an antigen to be included in vaccine formulation.

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