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The Mitochondrial Peroxiredoxin of *Leishmania* - a novel member of the parasite chaperone network, crucial for infection

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À Minha Mãe

SUMMARY

Leishmania, the causative agent of leishmaniasis, are transmitted to humans and dogs by the bite of an insect vector. Several factors contribute to parasite infectivity and, ultimately, to disease development. During this PhD project we have investigated the functional importance of the mitochondrial tryparedoxin peroxidase (mTXNPx) of *Leishmania infantum*, a member of the 2-Cys-peroxiredoxin (2-Cys-Prx) family of enzymes. Our motivation arose from the observation that, although *L. infantum* devoid of mTXNPx were unable to survive in the mammalian host, it was not the well-established peroxidase activity of this protein but an alternative unidentified function that was responsible for such noticeable phenotype. Besides working as peroxidases, some members of the 2-Cys-Prxs family display also chaperone activity *in vitro*. The results presented in this thesis inscribe TXNPxs as a novel member of the chaperone network of *Leishmania*, whose function appears decisive for parasites to withhold the shift in temperature during transition from the insect to the mammalian host.

Our data revealed that *L. infantum* devoid of mTXNPx have a growth defect and aggregate more proteins than controls when exposed to a temperature shift from 25°C to 37°C. Such thermosensitive phenotype is highly reminiscent of cells lacking molecular chaperones and consonant with the observed *in vitro* chaperone activity of mTXNPx. Conclusive evidence that the crucial function of mTXNPx during infection is that of a molecular chaperone, came from the observation that, similarly to parasites devoid of mTXNPx, *L. infantum* expressing a chaperone-inactive version of mTXNPx were unable to thrive in a murine model. Analysis of the underlying mechanism behind this novel function revealed that mTXNPx has chaperone activity when reduced and decameric, but not when oxidized and dimeric. While this finding establishes the ring-like shaped decamer as being essential for function, an additional level of regulation was shown necessary for mTXNPx to switch into an active chaperone. We discovered that, by entailing conformational changes in the protein structure, temperature can function as a regulator of the chaperone function. This is supported by an observed temperature-dependent increase in hydrophobicity and by preliminary cross linking data showing that interaction of mTXNPx with clients involves considerable conformational changes of the protein during heat-shock. Hence, the decamer represents the chaperone-activatable species, while temperature-induced structural rearrangements acting on this scaffold constitute the trigger for activation, *i.e.* for exposure of the client binding site. The client binding site appears to lie in the lumen of the decameric structure of mTXNPx as supported by i) EM microscopy of mTXNPx:client complexes, ii) activity studies showing that interference with

the N-terminus of the protein, which faces the inside of the decamer, abrogates chaperone function, and by iii) cross-linking data identifying one residue of the decamer lumen as being implicated in client binding. In terms of the position of mTXNPx within the cellular chaperone network we found that mTXNPx is a stress activated chaperone holdase which, upon return to non-stress conditions, is capable of cooperating with additional chaperones (known as chaperone foldases), ultimately promoting the refolding of client proteins.

In summary, by establishing that the chaperone function of mTXNPx is crucial for *Leishmania* pathogenicity, the results described in this thesis conclusively demonstrate that the chaperone function of 2-Cys Prxs is of physiological significance. Additionally, insights obtained into the mode of action of the mTXNPx chaperone are key to understand the mechanism of chaperone function of other members of the 2-Cys-Prx family.

RESUMO

Protozoários do género *Leishmania* são agentes patogénicos, causadores de leishmanioses, um conjunto de doenças do cão e do Homem, com sintomatologias e taxas de mortalidade variadas. A forma mais comum de transmissão destes parasitas é através da picada de um inseto flebótomo, a mosca da areia. A capacidade da *Leishmania* infetar mamíferos depende de diversos fatores, dos quais alguns são inerentes ao hospedeiro, outros ao próprio parasita. Um destes fatores do parasita foi escolhido como objeto de estudo desta tese de Doutoramento.

O objetivo geral do trabalho aqui apresentado é o de definir e caracterizar a função crucial desempenhada pela triparedoxina peroxidase mitocondrial (mTXNPx) de *Leishmania infantum,* uma enzima pertencente à família das peroxirredoxinas de 2 cisteínas (2-Cys-Prx). A importância da mTXNPx em *Leishmania* é evidenciada pelo fato de, na falta desta enzima, os parasitas serem incapazes de sobreviver no hospedeiro mamífero. Até à publicação dos trabalhos compilados nesta tese, esta enzima era conhecida apenas por atuar como peroxidase no parasita, ou seja pela sua capacidade de eliminar cataliticamente hidroperóxidos. No entanto, resultados do nosso grupo vieram questionar a relevância desta função para a sobrevivência da *Leishmania*, motivando assim este trabalho de investigação. Esses resultados demonstravam que não era a atividade de peroxidase da mTXNPx que dotava a *Leishmania* de capacidade infeciosa, mas sim uma outra qualquer função alternativa, até então desconhecida.

Para além da atividade de peroxidase, algumas 2-Cys-Prxs podem atuar como chaperones moleculares, evitando agregação irreversível de outras proteínas induzida por stress térmico ou por outro tipos de agressão. O trabalho aqui desenvolvido veio mostrar que também a mTXNPx de *L. infantum* é dotada dessa atividade e, mais, que é a função de chaperone desta molécula que determina o seu papel crucial para a sobrevivência dos parasitas no hospedeiro mamífero. Com base nos nossos resultados elaborámos um modelo segundo o qual a mTXNPx atua como um chaperone que protege os parasitas do choque térmico a que eles ficam sujeitos aquando da transmissão do inseto (25ºC) para o hospedeiro mamífero (37ºC).

Uma vez estabelecida a relevância funcional da actividade de chaperone da mTXNPx, fomos de seguida dissecar a base mecanística desta função. Resultados dos nossos estudos moleculares permitiram-nos estabelecer vários aspetos mecanísticos relevantes: i) a estrutura decamérica da mTXNPx em forma de anel é a base da sua atividade chaperone; ii) a ligação de proteínas cliente é feita no interior do anel decamérico; iii) a proteína decamérica sente flutuações de temperatura, respondendo

com alterações conformacionais que levam à exposição de resíduos às proteínas cliente e à ativação da função chaperone da mTXNPx; iv) é possível anular a atividade de chaperone da mTXNPx interferindo com a sua região N-terminal que se encontra voltada para o interior do anel decamérico; v) a mTXNPx é uma chaperone “holdase", isto é ela congela as proteínas cliente num estado desnaturado, evitando a sua agregação irreversível, e transfere-as posteriormente para outras chaperones com função de renaturação.

Em suma, os resultados descritos nesta tese são inovadores na medida em que i) demonstram que a atividade de chaperone da mTXNPx é essencial para sobrevivência de *L. infantum* nos hospedeiros mamíferos e ii) exploram a base mecanística da atividade de chaperone da mTXNPx, que pode possivelmente ser extrapolada para outras enzimas da família das 2-Cys Prxs.

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CHAPTER I

General Introduction

1. Leishmaniasis

General Introduction

In the middle of the second decade of the 21st century, infectious diseases are still accountable for over a fourth of all human deaths, mortalities being due not only to the advent of new devastating infections such as AIDS, but also to ancient maladies. Leishmaniasis, a set of multifaceted diseases caused by protozoan parasites of the genus *Leishmania,* are among such olden infections that still affect humankind [1]. Depictions of what is thought to be *Leishmania*-infected individuals date as far back as 2500 B.C. [2].

Leishmaniasis ranks ninth among infections in terms of disease burden (*i.e.* a metric that reflects both mortality and morbidity) measured as disability-adjusted life years (DALY - *i.e.* 1 DALY translates as the loss of one year of "healthy" existence [3]). Furthermore, amongst tropical diseases, leishmaniasis occupies the second place in terms of mortality (only exceeded by malaria) and the fourth in terms of morbidity. According to the World Health Organization (WHO), leishmaniasis is a global problem affecting 98 nations, 72 of which are developing countries [4,5]. Therefore, these maladies are strongly allied to poverty and, together with leprosy, schistosomiasis and dengue, among other conditions, are regarded as neglected tropical diseases [1]. Leishmaniais affects primarily tropical and subtropical areas of the globe, the Mediterranean basin countries (such as Portugal) included. Nowadays this parasitosis still accounts for nearly 50.000 deaths per year [4,5]. Moreover, globally, 350 million people are at risk of infection with 1.5 - 2 million new cases occurring every year and 12 million people estimated to be parasitized [4,5]. Nevertheless, according to the WHO, these figures are likely underestimated due to underreporting.

As for many other infectious diseases, the ethiological agent of leishmaniasis, *Leishmania*, was described for the first time in the beginning of the 20th century, subsequently to the establishment of the germ-theory of disease by Pasteur [2,6]. Since then, nearly 30 different species of *Leishmania* have been described of which over 20 are human pathogens. The requirement for a vector as part of the natural mode of transmission of this organism to humans was established only 40 years after its identification [2]. Thus, leishmaniasis is a vector-borne disease, meaning that *Leishmania* are transmitted to the vertebrate host by the bite of a parasitized insect vector (the female sandfly of the genus *Phlebotomus* and *Lutzomya*, depending on the region of the world) [7]. Over 90 sandfly species are known to transmit *Leishmania*. Inherent to this mode of transmission is the observed geographical distribution of the disease as well as the variability in the number of infections along the year and even during the day, which is a consequence of the distribution, abundance and activity of the sandflies, respectively [8]. Moreover, the transmission of *Leishmania* by its insect vector may be classified as

zoonotic (where a third party, an animal reservoir, is involved in the transmission to men) or anthroponotic (in which transmission occurs directly between humans). Reservoir hosts (frequently asymptomatic) include rodents, dogs and wild canids. These are considered of great epidemiological importance as they contribute to the persistence of *Leishmania* pools in nature, thus promoting the dissemination and maintenance of this disease [9].

Social Impact

Infectious diseases are often associated to poverty and, as mentioned before, leishmaniasis is no exception [1,10]. As outlined below, this fact is unsurprising.

The outcome of a *Leishmania* infection is multifactorial, extending from asymptomatic to life threatening, contingent both on the *Leishmania* species and on the host stamina. Asymptomatic infections, which appear to constitute the majority of *Leishmania* infections, may progress to disease if the fine-tuned equilibrium at the host-parasite interface is disturbed. Factors that may contribute to this unbalance include those that may lead to immune fatigue such as malnutrition, poor sanitation and public health conditions, multiple infections, among others factors [4,11]. While these issues are not unique to developing countries they co-exist in a despondent way in these regions. Other traits allied with poverty such as lack of resources, underprivileged and overcrowded households, absence of protective methods such as nets to shield form sandfly bites, also promote the risk of infection [4]. Moreover, although not necessarily injurious to the parasitized individual, asymptomatic infections are particularly relevant in terms of infection dynamics as its carriers constitute central reservoirs for the anthroponotic cycle of *Leishmania* transmission.

In addition to constituting a massive burden for developing countries, leishmaniasis might also represent a potential menace to developed countries. It has been postulated that factors such as globalization (which may expose individuals from non-endemic regions to infected sandflies or bring reservoirs, humans or animals, to areas where disease is under control), global warming (which might change the overall distribution of the insect vector), among others may re-shape the worldwide distribution of diseases such as leishmaniasis [4,12]. Thus, leishmaniasis may possibly affect countries where it did not exist before or where it was considered under control. Another aspect that may contribute not only to change the geographic distribution but also the epidemiology and awareness of this ancient malady is, interestingly, an emergent infection agent: the HIV virus, the reasons being twofold [13,14]. First, as referred to above, any factor affecting the balance between *Leishmania* and its host can contribute to progression of the infection from silent to disease. This has been shown to be especially true in AIDS patients, where the HIV-

infection increases several fold the risk of developing a life-threatening form of leishmaniasis (known as visceral leishmaniais, VL – see below). Second, HIV-infections even though a major problem of developing countries are also omnipresent in the developed ones. Indeed, data from 2013 predicts that worldwide 35 million people live with HIV infection. For example, nowadays, in Southern Europe, VL associated to HIV-infections constitute up to 70% of the cases reported in adults [5,15]. Moreover, reports of *Leishmania*-HIV co-infections have been increasing worldwide especially in Eastern Africa, South America and the Indian continent [4]. For all the stated reasons, the WHO considers HIV infections as an emerging risk for the control of VL. Thus, the cumulative influence of all the above stated factors has led to the re-definition of leishmaniasis from an olden to a re-emergent disease.

Clinical manifestations

Based on symptoms and clinical manifestations, three major forms of leishmaniasis can be defined: cutaneous (CL), mucocutaneous (MCL) and visceral leishmaniaisis (VL, often referred to as Kala-azar) [4,16,17].

Cutaneous leishmaniasis is the most ubiquitous form of the disease. It is characterized by the presence of lesions or ulcers in the skin, primarily on exposed parts of the body to which the sandfly can access. This condition is self-limiting although the wounds can leave long-lasting scars. Nevertheless, a non-healing form of CL, diffuse CL (DCL), in which lesions spread throughout the body, has been reported mainly among immunocompromised individuals. Most of CL cases are observed in South America, Middle East and Central Asia.

Mucocutaneous leishmaniasis, is a type of leishmaniasis that is often highly disfiguring as large areas of mucous membranes (from nose, mouth and throat) can be partial or totally mutilated. This form of the disease occurs when, upon transmission, *Leishmania* spreads from the skin to the mucosal areas via lymph or blood stream. Mucocutaneous leishmaniasis rarely heals spontaneously. MCL is usually restricted to some areas of South America.

Contrary to CL and MCL, VL, a systemic chronic disease, can be fatal if left untreated. In this form of the disease parasites disseminate to internal organs such as the liver, spleen, bone marrow and lymph nodes, ultimately leading to their failure. Symptoms include weight loss, recurrent fever, anemia pancytopenia, hypergammaglobulinemia and hepatosplenomegaly. Post-Kala-azar dermal leishmaniasis (PKDL) may arise as a consequence of VL treatment. This sequel, due to its dermal nature, is of great concern as its carriers represent putative reservoirs for the anthroponotic cycle of VL. Even though

different areas of the world are endemic for VL, 90% of the cases are confined to 6 countries: Bangladesh, Brazil, Ethiopia, India, South Sudan and Sudan. Among *Leishmania* species, *L. donovani* and *L. infantum* (known as *L. chagasi* in the new world) are known to visceralize.

As outlined above, leishmaniasis is a multifactorial disease with both host and parasite factors influencing the outcome. An obvious difference between the three types of the disease is that in the first two (*i.e.* CL and MCL) the parasite is mainly confined to the skin while in VL it disseminates to internal organs. Although not categorical, this difference is commonly associated to the species of *Leishmania*. In this sense, understanding why some *Leishmania* species are able to viscerlize while others remain restricted to the skin is key to understand both disease progression and *Leishmania* survival mechanisms. Additionally, this knowledge might be exploited to the development of new therapies. In this sense, the toolbox provided by the advent of the genome sequencing has proved instrumental as paralleling the genome of conventionally visceralizing and non-visceralizing species of *Leishmania* helps pinpointing genes that might be important for the dissemination to the organs [18]. This allowed the identification, for example, of A2 gene found only in visceralizing species as in non-visceralizing species this is a pseudogene. Further functional studies confirmed the requirement of A2 proteins for visceralization and parasite survival within macrophages of visceral organs [19].

Diagnosis, treatment and prevention of leishmaniasis

Diagnosis of leishmaniasis must take into consideration both clinical symptoms (mentioned above) and the results of laboratory tests [20,21,22]. These can detect *Leishmania* either directly or indirectly. Direct visualization of the parasite can be made by microscopic observation of *Leishmania* stained bodies in smears of different samples: skin lesions, lymph nodes, bone marrow aspirates or, even, spleen and liver biopsies. Previous *in vitro* cultivation or animal infection of these samples can facilitate parasite visualization and, hence, leishmaniasis diagnosis. Indirectly, *Leishmania* can be diagnosed i) serologically by detection and measurement of antibodies (humoral response) specifically produced against the parasite in the blood, serum or other body fluids using methods such as direct agglutination test (DAT), indirect fluorescence antibody test (IFAT) and enzyme-linked immunosorbent assay (ELISA). Additional indirect methods include detection of *Leishmania* ii) antigens (e.g. latex agglutination test using the patient urine [23]), iii) DNA (by PCR) or iv) host cellular immunity response using *Leishmania* antigens (known as Montenegro skin test [24]). One important drawback transversal to all these methodologies is their challenging implementation in the field, particularly when

considering their usage in the prime affected regions – the developing countries. Therefore, the development of a method that is both accurate and easy to use in the terrain is crucial for leishmaniasis diagnosis and ultimately to its control. In this sense, the development of the K39 test (an immunochromatographic test that detects circulating antibodies against the recombinant K39 protein impregnated in a nitrocellulose paper band) has brought some optimism [25,26]. This test, which detects viscerotropic species of *Leishmania*, appears to be sensitive, does not need laboratory equipment, is inexpensive and thus appropriate to use in large screening campaigns.

Once a *Leishmania* infection is confirmed, adequate treatment must be promptly initiated. Several chemotherapeutic options can be used for this effect none of which is ideal [4,16]. Through the years, lengthy, parenteral doses of pentavalent antimonials have been used as primary line medications. Besides problems related with the administration route, the duration of the treatment and its associated toxicity, the emergence of drug resistance cases precludes the usage of those compounds in some countries [16,17]. The second line drug, the efficient antifungal amphotericin B (AmpB), considered as an alternative to antimonials, is also toxic unless it is administered in its liposomal formulation; unfortunately, the high cost of liposomal AmpB renders it prohibitive in most countries affected by leismaniasis. Other therapeutics used to treat leishmaniasis include pentamidine and miltefosine [16,17]. Miltefosine primarily developed as an anticancer drug, was shown to be very effective against *Leishmania* in several clinical trials even in patients with antimonial resistance [23]. Importantly, being an oral drug, miltefosine circumvents the restrictions linked with the parenteral administration of other chemotherapeutics. Nevertheless, miltefosine has teratogenic potential and therefore its usage has to be taken into account especially among young children and pregnant women [27]. Overall, as highlighted above, all available therapeutics present drawbacks. Therefore, development of alternative therapeutics with focus on efficacy and ease of application in the affected countries should be a priority.

Diagnosis and treatment of leishmaniasis are important measures to control this malady. Ideally, however, all forms of the disease should be prevented and this would most adequately be achieved through vaccination [28,29]. Even though the development of a vaccine for leishmaniasis seems, in principle, feasible as resolution of a primary infectious frequently leads to resistance to re-infection, up to date no efficient and safe human vaccine has been developed against this parasitosis [29]. Efforts in this area are being made namely in what concerns the use of live-attenuated, DNA or protein-based recombinant vaccines [30,31]. Still, most approaches are far from being implemented on the field. Additional prophylactic measures that can help managing leishmaniasis include control of the populations of the insect vector and large-scale application of individual

protective actions against bites [4]. Also, the control of reservoir animals is fundamental as these are responsible for the maintenance of the zoonotic mode of transmission of the disease [4,32]. In some countries dog culling is used, however this raises imperative ethical questions especially now that moderately efficient vaccines against canine leishmaniasis (Leishmune and CaniLeish) exist [33,34,35]. Moreover, as referred to previously, effective disease management (*i.e.* early diagnosis and effective use of available drugs) are key towards reducing the prevalence of the disease. Perhaps, to ultimately achieve eradication of leishmaniasis and related maladies, an integrated vision of all components of the disease together with educational campaigns need to be implemented. In this sense the re-emergence of the holistic approach that underlies the “One-health” paradigm which is routed in a concerted action of different fields (public health policy makers, researchers, drug developerers, doctors, vector specialists, environmentalists, among others) may represent a fresh start for leishmaniasis control [36].

The Portuguese scenario

In Portugal, as in other countries of the Mediterranean basin, *Leishmania infantum* is the agent responsible for VL and CL. In the past most cases of leishmaniasis used to occur in children, owing to their inexperienced immune system however this scenario has recently changed [37]. While the number of children affected by visceral leishmaniasis has decreased from the eighties onwards the number of affected adults has increased. Between 1999 and 2014 the laboratory of leishmaniasis at IHMT (Instituto de Higiene e Medicina Tropical) confirmed the diagnostic of 266 cases of leishmaniasis [37]. Of these, 199 were visceral and 27 were of cutaneous leishmaniasis. As referred before, factors leading to immune fatigue may promote disease development and indeed the emergence of *Leishmania*/HIV co-infection is of epidemiological importance. Actually, of the 199 confirmed cases of visceral leishmaniasis in Portugal, 122 were found in immunocompromised individuals including those affected by AIDS. In fact, in some regions of Portugal and Spain, *Leishmania* is already the third most common opportunistic infection in HIV-infected individuals. Disturbingly, WHO estimates that in southern Europe 2-9% of the AIDS patients will develop VL [4,38].

In the Mediterranean region leishmaniasis caused by *L. infantum* is a zoonosis being the dog the main host and reservoir [37,39]. *Leishmania* infection can thus result in canine leishmaniasis (CVL), a chronic disease with viscerocutaneous symptoms. Recent studies suggest that the average prevalence of CVL is around 6%. However, in some areas prevalence can be as high as 17,4% [40]. Apart from being severely affected by the

disease, dogs constitute an important reservoir of *L. infantum* for the zoonotic transmission cycle to humans. As such, canine leishmaniasis is not only a veterinary but also a public-health problem. Moreover, a recent study showed that in Portugal, the prevelance of *Leishmania* infection among cats ranges between 0.3 and 30.4% suggesting that this animal might also be important for the maintencae of the parasite transmission cycle [41]. In Portugal the vectors responsible for transmission of *Leishmania* are *Phlebotomus pernicious and P. ariasi.* In a recent report 0.13% of the collected sandflies were PCR positive for presence of *Leishmania* DNA [42].

Although VL, CL and CVL are endemic in Portugal, the community has in general limited knowledge about this disease. With the goals of increasing awareness and of reducing the prevalence of VL, CL and specially CVL, a national platform for leishmaniasis was recently created, the ONLeish – Observatório Nacional das Leishmanioses (National Leishmaniasis Observatory) [37].

2. *Leishmania*: the causative agent of leishmaniasis

General Introduction

Leishmania is part of the order Kinetoplastida. This order, composed of several families of flagellated protozoa, is amongst the firsts to branch off of the eukaryotic lineage. Among kinetoplastid families, the Trypanosomatidae is particularly relevant from a medical point of view as some of its affiliates are responsible for major tropical diseases. Besides including several species of *Leishmania*, this family encompasses *Trypanosoma brucei*, the agent of African sleeping sickness and *Trypanosoma cruzi*, the agent of Chagas disease. Overall, the maladies caused by members of the Trypanosomatidae family cause over 100’000 deaths yearly (www.who.int). Additional kinetoplastid members include parasites of insects (*e.g.*, *Crithidia fasciculata*) and of plants (*e.g.*, species of the *Phytomonas* genus). In spite of the different pathologies and life cycles, these parasites share a vast number of orthologues (~75% of the gene pool) and a great degree of gene synteny (*i.e.* the same position within chromosomes) [18,43,44]. Thus, research done in a member of one genus has the potential of being transversely applied to others. In other words, studies carried out in *Trypanosoma sp.* are frequently extrapolated to *Leishmania s.p.* and vice-versa. It is important to highlight, nevertheless, that many of the genes present in these parasites’ genomes are classified as of unknown function, demonstrating the long path that research in this field still has ahead [18,43,44].

Kinetoplastids and their oddities

In terms of biology, Kinetoplastids share with other eukaryotes basic features including the presence of i) a nucleus, and ii) different organelles, that tear them apart from prokaryotes. However, they also possess unique characteristics that reflect their ancientness and distinguish them from other eukaryotes.

Perhaps the most conspicuous peculiarity of Kinetoplastids is the presence of a disk shaped structure near the basal body, next to the insertion of the flagella, the kinetoplast [45]. This unique structure, after which the order Kinetoplastida was coined, is composed of mitochondrial DNA, also known as kinetoplastid DNA or kDNA. The kDNA is an intertwined, massive network composed of DNA maxicircles and minicircles which form a discrete structure in the cell. kDNA encodes some mitochondrial proteins and also small RNAs responsible for RNA edition (guide RNAs), however it appears not to encode transfer RNAs (tRNAS), implying that these need to be imported from the cytosol. Besides encoding proteins and guide RNAs, kDNA also appears to have an additional structural function. The kDNA is not the sole oddity of these parasites mitochondria. In fact, the mitochondria itself is also peculiar for being a single, tubule-shaped structure occupying a large fraction of the cell that extends from the apical to the basal extremities of the cell [46].

The compartmentalization of the first seven glycolytic enzymes in a peroxisome-like organelle, the glycosome, constitutes another difference of Kinetoplastida towards other eukaryotes. This arrangement is considered key for the regulation of glycolysis. Due to the absence in these organism of negative feedback regulation of hexokinase activity, glycolytic compartmentalization provides a mechanism for preventing glycose exhaustion, *i.e.* for preventing parasites from consuming all glycose and dying from starvation [47,48]. In addition to being involved in glycolysis and its regulation, glycosomes are additionally implicated in fatty acid oxidation, pyrimidine biosynthesis and purine salvage.

The flagellar pocket, another distinctive organelle, is an invagination of the plasma membrane around the flagellum [49]. This area is the place where most endocytic and exocytic events occur. The confinement of vesicular traffic to this area occurs because the intricate net of microtubules present beneath the plasma membrane is looser around the flagellar pocket. Because of the importance of the flagellar pocket in cellular trafficking, other organelles, such as the Golgi apparatus, are polarized near this structure. As such, together these organelles constitute a multiorganellar complex that is involved in defining cell polarity.

Further differences towards other eukaryotes include high genomic plasticity and high recombination rates [50,51,52]. On one hand, these features allow the production of genetically manipulated mutants by homologous recombination, a tool that is key for

functional studies. On the other hand, the high genomic plasticity also enables these protozoans to amplify specific regions of their genome forming amplicons which might impair knockout generation, especially when the gene to be deleted is essential to the parasite [52]. A reflex of the genomic plasticity of these cells is the presence of multiple copies of the same gene, a feature that might preclude the generation of double mutant parasites. This is particularly true for certain families of proteins such as heat shock proteins (for example *Leishmania* encodes a total of 18 HSP90, 16 HSP70 and 66 HSP40 molecules) [53]. This problem, while easily circumvented in higher eukaryotes by means of RNA interference (RNAi), poses technical difficulties in most *Leishmania* species as these lack key enzymes indispensable for RNAi (*e.g.* Dicer) [54]. Additionally, although considered diploid organisms, the number and size of chromosomes actually varies within a given *Leishmania* population, further illustrating the high genomic plasticity of these protozoa. DNA transcription and regulation is also remarkably atypical in *Leishmania* [55,56,57]. Most genes lack introns and promoters, and are typically co-transcribed into polycistronic units [55,56]. This array of functional unrelated genes is then processed by trans-splicing and polyadenylation to yield mature, individual transcripts. Owing to these features, gene expression is not controlled at a transcriptional level, rather it relies on RNA half-lives and protein stability. This mode of gene regulation is in sharp contrast with that of most eukaryotes. In the latter, gene promoters are crucial for incorporating and coordinating internal or external signals and translating them in an orchestrated expression of functional related genes that allow the cell to respond to any given environmental cue. The absence of classical promoters in kinetoplastids (the only exception being the promoters of the spliced leader genes) precludes such concerted response to be regulated at the transcriptional level and implys that these parasites employ other levels of regulation.

The thiol-dependent metabolism of kinetopastids constitutes yet another difference towards other eukaryotes [58,59]. *Leishmania* and related organisms rely on a unique thiol, trypanothione (a conjugate of two gluthatione molecules linked by one spermidine molecule), that replaces most of the functions that in other organisms are taken over by glutathione [59,60]. Trypanothione and its biosynthetic pathway have been shown crucial in these organisms. Importantly, their antioxidant defense system is primarily trypanothione-dependent [58]. Moreover, due to this biochemical divergence, some of the molecules that are involved in kinetopastid redox biology are also unique, namely trypanothione reductase (that maintains the pools of reduced trypanothione at the expenses of NADPH) and tryparedoxin (a thioredoxin-like protein that reduces other proteins, such as peroxidases, at the expense of reduced trypanothione). In line with the distinctive character of their redox metabolism, trypanosomatids lack antioxidant enzymes

that are commonly found in other eukaryotes, as are the cases of glutathione reductase, thioredoxin reductase, catalase and selenocysteine-containing gluthathione peroxidases. Due to its uniqueness and essentiality, the trypanothione-dependent redox system of trypanosomatids has been considered over the years very attractive in terms of research and drug development. The protein under study in this thesis is a peroxidase whose reduction is coupled through tryparedoxin to this unique thiol system.

***Leishmania*: intricacies of managing a double life**

As already pointed out in the first part of this introduction, trypanosomatids are part of a group of organisms that live a double life. These so-called heteroxenous or digenetic organisms cycle between two distinct hosts as part of their life cycle (detailed below). In the specific case of *Leishmania*, the parasite shuttles between the intestinal tract of an insect vector (the sandfly) and the acidic phagolysosome of mammalian host macrophages. As a consequence, *Leishmania* unavoidably encounter remarkable changes in their environment as they transit between hosts. These include i) a rise in temperature (from ambient temperature to ~37ºC), ii) a drop in pH (as they colonize the acidic phagolysosome of macrophages), iii) changes in nutrient availability, and in osmotic and oxygen pressures. Moreover, *Leishmania* are exposed to the immune attack by its mammalian host by means, for example, of production of reactive oxygen and nitrogen species. Therefore, the ability of these parasites to quickly sense and respond to different environmental cues is of paramount importance for their viability and, ultimately, for pathogenesis. While these striking changes of milieu are stressful, they also constitute the trigger for the ultimate adaptation of the parasite to each host, *i.e.* shuttling between two distinct developmental forms: i) the promastigote which is found in the arthropod vector and ii) the amastigote found in the vertebrate host [61]. As detailed next, each parasite form differs from the other in terms of morphology and physiology, and is fully adapted to colonize each host.

Promastigotes are spindle-like shaped, extracellular, flagellated and highly motile cells that reside in a sugar rich, slightly alkaline milieu of the gut of a poikilothermic organism. Opposed to this, amastigotes are ovoid shaped, intracellular, aflagellated (actually, they possess a rudimentary flagellum) and non-motile forms that colonize the fatty acid- and amino acid-rich, acidic environment of the phagolysosome of a homoeothermic host. Despite these seemingly opposing phenotypes, promastigotes and amastigotes specific transcripts only differ by 1-3,5% [62,63,64,65]. This negligible difference in terms of gene regulation is not startling if one takes into account the very peculiar and promoter-independent mode that *Leishmania* and related trypanosomatids

employ to transcribe their genes [55,56]. The surprising figure is that, in terms of final gene products, the difference between promastigotes and amastigotes is still rather small, the percentage of differentially expressed proteins representing only 3-9% of the identified proteins in proteomic studies [65,66,67,68,69]. These differentially expressed gene products, which cover several classes of enzymes and other proteins, reflect the above mentioned adaptations of the parasite to the environmental differences of the host, such as the carbohydrate and amino acid metabolism, stress response and heat-shock proteins, proteolysis and detoxification systems. Apart from these few cases, most of the *Leishmania* genome is constitutively expressed in both life cycle stages, thus raising the question as to how *Leishmania* manage to quickly sense and adapt to differences in milieu as they cycle between both hosts. A possibility is that this phenomenon is post-translationally driven. In support of this, a comparative study by Morales *et al* has shown that there are major differences between the phosphoproteomes of both promastigotes and amastigotes [70,71]. Of the identified phosphorylated proteins, 38% were shown to be differentially represented between both forms, the phosphoproteome being significantly enriched in amastigotes. Interestingly, chaperones were among the phosphorylated proteins found to be overrepresented in amastigotes. The amastigote-specific phosphorylation of a sub-set of chaperones, which in some cases impacts on their activities [71], may represent an adaptive response of *Leishmania* during its passage from ambient temperature (the poikilothermic vector) into a warmer millieu (the homeothermic host at 37ºC).

Apart from phosphorylation, other post-translational modifications (PTMs), including glycosylation and acetylation, also occur during amastigote differentiation [72]. In a broader sense, PTMs may represent an important developmental strategy in organisms that are suddenly exposed to a new environment to which they have to quickly adapt without the requirement of *de novo* protein synthesis. Besides PTMs, also changes in the overall abundance of a given sub-set of proteins might dictate the promastigote-amastigote differentiation and/or stress response to the environmental changes. In organisms that lack canonical regulatory control of gene expression, these changes in protein level must occur at a post-transcriptional level. Indeed, Saxena *et al.* have demonstrated that over the course of promastigote to amastigote transition, the mRNA abundance of a pool of genes undergoes either transient or permanent changes in abundance [64]. Among genes transiently regulated are those that code for heat-shock proteins. This observation, together with the abovementioned phosphoproteomic study, underscores the importance of chaperones in the transition between promastigote-amastigote differentiation. In further support of this, protein phosphatases and kinases are also shown to be differentially regulated. Genes that are permanently regulated include

those encoding surface proteins and transporters, and proteins involved in cell maintenance, motility and growth.

For all it was said, the environmental changes parasites encounter during their life cycle can be perceived as a two-edged sword, in the sense that they are both stressfull and critical for differentiation [73]. As a consequence, the responses mounted can be seen on one hand as protective (the cases of chaperones and proteolytic systems which are important to deal with heat-induced protein unfolding and aggregation, and also of detoxification systems that are key to counteract the production of biotoxic products resultant from host-induced oxidative burst) and, on the other hand, as adaptative *i.e.* necessary for *Leishmania* differentiation as promastigote-amastigote reversion. Although impossible to untangle from each other, these different cellular responses ultimately contribute to life cycle progression and thus pathology.

Both promastigotes and amastigotes can be grown *in vitro* under conditions that mimic their natural environments, an attribute that greatly facilitates research of both life cycle stages of *Leishmania* in the laboratory context. Indeed, most studies mentioned to above were conducted using *in vitro* cultures of both parasite forms. Compared with amastigotes, it is far easier to handle and maintain *in vitro* cultures of promastigotes. As a consequence of this, most tools to genetically manipulate *Leishmania* (*e.g.* to generate knockout or overexpressing mutants) were optimized in the insect stage of the parasite. Transgenic promastigotes can then be cytodifferentiated into amastigotes. *In vitro*, this can be accomplished by mimicking natural phenomena (*e.g.* rising the temperature and lowering the pH). This method was actually used in the studies referred to above, which aimed at dissecting the changes that take place as promastigotes develop into amastigotes. Nonetheless, this procedure can be technically tricky and amastigotes obtained this way (known as "axenic" amastigotes for being "host cell-free") are often claimed not to fully mimick "true" amastigotes. A way to overcome this limitation is by using intramacrophagic amastigotes, which can be obtained either by *ex vitro* (macrophage monolayers) or *in vivo* (murine models) infections.

Life cycle

Leishmania are vector-borne pathogens whose transmission to the mammalian host is contingent on the insect feeding behavior. An overview of the life cycle of *Leishmania* is depicted in Fig. 1. During a blood meal, the insect vector inoculates virulent promastigotes (also known as metacyclic promastigotes) into the skin of the mammal alongside with immunomodulatory components present in the saliva [7,74,75]. Upon transmission and once in the dermis of the mammalian host, professional phagocytes, namely

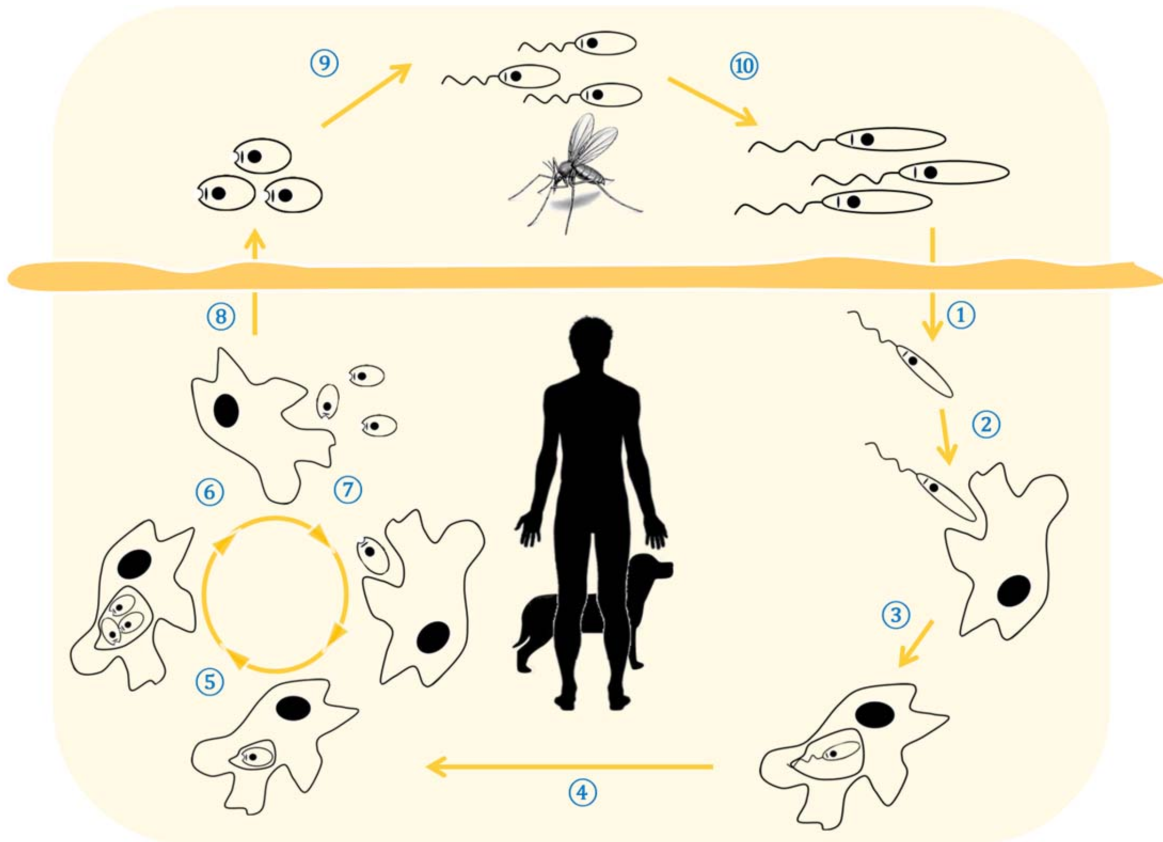

Figure 1: The *Leishmania* life cycle. During blood meal, *Leishmania*-colonized sandflies inoculate promastigotes into the dermis of mammalian hosts (1). Promastigotes are phagocytized by macrophages (or additional phagocytic cells) (2 and 3). Phagosomes containing promastigotes later on fuse with lysosomes or late endosomes forming phagolysosomes, *Leishmania*'s final niche inside macrophages. Inside these compartments promastigotes differentiate into amastigotes (4) which are adapted and replicate in the acidic content of phagolysosomes. (5). By a still uncompletely defined process amastigotes infect naïve macrophages (6, 7). Once a sandfly forages on an infected mammal it will acquire infected macrophages or free amastigotes along with other blood components (8). In the insect vector *Leishmania* reverts back to the promastigote form, which is adapted to live in this new environment (9). Through a series of developmented regulated modifications (known as metacyclogenesis) these promastigotes will differentiate into infective promastigotes which are pre-adapted to colonize mammals. The cycle restarts once an infected sandfly transmits promastigotes to a new host (1).

macrophages, denditric cells, and neutrophils, enclose these parasites in a phagocytic-mediated manner [45,76,77,78]. Various cells of the immune system interact with the parasite during the initial moments of infection, but the ultimate hosts of *Leishmania* are macrophages. The differentiation of promastigotes into amastigotes which allows the establishment of the parasite in the new host and, thus, the progression of the life cycle, occurs only in macrophages [79]. As for the other phagocytic cells that are initially colonized, such as the neutrophils, they may serve as transient hosts [80]. Inside macrophages, *Leishmania* reside within the phagolysosome. This compartment, which

originates from the fusion of the phagosome with late endosomes and/or lysosomes, is very acidic and enriched in hydrolytic enzymes. Contrary to promastigotes, amastigotes are well-adapted to living in phagolysosomes and it is inside these subcellular compartments that *Leishmania* replicate [81]. By a process still poorly elucidated which either depends on amastigotes being released from infected macrophages or on infected macrophages being taken up by other macrophages, amastigotes are able to further infect naïve macrophages thus, establishing an infection [82,83]. Recently, a model in which amastigotes are transferred between cells by immunomodulatory extrusions of the parasitophorous vacule was proposed [84]. Sandflies foraging on the blood of an infected mammal can then acquire colonized macrophages together with other blood components. Once inside the sandfly, amastigotes sense the different environment and revert back to promastigotes. These newly reverted promastigotes (alias, procyclic promastigotes) are fast dividing and non-infectious. Through a developmental process (known as metacyclogenesis, which in general lasts 6-10 days), non-infectious promastigotes undergo several developmental states which culminate with the formation of non-dividing, highly mobile and infectious promastigotes that are pre-adapted to successfully invade a new mammalian host [85]. In order to ensure completion of its life cycle, *Leishmania* have evolved several strategies [81]. These operate at different stages of the life cycle and include: i) alteration of the insect feeding behavior [86], ii) evasion of the host immune response (*e.g.* by accessing macrophages through infected neutrophils or via opsonic-dependent ways) [80,87,88], iii) delay of the biogenesis of the phagolysosome (for the time period required for the recently phagocytized promastigotes to revert to amastigotes, the parasite form fully equipped to survive the harsh milieu of the phagolysosome) [89,90], and iv) hijacking of host signaling pathways [91]. Moreover, the protein under study in this thesis was found essential for *Leishmania* to survive inside the mammal and thus key for the parasite to maintain its life cycle. This protein is a peroxidase that belongs to the 2-Cys-Peroxiredoxin family. As such, specificities on this family of proteins will be explored next.

3. 2-Cys-Peroxiredoxins

General introduction

Peroxiredoxins (Prxs) are ubiquitous, ancient proteins found in every biological kingdom [93]. In addition to being phylogenetically widespread, multiple isoforms of Prxs are commonly found within the same cell [93]. These different isoforms are present in the cytosol and in various cellular compartments. Prxs constitute one of the most abundant proteins in *E. coli*, make up ~ 0.8% of the soluble proteome in mammalian cells, and are the third most expressed proteins in erythrocytes [94]. Expression of Prxs has been shown to increase further under certain stress conditions or disease states [94].

Prxs have been ascribed with distinct cellular activities ranging from peroxidases that act either as general antioxidant devices or as sophisticated regulators of peroxide-dependent cell signaling pathways, to thiol oxidases and molecular chaperones [95]. Since these activities are at the core of many cellular functions, either by controlling peroxide levels, transferring peroxide signals or by chaperoning other proteins, Prx disturbance has been shown to have dramatic impacts on cellular homeostasis [96]. Indeed, knockdown or knockout of Prx leads to a plethora of phenotypes, which range from increased sensitivity towards oxidative damage of proteins, lipids or DNA (attributed to abrogation of the peroxidase activity) and heat stress (attributed to disruption of the chaperone activity), to downstream phenotypes, including tumor development, shortened lifespan, and enhanced T cell proliferation [96]. Importantly, these pleiotropic phenotypes suggest that Prxs act as cellular hubs and exert functions that do not overlap with other known peroxidases or molecular chaperones.

Of all the activities that have been attributed to Prxs, the peroxide-scavenging function of Prx is undoubtedly the best characterized [94]. Nevertheless, the impact of this activity on the modulation of peroxide-dependent signaling cascades is still only emerging [92,97,98]. Moreover, an increasing number of cases have been reported that illustrate the ability of Prxs to act as thiol oxidases, thereby modulating the redox status and activity of downstream proteins [99,100,101,102,103]. Finally, the chaperone activity has been reported for different Prxs [104,105,106]. Yet, neither the mechanism of chaperone function nor the physiological relevance of this activity is known.

Peroxidase function of peroxiredoxins

Prxs form a highly conserved superfamily of antioxidant enzymes, thought to have evolved from an ancestor encompassing a thioredoxin-fold [107]. Peroxiredoxins are broad-spectrum peroxidases, which reduce a variety of different peroxides including H_2O_2,

organic hydroperoxides and peroxynitrite, with varying efficiencies [94,108,109]. Due to their role in peroxide detoxification, Prxs are considered major players in protecting cells not only from the toxic effects of oxidants produced endogenously but also against those of exogenous origin. One example is during the oxidative burst when cells of the host immune system produce high concentrations of reactive oxygen and nitrogen species to kill off pathogens [110]. Prxs have been found to be vital enzymes in parasites such as *Leishmania*, whose survival and persistence in mammals is largely dependent on their ability to cope with host-derived oxidants produced during macrophage invasion [110].

The mechanism of action employed by peroxiredoxins was not immediately evident since Prxs did not resemble any well-established peroxidases such as catalase or classical glutathione peroxidases. While catalases and glutathione peroxidases rely on the presence of heme and selenocysteine, respectively to reduce peroxides, peroxiredoxins were found to depend solely on one, universally conserved thiol (known as peroxidatic cysteine, Cp). Although reaction rates between peroxides and thiolate anions ($R\text{-}S^-$) are generally very low (~18–26 M^{-1} s^{-1}) [111,112,113], the catalytic efficiency of Prx's active site cysteine (10^5 - 10^7 M^{-1} s^{-1}) was found akin to that of catalases (10^6 M^{-1} s^{-1}) and glutathione peroxidases (10^8 M^{-1} s^{-1}) [109]. The structural environment around the peroxidatic cysteine provides a rational for the approximately 10^5 - 10^7 - fold increase in k_{cat} observed for the thiol-based peroxidase function of Prxs as compared to most other cysteine thiols. Immediately adjacent to the active site Cp, Prx contains four additional, highly conserved amino acids (Pro, Thr, Arg, and Glu/Gln/His) [114]. This microenvironment around Cp appears to lower the pK_a of the cysteine from approximately 8.6 to 5-6, which is fundamental to deprotonate the cysteine and generate the more reactive thiolate anion [115]. Moreover, it generates a hydrogen bond network that is thought to be important for properly positioning the peroxide within the active site and influence its chemistry and thus its reduction [116].

The high cellular concentration of most known Prxs together with their high catalytic rates, make these peroxidases major players in the maintenance of the cellular redox homeostasis. Indeed, it has been estimated that Prxs alone reduce up to 90% of the peroxide content of the cytosol. However, in recent years the vision of oxidants solely as damaging molecules has changed, and so has the perception of peroxidases as mere peroxide-eliminating devices. As oxidants, such as H_2O_2 become increasingly recognized as second messengers, Prxs are also gradually accepted to serve as hubs in the regulation of peroxide-dependent cell transducing pathways [117]. This role of Prxs as intermediaries of cell signaling pathways has thus the possibility of influencing major cellular events.

Catalytic cycle of the peroxidase activity of peroxiredoxins

All peroxiredoxins contain one conserved cysteine at their active site, the peroxidatic cysteine (C_P), which is absolutely crucial for peroxide detoxification [94]. Thus, any mutation in C_P abolishes the peroxidase function of these proteins. The thiolate anion of C_P (S_P^-) performs a nucleophilic attack on the O–O bond of the peroxide (ROOH), thus catalyzing its direct reduction to the corresponding alcohol (ROH). During this process, C_P becomes oxidized to sulfenic acid (Prx-S_POH). Sulfenic acids are usually unstable and have the tendency to condense with other thiols (known in this cycle as resolving thiols - R-S_RH), resulting in the formation of a disulfide bond (Prx-S_P-S_R-R) and the release of water. Reduction of the disulfide bond depends on cell-specific oxidoreductases, such as thioredoxin or thioredoxin-like proteins (*e.g.* tryparedoxin in *Leishmania* and related trypanosomatids – see section Prxs of *Leishmania*) [118,119]. Ultimately, thioredoxin, which is part of an enzymatic cascade that uses thiol-disulfide exchange reactions, bridges peroxiredoxin recycling to the NADPH pools of the cell. The catalytic cycle of peroxiredoxins can thus be divided into three consecutive events: i) oxidation of C_P, ii) resolution of C_P by a resolving thiol, and iii) recycling of Prx-S_P-S_R-R by a thioredoxin-like molecule (for overview of the Prx catalytical cycle see Figure 2, top panel) [120,123].

Depending on the source of the resolving thiol (R-S_RH), peroxiredoxins are traditionally subdivided into two major families, 1-Cys-Prx and 2-Cys-Prx. Whereas the second cysteine in 1-Cys-Prx is provided by a different molecule (*e.g.*, thioredoxin or glutathione), it is supplied in 2-Cys-Prx by a conserved C- terminal cysteine, which is known as the resolving cysteine (C_R). 2-Cys-Prxs are further divided into atypical or typical 2-Cys-Prxs depending on whether C_P and C_R form an intramolecular or an intermolecular disulfide bond, respectively. Consequently, typical 2-Cys-Prxs are obligate homodimers, which are arranged in a head to tail fashion. This arrangement brings C_P from one monomer into close proximity to the C_R of the other subunit of the dimer. Albeit traditionally used, the division into 1-Cys-Prx and typical and atypical 2-Cys-Prx is not phylogenetically informative since peroxiredoxins that use the same mechanism are not necessarily evolutionarily closer than Prxs that use different mechanisms [114]. To overcome this phylogenetic inconsistency, six sub-families of peroxiredoxins have been established based on their patterns of conservation: Prx1, Prx6, Prx5, PrxQ, Tpx and AhpE [114]. To determine to which family a given Prxs belongs, a database known as the PeroxiRedoxin classification index (PREX) was developed [121]. Peroxiredoxins found in *Leishmania* and related trypanosomatids are typical 2-Cys-Prxs, which belong to the Prx1 subfamily [110]. For this reason, the forthcoming sections will focus only on this sub-group of peroxiredoxins.

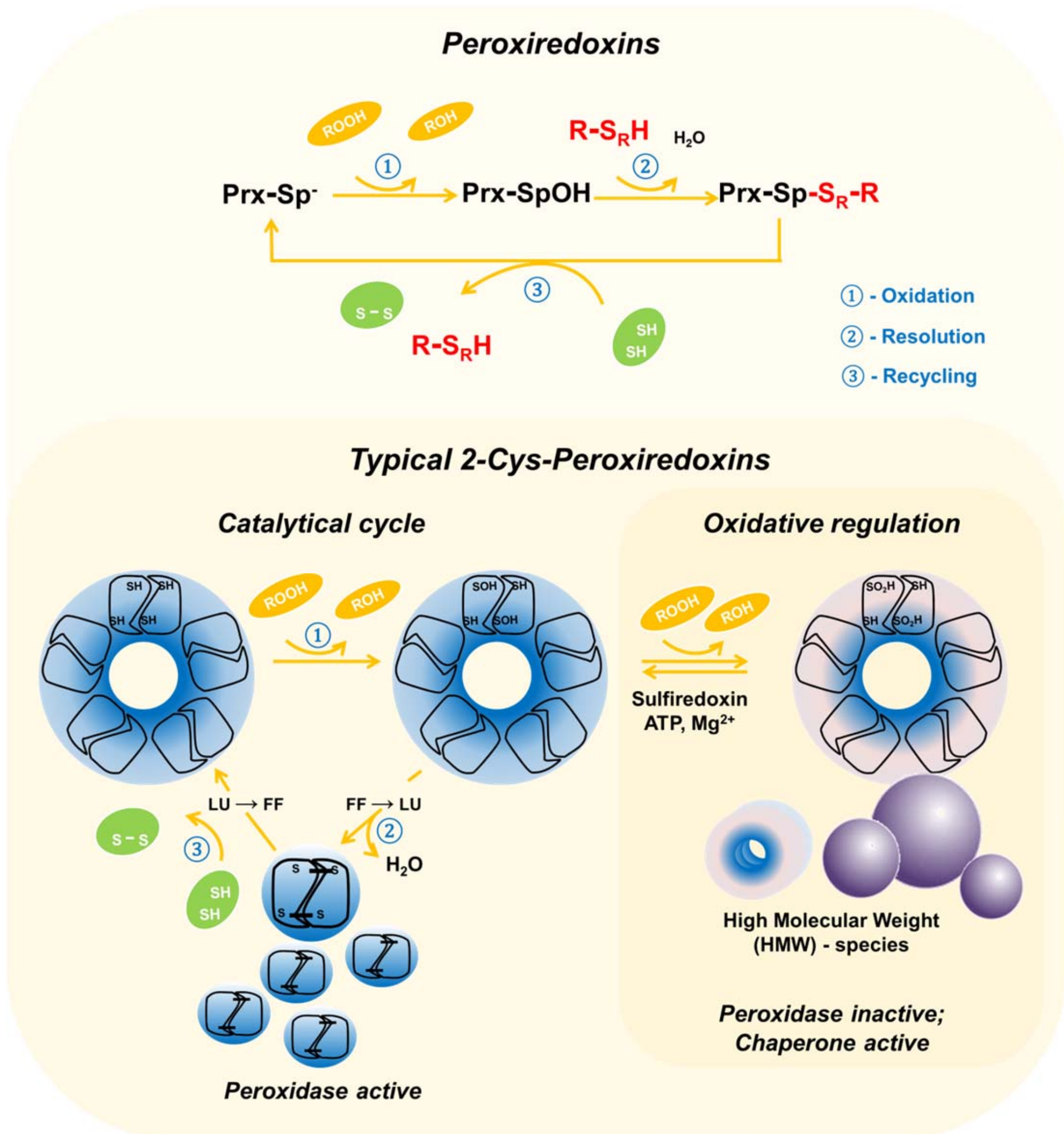

Figure 2: Mechanism employed by peroxiredoxins for peroxide reduction. The ubiquitous peroxidatic thiolate anion of Prx (Prx-S_P^-) performs a nucleophilic attack to the peroxide (ROOH) reducing it to the corresponding alcohol (ROH) and becoming in turn oxidized to a sulfenic acid (Prx-S_POH). Subsequently, Prx-S_POH condenses with an additional thiol (known in this cycle as the resolving thiol – R-S_RH) forming a disulfide bond (Prx-S_P- S_R-R). Reduction of Prx-S_P- S_R-R by thioredoxin (or thioredoxin-like molecules) restores Prx back to its thiol form. Based on this, peroxide detoxification by Prxs can be summarized to three consecutive chemical steps: oxidation of C_P (1), resolution of Prx-S_POH by an additional resolving thiol resulting in a disulfide bond (2) and recycling of Prx back to its thiol form by an oxidoreductase (3). Depending on the source of the resolving thiol (R-S_RH) Prxs are subdivided in 1-cys-Prx if R-S_RH, is provided by an additional molecule (such as glutathione or thioredoxin), and 2-cys-Prx if the R-S_RH is found in a Prx molecule, either within the same polypeptide or on an additional Prx peptide (in this case R-S_RH is known as resolving cysteine - C_R). In typical 2-cys-Prxs (mechanism detailed in the botton panel) Prx-S_P- S_R-R results in the formation of an intermolecular disulfide bond. In order to allow disulfide bond formation these molecules must shuttle between a fully to a local unfolding conformation (FF→LU). This change in conformation impacts on the oligomeric state of typical 2-cys-Prx and cause the dissembling of the decamer into its composing dimers. (Figure legend continues in the next page)

Overoxidation of peroxiredoxins

As outlined above, oxidation of C_P leads to the formation of an unstable sulfenic acid, which rapidly condenses with C_R to form a disulfide bond. However, some peroxiredoxins (*i.e.*, "sensitive" Prxs) experience further oxidation of C_P by a second or even third peroxide molecule, forming a stable sulfinic (Prx-S_PO_2H) or sulfonic acid (Prx-S_PO_3H), respectively (Figure 2) [122]. For many years, these higher sulfur oxidation states of Prxs, which are commonly referred to as overoxidized or hyperoxidized states, were considered irreversible modifications that precluded disulfide bond formation between C_P and C_R, and hence inactivated the peroxidase function [117]. In contrast, Prxs that are resistant to overoxidation are termed "robust" Prxs. It is now thought that structural motifs that pack alongside the active site cysteine contribute to the oxidation sensitivity of these different Prxs [92].

Overoxidized Prxs were long thought to be dead-end products of the Prx catalytic cycle [123]. This point of view changed, however, with the discovery of sulfiredoxin, an ATP- and magnesium-dependent protein, which reduces sulfinic acid back to the reduced thiol form (Figure 2) [124]. In their seminal study, Biteau *et al.* demonstrated that sulfiredoxin was capable of reducing overoxidized yeast Prx (Tsa1) *in vivo* without *de novo* protein synthesis [124]. Hence, this result clearly showed that overoxidation of Prxs was an *in vivo* reversible modification and not a wasteful dead-end product. The discovery of sulfinic acid reductases, such as sulfiredoxin raised the exciting possibility that overoxidation possessed physiological relevance. However, although conserved across many lower and higher eukaryotes and some cyanobacteria, sulfiredoxin is not present in all organisms whose Prxs experience overoxidation [125]. More recently, sestrin, a protein distantly related to the bacterial oxidoreductase AhpD was also proposed to display sulfinic acid reductase activity, and was, for that reason, considered an alternative to sulfiredoxin [126]. However, the role of sestrin in rescuing overoxidized species of Prx is

Figure 2 (cont.) Once reduction of Prx-S_P- S_R-R is accomplished by thioredoxin (or thioredoxin-like molecule), Prx refolds back to its fully folded state (LU→FF) which concomitantly results in the reassembly of the decamer. As an alternative to this catalytic cycle of peroxiredoxin, these molecules might experience oxidative regulation of its peroxidase function. This happens when Prx-S_POH is further oxidized by an additional peroxide molecule (Prx-S_PO_2H) before Prx-S_P- S_R-R formation. Generally, Prx-S_PO_2H, which are mostly found in eukaryotes, are designated as overoxidized forms and represent an inactive form of the catalytical cycle of Prx. Still, these forms are thought to be physiological relevant as they allow peroxide-mediated signaling pathways (known as "floodgate" model) or trigger chaperone activity of Prxs. The latter is thought to depend on the ability of Prx-S_PO_2H to stabilize the decamer or even promote the formation of HMW-species. The reversion of peroxidase inactive Prx-S_PO_2H back to its active form is accomplished by a sulfenic-acid reductase, sulfiredoxin at the expenses of ATP and Mg^{2+}.

still highly controversial as a number of studies subsequently refuted such activity [127,128]. If sestrins are excluded as sulfinic acid reductases, then the search for an alternative molecule in organisms such as *Leishmania* that lack sulfiredoxin is still open. Up to now, sulfonic acid formation of C_P, which happens after the attack of a third peroxide molecule, is still considered an irreversible modification of Prx in all organisms.

The evolutionary advantage of Prx overoxidation is still a matter of debate [96,123]. One hypothesis is that overoxidation and concomitant inactivation of the peroxidase function of Prx allows peroxide levels to transiently increase above a certain threshold that is necessary to oxidize downstream proteins [92,97]. By allowing the oxidation of those downstream proteins, inhibition of Prx would promote peroxide-mediated signaling. This hypothesis, frequently referred to as the "floodgate" model, provides a rational for how stress and non-stress related peroxide signaling events occur even in the presence of efficient peroxidases. Another model for the potential evolutionary advantage of Prx inactivation by overoxidation was recently proposed by Day *et al.* in *Schizosaccharomyces pombe* [129]. In this study, the authors showed that overoxidation of Prx allowed the pool of reduced thioredoxin to increase and become available to reduce essential proteins other than Prx (*e.g.*, methionine sulfoxide reductase), ultimately contributing to the survival of *S. pombe* under severe oxidative stress conditions. Both hypotheses are based on the idea that overoxidation causes the loss of peroxidase function. However, overoxidation of Prxs may also result in a gain-of-function. Jang *et al.* showed that sulfinic acid formation has the potential of acting as a redox switch, converting peroxiredoxins from peroxidases into molecular chaperones [104]. Under oxidative stress conditions, this new chaperone function was claimed to be important for protecting proteins from stress-mediated aggregation, an activity that ultimately protects the cell from death [104,130]. Since the chaperone function of Prxs is at the core of this thesis, it will be discussed in more detail bellow.

The redox state of peroxiredoxins is linked to structural rearrangements

During peroxide catalysis, Prxs undergo major structural changes that are intimately connected to their redox states (see Figure 2) [131,132]. Upon oxidation of C_P, major conformational changes must occur in Prx that allow for the formation of the C_P-C_R disulfide bond since the two cysteines are located 14 Å apart in the reduced conformation. The structural change involves the partial unfolding of the helix containing C_P [132], which places C_P in a more accessible position – also known as the C_P loop – for C_R attack and formation of a disulfide. Additionally, rearrangements in the region containing C_R might also be necessary for C_P-C_R reaction. Consequently, disulfide bond formation locks Prxs

in a partially unfolded conformation. Reduction of the C_P-C_R disulfide bond by thioredoxin allows the C_P loop, and consequently Prx, to refold. Thus, Prxs cycle between a fully folded (FF) and a locally unfolded (LU) conformation [123]. The rate of unfolding of the C_P-containing helix, which depends on the equilibrium constant between both conformations (FF and LU) and on the type of peroxiredoxin, constitutes a kinetic pause for C_P-C_R formation. The longer this kinetic pause, the more likely it is that C_P reacts with additional peroxide molecules and becomes overoxidized. Disulfide bond formation and overoxidation of C_P can thus be seen as competitive reactions in the Prxs cycle. Any structural feature of Prxs that delays the FF to LU transition facilitates overoxidation. Indeed, by comparing overoxidation-sensitive with overoxidation-robust Prx variants, it became evident that sensitive variants have two motifs that are positioned close to the FF active site: a GGLG and a C-terminal YF motif [92,133]. However, additional, yet to be identified residues seem to contribute also to Prx overoxidation; this conclusion is supported by the observation that *Homo sapiens* PrxII and PrxIII, despite displaying both the GGLG and YF motifs, exhibit a 10-fold difference towards sensitivity to overoxidation [133].

The redox state of Prxs not only dictates the FF-LU transition but also impacts the quaternary structure of the protein (see Figure 2) [131]. Prxs transition between obligate homodimers and higher oligomeric species, including octamers, decamers, dodecamers or even higher molecular weight species, such as filaments made up of Prxs decamers or large ball-shaped structures. All of these oligomers result from the reversible assembly of dimers. The reversible association of dimers into higher molecular species is governed by the redox status of the protein and the concomitant FF-LU switch. Generally, five reduced Prx dimers associate into decamers with ring-like structure [131]. Oxidation and disulfide bond formation results in the dissociation of the decamer into the individual oxidized dimers. Even though disulfide bonds are commonly regarded as structure-stabilizing components rather than disrupting modifications, disulfide bond formation between C_P and C_R actually leads to major structural rearrangements that disrupt the interactions between individual dimers and subsequently lead to disassembly of the decamer. Upon reduction of C_P-C_R and refolding of the C_P-loop, Prxs return back to their decameric form. Parsonage *et al.*, demonstrated that peroxidase activity decreases in Prx mutants that are unable to decamerize [134], indicating that assembly into decamers is important for peroxidase activity. Hyperoxidation of C_P, on the other hand, was shown to stabilize the decameric structure of Prxs as well as higher molecular weight (HMW) species and appeared to be important for the chaperone function of Prxs [104,135]. Reduction of the overoxidation by sulfiredoxin was found to reverse both functional and conformational changes [104]. Other factors such as ionic strength, pH, presence of tags and mutations

were also shown to impact the quaternary structure of Prxs [131]. Nevertheless, *in vivo* the redox status is likely to be the main modulator of Prx oligomerization.

Chaperone function of peroxiredoxins

The idea that Prxs moonlight as molecular chaperones and confer cytoprotection against protein unfolding stress conditions arose from the observations that i) PrxI and PrxII deletion mutants of yeast display heat-shock sensitivity, a phenotype frequently observed in cells lacking molecular chaperones and ii) Prxs share their basic thioredoxin-fold with other proteins known to display chaperone activity [104]. *In vitro* chaperone assays then confirmed the hypothesis that yeast Prxs work as chaperones [104]. Since then, *in vitro* chaperone activity has been demonstrated for multiple Prxs, and has been suggested to be important under different stress conditions such as oxidative stress, heat-shock or zinc deficiency [104,130,135].

Biochemical and functional studies revealed that the chaperone function of Prxs was linked to the formation of decamers or HMW species [104,105,135]. Indeed, HMW species that resemble ball-like structures were even considered super-chaperone versions of Prxs. As mentioned before, assembly into chaperone-active HMW-species was suggested to be overoxidation-mediated, causing Prxs to lose peroxidase activity as they gained chaperone function (Figure 2). Several studies helped to disentangle the connection between overoxidation, HMW species formation and chaperone function of Prxs. Among other findings, these studies have shown that neither overoxidation nor HMW formation are in fact mandatory for Prxs' chaperone function. Indeed, a number of alternative, overoxidation-independent factors can also trigger the chaperone activity of Prxs. These include i) temperature-mediated activation [104], ii) phosphorylation of a conserved threonine found at the dimer/dimer interface of human PrxI [105], iii) irreversible overoxidation of C_P to sulfonic acid [136] and more recently, iv) changes in pH [106] and v) lysine acetylation [137]. Furthermore, non-physiological stimuli were also identified, including gamma-ray and proton irradiation [138]. The finding that a wide array of apparently unrelated signals can trigger chaperone activity raised the obvious question as to how these signals are structurally integrated and translated into the same activity. Finding the answer for this important aspect will likely reveal the nature of the structural rearrangements that are necessary for Prx chaperone activity. Of notice, many of the conditions that activate the chaperone function of Prxs have the potential to cause local unfolding [104,135]. One possible consequence of this phenomenon may be the exposure of hydrophobic areas in the Prx protein, a hallmark of client binding sites in molecular chaperones [139].

The assembly of Prxs into HMW products, such as the ball-like structures initially reported, is considered a feature of Prxs functioning as molecular chaperones [105]. However, Prx decamers were also found to display chaperone function indicating that HMW formation is not necessary for chaperone activity [136,140]. That the decamer is likely the minimal oligomeric state endowed with chaperone function became evident in studies with hPrxI, which showed that disturbance of the decamer by glutathionylation abolishes chaperone activity [141]. Moreover, the presence of a Cys at the dimer/dimer interface of Prxs was shown to be important for the chaperone function of human PrxI, possibly because it forms an intra-dimer disulfide bond that stabilizes the decamer [140]. So far it is not clear why the decameric form of Prx is critical for chaperone function.

Saccoccia *et al.* compared the X-ray structure of a chaperone-active and inactive version of *Schistosoma mansoni* Prx1 [106]. In the specific case of SmPrx1, chaperone activity appeared to correlate with the pH-dependent stacking of two decamers. Based on conformational differences between the chaperone-active and inactive form, the authors concluded that unfolding of both, the helix containing C_P and the C-terminus of the protein, was important for the stacking of the two decamers, and thus for the activation of the chaperone function. Mutational follow-up studies led the authors to postulate that unfolding of the C-terminus leads to the exposure of a client-binding site, which would be adjacent to the active site of Prxs and face the outside of the decameric ring structure [106,142]. These results were recently challenged by a cryo-electron microscopy (EM) - based study by Radjainia *et al.*, which revealed that the C-terminus of the chaperone active form of the human mPrx3 protein is partially folded [143]. Hence, unfolding of C-terminus for activation of Prxs does not appear to be mandatory for chaperone activity. Based on the analysis of the hydrophobic patches found in the HMW species, Radjainia *et al* further suggested that the client-binding site of mPrx3 lies within the lumen of the protein [143]. From a chaperone-mechanistic standpoint, it is important to determine which structural rearrangements are necessary for chaperone activation of Prxs as well as to define the client-binding site of these molecular chaperones.

Traditionally, chaperones are categorized into different classes and serve different purposes in cells [144]. Some chaperones assist proteins to acquire their native structure (*e.g.* DnaK, Hsp70 and GroEL) while others prevent proteins from aggregation (*e.g.* Hsp33, Hsp31) or promote the resolubilization of aggregated proteins (*e.g.* ClpB). While Prxs have been convincingly shown to prevent the aggregation of different client proteins *in vitro*, none of the published studies tested whether Prxs have intrinsic capacity to promote the refolding of their client proteins or whether they are able to interact and transfer clients to additional chaperone for proper refolding. Alternatively, it is possible that Prxs target their clients for proteolysis in a controlled manner. Moreover, it will be

important to study exactly under which cellular conditions Prxs are chaperone-active as they will help us understand the position that Prx occupies within the cellular chaperone network and thus its relevance in the cell.

Peroxiredoxins of *Leishmania*

Peroxiredoxins of *Leishmania* and other trypanosomatids are known as tryparedoxin peroxidases (TXNPx) owing to their dependence on tryparedoxin (TXN), a thioredoxin-like molecule found exclusively in these organisms, as reducing agent [58]. Due to this unique reductant, the structural features of TXNPxs that are thought to be responsible for interaction with TXN are exclusive to these parasites [59]. Another trypanosomatid-specific feature is that TXN couples TXNPx recycling to the cellular NADPH pools through their distinctive dithiol trypanothione (TS_2) [59]. Overall, the enzymatic cascade that leads to peroxide detoxification in trypanosomatids is therefore composed of: TXNPx, TXN, TS_2, trypanothione reductase (TR) and NADPH.

Trypanosomatids encode different TXNPx isoforms although the exact number varies according to the species [58]. In the specific case of *Leishmania infantum*, four *TXNPx* ORFs are present in the genome. These ORFs encode two cytosolic (cTXNPx) and one mitochondrial (mTXNPx) isoform as well as a recently identified glycosomal molecule (gTXNPs) [145, H. Castro and A.M. Tomás, unpublished results]. TXNPxs have been primarily studied due to their potential role as antioxidant that provide protection against both endogenous peroxides and those resultant from the oxidative burst mounted by the host phagocytic cells during parasite invasion. It is therefore not surprising that the peroxidase activity of TXNPxs has been extensively studied both on a biochemical and on a functional level [58,59,110]. *In vitro* peroxidase assays demonstrated that TXNPxs reduce a broad array of peroxides and peroxynitrite with efficiencies ranging from ~10^4 M^{-1} s^{-1} to 10^7 M^{-1} s^{-1} [146,147,148,149]. Moreover, overexpression of either cytosolic or mitochondrial TXNPxs was shown to protect trypanosomatids from exogenously added oxidants and promote survival inside macrophages [145,150,151,152]. In line with these findings, depletion of cytosolic TXNPx by either iRNA in *T. brucei* [153] or antisense RNA in *L. amazonensis* [152] had a negative impact on parasite resistance towards a bolus of H_2O_2.

TXNPxs are typical 2-Cys-Prxs that belong to the PrxI sub-family [110]. Some members of this sub-family, which also includes human PrxI-IV, yeast TSA and bacterial AhpC, have been shown to undergo overoxidation. Almost all TXNPxs contain the structural motifs typically associated with sensitivity towards overoxidation, including the GGLG sequence and the YF- or FF-related C-terminal motif [58]. Moreover kinetic studies suggested that the peroxidase activity of mTXNPx is rapidly inhibited in the presence of

lipid hydroperoxides [147]. So far, however, the only direct evidence that TXNPxs are overoxidized *in vivo* came from studies in TXNPxs-overexpressing *T. cruzi*, where cells were challenged with a high bolus of peroxinitrite [154]. Even if TXNPx overoxidation is confirmed in the parasite context, the lack of an obvious sulfiredoxin encoding sequence in the genome of trypanosomatids raises the question about the physiological relevance of this post-translational modification.

Like other Prxs, TXNPxs cycle between oxidized dimers and reduced decamers [155,156]. In addition, however, oxidized decamers have also been reported. Yet this result is potentially due to an *in vitro* artifact since many non-physiological factors, including ionic strength, pH and presence of tags affect the oligomerization state of Prxs [131,147]. Very recently, Morais *et al.* showed that acidic pH promotes decamerization of the *L. braziliensis* mTXNPx, apparently through the protonation of a conserved His placed at the dimer/dimer interface of mTXNPx [155]. While this finding might have physiological relevance owing to the fact that *Leishmania* parasites experience changes in environmental pH as part of their life cycle, its actual meaning is yet to be addressed.

As the focus of this thesis is the mitochondrial isoform of TXNPx, the next chapter will consider information available for this protein.

Mitochondrial TXNPx (mTXNPx)

The genome of trypanosomatid's encodes for one mitochondrial TXNPx (mTXNPx). Immunolocalization studies in *Leishmania chagasi* have shown that the pattern of mTXNPx expression changed according to the physiological state of the parasite; whereas mTXNPx was restricted to the area of the kinetoplast (kDNA) in fast-dividing promastigotes, it spread throughout the mitochondria in non-dividing promastigotes and amastigotes [157]. However, such dramatic change in localization pattern has not been observed for *Leishmania infantum*, where mTXNPx is found over the kinetoplast and dispersed throughout the mitochondria irrespective of the growth stage [145]. Due to its sub-cellular localization, mitochondrial TXNPx was considered to be a very important scavenger of respiration-derived reactive oxygen species [58]. Nevertheless, the observation that overexpression of mTXNPx protects parasites also against a bolus of exogenous t-butyl hydroperoxides led to the idea that mTXNPx also constituted an important barrier against host-derived oxidants [145,152]. Another role attributed to mTXNPx was its involvement in the prevention of programmed cell death induced by H_2O_2 [157]. Additionally, mTXNPx was found to be associated with parasite resistance to miltefosine, a drug used to treat leishmaniasis and whose mode of action is linked to apoptosis and disturbance of lipid-mediated cell signaling [158]. Finally, mTXNPx was

reported to be responsible for oxidizing the *U*niversal *M*inicircle *S*equence *B*inding *P*rotein (UMSBP) [103]. When reduced, UMSBP binds to the replication origin present in the minicircles of kDNA, and initiates their replication. Upon mTXNPx-mediated oxidation, UMSBP is released and replication of kDNA is terminated. The postulated role of mTXNPx in oxidation of UMSBP is of particular importance since it constitutes an example of the ability of Prx to act as oxidase that transfers the peroxide signal to downstream proteins. Given that all of the physiological functions attributed to mTXNPx were based on its peroxidase activity (or at least on the presence of C_P), it came as a surprise the finding that mitochondrial TXN (TXN2), the presumed primary redox partner of mTXNPx was not essential for *Leishmania* survival [159]. This result not only raised the possibility that an alternative partner reduced mTXNPx but also questioned the importance of the mitochondrial redox metabolism for parasite physiology. Indeed, follow up studies from our lab revealed that while mTXNPx is crucial for parasite survival in the mammalian host, its peroxidase function is not [160]. Parasites devoid of mTXNPx were unable to survive inside the mice, whereas knockout cells complemented with a peroxidase inactive mTXNPx variant were fully competent to infect and thrive in mice. This result, which constituted the starting point of the present thesis, indicated that the role of mTXNPx in *Leishmania* survival and infectivity was not linked to its activity as peroxidase or oxidase, as previously thought, and raised the question as to why mTXNPx is essential for *Leishmania* in its mammalian host. The work developed in the context of this thesis aimed at clarifying this aspect.

Scope and outline of the project

The aim of this PhD project was to decipher the importance of the *Leishmania infantum* mitochondrial tryparedoxin peroxidase (mTXNPx), member of the 2-Cys Prx family, for parasite infectivity. To achieve this goal, the following specific objectives were defined: i) to investigate if mTXNPx presents chaperone activity *in vitro* and, if so, ii) to dissect its molecular mechanism, and iii) to address the relevance of this activity for *L. infantum* infectivity. The present thesis describes the work performed during this project to accomplish the proposed objectives and is divided in the following sections:

Chapter II includes data from a published article (Castro *et al.*, 2011) demonstrating for the first time that *Leishmania* 2-Cys-Prxs can behave *in vitro* as molecular chaperones, a function previously attributed to some of the members of this family. This part of the thesis also shows that parasites devoid of mTXNPx present a heat-shock phenotype which is common to cells lacking chaperones. In view of these results, we tentatively suggested in this first paper that the chaperone activity of mTXNPx could be responsible for the crucial function played by this enzyme *in vivo*.

Chapter III includes published data from Teixeira *et al.*, 2015, where we confirmed the chaperone function of mTXNPx and shed light into its molecular mechanism of action. Additionally, we categorically defined that the crucial role of mTXNPx during *Leishmania* infection was that of a chaperone.

Chapter IV consists of preliminary data investigating the mTXNPx residues directly involved in client binding.

The last chapter of the thesis, **Chapter V**, considers the main results of obtained and their implications in what concerns *Leishmania* physiology and the mechanism of function of chaperones of the 2-Cys Prx family.

CHAPTER II

Leishmania Mitochondrial 2-Cys-Peroxiredoxin: Insight Into Its Novel Chaperone Activity

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The results described in this chapter were published as part of the following paper: Castro H, et al 2011. Leishmania mitochondrial peroxiredoxin plays a crucial peroxidase-unrelated role during infection: insight into its novel chaperone activity. PLoS Pathog. (10):e1002325. (The complete paper can be found in appendix).

Abstract

Two-cysteine peroxiredoxins (2-Cys-Prxs) are ubiquitous peroxidases that play various functions in cells. In *Leishmania* and related trypanosomatids, these molecules were long considered important antioxidant devices protecting the cell against reactive oxygen and nitrogen species (ROS and RNS). Therefore, the discovery that the mitochondrial 2-Cys-Prx (mTXNPx) plays a crucial function during parasite development in mice that is independent of its peroxidase activity was largely unforeseen. This finding implied that mTXNPx must have an additional and peroxidase-unrelated function. Here, a novel function is proposed for mTXNPx, that of a molecular chaperone, which may explain the impaired infectivity of the null mutants in mice. This premise is based on the observation that i) the enzyme is able to suppress the thermal aggregation of citrate synthase *in vitro* and that ii) parasites devoid of mTXNPx (*mtxnpx*$^{-}$) were more sensitive than controls to a temperature shift from 25°C to 37°C, a phenotype reminiscent of organisms lacking specific chaperone genes. In conclusion, the findings reported here change the paradigm which regards all trypanosomatid 2-Cys peroxiredoxins solely as peroxide-eliminating devices.

Introduction

Leishmaniasis is a malady caused by organisms of the genus *Leishmania*. These are vector-borne protozoan parasites whose life cycle alternates between a sandfly and a mammalian host. Leishmaniasis is a widespread disease affecting mainly tropical and subtropical regions. In Portugal, as well as in other Mediterranean countries, *L. infantum* is the agent responsible for human and canine leishmaniasis. Presently, treatment of leishmaniasis is not ideal presenting many drawbacks. In order to develop new strategies to fight this disease, the study of fundamental and unique aspects of the *Leishmania* biology is of paramount importance. The *Leishmania* thiol-dependent metabolism, due to its dependence on the unique thiol thrypanothione, is regarded as promissing as it meets criteria of essentiality and uniqueness. Among cellular pathways that dependent on trypanothione, is peroxide elimination. Contrary to mammals, *Leishmania* and related trypanosomatids rely on trypanothione instead of glutathione to tie peroxide detoxification to the cellular NADPH pools.

2-Cys-Prxs, known as TXNPxs in trypanosomatids, are trypanothione-fuelled peroxidases. 2-Cys-Prxs are ubiquitous peroxidases that rely on an absolutely conserved and critical cysteine (also known as peroxidatic cysteine –C_P) for peroxide detoxification. TXNPxs are kept reduced by tryparedoxin (a trypanothione-dependent oxidoreductase) instead of the ubiquitous thioredoxin (present in the *Leishmania* mammalian host, for example). *Leishmania* and other trypanosomatids encode several TXNPx isoforms found in different cellular compartments. Both cytosolic (cTXNPx) and mitochondrial TXNPx (mTXNPx) were shown to be essential for these parasites's survival [153,160]. Nevertheless, the crucial role played by both isoforms appears to be dissimilar. While cytosolic TXNPx appears to be essential for the parasite due to its role in peroxide detoxification [153], and thus antioxidant defense, mitochondrial TXNPx, was surprisingly found to have a crucial, peroxidase-unrelated, function [160]. This was based on the observation that while *mtxpx*$^-$ parasites were unable to survive inside mice, *mtxpx*$^-$ complemented with a peroxidase inactive version of mTXNPx (mTXNPxC81S), in which the highly required peroxidatic cysteine was substituted, were fully competent in rescuing the *mtxnpx*$^-$ essential phenotype [160]. Therefore, this study not only established mTXNPx as a crucial factor for *Leishmania* infectivity as it also, implied that mTXNPx must have an additional function, thus opening novel avenues regarding the physiological role of mTXNPx.

In the present study we sought to study alternative functions for the mitochondrial TXNPx (mTXNPx) that might explain its essentiality. Besides working as peroxidases, 2-Cys-Prxs were also ascribed with chaperone activity [104]. Hence, we have tested if

mTXNPx has chaperone activity *in vitro* and if this function is relevant in the context of the cell. Our data demonstrates that mTXNPx functions as a molecular chaperone *in vitro* and that this function might contribute to the fitness of the parasite as it transits from the insect to the mammalian host temperature. Based on these results we hypothesize that the molecular chaperone activity of mTXNPx might be the previously uncharacterized function of the protein responsible for *Leishmania* persistence in the mammal host.

Materials and Methods

Parasite cultures - *Leishmania infantum* promastigostes (MHOM MA67ITMAP263) were cultured at 25°C in RPMI 1640 Glutamax medium, supplemented with 10% inactivated fetal bovine serum (FBSi), 50 U ml^{-1} penicillin, 50 µg ml^{-1} streptomycin (all from Gibco) and 25 mM hepes sodium salt pH 7.4 (Sigma).

Production of the mTXNPxC81S mutein - A site directed mutagenesis strategy was employed to introduce the Cys81 to Ser (C81S) mutation in the mTXNPx ORF cloned into pSSU-PHLEO-infantum [160] and pET28c [160]. For this, the full-length plasmids were PCR-amplified with sense and antisense mutated primers (P1 and P2 in Table S1) using *Pfu* polymerase (Stratagene). Upon digestion of parental (wild type) DNA with 10 units of *Dpn*I (New England Biolabs) for 1 h at 37ºC, the reaction (4 ml) was used directly to transform *Escherichia coli* DH5α strain. Mutant colonies were confirmed to carry the site-directed mutation by sequencing both strands.

Heterologous expression and purification of ΔmTXNPx and ΔmTXNPxC81S - To mimic the mature mitochondrial protein, truncated wild type mTXNPx (ΔmTXNPx), *i.e.* lacking the first 26 amino acids that compose the mitochondrial targeting peptide [145], was produced in the *E. coli* strain BL21 (DE3) Tuner upon transformation a pET28c (Novagen) construct containing the ΔmTXNPx gene fragment (PCR-amplified with primers P3/P4 in Table S1). To produce the mutant ΔmTXNPxC81S protein, the pET28c-Δ*mTXNPxC81S* was generated by site directed mutagenesis (detailed above) and used to transform the same *E. coli* strain. Both enzymes were expressed in bacteria as fusion proteins carrying an N-terminal six histidine tag. Upon induction for 3 hrs at 30°C in the presence of 50 µg ml^{-1} kanamycin and 0.1 mM isopropyl-*β*-D-thiogalactopyranoside (IPTG), bacteria were pelleted, suspended in 500 mM NaCl, 20 mM Tris-HCl pH 7.6, disrupted by sonication and centrifuged at 30,000×*g* for 30 min at 4°C. The supernatant was applied to a His Bind resin (Novagen) column and the recombinant protein eluted with an imidazole gradient (5 to 1000 mM) at a flow rate of 1 ml min^{-1}. Fractions confirmed to

contain the protein by SDS-PAGE were pooled, applied to PD-10 columns (Amersham) and eluted in 50 mM sodium phosphate buffer pH 8.0. For removal of the His tag, enzymes were first digested with biotinylated thrombin (Novagen), and then incubated with immobilized streptavidin for removal of the protease. Proteins were concentrated by ultrafiltration, recovered in 40 mM hepes pH 7.5 and quantified by the bicinchoninic acid (BCA) protein assay (Pierce), using bovine serum albumin (BSA) as standard.

Determination of chaperone activity - The chaperone activity of ΔmTXNPx and ΔmTXNPxC81S was measured using citrate synthase (CS) from porcine heart (Sigma) as substrate, as described before [161]. Each enzyme was diluted in 40 mM hepes sodium salt pH 7.5 to reach final concentrations of 0.75 or 1.5 µM. A cocktail of protease inhibitors and 100 µM DTT were added to the reaction mixture to prevent degradation of CS and disulfide cross-linking between the peroxiredoxin and CS molecules, respectively, both of which could result in an apparent chaperone-like activity. Samples were pre-incubated at 43°C for 5 min and the reaction started by addition of CS to a final concentration of 0.15 µM. Light scattering due to CS aggregation at 43°C was monitored using a FluoroMax-4 spectrofluorometer, with excitation and emission wavelengths set to 500 nm and excitation and emission slits set to 2 nm. Data were recorded for 20 min.

Thermotolerance assays – Previously generated *L. infantum* promastigotes lines [160], pre-synchronized by 4–5 daily passages of $5{\times}10^5$cells ml^{-1}, were seeded at 10^6 ml^{-1} and allowed to grow for 4 days at either 25°C or 37°C. Every 24 hours cell densities were determined with a Neubauer-counting chamber for growth curve determination.

Results

Is mTXNPx functioning *in vivo* as a molecular chaperone?

In addition to acting as peroxidases, 2-Cys-Prxs may in some cases exhibit chaperone activity [104,135], an attribute that, to date, has never been described for any TXNPx. The possibility of mTXNPx acting as a chaperone was therefore investigated as an attempt to provide an alternative function for this enzyme in *Leishmania* amastigotes. The chaperone activity of purified recombinant ΔmTXNPx was assessed *in vitro* by testing its ability to suppress the aggregation of thermally denatured citrate synthase (CS). When incubated at 43°C citrate synthase unfolds, leading to significant aggregation that can be monitored by measuring light scattering spectrofluorometically [161]. Addition of

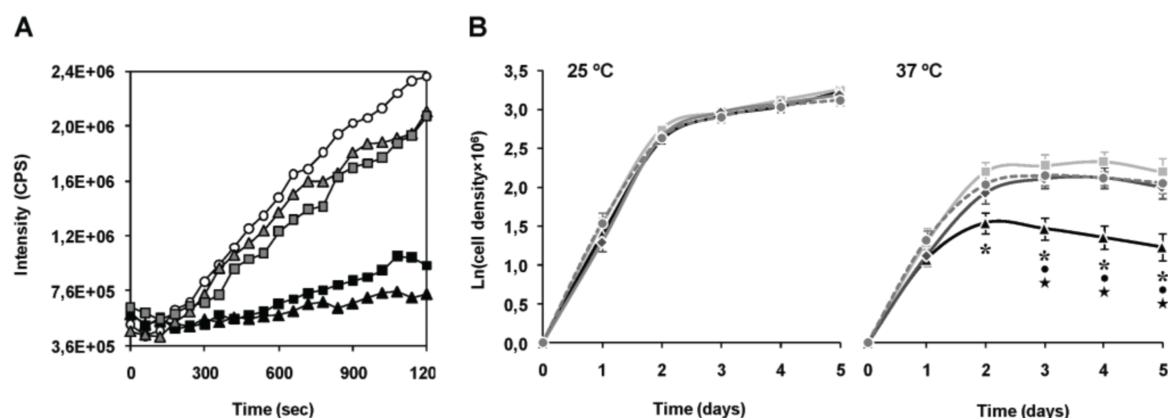

Figure 1: Evidence for the chaperone activity of mTXNPx. A. Chaperone activity of purified recombinant ΔmTXNPx and ΔmTXNPxC81S was analyzed *in vitro* by assessing the enzymes' ability to suppress the thermal aggregation of citrate synthase (CS). Aggregation of CS was induced at 43°C and monitored by measuring light scattering at 500 nm in the absence (open circles) or presence of 5:1 (grey-filled squares) or 10:1 (black squares) ratios of ΔmTXNPx monomers to CS monomers. Alternatively, the mutein ΔTXNPxC81S was added to the reaction at 5:1 (grey-filled triangles) or 10:1 (black triangles) ratios. **B**. Wild type (light grey squares), *mtxnpx*$^{-}$ (black triangles) *mtxnpx*$^{-}$*/+mTXNPx* (dark grey diamonds) and *mtxnpx*$^{-}$*/mTXNPxC81S* (dark grey circles and dashed line) *L. infantum* promastigotes were seeded at 10^{6} ml^{-1} in complete RPMI medium and incubated at 25°C (*left*) and 37°C (*right*). The Y axis represents ln (or log_e) of cell densities recorded throughout 5 days. Values represent mean and standard error of the mean of 4 to 8 independent curves. Also indicated are the statistically significant differences (i.e. p<0.05) between *mtxnpx*$^{-}$and each of the control parasite lines, *i.e.* wild type (*), *mtxnpx*$^{-}$*/+mTXNPx* (•) and*mtxnpx*$^{-}$*/+mTXNPxC81S* (★).

ΔmTXNPx to the reaction at a 10-fold molar excess completely suppressed the thermal aggregation of CS (Figure 1A). This effect was much less pronounced when ΔmTXNPx was added at 5-fold molar excess. Importantly, the ΔmTXNPxC81S mutein was also capable of preventing CS aggregation, exhibiting the same behavior as the wild type enzyme (Figure 1A). To gain insight into the physiological significance of the chaperone activity of mTXNPx a thermotolerance assay was conducted. The experiment consisted in monitoring cell growth of *mtxnpx*$^{-}$ and control parasites (wild type, *mtxnpx*$^{-}$/*+mTXNPx*, and *mtxnpx*$^{-}$/*+mTXNPxC81S*) at both 25°C and 37°C, which are the temperatures encountered by the parasite in the insect vector and in the mammalian host, respectively. The cell growth curves depicted in Figure 1B show that, while at 25°C the proliferation rates of all parasite lines were indistinguishable, at 37°C the growth rate of *mtxnpx*$^{-}$ was significantly lower than that of wild type parasites. The thermo-sensitive phenotype observed for *mtxnpx*$^{-}$ was recovered in the rescued *mtxnpx*$^{-}$*/+mTXNPx* and *mtxnpx*$^{-}$/*+mTXNPxC81S* mutants. These observations show that expression of mTXNPx renders promastigotes more permissive to 37°C, *i.e.* this enzyme confers thermotolerance to *L. infantum* irrespective of its peroxidase activity. It is thus reasonable to speculate that

the crucial role played by mTXNPx during amastigote development in the mammalian host is that of a molecular chaperone.

Discussion

This work has, for the first time, uncovered that trypanosomatid 2-Cys-Prxs can function as molecular chaperones and that this activity might be relevant for parasite survival *in vivo*. Several pieces of evidence point into this direction. i) Purified recombinant mTXNPx was shown to prevent the thermal aggregation of CS, a well-known chaperone substrate [161]. This activity does not depend on the peroxidatic capacity of mTXNPx, as the mTXNPxC81S mutein was also active in this assay, thus providing a rationale for the recovery of virulence observed for *mtxnpx*$^{-}$*/+mTXNPxC81S*. This is in line with observations reported by Jang *et al.* [104]. ii) The chaperone activity of 2-Cys-Prxs is known to depend on their quaternary structure and, as shown before, mTXNPx is capable of associating into decameric ring-like structures [147], which are the minimal oligomeric arrangements required for such function [141]. iii) Gain of chaperone function and loss of peroxidase activity of 2-Cys-Prxs are, in most organisms, accompanied with the overoxidized form of the protein [95] and mTXNPx undergoes overoxidation when promastigotes are exposed to 37°C (Fig. S1). Since *Leishmania* do not possess sulfiredoxins to enzymatically regenerate overoxidized 2-Cys-Prxs [58] as occurs in higher eukaryotes, such peroxidase-inactive form of mTXNPx is likely to persist until the protein is synthesized *de novo*. iv) Additional support consubstantiating a role for mTXNPx as a chaperone came from the observation that lack of mTXNPx expression rendered promastigotes more sensitive to 37°C, the temperature encountered in the mammalian host. Such thermo-sensitive phenotype is reminiscent of some chaperone-deficient mutants [162,163,164]. Accepting that mTXNPx is a molecular chaperone *in vivo*, one can only speculate how this function could impact on parasite survival under the conditions met in the host. In mitochondria, molecular chaperones are associated with a multitude of functions. They participate in the biogenesis of new mitochondrial peptides by assisting their trans-membrane transport and their refolding to the native conformation inside the matrix. In addition, they are part of the mitochondrial protein quality control system that repairs damaged or misfolded proteins or mediates their removal by proteolysis [165]. Under situations of cellular stress (including elevated temperatures), the activity of mitochondrial chaperones becomes even more imperative for protein homeostasis [165]. Accordingly, it is reasonable to assume that mTXNPx functions as a molecular chaperone

that ensures integrity of mitochondrial functions, its activity being particularly relevant when parasites reside in vertebrate hosts.

A novel peroxidase-unrelated function, as a molecular chaperone, is proposed for this enzyme, which could be critical for mitochondrial functionality under the conditions encountered by the parasite in the mammalian host. This finding thus constitutes a turning point into the current state of knowledge regarding the physiologic role of peroxiredoxins in trypanosomatids.

Acknowledgments

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Author Contributions

Conceived and designed the experiments: H.C., A.M.T. Performed the experiments: H.C., **F.T.**, S.R., M.S., T.C. Analyzed the data: H.C., **F.T.**, F.FdS, P.O., A.M.T. Contributed reagents/materials/analysis tools: M.F. and R.A. Wrote the paper: H.C., A.M.T. (Note: the abstract and introduction, written specifically for this thesis by **F.T.**, differ from the plublished paper.)

Supporting Information

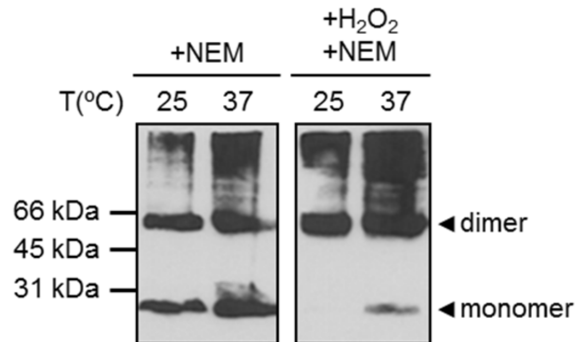

Figure S1: Susceptibility of mTXNPx to overoxidation in promastigotes exposed to 37°C. *Leishmania infantum* promastigotes grown for 4 days at either 25°C or 37°C (as in Figure 1B) were collected (1.4×10^7 cells) and lysed in the presence of 200 mM *N*-ethylmaleimide (NEM) (*left*). Alternatively, cell lysates were pre-treated with H_2O_2 (50 µM H_2O_2 for 30 min, followed by a further addition of 50 µM H_2O_2) prior to fixation with NEM (*right*). Extracts were examined by western blot with the anti-mTXNPx antibody. The dimer corresponds to the oxidized form of mTXNPx, whereas the monomer corresponds to either the reduced or the overoxidized protein. In cell lysates of parasites grown at 37°C the monomeric form of mTXNPx persists even upon H_2O_2 pre-treatment, indicating that this is the overoxidized (peroxidase inactive) form of the enzyme. This western blot is representative of two independent experiments. Protein loading was controlled by Ponceau staining.

Table S1: List of oligonucleotides used in this work

Primer name	Primer sequence
P1	5’-CTTCACCTTCGTTTCCCCGACCGAGATCA-3’
P2	5’-TGATCTCGGTCGGGGAAACGAAGGTGAAG-3’
P3	5’-ccgcgca*catatg*AATCTGGACTATCAGATGTAC-3’
P4	5’-caccg*ctcgag*TCACATGTTCTTCTCGAAAAAC-3’

Clamp sequences are indicated in lower cases, restriction sites in bold/italic, and mutations introduced by site directed mutagenesis underlined.

CHAPTER III

Mitochondrial Peroxiredoxin Functions as Crucial Chaperone Reservoir in *Leishmania infantum*

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Running Title: Chaperone Function of mTXNPx is Crucial for Parasite Infectivity

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Abstract

Cytosolic eukaryotic 2-Cys-peroxiredoxins have been widely reported to act as dual-function proteins, either detoxifying reactive oxygen species or acting as chaperones to prevent protein aggregation. Several stimuli, including peroxide-mediated sulfinic acid formation at the active site cysteine, have been proposed to trigger the chaperone activity. However, the mechanism underlying this activation and the extent to which the chaperone function is crucial under physiological conditions *in vivo* remained unknown. Here we demonstrate that in the vector-borne protozoan parasite *Leishmania infantum*, mitochondrial Prx (mTXNPx) exerts intrinsic ATP-independent chaperone activity, protecting a wide variety of different proteins against heat stress-mediated unfolding *in vitro* and *in vivo*. Activation of the chaperone function appears to be induced by temperature-mediated restructuring of the reduced decamers, promoting binding of unfolding client proteins in the center of Prx's ring-like structure. Client proteins are maintained in a folding competent conformation until restoration of non-stress conditions, upon which they are released and transferred to ATP-dependent chaperones for refolding. Interference with client binding impairs parasite infectivity, providing compelling evidence for the *in vivo* importance of Prx's chaperone function. Our results suggest that reduced Prx provides a mitochondrial chaperone reservoir, which allows *L. infantum* to successfully deal with protein unfolding conditions during transition from insects to the mammalian hosts, and generate viable parasites capable of perpetuating infection.

Significance

Peroxiredoxins are highly abundant proteins, which serve two seemingly mutually exclusive roles; as peroxidases and molecular chaperones. So far, little is known about the precise mechanism of Prx's activation as chaperone and the physiological significance of this second function. Here we demonstrate that in *Leishmania infantum*, reduced peroxiredoxin provides a crucial stress-specific chaperone reservoir, which is rapidly activated upon exposure to unfolding stress conditions. Once activated, peroxiredoxin protects a wide range of different clients against protein unfolding. Clients are bound in the center of the decameric ring, providing experimental evidence for previous claims that peroxiredoxins serve as likely ancestors of chaperonins. Interference with client binding impairs *Leishmania* infectivity, providing compelling evidence for the *in vivo* importance of Prx's chaperone function.

Introduction

Peroxiredoxins (Prxs) are highly conserved and ubiquitous peroxidases that constitute some of the most abundant proteins in the cell [123]. Typical 2-Cys-Prxs, the sub-family of peroxiredoxins addressed in this study, are obligate homodimers of two inverted subunits that rely on two cysteines for hydroperoxide detoxification: the peroxidatic cysteine C_P, which interacts directly with peroxides, and the resolving cysteine C_R, which condenses with the oxidized C_P of the other subunit by forming a disulfide bond. The disulfide bond is subsequently regenerated by a thiol-containing oxidoreductase, such as thioredoxin or another member of the thioredoxin family [118]. 2-Cys-Prxs have been extensively studied in regards to their role in the detoxification of reactive oxygen and nitrogen species [94]. More recently, however, they have also been implicated in other biological activities, including H_2O_2-signaling, protein oxidation and chaperoning functions under stress conditions [97,117]. The chaperone activity of 2-Cys-Prxs was first reported in 2004, when Jang *et al* proposed that peroxide-mediated overoxidation of the active cysteine C_P in yeast peroxiredoxin Tsa1 inactivates the peroxidase function and triggers its conversion into high molecular weight oligomers that prevent *in vitro* protein aggregation [104]. Since this initial discovery, other factors, including phosphorylation or exposure to low pH have been shown to prompt the functional switch from peroxidases to chaperones in various organisms [105,106,142], raising the possibility that cysteine overoxidation is only one of several mechanisms that activate the chaperone function of eukaryotic Prx.

Leishmania infantum, an intracellular protozoan parasite with a digenetic life cycle (*i.e.*, it shuttles between two hosts, insects and mammals), encodes several 2-Cys-peroxiredoxins [58,110,145], one of which is the mitochondrial, mTXNPx [160]. We showed that mTXNPx-deficient promastigotes (*i.e.*, the insect form) are significantly more sensitive to a temperature shift from 25°C to 37°C than promastigotes harboring functional mTXNPx (Chapter II of this thesis). This shift in temperature is similar to the one that parasites encounter upon transitioning from the insect to the mammalian host. Consistent with this result, we found that mTXNPx-deficient amastigotes (*i.e.*, the mammalian form) are unable to survive within their mammalian host [160]. These phenotypes could not be attributed to the peroxidase function of mTXNPx since a *L. infantum* line expressing a peroxidase-inactive mutant variant of mTXNPx lacking C_P (mTXNPxC81S) was fully capable of surviving the temperature shift to 37°C and infecting mice [160]. These results suggested that the essential function observed *in vivo* is not based on mTXNPx's peroxidase activity but more likely involves a second function of mTXNPx, potentially as molecular chaperone (chapter II of this thesis). Moreover, the fact that the mTXNPxC81S

variant was able to rescue the *in vivo* non-infective phenotype of mTXNPx-depleted parasites excluded the possibility that overoxidation of C_P was responsible for the functional switch and raised the intriguing question as to how the dual functions of mTXNPx might be regulated in *Leishmania* mitochondria.

Here we demonstrate that reduced, decameric mTXNPx acts as powerful molecular chaperone, specifically protecting proteins against temperature-induced aggregation. Our studies provide compelling experimental evidence that mitochondrial mTXNPx is a fully integrated member of the mitochondrial proteostasis network of parasites and that its role as chaperone is crucial for parasite infectivity.

Material and Methods

***Protein purification and preparation of oxidized and reduced mTXNPx* –** Mature mTXNPx (*i.e.*, lacking the first 26 amino acids that compose the mitochondrial targeting sequence MTS) was purified from *Escherichia coli* under non-reducing conditions. It was subjected to thrombin treatment to remove the N-terminal His tag, leaving behind a four-amino acid scar (MetGlySerHis) at the N-terminus. The protein was >90% oxidized, dimeric and fully peroxidase-active (*i.e.*, $mTXNPx_{ox}$). To prepare $mTXNPx_{red}$, $mTXNPx_{ox}$ was incubated in the presence of 5 mM DTT or its physiological reducing system consisting of 200 μM NADPH, 0.5 U/ml *L. infantum* trypanothione reductase (TR), 50 μM trypanothione disulfide (TS_2, Bachem) and 2.5 μM *L. infantum* tryparedoxin 2 (TXN2) for 30 min at 30°C. TR and TXN2 were purified as previously described [166]. Upon reduction, mTXNPx was diluted into the different assay buffers maintaining a DTT concentration in the final activity assay between 0.2 and 1.5 mM. Equivalent concentrations of DTT or components of the reducing enzymatic cascade were added to control samples lacking mTXNPx. DnaK, DnaJ, GrpE were purified as previously described [167].

***Chaperone activity assays* -** To investigate the chaperone activity of reduced and oxidized mTXNPx, 100 nM luciferase (Promega) was incubated in 40 mM HEPES, pH 7.5 at 41.5°C either in the absence or presence of different molar ratios of mTXNPx. Light scattering was measured using a fluorescence spectrophotometer (Hitachi F4500) equipped with a temperature-controlled cuvette holder and stirrer ($\lambda_{ex/em}$ 360 nm, slit widths 2.5 nm). To determine the effect of $mTXNPx_{red}$ on the reactivation of luciferase, 100 nM of luciferase was incubated either alone or in the presence of different ratios of $mTXNPx_{red}$ in 40 mM HEPES, pH 7.5, 50 mM KCl for 20 min at 41.5°C. The reaction was

then cooled to 25°C. After 10 min, the samples were supplemented with 2 mM MgATP, 0.1 mg/ml BSA and, where indicated, with 2 µM DnaK, 0.4 µM DnaJ and 2 µM GrpE. At defined time-points, luciferase activity was measured by luminescence using a FLUOstar Omega microplate reader (BMG Labteck) [168]. To determine the influence of mTXNPx on *ex vivo* protein aggregation, 200 µg of a soluble cell lysate from *E. coli ∆rpoH* strain was prepared as described [169], supplemented with either 25 or 50 µg of mTXNPx, and incubated at 45°C for 60 min under constant shaking at 500 rpm. Samples containing $mTXNPx_{red}$ were supplemented with 1.5 mM DTT to prevent the re-oxidation of mTXNPx. The insoluble protein fraction was separated by centrifugation at 16,100 rpm for 30 min and analyzed by reducing SDS-PAGE.

Preparation of $mTXNPx_{red}$ - client complexes and electron microscopy (EM) – Chaperone active $mTXNPx_{red}$ (10 µM) alone or in the presence of luciferase (1 µM) was incubated for 1 min on ice and 2 min at room temperature. Samples were then transferred to an incubator and slowly heated from 30°C to 42°C for 10 min to allow complex formation. Samples were centrifuged at 16,100 *xg* for 30 min at 4°C to remove large aggregates. Proteins in the soluble supernatant were negatively stained with 0.75% (w/v) uranyl formate (pH 5.5-6.0) on 400-mesh carbon-coated copper grids (Pelco) as previously described [170]. Samples were imaged under low dose conditions using a G2 Spirit TEM (FEI) operated at 120 keV. Micrographs were taken at 52,000x magnification with 2.16 Å per pixel using a 4k x 4k CCD camera (Gatan). Single particles of $mTXNPx_{red}$ and $mTXNPx_{red}$:luciferase complexes were manually selected using Boxer in the EMAN software package [171] and totaled 9,963 and 10,222, respectively. Reference-free 2D classification and projection averages were determined using SPIDER [172].

Spectroscopic properties of reduced and oxidized mTXNPx - To determine temperature-dependent changes in the surface hydrophobicity of mTXNPx, 3 µM of $mTXNPx_{red}$ or $mTXNPx_{ox}$ were incubated with 25 µM 4,4′-dianilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) in 40 mM HEPES, pH 7.5, and fluorescence emission spectra were recorded at either 25°C or 42°C as described [173]. To determine bis-ANS binding as a function of temperature, the protein:bis-ANS mixtures were heated from 25 to 70°C (1°C per minute), and changes in bis-ANS fluorescence were continuously monitored at 500 nm. To determine temperature-mediated changes in the secondary structure of the mTXNPx, far-UV circular dichroism (CD) spectra of 0.1 mg/ml $mTXNPx_{red}$ or $mTXNPx_{ox}$ were recorded in 10 mM sodium phosphate buffer, pH 8 at 25°C and 42°C using a Jasco-J810 spectropolarimeter. To monitor the thermostability of mTXNPx, the CD signal at 197 nm was followed from 20 to 80°C. The temperature was increased at a rate of 1°C per minute, and controlled by a Jasco Peltier device. All spectra were buffer-corrected.

Protein aggregation in vivo **-** Aggregated proteins from *L. infantum* promastigotes exposed to either 25°C or 37°C were prepared as previously described [174]. Briefly, 10^7 promastigote cells were collected, washed with lysis buffer (50 mM potassium phosphate buffer pH 7, 1 mM EDTA, 5% v/v glycerol) and stored at -80°C. Cell lysis was achieved by thawing samples at 37°C and subsequent sonication. Upon removal of intact cells by centrifugation (775 *xg*, 10 min, 4°C), total cell extracts were centrifuged again (11,385 *xg*, 20 min, 4°C), and pellets were resuspended in lysis buffer. Membrane proteins were then solubilized by addition of 20% v/v NP-40, and separated from aggregates by centrifugation (11,385 *xg*, 20 min, 4°C). Pellets containing aggregated proteins were analyzed by SDS-PAGE.

Parasite culture and genetic manipulation – *L. infantum* promastigotes (MHOM MA67ITMAP263) were cultured at 25°C in RPMI 1640 medium with GlutaMAXTM-I (Gibco) supplemented with 10% v/v inactivated fetal bovine serum (FBSi, Gibco), 50 U/ml penicillin (Gibco), 50 mg/ml streptomycin (Gibco) and 25 mM HEPES sodium salt, pH 7.4 (Sigma). For growth rate determination, *L. infantum* promastigotes, previously synchronized by 3–4 daily passages at 10^6 cells/ml, were seeded at 10^6 cells/ml and their density examined over time in Neubauer counting chambers. Transfection of promastigotes was performed as described [175]. Isolated clones were transferred from agar plates containing selective drug (17.5 mg/ml phleomycin (Sigma)) into liquid medium.

Generation of mtxnpx⁻/MTS.His.THR-mTXNPx mutants **-** The pSSU-*PHLEO-infantum-MTS.His.THR-mTXNPx* fragment to complement *mtxnpx⁻ L. infantum* promastigotes was assembled according to the scheme in Fig. S5. To generate this construct, a DNA fragment corresponding to *His.THR-mTXNPx* was PCR amplified with primers P1 and P2 (Table S1) from pET28c-*mTXNPx* [160], which harbors the *mTXNPx* ORF in frame with an N-terminal His/thrombin tag. The resulting PCR product was cloned into the *Sac*II and *Xba*I sites of pSSU-*PHLEO-infantum-mTXNPx* [160]. Prior to this cloning step, the pSSU-*PHLEO-infantum-mTXNPx* [160] vector was manipulated by site directed mutagenesis with primers P3 and P4 to eliminate the *Sac*II restriction site located in the 5'SSU fragment of the plasmid. The resulting pSSU-*PHLEO-infantum-MTS.His.THR-mTXNPx* construct was further subjected to site direct mutagenesis with primers P5 and P6 to remove an undesired *Nde*I restriction site in the *His.THR-mTXNPx* ORF. Before transfection of *mtxnpx⁻* promastigotes, the construct was linearized by digestion with *Nde*I-*Pme*I and purified from agarose gel by electroelution. Expression of His.THR-mTXNPx in the resulting *mtxnpx⁻/+MTS.His.THR-mTXNPx* clones was confirmed by western blot and indirect immunofluorescence according to previously described procedures [160,175].

Animals and ethics statement - BALB/c and National Marine Research Institute (NMRI) mice were purchased from the animal facility house at IBMC. All mice used in this study were raised in specific pathogen free conditions. Euthanasia was performed in a 20% isofluorane atmosphere. The experimental animal procedures were approved by the Local animal Ethics Committee of IBMC, University of Porto, Portugal and licensed by DGV (General Directory of Veterinary, Ministry of Agriculture, Rural Development and Fishing, Govt. of Portugal), in May 18 2006 with reference 520/000/000/2006. All animals were handled in strict accordance with good animal practice as defined by national authorities (DGV, Law nu1005/92 from 23rd October) and European legislation EEC/86/609.

Determination of parasite burden by limiting dilution assay - Parasite lines were passaged through NMRI mice prior to the infection experiment. BALB/c male mice were then inoculated intraperitoneally with 10^8 *L. infantum* stationary phase promastigotes. Experimental groups were randomized to mice age, cage, and cage location. Two and eight weeks after infection, mice were sacrificed. The spleens were excised, weighed and homogenized in Schneider's medium (Sigma) supplemented with 10% v/v FBSi, 100 U/ml penicillin, 100 mg/ml streptomycin, 5 mM HEPES, pH 7.4, 5 mg/ml phenol-red (Sigma) and 2% v/v sterile human urine. The number of parasites per gram of organ (parasite burden) was calculated as described by [176]. Briefly, homogenates were diluted to 10 mg/ml and subsequently titrated in quadruplicates across a 96-well plate in serial four-fold dilutions (four titrations per spleen). After two weeks of growth at 25°C, the last dilution containing promastigotes was recorded and the parasite burden calculated.

Results

Reduced mTXNPx functions as efficient molecular chaperone *in vitro*

Previous findings indicated that mTXNPx displays ATP-independent chaperone activity *in vitro*, preventing citrate synthase (CS) from non-specific aggregation at elevated temperatures (chapter II of this thesis). To further characterize this activity and gain insights into the mechanism by which this chaperone function is regulated, we purified mature mTXNPx from *E. coli* and analyzed its ability to protect several *in vitro* chaperone client proteins from thermal aggregation. Since *Leishmania* lines expressing the peroxidase-inactive variant mTXNPxC81S retained the capacity to infect mice and withstand heat shock treatment [160 and Chapter II], we reasoned that overoxidation of the active site cysteine is unlikely the physiological stimulus triggering mTXNPx's

conversion into a chaperone. We therefore focused only on the reduced and oxidized subpopulations of wild-type mTXNPx, knowing that substantial amounts of both species are constitutively present in *Leishmania* promastigotes [160]. Like observed with other peroxiredoxins, purification of mTXNPx in the absence of reducing agents yields >90% disulfide-bonded, dimeric protein (mTXNPx$_{ox}$), indicative of its high propensity to air-oxidize (Figure S1A). We found that large quantities of the reducing agent dithiothreitol (DTT) were necessary to generate mTXNPx$_{red}$ (Figure S1A), and that the constant presence of moderate amounts of DTT (0.2 mM) was required to prevent spontaneous re-oxidation of mTXNPx$_{red}$ during activity assays. To directly compare the chaperone function of mTXNPx$_{red}$ and mTXNPx$_{ox}$, we tested their influence on the aggregation of thermally unfolding luciferase, which rapidly aggregates in the absence of Prx (Figure 1A, 0:1). In the presence of a 10-fold molar excess of mTXNPx$_{red}$, the thermal aggregation of luciferase was significantly decreased, and with a 20-fold excess of mTXNPx$_{red}$ aggregation was completely suppressed (Figure 1A). Similarly, when purified mTXNPx was reduced by its physiological reducing system, it almost completely suppressed aggregation of luciferase (Figure S1B). In contrast, however, when the same amount of mTXNPx$_{ox}$ was added to the incubation reaction, no significant effect on luciferase aggregation was detected (Figure 1B). Similar results were obtained when two other classical chaperone client proteins, malate dehydrogenase (Figure S1C) and citrate synthase (Figure S1D) were tested as client proteins. In each case, mTXNPx$_{red}$ prevented thermal aggregation of the model proteins, whereas mTXNPx$_{ox}$ had only negligible effects. We concluded from these results that reduced mTXNPx works as effective ATP-independent molecular chaperone, and that overoxidation of the active site cysteine is not a crucial factor in the activation process.

The mTXNPx chaperone-active species is the decamer

Leishmania mTXNPx, like most other typical 2-Cys-Prxs [131], undergoes redox-dependent changes in its quaternary structure, cycling between reduced ~230 kDa decamers and oxidized 46 kDa disulfide-bonded dimers (Figure S2A). We conducted isothermal titration calorimetry (ITC) of both oxidized and reduced mTXNP and confirmed that the reduced mTXNPx undergoes the typical, highly cooperative decamerization process observed also for other members of the Prx family. We determined a critical transition concentration CTC (*i.e.*, the minimal concentration at which mTXNPx$_{red}$ forms decamers) [117,177] of 0.75 µM (Figure S2B), which is well below the concentration of mTXNPx in promastigotes (2-6 µM) or amastigotes (49-102 µM) (Fig. S2C). Because the CTC was found to be also below the concentration of mTXNPx$_{red}$ that we typically use for our activity assays, we concluded that mTXNPx$_{red}$ is decameric in our assays. In contrast,

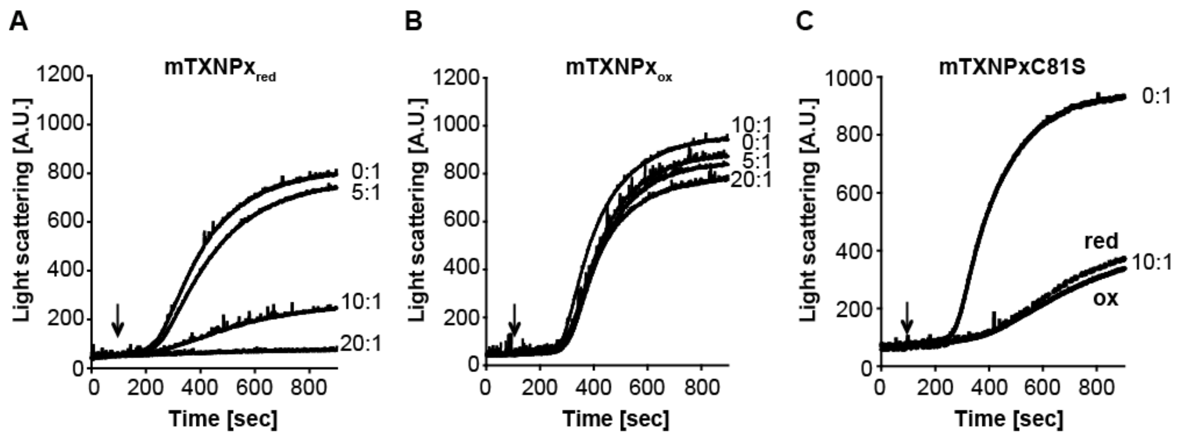

Figure 1: Reduced mTXNPx prevents thermal aggregation of luciferase. Influence of **A**. $mTXNPx_{red}$ or **B**. $mTXNPx_{ox}$ on the thermal aggregation of luciferase. Native luciferase (0.1 µM) was incubated in the absence (0:1) or presence of different ratios of mTXNPx at 41.5°C and light scattering (expressed in arbitrary units, A.U.) was monitored at 360 nm. Upon dilution of $mTXNPx_{red}$ into the assay buffer, the concentration of residual DTT was 0.2 mM. The same amount of DTT was added to the respective controls. **C**. Influence of a 10-fold molar excess of reduced or oxidized mTXNPxC81S mutant variant on the *in vitro* aggregation of thermally unfolding luciferase. Addition of 0.2 mM DTT did not significantly affect luciferase aggregation in the absence of chaperones. The ratio of chaperone to client protein was calculated based on the monomeric form of mTXNPx. Arrows indicate the time point at which luciferase was added to the reactions.

we did not observe any decamerization for $mTXNPx_{ox}$ even at concentrations as high as 100 µM, indicating that $mTXNPx_{ox}$ is dimeric in our assays (Figure S2B). This observation, together with the data shown above, implied that the minimal chaperone-active peroxiredoxin species is likely the reduced decamer. Absence of the active site cysteine, as is the case in the peroxidase-inactive mutant mTXNPxC81S [160], maintains the protein in a mixture of decameric and higher oligomeric conformations under non-reducing conditions, and in a purely decameric conformation upon its reduction (Figure S2A). Analysis of the *in vitro* chaperone function of purified mTXNPxC81S revealed that this mutant variant is constitutively chaperone active, exerting a very similar propensity to prevent protein aggregation both in the reduced and oxidized form (Figure 1C). These results are in excellent agreement with our previous *in vivo* studies showing that this mutant variant rescues the heat shock phenotype of mTXNPx-deficient promastigotes and restores their infectivity [160]. These findings also suggest that no oligomeric structure higher than the reduced decamer is necessary to confer chaperone activity to *Leishmania* mTXNPx. Our titration experiments using increasing amounts of wild-type $mTXNPx_{red}$ were consistent with this conclusion and showed that a 10-fold molar excess of mTXNPx monomers is both necessary and sufficient to significantly suppress the aggregation of one molecule of client protein (Figure 1A). Together, our data demonstrate that the redox

status of mTXNPx is crucial for its chaperone function, most likely via its influence on the oligomeric structure of the Prx.

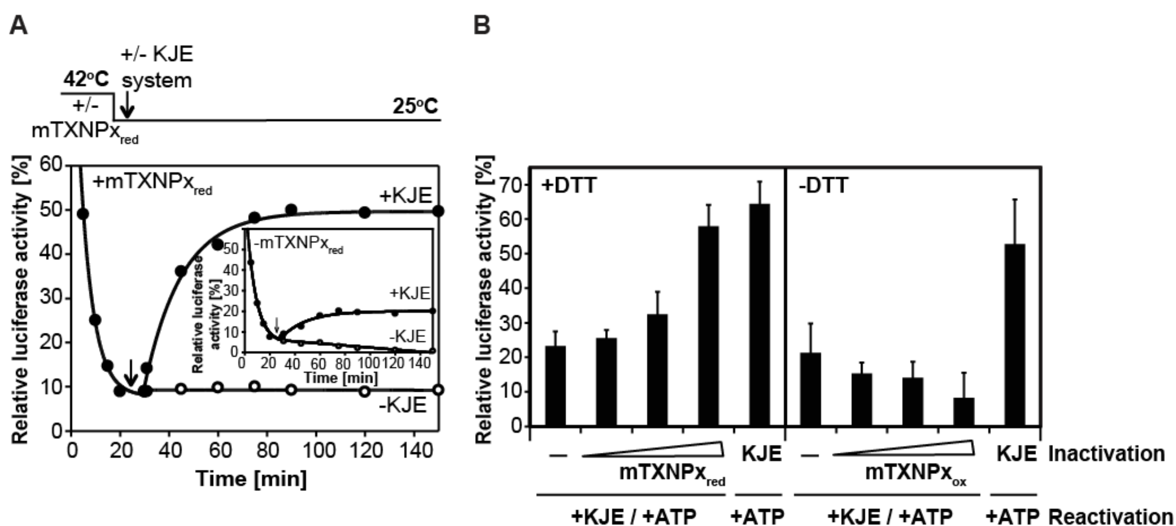

Figure 2: mTXNPx$_{red}$ maintains luciferase in a refolding competent state. A. Luciferase (0.1 µM) was incubated in the absence (inset) or presence of a 40:1 molar ratio of mTXNPx$_{red}$ for 20 min at 42°C. Samples were cooled to 25°C. After 10 min (as indicated by the arrow), the samples were supplemented with 2 mM MgATP, 0.1 mg/ml BSA and, where indicated, with a 20:4:20:1 ratio of DnaK:DnaJ:GrpE (KJE) system to luciferase. At the indicated time points, aliquots were removed and luciferase activity was determined. **B**. Luciferase (0.1 µM) was inactivated either in the absence of any chaperone system (-), in the presence of a 10:1, 20:1 or 40:1 molar ratio of mTXNPx$_{red}$ / mTXNPx$_{ox}$ to luciferase, or in the presence of a 20:4:20:1 ratio of DnaK:DnaJ:GrpE (KJE) to luciferase at 42°C. All samples shown in the left panel contained 0.2 mM DTT, whereas samples shown in the right panel did not contain any reducing agents. After 20 min, the temperature was shifted to 25°C for 10 min, and 2 mM MgATP and 0.1 mg/ml BSA was added to all samples. In addition, all samples except those that already contained the KJE-system, were supplemented with a 20:4:20:1 ratio of DnaK:DnaJ: GrpE:luciferase. After 120 min of incubation at 25°C, luciferase activity was measured. Luciferase activity before incubation at 42°C was set to 100%.

Reduced mTXNPx maintains clients in folding-competent conformation

Several studies have shown that Prxs can suppress aggregation of different client proteins *in vitro*. However, to our knowledge, few mechanistic studies have addressed the fate of client proteins once bound to 2-Cys-Prxs.To investigate whether mTXNPx$_{red}$ has the ability to delay the unfolding process and/or refold its unfolded client proteins, we examined the influence of mTXNPx$_{red}$ on the inactivation and reactivation of thermally denatured luciferase. We incubated luciferase in the absence or presence of chaperone-active mTXNPx$_{red}$ at 42°C and monitored luciferase activity over time. Within 20 min of incubation, luciferase activity was below 10%, independently of the absence (Figure 2A,

inset) or presence of $mTXNPx_{red}$ (Figure 2A). This result is reminiscent of other chaperones, such as Hsp33, which prevent protein aggregation without slowing protein unfolding. We then shifted the temperature to 25°C and further monitored luciferase activity. However, we did not observe any significant luciferase reactivation (Figure 2A, open circles), indicating that $mTXNPx_{red}$ is either unable to release its client proteins or releases them in a refolding-incompetent form. Because many ATP-independent chaperones like Prx are unable to refold their client proteins and instead transfer them to ATP-dependent foldases, such as the Hsp70- system [178], we tested whether $mTXNPx_{red}$ could also cooperate with other chaperone systems in the folding of its client proteins. We supplemented the incubation reaction after its shift to 25°C with the bacterial DnaK/DnaJ/GrpE (KJE) system, which is highly homologous to the mitochondrial Hsp70-system [179,180]. Addition of the KJE-system to pre-formed $mTXNPx_{red}$:luciferase complexes resulted in a substantial increase in luciferase reactivation (Figure 2A, closed circles), which was dependent on the amount of $mTXNPx_{red}$ present during thermal inactivation (Figure 2B). In the presence of a 4-fold molar excess of $mTXNPx_{red}$ decamers to luciferase, nearly 40% of luciferase molecules reactivated upon shift to non-heat shock temperatures and addition of the KJE-system (Figure 2A, B). In contrast, when the thermal inactivation of luciferase was conducted in the absence of $mTXNPx_{red}$ (Figure 2A inset) or in the presence of $mTXNPx_{ox}$ (Figure 2B), KJE-mediated reactivation of luciferase was less than 10%. We concluded from these results that chaperone-active $mTXNPx_{red}$ maintains its client proteins in a folding-competent conformation.

mTXNPx functions as general chaperone both *in vitro* and *in vivo*

Little is known about the client specificity of peroxiredoxins or the extent to which they affect protein aggregation *in vivo*. To address the potential client specificity of mTXNPx, we first investigated the influence of purified mTXNPx on the heat-induced aggregation of proteins within a soluble bacterial extract under both reducing and non-reducing conditions. We used extracts of a bacterial strain that lacks the heat shock sigma factor σ32 (gene name *rpoH),* and therefore contains reduced levels of most *E. coli* chaperones. This approach allowed us to minimize the influence of other chaperones in the assay, and primarily focus on the chaperone function of mTXNPx. We prepared cell lysates of the *rpoH*-deletion strain (Figure 3A, lane T) and supplemented these with different amounts of oxidized or reduced mTXNPx. We then incubated the lysates at either 30°C or 45°C for 60 min, separated the aggregated from the soluble proteins by centrifugation and analyzed the precipitated proteins on a reducing SDS-PAGE. As shown in Figure 3A, $mTXNPx_{red}$ exerts a highly promiscuous protein-protective effect, protecting the vast majority of thermolabile *E. coli* proteins against temperature-induced protein aggregation. In contrast

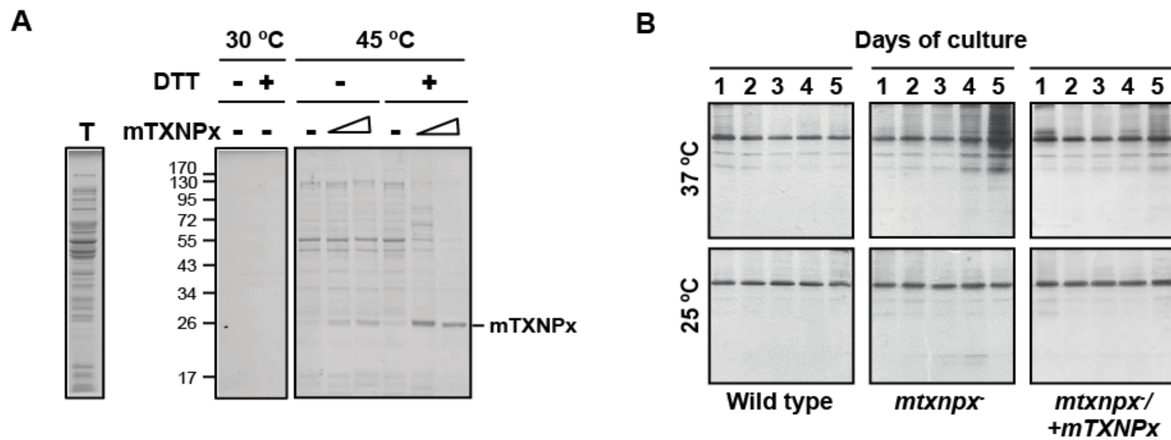

Figure 3: mTXNPx displays general chaperone activity. **A**. Aliquots of a soluble extract (T) from the chaperone-deficient *E. coli rpoH* mutant strain were prepared, supplemented with increasing amounts of $mTXNPx_{red}$ (in the presence of 1.5 mM DTT) or $mTXNPx_{ox}$ (in the absence of DTT) and heat-treated for 60 min at 45°C. Samples without mTXNPx served as controls. The insoluble proteins were then separated from the soluble supernatant by centrifugation, and analyzed on reducing SDS-PAGE. The order of the lanes in the gel was electronically rearranged to facilitate data interpretation. **B**. Wild type, *mtxnpx*− or *mtxnpx*−/+mTXNPx *L. infantum* promastigotes were incubated at 25°C or 37°C. At the indicated time-points, parasites were collected and their aggregated proteins were isolated and analyzed by SDS-PAGE. The major protein band that is present in all lanes is likely GP63, a GPI-anchored membrane protein, and one of the most abundant protein in *Leishmania*.

and in agreement with our data shown above, $mTXNPx_{ox}$ was unable to affect the aggregation behavior of thermolabile *E. coli* proteins. These studies demonstrate that reduced mTXNPx functions as a highly effective general chaperone.

To determine whether mTXNPx exerts a similarly broad protein-protective effect in *Leishmania*, and if so under which physiological conditions, we analyzed protein aggregation in wild-type and *mtxnpx*⁻ promastigotes at 37°C, a temperature at which promastigotes lacking mTXNPx show a thermosensitive phenotype (Chapter II). Because temperature sensitive phenotypes are common in cells lacking chaperones and usually associated with protein aggregation, we reasoned that we should observe significantly increased protein aggregation in promastigotes of mTXNPx deletion lines compared to wild type promastigotes or parasites cultured at 25°C. We therefore grew wild-type and *mtxnpx*⁻ promastigotes at both 25°C and 37°C for up to 5 days, took aliquots at defined time points and separated soluble from insoluble proteins. We then analyzed the aggregated proteins on SDS-PAGE (Figure 3B). While we did not observe any significant protein aggregation in either strain background at 25°C (Figure 3B, lower panel), we detected substantially higher levels of protein aggregation in *mtxnpx*⁻ parasites than in wild type parasites cultivated at 37°C. Moreover, the proteins that were protected in wild-type

cells and aggregated in heat treated *mtxnpx⁻* promastigotes showed a wide variation in size, in full agreement with our bacterial lysates studies. Introduction of an ectopic copy of mTXNPx into the *mtxnpx⁻* deletion strain (*mtxnpx*−/+mTXNPx) largely diminished protein aggregation at 37°C (Figure 3B), consistent with our previous findings that an ectopic copy of mTXNPx completely rescues the temperature-sensitive phenotype (Chapter II). These results corroborate the general chaperone function of mTXNPx, and demonstrate that mTXNPx plays an important role in preventing temperature-induced protein aggregation in parasites.

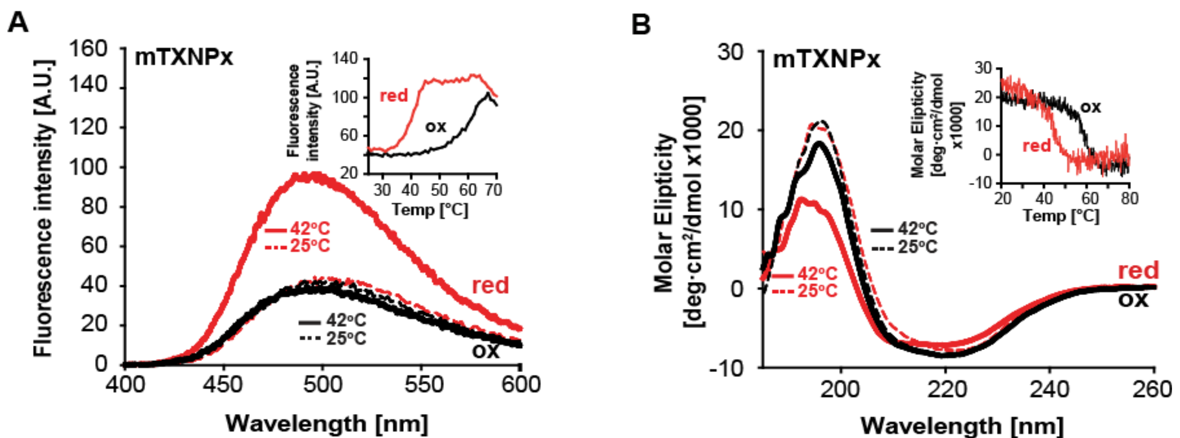

Figure 4: mTXNPx undergoes temperature-mediated structural changes. **A**. Bis-ANS binding to $mTXNPx_{red}$ (red lines) or $mTXNPx_{ox}$ (black lines) at either 25°C (dashed lines) or 42°C (solid lines)**. Inset**. Bis-ANS binding of $mTXNPx_{red}$ or $mTXNPx_{ox}$ as a function of temperature monitored at 500 nm. **B**. Far-UV circular dichroism spectra of $mTXNPx_{red}$ (red lines) or $mTXNPx_{ox}$ (black lines) at either 25°C (dashed lines) or 42°C (solid lines). **Inset**. Temperature-dependent changes in the secondary structure of $mTXNPx_{red}$ or $mTXNPx_{ox}$ recorded at 197 nm. Temperature was increased at a rate of 1°C per minute. All spectra were buffer corrected.

$mTXNPx_{red}$ undergoes structural rearrangements at heat shock temperatures

Our data showed that decameric $mTXNPx_{red}$ works as an efficient molecular chaperone under heat shock conditions both *in vivo* and *in vitro* but is unable to suppress aggregation of chemically-denatured client proteins at 30°C, even when present at very high molar excess (Figure S3A, B). These results made us wonder whether temperature-induced structural changes might contribute to the chaperone activity of reduced mTXNPx. To address the effect of temperature on mTXNPx structure and function, we incubated dimeric $mTXNPx_{ox}$ and decameric $mTXNPx_{red}$ at either 25°C or 42°C (*i.e.*, the temperature at which we observed chaperone activity *in vitro*), and compared surface hydrophobicity and secondary structure. We decided to focus on these two parameters since most molecular chaperones rely on the exposure of hydrophobic patches to bind

unfolding clients [139,173], and many ATP-independent chaperones, including Hsp33, sHsps and HdeA, have been shown to undergo significant unfolding as part of their stress-specific activation [181]. Analysis of the surface hydrophobicity of $mTXNPx_{red}$ using 4,4′-bis-anilino-1,1′-binaphthyl-5,5′-disulfonic acid (bis-ANS) binding revealed a significant increase in the fluorescence signal and a 30-40 nm blue shift in the emission maximum at 42°C compared to 25°C (Figure 4A, red traces), fully consistent with the temperature-induced exposure of hydrophobic surfaces [182]. In contrast, no significant increase in bis-ANS binding was detected for chaperone-inactive dimeric $mTXNPx_{ox}$ irrespective of the temperature (Figure 4A, black traces). Subsequent analysis of bis-ANS binding as function of the temperature confirmed these observations and revealed that $mTXNPx_{red}$ begins to expose hydrophobic surfaces at ~ 35°C with an apparent midpoint of transition at ~ 40°C. In contrast, incubation temperatures above 50°C were required to detect any increase in bis-ANS binding to $mTXNPx_{ox}$, likely coinciding with mTXNPx unfolding (Figure 4A, inset). Consistent with these results, circular dichroism analysis revealed a substantial decrease in the alpha-helical content of $mTXNPx_{red}$ but not in $mTXNPx_{ox}$ upon incubation at physiological heat shock temperatures (Figure 4B and inset). These results strongly suggest that reduced decameric but not oxidized dimeric mTXNPx undergoes major conformational changes in a physiologically relevant temperature range leading to increased surface hydrophobicity and coinciding with the activation of its chaperone function.

Reduced mTXNPx decamer binds unfolded luciferase in the center of its ring-like structure

The ring-like structure of chaperone-active Prx decamer, which has been extensively studied by electron microscopy (EM) and has been solved by X-ray crystallography [183,184,185], prompted our subsequent studies, which were aimed at directly visualizing client protein binding. For these experiments, we incubated $mTXNPx_{red}$ either alone or in the presence of luciferase at 30°C or 42°C, using a 1:1 ratio of $mTXNPx_{red}$ decamer to luciferase. We then analyzed its structure by EM. We found that $mTXNPx_{red}$ forms typical ring-like structures featuring a large central cavity at both 30°C and 42°C in the absence of any client proteins, as well as at 30°C when the client protein is native and presumably correctly folded (Figure 5A, S4). Top-down orientations are preferred, however many side-views are visible that identify a single ring arrangement. Stacked ring complexes, which have been previously characterized [92], are not observed under these conditions. Strikingly, upon incubation of $mTXNPx_{red}$ and luciferase at 42°C many of the $mTXNPx_{red}$ particles contained additional electron density in the center, suggesting that thermally unfolded luciferase is bound within the ring-like structure (Figure 5B). The side-views also

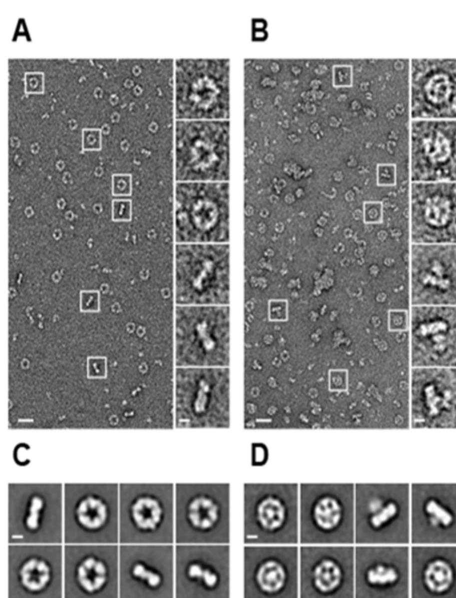

Figure 5: mTXNPx$_{red}$ decamer binds thermally unfolded luciferase in the center of its ring. Representative EM micrographs and selected single particle images of negatively stained mTXNPx$_{red}$ decamers **A**. alone or **B**. following incubation with thermally unfolded luciferase. **C**., **D**. Reference-free 2D projection averages showing top-down and side views of the mTXNPx$_{red}$ decamer alone or with luciferase. Percentage of mTXNPx$_{red}$ decamers in complex with luciferase varied between experiments. Scale bars equal 200 Å for the micrograph images and 50 Å for the boxed particles and averages.

showed additional density protruding out from one side of the ring. We collected single particle data sets for mTXNPx$_{red}$ alone (9,963 particles) and with luciferase (10,222 particles), and generated reference-free 2D projection averages. The 2D averages of mTXNPx$_{red}$ alone showed five well-defined lobes of density in the top-down views that are likely dimers arranged as the five-fold symmetric ring (Figure 5C). The diameter of the rings was measured to be approx. 130 Å with a 50 Å internal cavity and an overall width of approx. 40 Å, agreeing with previous crystal structures [186]. In the 2D averages of mTXNPx$_{red}$ with luciferase, the ring complex appears to directly contact a central, globular luciferase structure that is positioned in its center (Figure 5D). While the density for luciferase is structurally variable, we observed as many as five contact points in the complex, strongly suggesting that the interaction is based on mTXNPx$_{red}$ dimers. Notably, in the side-view projection averages, we observed that luciferase protrudes from one side of the decamer ring indicating that a preferential orientation for client binding likely exists. This result also shows that only portions of the client proteins are bound to mTXNPx$_{red}$, a finding that agrees well with our earlier results demonstrating that mTXNPx functions as a general chaperone, which does not seem to impose any size limitation on its client proteins. Overall, these data demonstrate that mTXNPx$_{red}$ forms single decameric rings that use direct contacts to position the luciferase client in the center of its ring. Based on the small interaction region, we concluded that only a defined set of residues that face the

center of the mTXNPx ring are likely involved in recognizing and binding unfolding client proteins.

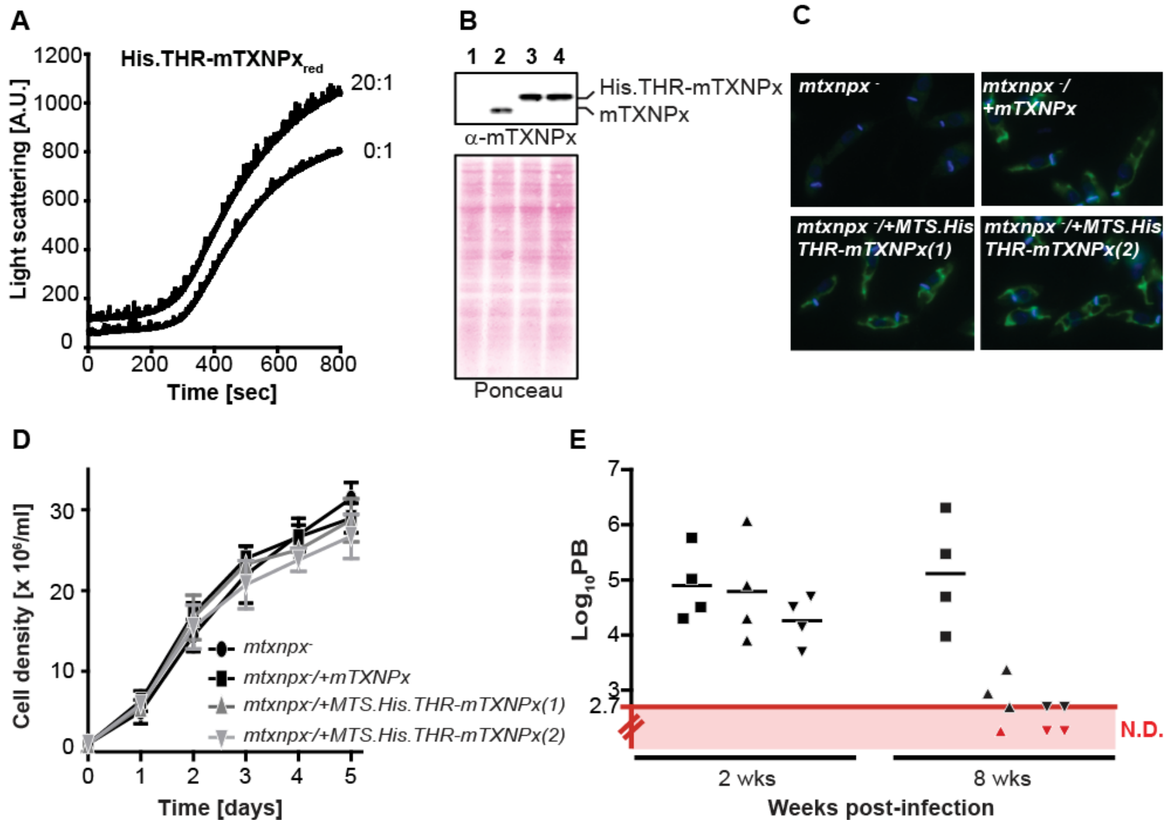

Figure 6: Chaperone activity of mTXNPxs is critical for *Leishmania* virulence. A. Influence of a 20-fold molar excess of His.THR-mTXNPx$_{red}$ on the *in vitro* thermal aggregation of luciferase. See the legend of Figure 1 for details. **B**. Analysis of mTXNPx expression in *mtxnpx*$^-$ (lane 1), *mtxnpx*$^-$*/+mTXNPx* (lane 2) and two *mtxnpx*$^-$*/+MTS.His.THR-mTXNPx* (lanes 3 and 4, respectively) promastigote clones using a polyclonal antibody raised against the recombinant His.THR-MTS.mTXNPx. Purified mTXNPx and His.THR-mTXNPx were used as controls for the expected size of the proteins and their position on the blot is indicated. Ponceau S staining of the blot is shown as loading control. **C**. Localization of the His.THR-mTXNPx chimera in *mtxnpx*$^-$ promastigotes using indirect immunofluorescence with the anti-mTXNPx antibody (green) merged with DAPI (blue). Controls as described above are included. **D**. Growth rate of *mtxnpx-* (black circles), *mtxnpx-/+mTXNPx* (black squares) and two *mtxnpx-/+MTS.His.THR-mTXNPx* clones (dark grey triangles). **E**. Parasite burden of BALB/c mice after 2 and 8 weeks of infection. BALB/c mice were inoculated intraperitoneally with stationary phase promastigotes of *mtxnpx*$^-$*/+mTXNPx* clone (squares) or two *mtxnpx*$^-$*/+MTS.His.THR-mTXNPx* clones (triangles). After 2 and 8 weeks of infection, the number of parasites per gram of spleen (parasite burden, PB) was determined. Each point represents one animal. The red line indicates the detection limit of the technique ($\log_{10}$=2.7). Animals with PBs below this limit are “not detected” (ND) and represented by red symbols.

mTXNPx chaperone function is critical for parasite infectivity

Analysis of the 2D class average particles of mTXNPx in complex with luciferase suggested that the N-terminal extensions of Prx, which face the interior of the decamer

[186], might serve a critical role for client binding and therefore for the chaperone function of mTXNPx. This idea was also fully consistent with an earlier observation in our lab that an N-terminal His-thrombin tag variant of mTXPNx (His.THR.mTXNPx) failed to protect luciferase against heat-induced aggregation *in vitro* (Figure 6A). These results confirmed the involvement of the N-terminus of mTXNPx in client interaction and suggested that addition of extra residues abolishes client binding. Fortuitously, His-thrombin tag variants containing the additional MTS were previously found to be decameric and exert full peroxidase activity [147]; we now were able to directly determine the specific role of mTXNPx's chaperone function in *Leishmania* infectivity and persistence in mammals. We transfected the previously established *mtxnpx*$^{-}$ knockout cell lines [160] with pSSU-*PHLEO-MTS.His.THR-mTXNPx*, a plasmid containing the coding sequence for a His-thrombin tag between the predicted mTXNPx mitochondrial targeting sequence (MTS) and the mature protein (Figure S5). Western blot analysis confirmed the presence of a slower migrating mTXNPx-species in the resulting transformants, indicative of the presence of a His-thrombin tag (Figure 6B). We ascertained the correct targeting of His.THR-mTXNPx into mitochondria by immunofluorescence (Figure 6C). To test whether the chaperone function of mTXNPx is indeed essential for *Leishmania* virulence, we then inoculated BALB/c mice with *mtxnpx*$^{-}$*/+mTXNPx* or two different lines of *mtxnpx*$^{-}$*/+MTS.His.THR-mTXNPx* transfectants, and evaluated the parasite burden in the spleens 2 and 8 weeks post infection. Despite permitting wild type-like growth of promastigotes at 25°C (Figure 6D), the chaperone-inactive His.THR-mTXNPx variant was unable to restore the virulence of the *mtxnpx*$^{-}$ parasites (Figure 6E). Based on these results, we conclude that the molecular chaperone activity is crucial for *Leishmania* survival within the mammalian host.

Discussion

The reduced decamer serves as the basic chaperone-activatable Prx species

Following the pioneering work of Jang et al. [104], several studies independently validated the conclusion that cytosolic 2-Cys peroxiredoxins serve a dual purpose: as peroxidases that detoxify hydroperoxide or as chaperones that protect proteins against irreversible aggregation. The latter function was associated with the formation of high molecular weight complexes of Prx. The switch between these two, apparently mutually exclusive functions of Prx was largely attributed to cysteine-overoxidation [104]. However, phosphorylation [105], exposure to low pH [106,187], and other stimuli [136,137] were found to also prompt chaperone activity. These results raised the obvious question as to

whether all of these mechanisms work in their own independent way to generate chaperone-active peroxiredoxin species, or whether they share a single, unifying activation mechanism. Our study now proposes that the reduced Prx decamer serves as the basic, chaperone-activatable scaffold, which is sensitive to structural rearrangements that are likely required for the chaperone to interact with unfolded clients. Our conclusion that mTXNPx does not have to be post-translationally modified or form high molecular weight (HMW) complexes to exert chaperone function explains earlier *in vivo* findings in yeast, which showed that both wild-type and the peroxidase-inactive Tsa1 variant lacking the active site cysteine, protect yeast cells against stress conditions at which no significant ROS production (and hence no overoxidation or HMW complex formation) was detected [104]. These results also help explain how organisms, such as *Leishmania*, which experience little Prx cysteine overoxidation [160] and lack the sulfinic acid-reductase sulfiredoxin, are able to exploit their peroxiredoxins as additional reservoir of chaperone activity under protein-unfolding stress conditions.

Our data clearly demonstrate that reduced mTXNPx decamers serve as potent molecular chaperones under heat shock conditions *in vitro*, disagreeing with earlier studies that reported no detectable chaperone activity in reduced Prx [106,177]. Our studies now show that to exert full chaperone activity, Prx needs to be i) maintained in a reduced, decameric form; and ii) devoid of N-terminal tags and iii) used at concentrations that promote decamerization. Therefore, simple absence of reducing agents in the assay buffer, presence of N-terminal tags or even usage of insufficient Prx concentrations (below the K_D of Prx decamerization) in the activity assays will abrogate the chaperone function of reduced Prx. In contrast, posttranslational modifications or active site mutations that have been shown to “induce chaperone-activity” are known to stabilize Prx’s decameric structure [105,136,177], and likely work by simply maintaining the chaperone activity of reduced Prx under the assay conditions used. Since intracellular conditions are highly reducing and Prx concentrations are typically much higher than the K_D of decamerization, much of the cellular Prx will be in its chaperone-activatable form, making posttranslational modifications likely unnecessary to achieve chaperone function *in vivo*. What appears to be necessary for chaperone activation of Prx, however, is a structural remodeling of the decameric structure, which can be induced by elevated temperatures (an inherent part of most *in vitro* chaperone assays) and, possibly, by low pH or other protein unfolding stress conditions, such as oxidative stress [130]. In conclusion, our data suggest that Prxs are capable of integrating different unfolding stimuli and translating them into structural rearrangements that trigger chaperone activation. This makes Prx a member of a growing class of stress-specific chaperones, whose activation demands local unfolding events [139,173,181,188]. What remains to be investigated is the extent to which these local

unfolding events influence the peroxidase activity of reduced Prx and whether this mode of regulation applies not only to mitochondrial Prx but apply also to the cytosolic members of the 2-Cys peroxiredoxin family.

Peroxiredoxins work as ring-like chaperones

Based on our biochemical and *in vivo* studies, we now provide the long-sought explanation for the functional relevance of Prx's oligomeric structures. We found that unfolding luciferase binds to the center of Prx's ring-like structure and that, by employing N-terminal residues that face the interior of the decamer, Prx binds and stabilizes client proteins. These results are in contrast to previous conclusions based on the analysis of the pH-activated *Schistosoma mansoni* Prx (*Sm*PrxI) structure, which suggested that client proteins might bind to a hydrophobic surface that is exposed upon unfolding of the C-terminus [106,142]. While we cannot exclude that this region is also involved in client interaction, it would require substantial rearrangements to achieve these additional interactions. In either case, the fact that Prx binds clients in the center of its ring-like structure places Prx in the same category of other ring-like chaperonins, such as GroEL, which equally accommodate their client proteins in a central cavity [189]. These results provide support for the previous notion that peroxiredoxins are the most likely origin of chaperonins [190].

Physiological implications of mTXNPx as temperature-sensitive chaperone

In this study, we demonstrated that mTXNPx prevents protein aggregation in *Leishmania* promastigotes under temperature conditions that resemble those found at the initiation of an infection within a mammalian host. This result confirms our previous findings that mTXNPx confers thermotolerance to parasites and suggests that the crucial *in vivo* function of this protein is that of a chaperone. Temperature shifts are part of *Leishmania* biology, and parasites evolved different mechanisms to cope with this stressful situation. According to our model (Figure 7), reduced mTXNPx decamers sense the environment changes that occur as promastigotes shuttle from 25°C to 37°C and use structural rearrangements to protect a wide range of thermolabile proteins against aggregation. Importantly, mTXNPx is a highly abundant protein in *Leishmania*, and at least 50% of the mTXNPx found in promastigotes exposed to 37°C is in its reduced and hence chaperone-activatable decameric form (Chapter II, Fig. S1). This provides a substantial pool of mitochondrial chaperone activity that can be instantaneously called upon when needed, while avoiding time-consuming protein translation and mitochondrial trafficking processes. Although the chaperone function of mTXNPx is likely to be important

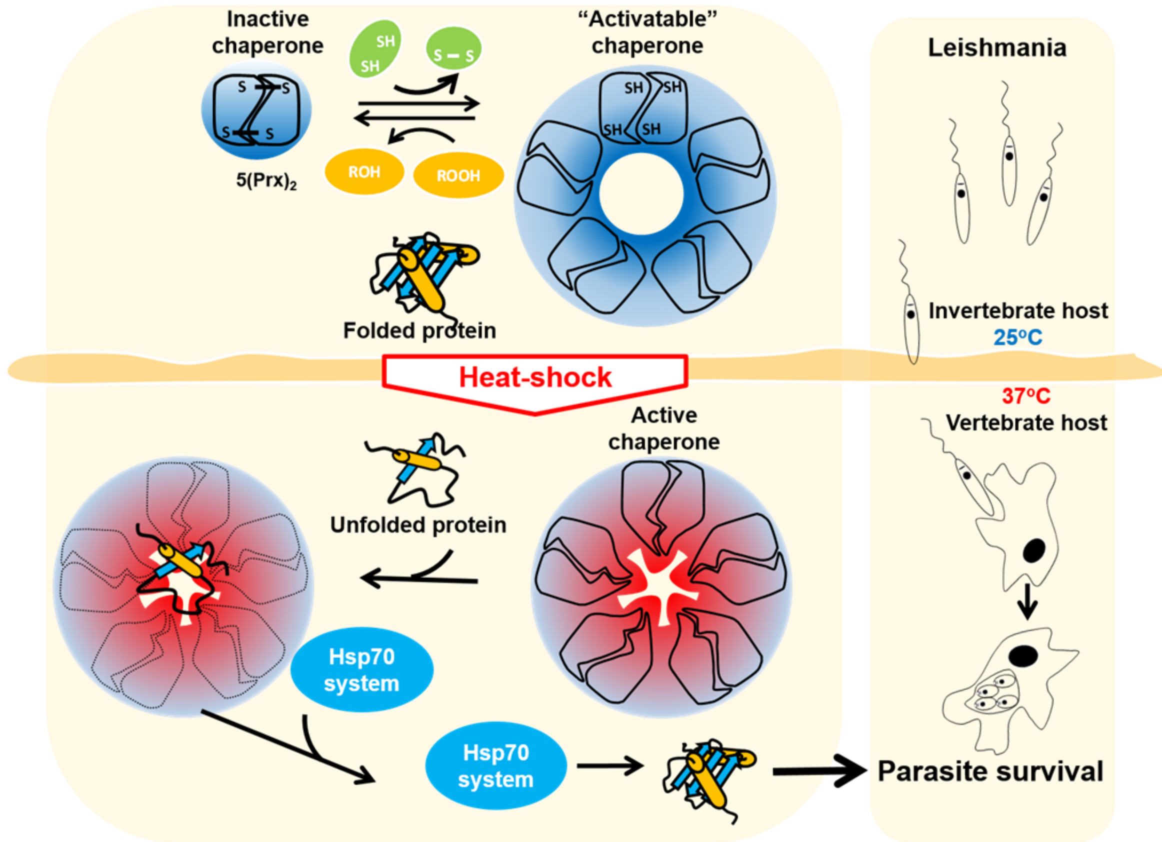

Figure 7: Physiological role of mTXPNx's chaperone function in *Leishmania*. Exposure of *Leishmania* promastigotes (the insect stage) to mammalian body temperature of 37°C leads to the protein unfolding. This change in temperature is sensed by reduced mTXNPx decamers and translated into structural rearrangements, which likely contribute to the activation of its chaperone function. Once chaperone active, mTXNPx decamers bind the client proteins in the center of their ring-like structure preventing the client proteins from forming cytotoxic aggregates. Client proteins are maintained in a refolding-competent state and can be reactivated by members of the Hsp70-system in the presence of ATP. The working cycle of mTXNPx chaperone appears crucial for *Leishmania* to adapt to and survive the temperature encountered in the mammalian host and hence to generate viable amastigotes.

for differentiation and, consequently, formation of viable amastigotes, it is worth noting that mTXNPx is expressed all along the infective process. Hence it is likely that this function provides additional protection to the parasite in other circumstances, such as during fever episodes. In conclusion, this study sheds light on previously undefined aspects of the chaperone function of Prxs, and provides unambiguous evidence for the importance of mTXNPx's chaperone function on *Leishmania* biology and infectivity.

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Author Contributions

F.T., H.C., A.M.T., and U.J. designed research; **F.T.**, H.C., T.C., E.T., P.K. and D.R.S. performed research; **F.T.**, H.C., E.T., P.K., D.R.S., A.M.T., and U.J. analyzed data; and **F.T.**, H.C., D.R.S., A.M.T., and U.J. wrote the paper.

Supporting Information

Material and Methods

Chaperone activity assays - To investigate the chaperone activity of mTXNPx, 100 nM luciferase (Promega), 100 nM citrate synthase (Sigma-Aldrich) or 150 nM malate dehydrogenase (Roche) were incubated in 40 mM HEPES, pH 7.5 at 41.5°C, 43°C or 45°C, respectively, either in the absence or presence of a 20:1 molar ratio of mTXNPx$_{re}$d or mTXNPx$_{ox}$. To prepare mTXNPx$_{red}$, mTXNPx$_{ox}$ was incubated in the presence of 5 mM DTT or its physiological reducing system consisting of 200 µM NADPH, 0.5 U/ml *L. infantum* trypanothione reductase (TR), 50 µM trypanothione disulfide (TS_2, Bachem) and 2.5 µM *L. infantum* tryparedoxin 2 (TXN2) for 30 min at 30°C. To test the influence of mTXNPx$_{red}$ on chemically denatured client proteins at non-stress temperatures, 12 µM citrate cynthase (CS) was incubated for 12 h in 6 M Gnd-HCl or for 4-5 hours in 5 M urea using 40 mM HEPES, pH 7.4 as buffer. Then, CS was diluted to 75 nM (from Gnd-HCl) or 80 nM (from urea) into 40 mM HEPES, pH 7.5 at 30°C in the absence or presence of mTXNPx$_{red}$. Light scattering was measured using a fluorescence spectrophotometer (Hitachi F4500) equipped with a temperaturecontrolled cuvette holder and stirrer ($\lambda_{ex/em}$ 360 nm, slit widths 2.5 nm).

Size exclusion chromatography - To determine the redox dependent oligomerization state of mTXNPx, size-exclusion chromatography (SEC) was performed. Briefly, 125 µg wild-type or mutant mTXNPx$_{red}$ or mTXNPx$_{ox}$ was applied onto a Superose 12 column, pre-equilibrated with 0.1 M sodium phosphate buffer pH 7.2, 150 mM NaCl. In the case of mTXNPx$_{red}$, the buffer was supplemented with 1 mM DTT to prevent reoxidation of the proteins. Calibration of the column was performed using commercially available molecular mass standards. The concentration of reduced and oxidized wild-type mTXNPx in the peak area was calculated. Briefly, the average peak concentration of each eluting species on the chromatogram was calculated integrating the area below each peak (at the peak maximum ± 0.5 ml retention volume) using the Origin software (OriginLab, Northampton, MA). Then the percent area (%p) of each peak compared to the total area of the entire chromatogram was determined, and the average peak concentration (ca) of monomeric mTXNPx was calculated using the following equation:

$$c_a = \frac{\%p * n_t}{M_r * 0.001l}$$

where nt is the total amount of loaded mTXNPx (*i.e.*,125 µg) and Mr depicts the molecular weight of monomeric mTXNPx.

Isothermal Titration Calorimetry (ITC) - ITC experiments of $mTXNPx_{red}$ and $mTXNPx_{ox}$ were conducted as previously described for other Prx members [191] using an iTC200 (Malvern Instruments Ltd., Malvern, UK). $mTXNPx_{red}$ or $mTXNPx_{ox}$ were dialysed overnight against 50 mM sodium phosphate buffer, pH 8 supplemented with 1 mM DTT in case of $mTXNPx_{red}$. $mTXNPx_{red}$ (50 µM) or $mTXNPx_{ox}$ (100 µM) were loaded into a syringe and titrated into a cell containing the same buffer equilibrated at 25°C. In order to allow the dissociation of mTXNPx to reach equilibrium in the cell, the injection interval was set to 840 sec for the reduced and 600 sec for the oxidized protein, with an initial delay time of 60 sec. The solution was stirred with 1,000 rpm, and the reference power was set to 10 µcal s^{-1} at high feedback mode. After each experiment, the instrument was washed as recommended by the manufacturer. Data analysis was conducted using a plugin for the software Origin (OriginLab, Northampton, MA) provided by the manufacturer. The heat of dilution from a buffer to buffer injection was not subtracted from the data since no heat change was observed. The critical transition concentration (CTC) was calculated as described [131,191]. The reaction enthalpy of the decamer dissociation was calculated as the average of the integrated heat pulses (cal per mol mTXNPx monomer) of all injections before reaching the CTC. The first injection was incomplete and therefore disregarded.

Determination of the intracellular concentration of mTXNPx - Protein extracts of *L. infantum* stationary phase promastigotes (cultured as described in the main text) and axenic amastigotes [192] were used to estimate the total *in vivo* concentration of mTXNPx. Recombinant purified mTXNPx was used as standard. Proteins were separated using a reducing SDS-PAGE and blotted onto a nitrocellulose membrane. The blot was then incubated with polyclonal anti-mTXNPx rabbit serum (dilution 1:1000). Anti-rabbit IgG conjugated with horseradish peroxidase (dilution 1:10000; Molecular Probes) was used as secondary antibody. The immuno-reaction was detected with ClarityTM western ECL substrate (Biorad) and imaged in a ChemiDocTM XRS^+ imagining system (BioRad). Band intensities were quantified using the Image LabTM software (BioRad). Concentration of mTXNPx in the different stages of parasite life cycle was estimated based on reported cell volumes [193,194].

Negative Staining EM - $mTXNPx_{red}$ (10 µM) alone, luciferase (1 µM) alone, or a mixture of $mTXNPx_{red}$ and luciferase were first incubated on ice. Then, the samples were split and incubated for 2 min at room temperature before they were transferred either to 30°C or slowly heated from 30°C to 42°C for 10 min to allow complex formation. All samples were centrifuged at 16,100 *xg* for 30 min at 4°C to remove potential aggregates.

Proteins in the soluble supernatant were then negatively stained using 0.75% (w/v) uranyl formate (pH 5.5-6.0) on 400-mesh carbon-coated copper grids (Pelco) as described in the main text. Samples were imaged under low dose conditions using a G2 Spirit TEM (FEI) operated at 120 keV. Micrographs were taken at 52,000x magnification with 2.16 Å per pixel using a 4k x 4k CCD camera (Gatan).

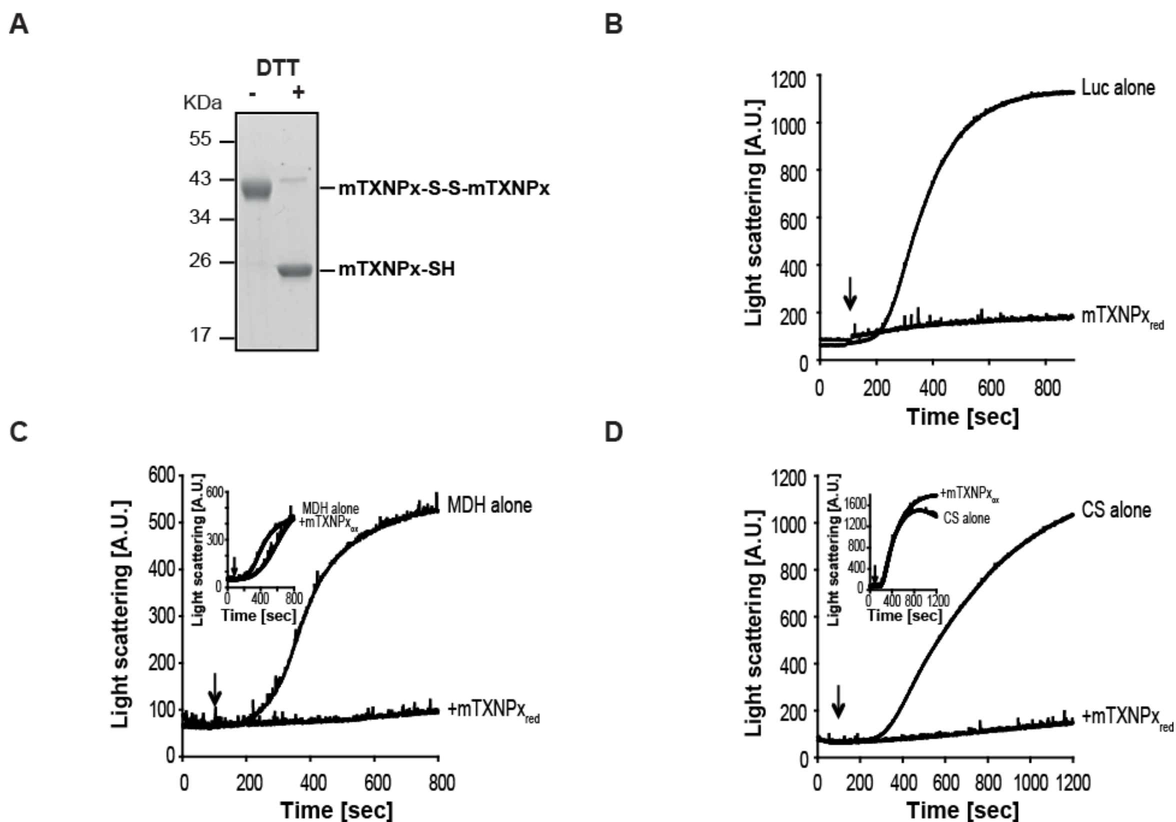

Figure S1: Reduced mTXNPx prevents thermal aggregation of different client proteins. A. Non-reducing SDS-PAGE of purified recombinant mTXNPx in the absence and presence of DTT. **B**. Influence of mTXNPx$_{ox}$ upon incubation with its physiologic NADPH/TR/TS_2/TXN2 reductant-system on the aggregation of luciferase at 41.5°C. A 20:1 molar ratio of mTXNPx to luciferase was used. The control reaction consisted of luciferase incubated with the components of the reducing system in the absence of mTXNPx. The arrow indicates the time point when luciferase was added to the reaction mixture. **C - D**. Influence of DTT-reduced or oxidized mTXNPx (inset) on the thermal aggregation of malate dehydrogenase (MDH) or citrate synthase (CS). Aggregation of MDH and CS was induced at 45°C and 43°C, respectively. A 20:1 molar ratio of mTXNPx to client proteins was used. Control reactions conducted in the absence of mTXNPx$_{red}$ were also supplemented with 0.2 mM DTT.

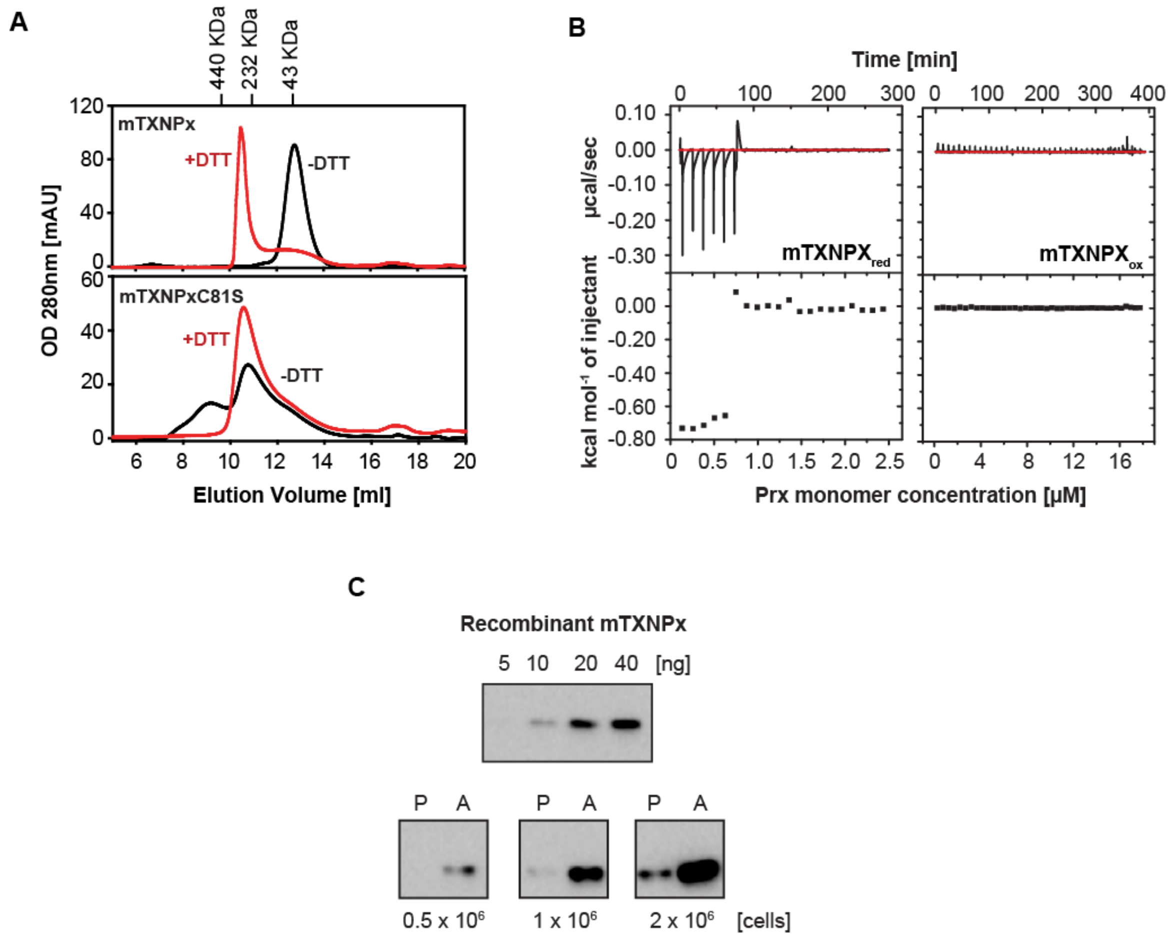

Figure S2. Oligomerization state, K_D and *in vivo* concentration of mTXNPx. A. The oligomeric states of oxidized (red traces) and reduced (black traces) mTXNPx WT or mTXNPxC81S as determined by size exclusion chromatography. The concentration of wild-type $mTXNPx_{red}$ and $mTXNPx_{ox}$ in the elution peak was determined to be 3 µM and 4 µM, respectively. Size exclusion of reduced mTXNPx was conducted in the presence of 1 mM DTT to prevent re-oxidation. The elution volumes of three standard proteins (43, 232 and 440 KDa) are shown. **B**. Determination of the decamer-dimer transition using isothermal titration microcalorimetry (ITC). Representative raw data output (top panel) and integrated heat pulses (bottom panel) of $mTXNPx_{red}$ (left panels) and $mTXNPx_{ox}$ (right panels) upon dilution into buffer. In the case of $mTXNPx_{red}$, exothermic heat pulses indicative of a decamer to dimer dissociation were monitored until a critical transition concentration (CTC) of 0.75 µM was reached in the cell. The average reaction enthalpy ΔH of $mTXNPx_{red}$ decamer dissociation was determined to be 644 ± 61 cal/mol monomer. In the case of $mTXNPx_{ox}$, no transition was observed indicating that $mTXNPx_{ox}$ remains dimeric up to concentrations of at least 100 µM. Averages of CTC as well as reaction enthalpy ΔH were derived from three independent experiments. **C**. Estimation of mTXNPx concentration in *Leishmania infantum* stationary-phase promastigotes and axenic amastigotes. Western blot of increasing quantities of recombinant mTXNPx was used as standard to estimate the concentration of the mTXNPx in stationary-phase promastigotes (P) and axenic amastigotes (A). The respective number of cells used for each experiment is stated below each panel. Protein bands were analyzed by densitometry and protein concentration was calculated using a reported volume of 75 fl and 15 fl for promastigotes and amastigotes, respectively [193,194]. The concentration of mTXNPx for the whole cell was estimated to be 1.6 – 6.1 µM and 49.1 – 102.0 µM for stationary phase promastigotes and amastigotes, respectively. However, since mTXNPx is exclusively mitochondrial, the intracellular concentration reached in mitochondria will be significantly higher. (Figure legend continues in the next page.)

Figure S2 (cont). Protein concentration was obtained with two independent experiments. Blots refer to one experiment. Densitometry values that were beyond the values of the standard curve were excluded from the analysis.

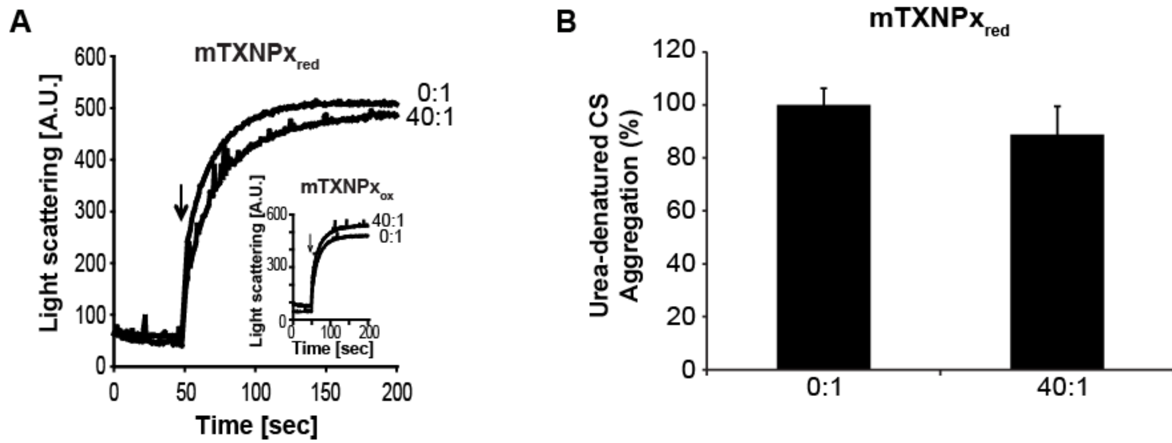

Figure S2: Reduced mTXNPx fails to protect chemically denatured CS at 30°C. Influence of $mTXNPx_{red}$ on the aggregation of chemically denatured citrate synthase (CS) at 30°C. **A**. GndHCl-denatured CS was diluted into a reaction system containing a 40:1 molar ratio of reduced or oxidized (inset) mTXNPx to CS. Aggregation of CS was followed by light scattering at 360 nm. The ratio of chaperone to client protein was calculated based on the monomeric form of mTXNPx. Arrow indicates the point at which the client protein was added to the reactions. **B**. Urea-denatured CS was diluted into a reaction system containing a 40:1 molar ratio of mTXNPxred to CS. The difference in light scattering signal before and 140 sec after addition of urea-denatured CS was set to 100% aggregation. At least three independent experiments were conducted and the StdDev of three individual measurements is shown.

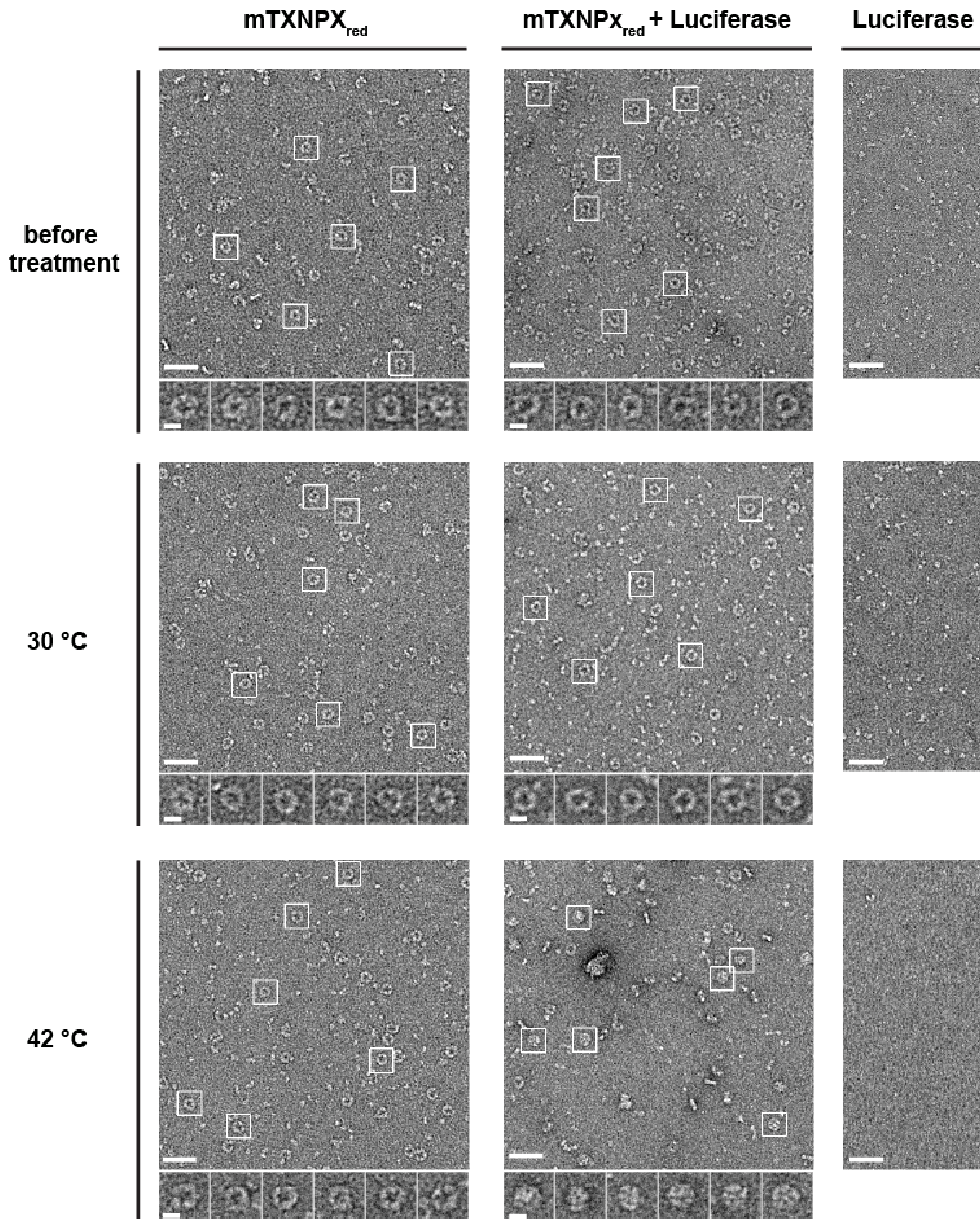

Figure S3: Reduced, decameric mTXNPx interacts preferentially with thermally destabilized luciferase. Negative stain EM of mTXNPx$_{red}$ alone or in the presence of luciferase before (*i.e.*, before treatment) or after 10 min incubation at either 30°C or 42°C. Luciferase alone was treated under the same conditions. Samples were centrifuged to remove all aggregated proteins and the supernatant was used for negative stain EM. Representative micrographs and selected single particle projections are shown with scale bars equal to 200 Å for the micrograph images and 50 Å for the boxed particles. This experiment was performed independently of the one shown in Figure 5. The percentage of mTXNPx$_{red}$ decamers in complex with luciferase varied between experiments.

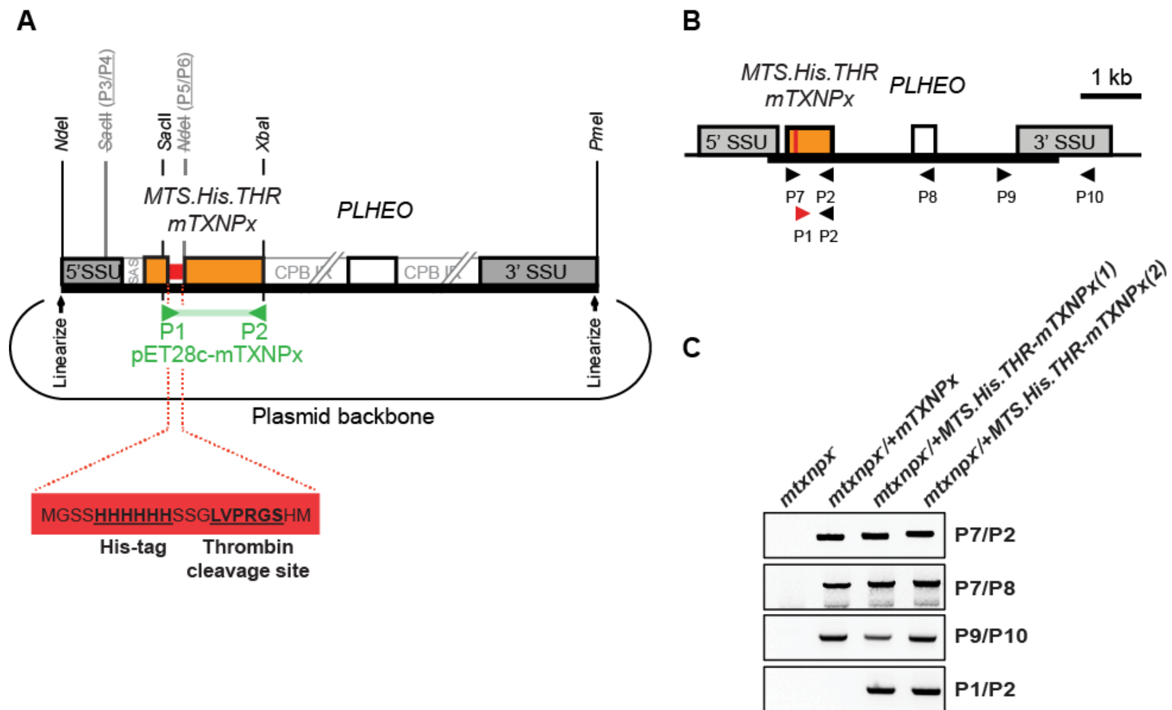

Figure S5: Generation of *L. infantum* transfectants expressing an N-terminally tagged mTXNPx mutant variant. A. Map of the pSSU-PHLEO-*infantum* MTS.His.THR-mTXNPx construct used for complementation of previously generated *mtxnpx*⁻ *L. infantum* parasites. The His.THR-mTXNPx fragment was PCR amplified from pET28c-*His.THR-mTXNPx* with primers P1 and P2 (Table S1), and cloned into the *Sac*II/*Xba*I sites of *pSSU-PHLEO-infantum-mTXNPx*. The resulting construct was linearized by digestion with *Nde*I+*Pme*I prior to electroporation of *mtxnpx*⁻ promastigotes. Depicted in the scheme are the restriction sites (*Nde*I, *Sac*II, *Xba*I and *Pme*I) relevant for molecular biology procedures. The endogenous mitochondrial targeting sequence (MTS) and the mTXNPx ORF encoding the mature protein are shown in orange, and the histidine-thrombin (His.THR) in red. Also illustrated is the amino acid sequence of His.THR. Grey boxes represent the 5' and 3' coding regions of the small subunit 18S rRNA (SSU) of *L. infantum*, required for integration of the construct into the SSU locus by homologous recombination. Grey framed white boxes refer to the splice leader acceptor site (SAS), as well as to the cysteine proteinase B intergenic region (CPB IR) cloned downstream of MTS.His.THR-mTXNPx and of PHLEO to guarantee adequate expression of both genes. **B**. Genomic organization of the small subunit 18S rRNA (SSU) locus of *mtxnpx*⁻*/+MTS.His.THR-mTXNPx* mutants with the integrated PHLEO construct (represented by the thick line). Arrowheads show the location of primers used for PCR diagnosis of the transfectants (in C.). **C**. PCR analysis of genomic DNA from *mtxnpx*⁻, *mtxnpx*⁻*/+mTXNPx*, and two independent *mtxnpx*⁻*/+MTS.His.THR-mTXNPx* promastigote clones, using specific primers to amplify the mTXNPx ORF (P7/P2), to diagnose for the presence of the PHLEO cassette (P7/P8) and its correct integration into the ribosomal locus (P9/P10), and to detect the MTS.His.THR-mTXNPx chimera (P1/P2) (primer location is represented in A.).

Table S1. List of oligonucleotides used in this work.

Primer number	Primer sequence
P1	5'GCAGTT***CCGCGG***GTTCGCGGCTACGTCGCCGCTTTTGATGGGCAGCAGCCATCATCATC-3'
P2	5'-tgc***tctaga***TCACATGTTCTTCTCGAAAAAC-3'
P3	5'-CTGGTGCCAGCACCAGCGGTAATTCCAGC-3'
P4	5'-GCTGGAATTACCGCTGGTGCTGGCACCAG-3'
P5	5'-CCGCGCGGCAGCCACATGAATCTGGACTA-3'
P6	5'-TAGTCCAGATTCATGTGGCTGCCGCGCGG-3'
P7	5'-cgc***ggatcc***ATGCTCCGCCGTCTTCCCA-3'
P8	5'-CCACTGCCGCAGCGTT-3'
P9	5'-GCGGTGTGTACAAATGGC-3'
P10	5'-gg***actagt***TCAGTCCTGCTCCTCGGC-3'

Clamp sequences are indicated in lower case, restriction sites in bold/italic, and mutations introduced by site directed mutagenesis underlined.

CHAPTER IV

Identification of the Client Binding Site of mTXNPx

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Unpublished data.

Abstract

Two-Cys-peroxiredoxins (2-Cys-Prxs) are ubiquitous and highly abundant proteins endowed with two functions, that of peroxidase and of chaperone. While the peroxidase activity has been extensively studied and characterized, the mechanisms underlying the chaperone function of 2-Cys-Prxs are not so well defined. In this study we used the mitochondrial Prx (mTXNPx) from *Leishmania*, whose chaperone function is crucial for parasite infectivity, to dissect the regions involved in client binding. Our approach involved the genetic incorporation of a photo-crosslinkable amino acid at various positions of the mTXNPx sequence, followed by *in vivo* and *in vitro* crosslinking with client proteins. Our preliminary results indicate that temperature-dependent structural rearrangements of the reduced mTXNPx decamer are required for exposure of the client binding site. Furthermore, they support previous results showing that mTXNPx binds clients in the middle of its decameric structure by providing experimental evidence that at least one putative binding site for clients appears to face the lumen of the decamer. This result supports previous claims that peroxiredoxins are the likely ancestors of chaperonins such as GroEL, whose mode of action also involves client binding inside a cavity. Regardless, data presented here also suggests that additional client binding sites, found in other regions of the mTXNPx decamer, might exist.

Introduction

Some members of the 2-Cys-peroxiredoxin (2-Cys-Prxs) family of proteins display chaperone activity [95,104]. This function, first discovered by Jang *et al.* in 2004, appears to protect cells from different stress conditions such as oxidative stress, heat-shock temperatures or zinc deficiency [104,130,135]. The cytoprotective role of 2-Cys-Prxs is likely dependent on their ability to protect a wide variety of proteins (also known as client proteins) from stress-induced unfolding and aggregation [104].

The mitochondrial Prx of *Leishmania* (mTXNPx) is a molecular chaperone essential for survival of these protozoan parasites in mammalian hosts (chapter III of this thesis). Data presented in chapter III revealed that mTXNPx in its reduced, decameric, ring-like conformation functions as an effective chaperone holdase that protects a range of proteins from heat-induced aggregation. Upon return to non-stress conditions, mTXNPx interacts with other chaperones and transfers its client proteins for proper refolding. Activation of the chaperone function of mTXNPx appears to be mediated by heat-induced structural rearrangements of the protein that lead to the exposure of hydrophobic surfaces, which are likely the binding sites for unfolding client proteins. Our previous study further suggested that the client binding site of mTXNPx faces the inside of its decameric ring. Two pieces of data supported this conclusion: i) electron microscopy (EM) analysis of mTXNPx-client complexes revealed mTXNPx decamers with dense particles in their center, consistent with the presence of the client protein; ii) interfering with the N'-terminus of mTXNPx, which faces the interior of the decamer, abrogated its chaperone function.

The present study aims at gaining further insight into the mechanism of chaperone activity of mTXNPx by defining which residues of the protein are involved in client binding. To accomplish this, we constructed several mTXNPx variants harboring the unnatural photo-crosslinkable amino acid *p*-benzoyl-L-phenylalanine (Bpa) at different positions in the mTXNPx sequence. We then analyzed the mTXNPx muteins which crosslinked with client proteins under protein unfolding heat-shock conditions. Following this approach we could identify five residues with probability of being directly implicated in interaction with client proteins. Moreover, our preliminary results support the idea that residues facing the interior of the decamer could be involved in client binding. However, additional binding sites may also co-exist. Furthermore, the results presented here pinpoint the importance of the heat-induced structural rearrangements necessary for the exposure of the client binding site of mTXNPx as many of the residues found to crosslink with client proteins under heat-stress conditions are found buried in the structure of the resting protein.

Overall, this study represents the first mechanistic-oriented approach to study the client binding site of a member of the 2-Cys-Prxs family.

Materials and Methods

Plasmid construction – In order to express mTXNPx without the mitochondrial import sequence or additional tags, pET28c6His-THR-TmTXNPx was PCR-amplified using *Pfu* polymerase and primers ccgcgca*catatg*AATCTGGACTATCAGATGTAC and gca*catatg*AGAAATAATTTTGTTTAACTTTAAGAAGGAGATATA (clamp sequences are indicated in lower case and restricition site in italic). The PCR product was digested with *Nde*I and subsequently re-ligated to originate a plasmid encoding mTXNPx devoid of any tag – pET28cTmTXNPx. The plasmid was further sequenced to ensure that no mutations on mTXNPx ORF had occurred and that the N-terminal 6His-Thr tag had been successfully removed.

Production of the mTXNPx-Bpa variants – For the construction of mTXNPx-Bpa variants a site-directed mutagenesis approach was used to substitute phenylalanine or tyrosine codons by an amber stop codon (TAG). For this, pET28cTmTXNPx was PCR-amplified using *Pfu* polymerase in the presence of a sense primer containing the desired mutations (see table S1). Following *Dpn*I digestion of the parental DNA for 1h at 37°C and desalting, the PCR product was used to transform *E. coli* (XL1-Blue strain). Mutant colonies were sequenced and confirmed to carry the desired site-directed mutation.

In vivo Bpa-mediated photo-cross-linking – *E. coli* cells (BL21 strain) were cotransformed with the plasmid encoding different mTXNPx-Bpa variants (pET28cTmTXNPx-Bpa) and the plasmid encoding the orthogonal aminoacyl-tRNA synthetase/tRNA pair (pEVOL) necessary for Bpa incorporation [195]. Cells were then plated in the presence of appropriate selective drugs and colonies allowed to grow overnight. Colonies were disloged using 2ml LB and cells further diluted to OD_{600}=0.4 into LB containing the adequate combination of antibiotics and 1mM Bpa (Bachem AG, Bubendorf, Switzerland) and further grown at 37°C for 1h. Afterwards, cells were cooled down to 30°C and expression of mTXNPx-Bpa variants and tRNA synthetase/tRNA pair induced with 10µM IPTG and 0.1% L-arabinose, respectively. Cells were grown for 3h at 30ºC after which they were harvested and suspended to 1OD_{600}=100µl. Each sample was then subjected to 30 min treatment at either 30ºC or 45ºC and further subjected, on ice, to UV irradiation (366nm) with a lamp distance of 2.5 cm for 10 min. Cells were then lysed by 3 cycles of sonication and 2 cycles of freeze-thaw. Total extracts were then suspended in SDS sample loading buffer before analysis by SDS-PAGE and immunoblotting using an anti-mTXNPx antibody.

In vitro Bpa-mediated photo-cross-linking – Total extracts of each strain expressing different Bpa variants of mTXNPx obtained as detailed above, were centrifuged for 30 min at max speed at 4ºC in order to obtain total soluble extract. Then,

20µl of this extract was diluted in 180 µl of 40 mM Hepes pH7.5, 10 mM NaCl, 1.1 mM DTT and incubated for 30 min at 30ºC for mTXNPx reduction. Subsequently, 150 nM recombinant luciferase was added to each protein extract and these further treated at 30ºC or 45ºC for 15 min. Samples were cooled down on ice and UV irradiated (366nm) with a lamp distance of 2.5 cm for 10 min and suspended in SDS sample loading buffer prior to being analyzed by SDS-PAGE and immunoblotting using an anti-luciferase antibody.

Results and Discussion

Genetic Incorporation of *p*-benzoyl-*L*-phenylalanine (Bpa) Into mTXNPx

In order to identify mTXNPx regions directly involved in client binding, we employed a technique originally developed by Schultz *et al.* [196]. This method involves the genetically-targeted incorporation of an unnatural, photocrosslinkable amino acid at precise positions in the protein. In order to accomplish this, the codon of a given amino acid is substituted by an amber suppressor stop codon, which is recognized by an orthogonal aminoacyl-tRNA synthetase/suppressor tRNA pair that specifically incorporates the unnatural amino acid at those positions during protein translation [195]. In the absence of the orthogonal aminoacyl-tRNA synthetase/suppressor tRNA pair or of the unnatural amino acid, translation stops at the genetically introduced stop codon causing premature chain termination. The unnatural amino acid that we have selected for this study is *p*-benzoyl-*L*-phenylalanine (Bpa), a photoreactive amino acid that acts as zero-length cross-linker upon exposure to UV light.

Using site-specific mutagenesis, we constructed 19 different mTXNPx variants, harboring Bpa at specific sites in mTXNPx (Fig. S1, residues that were substituted are highlighted in red). We individually replaced the codon sequences of either phenylalanine or related tyrosine residues with a TAG amber stop codon, reasoning that mTXNPx will likely tolerate these substitutions with the structural analogue Bpa. The 19 substitutions are fairly randomly distributed throughout the amino acid sequence of mTXNPx (Fig. S1). The resulting muteins were then introduced in *E. coli*. The Y169Bpa and F197Bpa muteins were not used in this study due to problems with strain growth after transformation. Therefore, out of the 19 mTXNPx variants generated, only 17 were tested *in vivo* for Bpa incorporation. *E. coli* cells individually expressing each of the mTXNPx variants as well as the aminoacyl-tRNA synthetase/suppressor tRNA pair were grown in the presence of exogenously added Bpa at 30°C for 3 hours. Subsequently, expression of

full-length mTXNPx, indicative of successful Bpa incorporation, was assessed by western blot analysis with an anti-mTXNPx antibody. As can be seen in Fig. 1, all 17 *E. coli* strains expressed the corresponding full-length mTXNPx (mTXNPx), indicating that Bpa was incorporated during the translation process. Bands smaller than the mTXNPx monomer can also be detected and likely represent truncated forms of the protein. Of notice, the Y38Bpa variant had markedly lower protein levels compared with the other variants.

Determination of mTXNPx residues putatively involved *in vivo* in client binding

In order to determine which residues are involved in client binding, bacteria expressing each of the 17 mTXNPx-Bpa variants were treated for 30 minutes at either 30°C or 45°C and subsequently subjected to UV light for 10 min to trigger crosslinking between mTXNPx and potential client proteins (Fig. 1). Treatment at 45°C was chosen not only as a cellular stressor that promotes protein aggregation *in vivo*, but also because earlier data suggested that high temperatures act as a trigger to activate the chaperone activity of mTXNPx (Chapter III of this thesis). At 30°C, mTXNPx is predicted to be chaperone-inactive and no protein unfolding intermediates are expected to accumulate. However, we identified a number of higher molecular weight bands upon UV treatment (+) for most mTXNPx-Bpa variants at 30°C, which likely result from spontaneous interaction of mTXNPx with *E. coli* proteins (Fig. 1). Of notice, the major band observed apart from the mTXNPx monomer (mTXNPx) likely corresponds to the dimeric form of mTXNPx (mTXNPx-mTXNPx). This band appears only after UV treatment indicating that it is Bpa-mediated and not disulfide-bonded mTXNPx. This result confirms the obligate homodimeric nature of mTXNPx. Of notice, the abundance of such UV-crosslinked dimer varies according to the mTXNPx mutein. The other bands present in the immunoblot and appearing as a smear above the dimeric mTXNPx band likely correspond to mTXNPx molecules crosslinked to *E. coli* proteins (mTXNPx-X). By comparing the crosslinking pattern and efficiency of the different variants at 30°C and 45°C, we identified at least three different groups of mTXNPx-Bpa mutants: Group I) variants crosslinking preferentially at 45°C and presenting distinct mTXNPx-X patterns at both temperatures (Y48Bpa, F54Bpa, F63Bpa, F70Bpa, F135Bpa); Group II) mutants crosslinking mostly at 45°C, but showing similar mTXNPx-X patterns at both temperatures (F20Bpa, Y42Bpa, F46Bpa, F47Bpa, F52Bpa), and Group III) variants not crosslinking (Y120Bpa, F167Bpa, F196Bpa) or crosslinking with similar efficiencies and patterns at 30 and 45ºC (Y5Bpa, Y8Bpa, Y38Bpa, Y86Bpa). Since the chaperone activity of mTXNPx is temperature activated, group I and II likely represent amino acids putatively involved in heat-induced client binding while group III encompasses amino acids that appear to crosslink non-specifically with *E. coli* proteins *in vivo*.

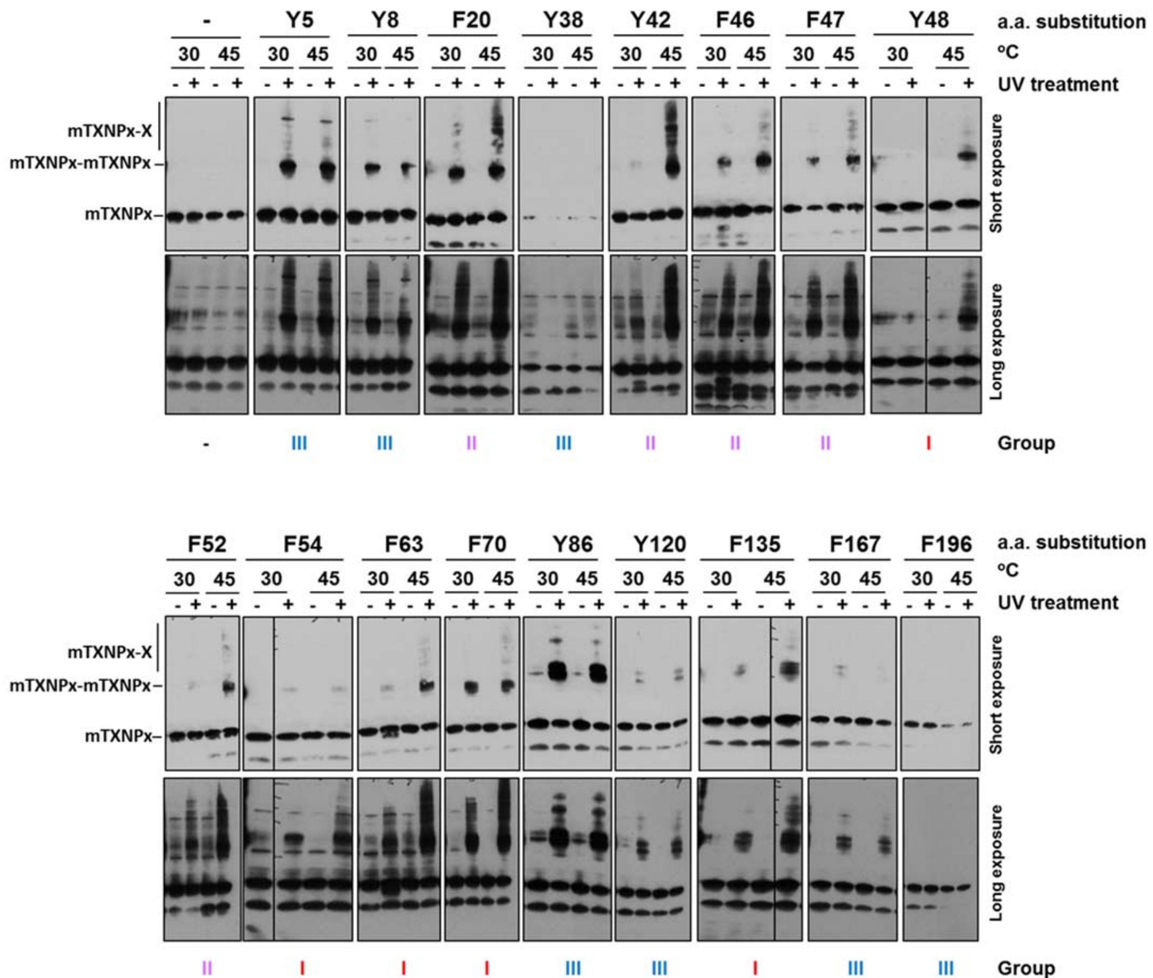

Figure 1: Identification of amino acids involved in mTXNPx interaction with client proteins *in vivo*. *Escherichia coli* expressing mTXNPx variants, in which specific amino acids were substituted by Bpa, were treated either at 30°C or 45°C for 30 min. Upon temperature treatment, cells were transferred to ice and either exposed (+) or not (-) to UV light for 10 min to induce crosslinking. Subsequently, bacteria were lysed and cell extracts analyzed by western blot with an anti-mTXNPx antibody. The two most prominent bands correspond to monomeric (mTXNPx) and dimeric mTXNPx mTXNPx-mTXNPx). Additional bands, seen as a smear, correspond to mTXNPx crosslinked to *E. coli* proteins (mTXNPx-X). Western blot signals detected at different intervals (short and long exposures) are shown. Wild-type mTXNPx, in which no amino acid was substituted, was used as negative control, and so were Bpa variants not irradiated with UV (-).

Exposure of the client binding site might involve the redisposition of several aminoacids

In order to depict which region(s) of mTXNPx might be involved in client binding, we mapped the three groups of amino acids onto the structure of the reduced mTXNPx decamer (Fig. 2). In order to facilitate visualization, only one dimeric unit of the decamer is shown (Fig. S2). Moreover, since mTXNPx has a ring-like shape (Fig. S2), four views of the structure were chosen: two top views (one from each side), and two side views [one

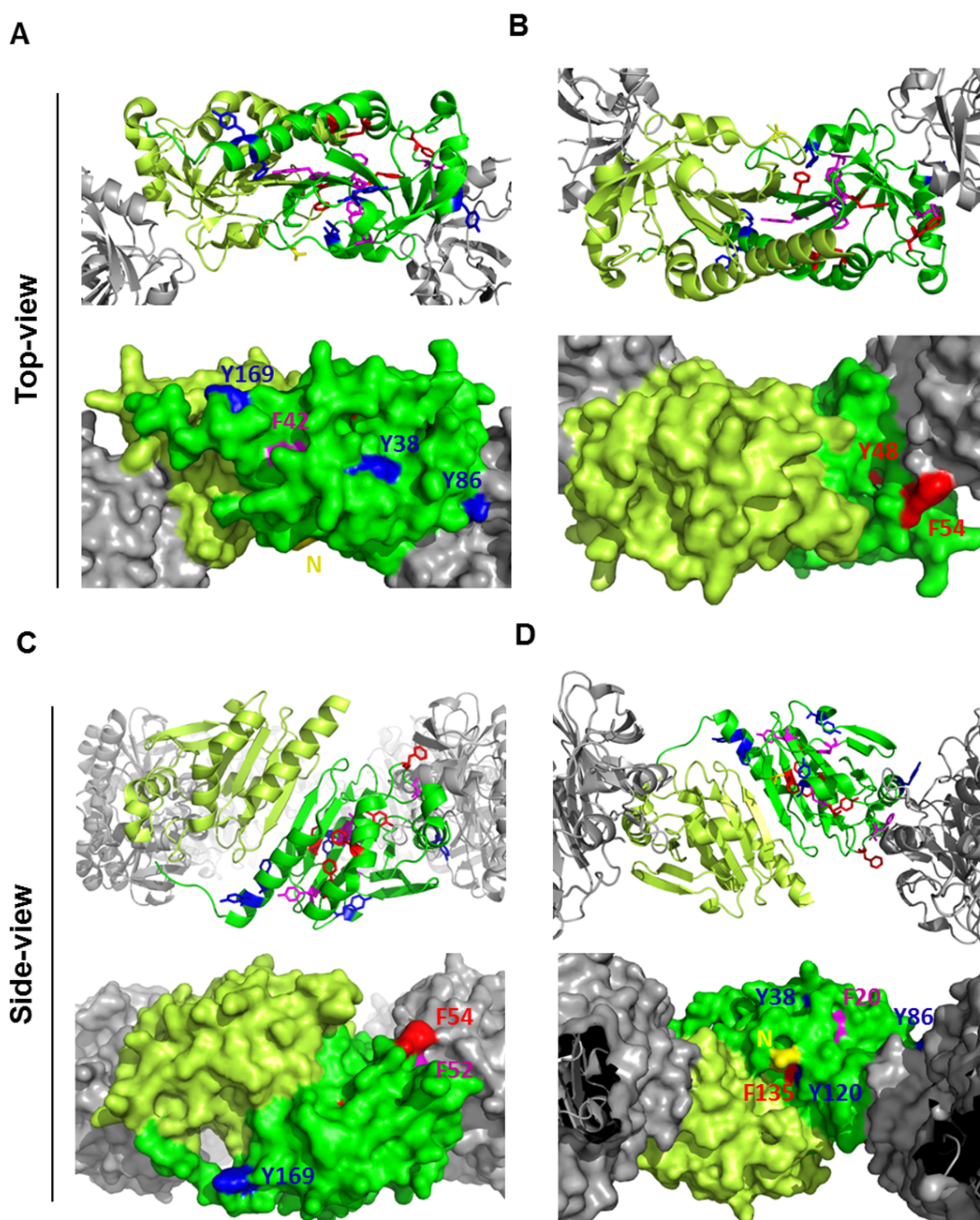

Figure 2: Mapping of Bpa-substituted residues in the quaternary structure of mTXNPx. Pictures show the quaternary structure of one mTXNPx dimer (in which each monomer is colored in two tones of green) within the context of the ring-like decamer (colored in grey) (see Fig. S2). Different views of the selected mTXNPx dimer are presented: two top views (**A** and **B**, which are 180º rotations of each other) and two side perspectives [one from the outside (**C**) and one from the inside (**D**) of the decameric ring]. Cartoon (top panels) and surface (bottom panels) representations of the structure are shown. Amino acids are colored differentially according to their ability to crosslink *E. coli* proteins at 30°C and 45°C. Accordingly, residues that i) crosslink only at 45°C are marked in red; ii) crosslink preferentially at 45°C, but also show some crosslinking at 30°C are highlighted in purple; iii) either do not crosslink or crosslink with similar efficiencies at both temperatures are shown in blue. The structure of the mTXNPx homologue from *L. brasiliensis* was used to highlight the residues involved in binding (4KB3) using pymol software.

from the outside (left panel) and the other from the inside (right panel) of the ring] (Fig. 2). Group I Bpa substitutions, which contain the residues most likely involved in client binding, are marked in red. Members of group II, which are potentially involved in binding, are marked in purple and members of group III which represent residues with the least likelihood of being involved in specific temperature-dependent interactions with other proteins are marked in blue.

As depicted in Fig. 2, the amino acids identified as candidates for mediating crosslinking with other proteins, i.e, members of groups I and II, are locate either buried in the mTXNPx structure or in its surface (as indeed also occurs with amino acids found not to crosslink- members of group III). Amino acids buried in the mTXNPx dimeric structure (top panels), are not visible in surface structure cartoons (bottom panels). Since the mTXNPx structure depicted in Fig. 2 was modeled based on an orthologue crystallized under non-stress conditions [155], it is reasonable to assume that the position of the buried residues identified as being involved in *in vivo* crosslinking (red and purple residues) does not reflect their real position in the temperature-activated chaperone species, which is known to be conformationally different from the resting molecule (Chapter III). Thus delineation of the client binding site according to the position of group I and II residues in the chaperone inactive structure is not straightforward. Previous findings suggested that heat-shock dependent structural rearrangements of mTXNPx are crucial for chaperone function, presumably by exposing an otherwise buried hydrophobic client binding site (Chapter III, Fig.4) Strikingly, the temperature-dependent structural rearrangements that mTXNPx undergoes seem not only to impact on its secondary structure (as seen on Chapter III, Fig.4), but also to translate into changes in the quaternary structure of the protein (Fig. S3). Preliminary negative staining electron microscopy (EM) data suggest that mTXNPx decamers reversibly disassemble at high temperatures (Fig. S3, right panels). This phenomenon, which is not observed when a client protein is present during heat-shock treatment (Fig. S3, left panels), is consistent with the idea that reorganization of the decamer might be important to expose the otherwise buried client binding site. In support of this suggestion, residues such as F52 and F54 and potentially Y48 which are close to the dimer/dimer interface appear to be involved in client binding. Follow-up studies are necessary to further test the hypothesis that restructuring of the decamer leads to exposure of the client binding site.

A group of mTXNPx residues putatively involved in client binding is surface-exposed (Fig. 2, bottom panels). We hypothesize that surface exposed residues that crosslinked *in vivo* under heat-stress conditions (Group I and II, marked in red and purple, respectively) may represent the amino acids that first interact with client proteins and initiate the structural rearrangements that lead to a fully exposed client binding site. As can be seen

in Fig. 2 (bottom panel), six of the residues involved in binding appear to be surface exposed (three red and three purple residues). Two of these, residues F52 and F54, sit on the edge of the dimer/dimer interface, almost facing the outer surface of the decamer (Fig. 2, view C). Two residues are on the side of the protein, Y48, that is adjacent to F54 (Fig. 2, view A and B), and F42 that is on the opposite side; both Y48 and F42 appear not to be fully exposed. The other two residues presumably interacting with clients, F20 and F135, face the lumen of the decamer (Fig. 2, view D). Based on the almost opposed distribution in the decamer of these groups of surface exposed residues it is difficult to envision how they constitute the same client-binding site. One possible explanation is that these groups of amino acids constitute different client binding sites. This mechanism was suggested for the small heat-shock protein Hsp18.1, which was shown to differentially interact with two client proteins (luciferase and malate dehydrogenase) *in vitro* [197].

***In vitro* crosslinking supports the idea that mTXNPx binds clients in the middle of its decameric structure**

In other to overcome the complexity inherent to *in vivo* studies where many and different client proteins are present, the regions of mTXNPx involved in binding a thermally unfolded client were analyzed *in vitro*. Luciferase, a client found to interact with the interior of mTXNPx decameric structure under heat-stress, was chosen for this purpose. We therefore prepared soluble extracts of bacteria expressing selected Bpa-variants, focusing primarily on those that showed significant temperature-dependent crosslinking *in vivo* (Y48, Y42, F54, F63, F70 and F135, Fig. 1). Several non-crosslinking variants [Y5, Y86 and F196 as well as wild-type protein (-)] were used as negative controls. To each of these extracts, we added recombinant luciferase and incubated the samples at either 30°C or 45°C. We then exposed extracts to UV light to trigger crosslinking. In order to specifically identify mTXNPx residues involved in luciferase binding, we used an anti-luciferase antibody to visualize higher migrating luciferase crosslinks. Of the residues tested, only Y48Bpa and F135Bpa showed a weak, yet detectable higher migrating luciferase band at 45°C but not at 30°C (Fig. 3, band marked with *). This band likely corresponds to mTXNPx-Luc crosslinking products since it does not appear in samples that were not treated with UV light or incubated at non-denaturing temperatures (Fig. 3). The apparent molecular weight of mTXNPx-Luc (between 95 and 130 kDa) is consistent with more than one mTXNPx monomer, presumably two or three mTXNPxs monomers, crosslinked to one luciferase. This result is interesting given the observation that a maximum of 5 spokes appears to coordinate binding of luciferase in the center of the decamer, a result that implies coordination of the dimer in binding (Chapter III, Fig. 5). Residue F63 cannot be excluded as a potential client binding amino acid since

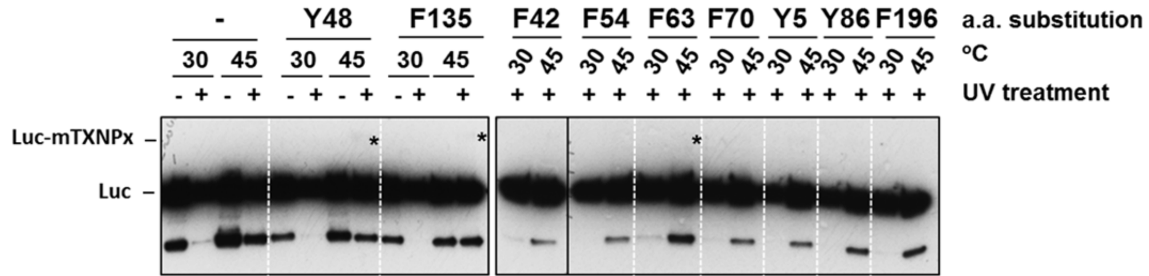

Figure 3: Amino acids Y48 and F135 of mTXNPx interact with thermally unfolded luciferase *in vitro*. Recombinant luciferase was added to total soluble extracts of cells expressing selected mTXNPx variants. Samples were then treated either at 30°C or 45°C for 30 min and subsequently exposed (+) or not (-) to UV light. Next, extracts were separated on an SDS-PAGE and immunoblotted with an anti-luciferase antibody. The prominent band corresponds to luciferase (Luc). Additionally, a faint band (marked with an asterisk) can be seen in some samples (Y48-Bpa, F135-Bpa and perhaps F63-Bpa) treated at 45°C. Importantly, for Y48-Bpa and F135-Bpa, this band only appears in samples treated at 45°C and UV irradiated showing that it likely corresponds to a complex between luciferase and mTXNPx (Luc-mTXNPx). Wild-type mTXNPx was used as negative control.

the presence of a very faint band of mTXNPx-Luc crosslinking was detected for the F63Bpa mutein as well although the non-UV treated control is missing. The residues found to be involved (marked in red and orange) in luciferase binding *in vitro* are highlighted in the mTXNPx structure (Fig. 4). Of the three amino acids (Y48, F63 and F135) found to crosslink with luciferase, F135 is surface exposed and faces the interior of the decamer (Fig. 4, view 4, bottom panel), Y48 is partially surface exposed and faces the top of the decamer (Fig. 4, view 2, bottom panel) and F63 is buried. Interestingly, the surface exposed residue F54 found as a potential crosslinker *in vivo* (Fig. 1 and 2) which faces the outside of the decamer (Fig. 2) does not appear to interact with recombinant luciferase under the conditions tested (Figure 3, 4). In part, these results could indicate that mTXNPx residues facing the lumen of the decamer (F135), and not those facing the outside (e.g. F54), are involved in luciferase binding *in vitro* supporting our previous EM data showing an additional density in the lumen of the decamer when mTXNPx is incubated with luciferase (chapter III, Fig. 5. and Fig. S4 and Fig. S3 of this chapter). Still, the involvement of other residues in luciferase binding (namely Y48 and F63) is not as straightforward to interpret in light of our EM data. Temperature-induced redisposition of these aminoacids may change their overall orientation on the structure allowing them to coordinate binding with F135 in the center of the structure, as suggested by EM data. However, in order to fully comprehend the crosslinking data, the structure of mTXNPx:luciferase complex would need to be solved. In line with this, efforts are being

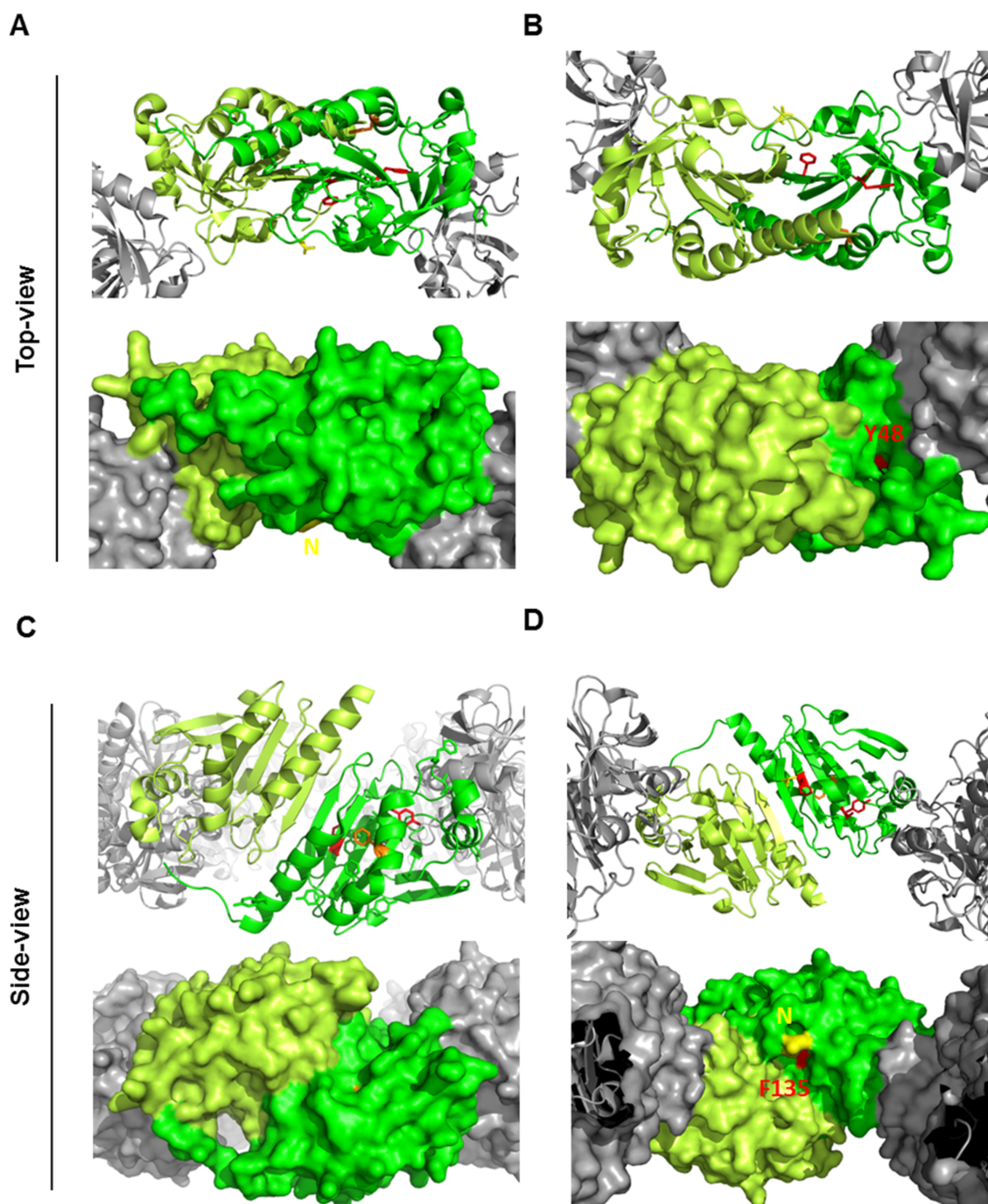

Figure 4: Residues involved in mTXNPx-luciferase interaction mapped on the structure of mTXNPx. Pictures show the quaternary structure of one mTXNPx dimer (in which each monomer is colored in two tones of green) within the context of the ring-like decamer (colored in grey) (see Fig. S2). Different views of the selected mTXNPx dimer are presented: two top views (**A** and **B**, which are 180º rotations of each other) and two side perspectives [one from the outside (**C**) and one from the inside (**D**) of the decameric ring]. Cartoon (top panels) and surface (bottom panels) representations of the structure are shown. Represented are mTXNPx aminoacids found, in vitro, to crosslink with recombinant luciferase upon exposure to 45°C. Aminoacids Y48 and F135 are colored in red while F63 is colored in orange. The structure of the mTXNPx homologue from *L. brasiliensis* was used to model to highlight the residues involved in binding (accession number: 4KB3) using pymol software.

made to obtain a sample of the mTXNPx:luciferase complex for cryo-EM studies.

Based on our previous data (chapter III) we suggested the N-terminus of mTXNPx, which faces the interior of the decamer, could play a role in client interaction. This hypothesis was consistent with the finding that addition of an N-terminal tag abrogated the chaperone function of mTXNPx *in vitro* and in the parasite context. In view of this model, it was striking to observe that N-terminus residues (namely Y5, Y8) were not involved in client binding (Fig. 1 and 3) raising the possibility that the N-terminus of the protein may have a different role than foreseen in mTXNPx chaperone function. Therefore in future studies it will be interesting to investigate the impact of N-terminal truncations on the chaperone activity of mTXNPx. Moreover, based on the *in vivo* and *in vitro* crosslinking data it will be necessary to compare the residues involved in binding luciferase to other model proteins such as malate dehydrogenase or citrate synthase. These studies will reveal whether interactions between mTXNPx and different clients are the same or vary according to the client (Fig. 2). It will also be important to test if the muteins found to be involved in crosslinking, both *in vivo* and *in vitro* display chaperone activity *in vitro*, are structurally similar to the wild type protein and, similarly to the latter, are able to reversible decamerize. Additionally, *in vitro* crosslinking experiments using the purified muteins need to be performed.

Overall, the results shown here represent the first step towards the characterization of the client-binding site in mTXNPx. This study implicated residues belonging to Group I (Y48Bpa, F54Bpa, F63Bpa, F70Bpa, F135Bpa) and to Group II (F20Bpa, Y42Bpa, F46Bpa, F47Bpa, F52Bpa) in mTXNPx client binding. Importantly, since the spacing between the substituted residues (Fig. S1) is, in some regions, larger than 30 residues, it will be worth conducting additional experiments focusing on residues around the potential client binding site(s). Although preliminary, these results support the idea that residues facing the lumen of mTXNPx ring-like structure might be involved in client binding. However, based on the *in vivo* crosslinking data, the presence of additional client binding sites cannot be disregarded. More importantly, this study highlights the significance of the structural rearrangements that mTXNPx undergoes at high temperatures which likely contribute to the exposure of the client binding site. Interestingly, these rearrangements may even involve the reorganization of the decameric structure of mTXNPx, a model that needs to be further explored. Uncovering the exact nature of such structural rearrangements as well as solving the structure of a chaperone-active form of mTXNPx will be crucial to define the client binding site and therefore constitute the next step of this project.

In a long term perspective, we expect that, by shedding light into the mechanistic aspects of mTXNPx chaperone activity, these findings aid in the development of inhibitors

of mTXNPx-client interactions. Inhibition of the chaperone function of mTXNPx is expected to negatively impact on *Leishmania* survival in their mammalian hosts, and might thus constitute a novel strategy to treat the devastating diseases elicited by these parasites.

Acknowledgements

We thank Bastian Groit for helpful discussions regarding *in vivo* crosslinking experiments and Philipp Koldewey for assistance with the software used to make Fig. 2 and 4. We also thank Prof. Schultz for providing the plasmid encoding the aminoacyl-tRNA synthetase/suppressor tRNA pair.

Supporting Information

5 8 20 38 42 46 47 48

MNLD**Y**QM**Y**RTATVREAAPQ**F**SGQAVVNGAIKDINMND**Y**KGK**Y**IVL**FFY**PM

52 54 63 70 86

D**F**T**F**VCPTEIIA**F**SDRHAD**F**EKLNTQVVAVSCDSV**Y**SHLAWVNTPRKKGGL

120 135

GEMHIPVLADKSMEIARD**Y**GVLIEESGIALRGL**F**IIDKKGILRHSTINDLPVGR

167 169 196 197

NVDEALRVLEA**F**Q**Y**ADENGDAIPCGWKPGQPTLDTTKAGE**FF**EKNM

Figure S1: Primary structure of mTXNPx showing the residues tested in the crosslinking studies. Highlighted in red are all phenylalanine and tyrosine residues that were genetically substituted by an amber stop codon that allows the incorporation of Bpa during protein synthesis.

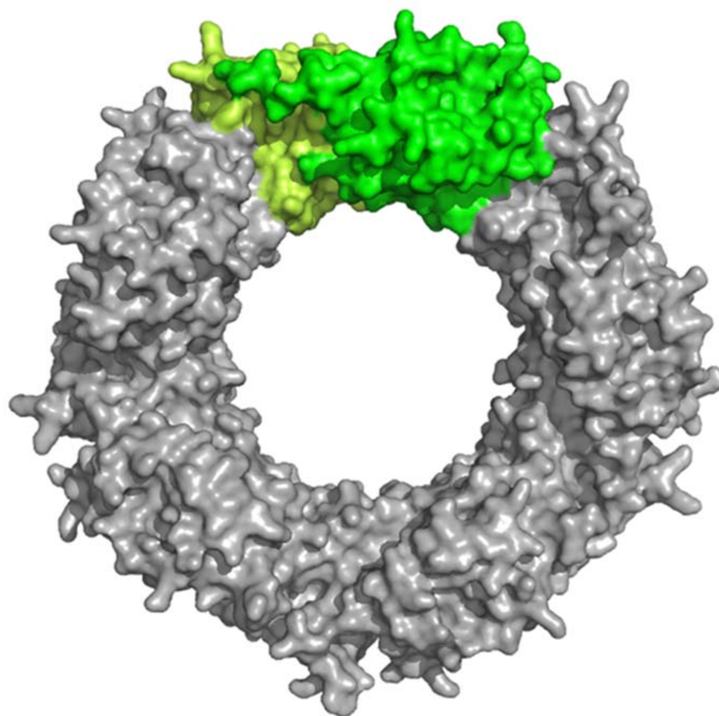

Figure S2: Structure of decameric mTXNPx. Shown is a top view of the surface of the decameric structure of mTXNPx. The structure used to create this cartoon is the solved structure of an mTXNPx homologue from *L. braziliensis* (accession number: 4KB3).

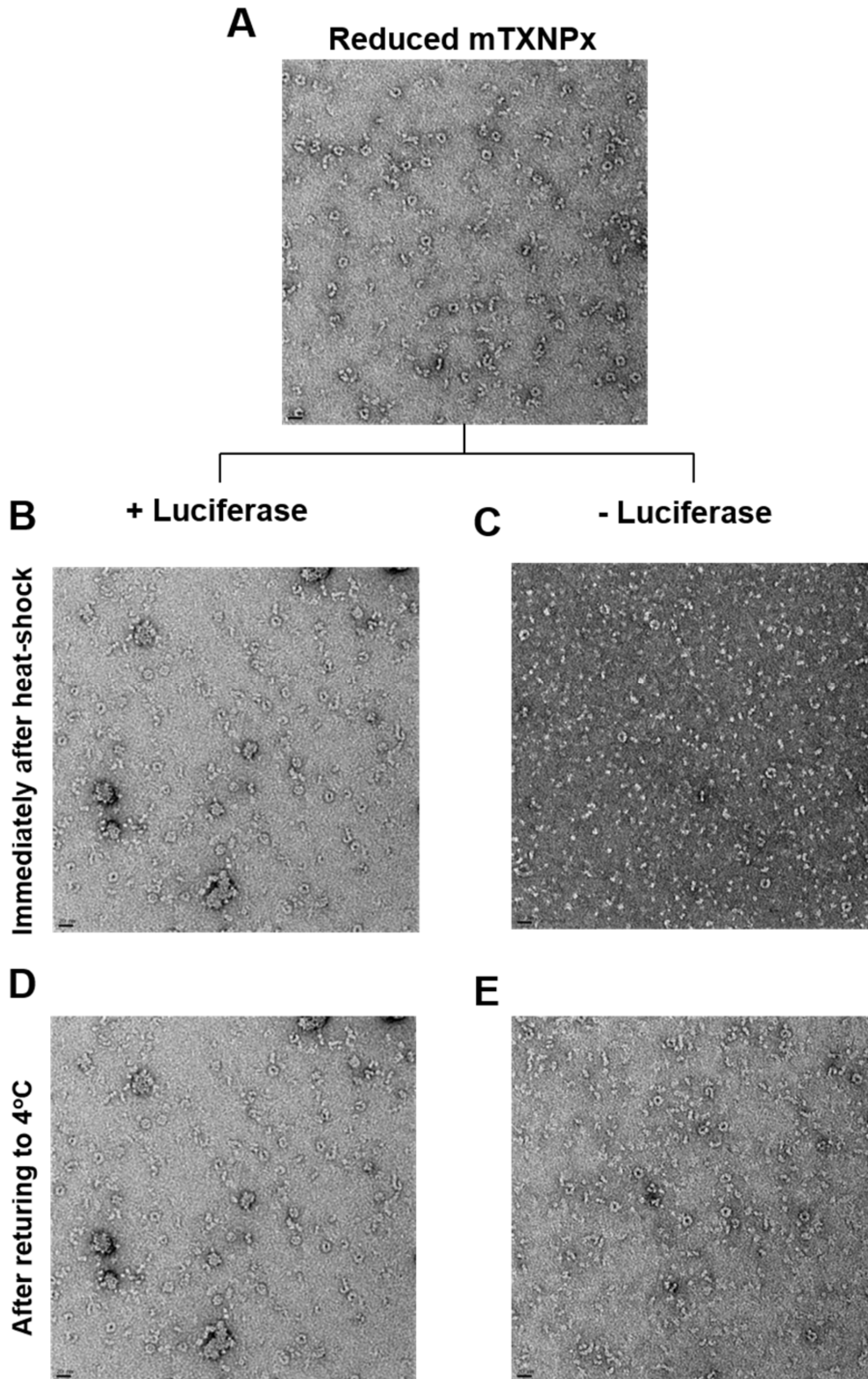

Figure S3: mTXNPx experiences heat-shock-induced rearrangements of its quaternary structure. Negative stain EM of mTXNPx upon reduction in the presence of 5mM DTT for 30min at 30°C (**A**). Following reduction, mTXNPx was incubated either alone (**C**) and or in the presence of luciferase before (**B**) for 10 min at 42°C and a sample applied immediately after treatment to an EM grid. Afterwards, both samples were centrifuged (max speed for 30 min at 4°C and the supernatant was used for negative stain EM (**D** and **E**). Representative micrographs are shown. Samples were and analyzed prepared as described on chapter **III**.

Table S1: List of oligonucleotides used in this work

A.A. Substitution	Primer sequence
Y5	5' ATGAATCTGGACTAGCAGATGTACCGTA 3'
Y8	5' CTGGACTATCAGATGTAGCGTACAGCGACTGTC 3'
F20	5' GAAGCTGCGCCACAGTAGTCCGGCCAGGCTGTC 3'
Y38	5' GATATAAACATGAACGACTAGAAGGGCAAGTACATTGTGC 3'
Y42	5' GAACGACTACAAGGGCAAGTAGATTGTGCTGTTTTTCTATC 3'
F46	5' GCAAGTACATTGTGCTGTAGTTCTATCCGATGGACTTC 3'
F47	5' CAAGTACATTGTGCTGTTTTAGTATCCGATGGACTTCACC 3'
Y48	5' GTACATTGTGCTGTTTTTCTAGCCGATGGACTTCACCTTC 3'
F52	5' GTTTTTCTATCCGATGGACTAGACCTTCGTTTGCCCGACC 3'
F54	5' CTATCCGATGGACTTCACCTAGGTTTGCCCGACCGAGATC 3'
F63	5' CCGAGATCATTGCGTAGTCGGATCGCCACG 3'
F70	5' GATCGCCACGCCGACTAGGAGAAGCTAAACAC 3'
Y86	5' CGTGCGATTCGGTGTAGTCTCACCTGGCGTG 3'
Y120	5' GAGATCGCTCGTGACTAGGGTGTGCTGATCGAG 3'
F135	5' GCTCTTCGAGGGCTCTAGATCATCGACAAGAAG 3'
F167	5' CGCGTGCTGGAGGCTTAGCAGTACGCGGACGAG 3'
Y169	5' CTGGAGGCTTTCCAGTAGGCGGACGAGAATGG 3'
F196	5' CACAAAAGCAGGCGAGTAGTTCGAGAAGAACATGTG 3'
F197	5' AAAGCAGGCGAGTTTTAGGAGAAGAACATGTGA 3'

Mutations introduced by site directed mutagenesis are underlined.

CHAPTER V

Concluding Remarks

At the beginning of this PhD project it was fully established that the mitochondrial tryparedoxin peroxidase (mTXNPx), a member of the 2-cys-peroxiredoxin family, was required for *Leishmania* to thrive in their mammalian hosts. Still, the crucial role provided by this protein during infection was incognito as it could not be credited to its well-characterized and long-thought important peroxidase activity. Consequently, the foundational question of this project was: Why is mTXNPx essential for *Leishmania* survival in mammals? Data gathered in this thesis provide an answer to this question by demonstrating that mTXNPx is important for amastigote endurance in mammals owing to its inherent capacity to act as a molecular chaperone. Apart from contributing towards the understanding of the role of mTXNPx in *Leishmania,* this study also reinforces the physiological significance of the chaperone function of 2-Cys-Prx family members. Moreover, based on molecular studies carried out on mTXNPx, the work presented here offers a mechanistic basis to interpret the chaperone activity of 2-Cys-Prxs in general.

The chaperone function of mTXNPx is important for *Leishmania* survival in mammalian hosts

Parasites of the genus *Leishmania* are responsible for an ancient disease that currently affects millions of people worldwide and for which no satisfactory treatment and prophylaxis are available [4, 16]. The establishment of a successful *Leishmania* infection is multifactorial depending both on host and on parasite traits. One of such factors is the capacity of *Leishmania* to withstand the severe environmental changes that accompany their transition from an insect vector into a mammalian host [73]. In this thesis, we compellingly demonstrate that the chaperone activity of mTXNPx plays a crucial role in *Leishmania* adaptation to the mammalian host environment, specifically to the rise in temperature associated to parasite transmission from insect vectors (ambient temperature) to mammals (temperature ~37°C). Furthermore, it is conceivable that the chaperoning effect of mTXNPx is also important whenever parasites undergo other thermal stresses such as those associated with host´s fever episodes (temperature ≥37°C). Consistent with the importance of mTXNPx for survival of the mammalian stage of *Leishmania*, expression of this chaperone is greatly increased in axenic amastigotes relative to promastigotes grown in culture (Chapter III, Fig. S2C). In the *in vivo* context, we could furthermore confirm, by immunofluorescence analysis of infected tissues, that mTXNPx was consistently expressed by *Leishmania* throughout the time course of a murine infection (for up to 30 days) (Teixeira F., Tomás A.M., unpublished results). Based on these observations, it is reasonable to hypothesize that the chaperone activity of mTXNPx is relevant for *Leishmania* fitness during the course of infection as well. If that is

indeed the case, then mTXNPx can be envisaged as a potential target for leishmaniasis drug development. Even though mTXNPx druggability was not addressed in this study, the possibility of abrogating chaperone activity by interfering with the N-terminus of the protein (shown in Chapter III) opens optimistic perspectives towards the design of structural inhibitors. The design of such inhibitors, which should conceptually mimic the effect of N-terminus interference, would be greatly facilitated by solving the structure of the chaperone-inactive protein (*i.e.* the protein carrying the N-terminal histidine-thrombin tag) and comparing it with the wild type version. Besides interference with the N-terminus, i) disruption of the mTXNPx decamer, ii) inhibition of the temperature-dependent structural rearrangements that appear to be required for chaperone activation, or iii) interference with the client binding site would, according to our data, also inhibit chaperone function and, consequently, arrest development of *Leishmania* amastigotes.

When considering the possibility of inhibiting the chaperone activity of mTXNPx for therapeutic purposes, it is important to recognize that peroxiredoxins are widespread and highly conserved proteins, present also in *Leishmania* hosts. Consequently, a drug targeting mTXNPx chaperone activity might affect mammalian Prxs as well with detrimental consequences for the host [123]. This will be particularly challenging if mammalian Prxs also prove to be crucial chaperones. Furthermore, inhibition of mTXNPx chaperone activity may, in some circumstances, perturb the peroxidase function of host Prxs. This is expected whenever inactivation entails disruption of the mTXNPx decamer, as it is well-established that this oligomeric state, although not absolutely crucial, is also important for peroxidase activity. A note of optimism to outline that potential side-effects associated with undesirable inhibition of host Prxs may be reduced if drugs are administered with modern systems (*e.g.* liposomes, nanoparticles) that deliver inhibitors to their specific target cells (e.g. *Leishmania*-harboring macrophages).

mTXNPx is part of the *Leishmania* stress response

The chaperone activity of mTXNPx confers thermotolerance to *Leishmania* promastigotes (insect stage) once these are transmitted to mammals, by protecting parasite molecules from heat-induced aggregation. mTXNPx can, therefore, be inscribed as a new component of the *Leishmania* stress response, *i.e.* of the set of reactions that helps parasites to respond and adapt to new, often stressful, environmental conditions as those found during transition between hosts [73]. Importantly, the observation that mTXNPx changes its structure in response to temperature fluctuations suggests that this chaperone is also capable of directly sensing heat. We hypothesize that, upon *Leishmania* transmission to the mammalian host, mTXNPx senses the increase of temperature and

changes its structure so as to become chaperone-active and, consequently, protect thermolabile proteins from aggregation. An advantage of this temperature-mediated regulation of mTXNPx chaperone activity is that it provides *Leishmania* an efficient and transcriptionally-independent mechanism to quickly respond to heat stress. As outlined in chapter I, in the case of digenetic parasites such as *Leishmania*, environmental changes can be felt not only as stresses but also as cues for parasite differentiation [73]. The cellular responses mounted during parasite transition mirror this dichotomy, being perceived as protective or adaptive. We believe that the response conferred by mTXNPx chaperone function is protective as parasites lacking this enzyme appear capable of differentiating into amastigote-like forms *ex vivo* (*i.e.* in *Leishmania*-infected macrophage monolayers), despite being unable to replicate and give rise to a successful infection in mice.

This work suggested that mTXNPx is part of the *Leishmania* stress response, assisting parasites to respond to heat stress when promastigotes are transmitted to mammals and, possibly also, when amastigotes experience episodes of host fever. Still, from our *in vivo* experimental data we could not ascertain whether, in addition to being required during the first moments after parasite entrance in the mammal, mTXNPx is crucial at later stages of infection or if the amastigote employes alternative mechanisms that allow adaptation to higher temperatures rendering the chaperone activity of mTXNPx non-essential. As outlined before, the answer to this question is very important when considering the use of mTXNPx as therapeutic target. In fact, if mTXNPx proves to be essential only during the first moments post-infection (*i.e.* during the adaptative period), its specific inhibition as a way of treating leishmaniasis will not apply. Instead, its use as a target for a prophylactic drug could be considered. To address the relevance of mTXNPx throughout infection an approach allowing the conditional expression of mTXNPx at different time-points would have to be employed. Such approach could encompass different strategies i) the conditional downregulation of mTXNPx using siRNA or ii) the inducible, promoter-dependent switch of mTXNPx expression. Unfortunately, these experiments cannot be easily performed as *L. infantum* (the species used in this study) lacks the machinery for RNAi studies and inducible systems are not robust [54,198]. One possibility to circumvent this technical limitation would be to perform such studies in *Trypanosoma brucei*, a member of the trypasomatid family where the RNAi machinery is fully functional [44,54]. First, though, it would be necessary to confirm that mTXNPx is also a crucial chaperone in this organism. Alternativelly, iii) mTXNPx expression could be coupled to a toxic protein allowing for negative selection of mTXNPx expression. Based on this approach *Leishmania* would favour the elimination of the plasmid unless the gene

of interest is essential. This approach was employed by Dacher *et al* to study the druggability of essential proteins in *Leishmania* promastigotes [198].

Discovery of the chaperone activity of mTXNPx constitutes a paradigm shift in the study of TXNPxs

The finding that a member of the tryparedoxin peroxidase family (TXNPx) can function as a chaperone, together with the conclusion that this activity, and not the peroxidase function, is crucial for *Leishmania* survival, constitutes a turning point in TXNPx research. For many years the paradigm was that TXNPxs were important antioxidant devices, their activity being crucial, for example, when trypanosomatids encounter the oxidative insult mounted by phagocytic cells during parasite invasion [110]. This conviction was consubstantiated by studies showing that modulation of TXNPx expression impacts on parasite sensitivity to peroxides [145,150,153,154]. In the specific case of mTXNPx-overexpressing parasites, these were shown to have an increased resistance towards exogenously added *t*-butyl hydroperoxide [145]. In light of our recent results showing that the peroxidase activity of mTXNPx is redundant all along the parasite life cycle, one has to recognize the limitation of such overexpression studies. In fact, if the levels of a peroxidase are artificially increased, an increment in peroxide resistance is not surprising; still, this does not necessarily convey physiological significance to the peroxidase activity. In this respect, knockout or knockdown experiments are more informative. Nevertheless, for proteins with two functions such as Prxs, subsequent validation of the essentiality of each possible activity (either the peroxidase or chaperone activity in the case of Prxs) is required to address the physiological relevance of that particular protein.

In view of our results it is important to reassess the function of TXNPxs in other trypanosomatid species. As many trypanosomatids (*e.g.*, the agents of Chagas and sleeping sickness diseases) are digenetic parasites, it is expected that the chaperone function of mTXNPx is also important for these organisms to cope with heat stress associated with transmission to mammals. Similarly, it is imperative to test if the cytosolic TXNPx isoforms also exhibit chaperone activity and, if so, to address its physiological relevance. Previous studies, reporting that downregulation of the cytosolic enzymes render parasites more sensitive towards peroxides appear to suggest that their peroxidase activity may indeed be crucial [153].

Study of mTXNPx conveys physiological relevance to the recently discovered chaperone function of Prxs

Over the last decade, members of the 2-Cys-peroxiredoxin family have been shown to work as molecular chaperones, a function that is thought to protect cells when these are exposed to stress conditions [104,130,135]. Although assumed to be important under such circumstances, the extent to which this function is essential for cells had never been directly tested. This was due to the fact that the mechanism underlying the chaperone activity of Prxs was not known and, as such, designing a chaperone-inactive Prx to test the essentiality of this function was not been possible. While studying mTXNPx, we have found that the N-terminally His.THR-tagged protein was, contrary to the wild-type protein, chaperone-inactive. Although the reasons for this inactivation are not yet fully understood, this chaperone-inactive version of mTXNPx provided us with one appropriate means to conclusively demonstrate that the second activity of this Prx is essential for *Leishmania* survival. Therefore, this result conveyed for the first time physiological significance to the chaperone function of members of the 2-Cys-Prxs family and showed that their chaperone activity does not overlap with that of other chaperones. Moreover, the finding that mTXNPx is a member of a growing group of stress-activated chaperones that upon stress are able to interact and transfer clients to additional chaperones for proper refolding, demonstrates that Prxs have the potential of being fully integrated members of the proteostasis network of a cell, a result that adds further functional relevance to the chaperone activity of Prxs.

Research on mTXNPx sheds light on the molecular basis of 2-Cys-Prxs chaperone function

The discovery in 2004 that 2-Cys-Prxs are endowed with chaperone activity represented a breakthrough in the field [104]. Since then, most studies have focused on establishing chaperone activity to other Prxs, and also on identifying the requisites for such function (*e.g.*, the trigger necessary for activation and the contribution of the oligomeric state for chaperone activity) [95, 104-106, 135-138]]. Regarding the activation mechanism, the literature is quite disperse. It suggests that a number of different and seemingly unrelated factors can trigger chaperone activity, and it also puts forward various structure-function relationships. Here we identified the decameric structure of mTXNPx as a requisite for chaperone activity. Importantly, we also suggested that this oligomeric species requires a heat stimulus to become a fully activate chaperone. In an attempt to conciliate our findings with those of others, we have proposed that the decamer, which is the minimal oligomeric state endowed with all the structural motifs

necessary for chaperone activity, represents the scaffold for function, and that unfolding conditions such as those induced by heat-shock temperatures represent the trigger for function. Based on our model, we foresee that overoxidation, phosphorylation, low pH and other factors previously suggested to be chaperone activators, might instead work as decamer stabilizers simply by decreasing the K_D for decamerization [131]. Even though we envisage the decamer as the framework necessary for chaperone activity, we cannot ignore that, under stress conditions, some Prxs preferentially assemble into HMW species endowed with "super-chaperone" activity. In this respect it will be important to understand why some Prxs are able to form these HMW species [104], how exactly they assemble and the importance of post-transcriptional modifications for this phenomenon. Moreover, for Prxs able to form HMW species it will be interesting to explore the physiological relevance of these structures and the extent to which their function can be substituted by chaperone-active Prxs not able to form HMW-species.

When studying molecular chaperones it is important to define the exact nature of their client binding site and the specific structural rearrangements necessary for its exposure. In the case of Prxs the client binding site was never directly explored. Here, using electron microscopy of mTXNPx-client complexes we showed that mTXNPx binds client proteins inside its decameric, ring-like structure. This result, besides constituting the first direct evidence of client binding by a member of the 2-Cys-Prx family, also provided a rational for the requirement of this oligomeric structure for function. To further explore the nature of the client binding site of mTXNPx at the molecular level, we are employing a strategy that involves the genetically imposed incorporation of a photo-crosslinkable amino acid at different positions of the mTXNPx sequence. Although preliminary, crosslinking results support the idea that residues facing the interior of the decamer might be involved in client binding, they also implicate amino acids located in other positions of the decamer as important for binding. Furthermore, the crosslinking data show that residues at the N-terminus of mTXNPx, which face the lumen of the decamer, appear to be not directly involved in client interaction. Importantly, by showing that under homeostasis many residues putatively implicated in binding are structurally buried, this approach highlighted the importance of the temperature-induced conformational changes that mTXNPx undergoes at heat-shock temperatures for exposure of the client binding site. The exact nature of these structural rearrangements is not known and needs to be further explored. Our preliminary EM data suggest, however, that these might involve the restructuring of the decamer. In a broader sense, it will be important to identify the residues involved in client binding in other Prxs known to display chaperone activity and the structural rearrangements necessary for such function. Having established that the decameric form of Prxs is the minimal oligomeric form displaying chaperone activity it would be important

that, for coherence, additional studies focus on this structure. Only after the systematic study of several Prxs one can start drawing conclusions about the basics of the chaperone activity of this highly conserved, widelyspread, and important family of proteins.

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LIST OF ABBREVIATIONS

2-Cys-Prxs – 2-Cysteine-Peroxiredoxins

AIDS – Acquired Immune Deficiency Syndrome

ATP – Adenosine triphosphate

bis-ANS - 4,4′-bis-anilino-1,1′-binaphthyl-5,5′-disulfonic acid

Bpa – *p*-benzoyl-*L*-phenylalanine

BSA – Bovine Serum Albumin

CD – circular dicroism

CL – Cutaneous Leishmaniasis

MCL – Mucocutaneous Leishmaniasis

C_P – Peroxidatic cysteine

C_R –Resolving cysteine

CTC – Critical Transition Concentration

cTXNPx – cytosolic Tryparedoxin Peroxidase

CVL – Canine Visceral Leishmaniasis

Cys - Cysteine

DALY – Disability-Adjusted Life Years

DCL – Diffuse Cutaneous Leishmaniasis

DNA – Deoxyribonucleic Acid

DTT – Dithiothreitol

ELISA - Enzyme-Linked Immunosorbent Assay

EM – Electron Microscopy

FBSi – inactivated Fetal Bovine Serum

FF – Fully Folded

gRNA – Guide RNA

gTXNPx – glycosomic Tryparedoxin Peroxidase

HIV – Human Immunodeficiency virus

HSP – Heat Shock Protein

IFAT – Immunofluorescence Antibody Test

IHMT – Instituto de Higiene e Medicina Tropical

IPTG – isopropyl-β-D-thiogalactopyranoside

kDNA – kinetoplastid DNA

KJE-system – DnaK/DnaJ/GrEp System

LU – Locally unfolded

MCL – Mucocutaneous Leishmaniasis

MTS – mitochondrial targeting sequence

mTXNPx – mitochondrial Tryparedoxin Peroxidase

NADPH – Nicotinamide Adenine Dinucleotide Phosphate

ONLeish – Observatório Nacional das Leishmanioses

ORF – Open Reading Frame

PCR – Polymerase Chain Reation

PKDL – Post-kala-azar dermal leishmaniasis

PREX – PeroxiRedoxin classification indEX

Prxs – Peroxiredoxin, frequently used indistinctively of 2-Cys-Orxs

PTM – Post-translational Modification

PV – Parasitophorous vacuole

RNA – Ribonucleic Acid

RNAi – interference RNA

SEC – size-exclusion chromatography

Ser – Serine

TR – Trypanothione Reductase

tRNA – transfer RNA

TS_2 – trypanothione disulfide

TXN – Tryparedoxin

TXNPx – Tryparedoxin peroxidase

VL – Visceral Leishmaniasis

WHO – World Health Organization

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"None of Us Is As Smart As *All of Us*"
"Nenhum de nós é tão inteligente quanto todos nós juntos."
Warren Bennis

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APPENDIX

List of publications and scientific communications

Publications related to the work in the report

2015 - Teixeira F, Castro H, Cruz T, Tse E, Koldewey P, Southworth DR, Tomás AM, Jakob U. Mitochondrial peroxiredoxin functions as crucial chaperone reservoir in Leishmania infantum. *Proc Natl Acad Sci U S A. 112(7):E616-24*

2011 - Castro H, **Teixeira F**, Romao S, Santos M, Cruz T, Fórido M, Apperlberg R, Oliveira P, Ferreira-da-Silva, F and Tomás AM. *Leishmania* mitochondrial peroxiredoxin plays a crucial peroxidase-unrelated role during infection. Insights into its novel chaperone activity. *PLoS Pathogens, 7(10):e1002325.*

Communications related to the work in the report

Oral presentations

2013 - **Teixeira F**, Castro H, Gray MJ Southworth D, Tomás AM, Jakob U. The *Leishmania infantum* mitochondrial tryparedoxin peroxidase – A temperature-regulated chaperone essential to parasite infectivity? WorldLeish 5. 13th to 17 th May, Porto de Galinhas, Pernambuco, Brazil

Posters in conferences

2012 - Teixeira F, Casto H, Southworth D, Tomás AM, Jakob U. Leishmania infantum Peroxiredoxin – A Temperature-Regulated Chaperone. FASEB Meeting. 4th- 9th July, Vermont, USA

Publications resultant from collaborations

2014 - Santos-Gomes GM, Rodrigues A*, **Teixeira F***, Carreira J, Pires GA, Carvalho S, Santos-Mateus D, Martins C, Marques C, Tomás AM. Immunization with the Leishmania infantum recombinant cyclophilin protein 1 confers partial protection to subsequent parasite infection and generates specific memory T cells. *Vaccine, 32(11):1247-53* (*these authors contributed equally to this work)

2012 - Rodrigues RF, Castro-Pinto D, Echevarria A, Dos Reis CM, Del Cistia CN, Sant'anna CM, **Teixeira F**, Castro H, Canto-Cavalheiro M, Leon LL, Tomás A.

Investigation of trypanothione reductase inhibitory activity by 1,3,4-thiadiazolium-2-aminide derivatives and molecular docking studies. *Bioorg Med Chem; 20(5):1760-6*

Courses

2012 - Biomolecular Interactions Analysis. INSTRUCT Course. 12th to the 16th of November, IBMC, University of Porto.

PLoS PATHOGENS

Leishmania Mitochondrial Peroxiredoxin Plays a Crucial Peroxidase-Unrelated Role during Infection: Insight into Its Novel Chaperone Activity

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Abstract

Two-cysteine peroxiredoxins are ubiquitous peroxidases that play various functions in cells. In *Leishmania* and related trypanosomatids, which lack catalase and selenium-glutathione peroxidases, the discovery of this family of enzymes provided the molecular basis for peroxide removal in these organisms. In this report the functional relevance of one of such enzymes, the mitochondrial 2-Cys peroxiredoxin (mTXNPx), was investigated along the *Leishmania infantum* life cycle. mTXNPx null mutants (*mtxnpx*$^{-}$) produced by a gene replacement strategy, while indistinguishable from wild type promastigotes, were found unable to thrive in a murine model of infection. Unexpectedly, however, the avirulent phenotype of *mtxnpx*$^{-}$ was not due to lack of the peroxidase activity of mTXNPx as these behaved like controls when exposed to oxidants added exogenously or generated by macrophages during phagocytosis *ex vivo*. In line with this, *mtxnpx*$^{-}$ were also avirulent when inoculated into murine hosts unable to mount an effective oxidative phagocyte response (B6.p47$^{phox-/-}$ and B6.RAG2$^{-/-}$ IFN-$\gamma^{-/-}$ mice). Definitive conclusion that the peroxidase activity of mTXNPx is not required for parasite survival in mice was obtained by showing that a peroxidase-inactive version of this protein was competent in rescuing the non-infective phenotype of *mtxnpx*$^{-}$. A novel function is thus proposed for mTXNPx, that of a molecular chaperone, which may explain the impaired infectivity of the null mutants. This premise is based on the observation that the enzyme is able to suppress the thermal aggregation of citrate synthase *in vitro*. Also, *mtxnpx*$^{-}$ were more sensitive than controls to a temperature shift from 25°C to 37°C, a phenotype reminiscent of organisms lacking specific chaperone genes. Collectively, the findings reported here change the paradigm which regards all trypanosomatid 2-Cys peroxiredoxins as peroxide-eliminating devices. Moreover, they demonstrate, for the first time, that these 2-Cys peroxiredoxins can be determinant for pathogenicity independently of their peroxidase activity.

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Introduction

The last 15 years have contributed decisively towards the dissection of the enzymatic pathways that lead to peroxide elimination in trypanosomatids, a group of organisms that includes *Leishmania* spp., *Trypanosoma brucei* and *Trypanosoma cruzi*, the causative agents of the different manifestations of leishmaniasis, African sleeping sickness and Chagas' disease, respectively. In these protozoan parasites, which lack the highly efficient enzymes catalase and selenium-containing glutathione peroxidases (GPXs), members of the peroxiredoxin family are regarded as key elements of the peroxide-reduction machinery [1].

Peroxiredoxins (PRXs) are ubiquitous enzymes that use a redox active cysteine residue (peroxidatic Cys) to reduce a broad spectrum of substrates, namely H_2O_2, organic hydroperoxides and peroxynitrite ($ONOO^-$). Upon reduction of the peroxide, the peroxidatic Cys-SH is oxidized to sulfenic acid (Cys-SOH). In PRXs harboring two active cysteines (known as 2-Cys PRXs), the sulfenic acid is reduced by another Cys residue (resolving Cys) to form a disulfide. According to the location of the resolving Cys, 2-Cys PRXs can be classified as typical or atypical [2]. In trypanosomatids all PRXs characterized to date fall in the category of typical 2-Cys [3]. Peroxiredoxins return to their reduced state upon reduction of the disulfide by an appropriate electron donor. In trypanosomatids such reductant is a unique oxidoreductase of the thioredoxin superfamily, known as tryparedoxin [4], which itself is reduced by these organisms' specific thiol trypanothione [$\mathcal{N}^1$,$\mathcal{N}^8$-bis(glutathionyl)spermidine; 1,3]. For that reason trypanosomatid PRXs are commonly referred to as tryparedoxin peroxidases or TXNPxs. Apart from participating in antioxidant defense, members of the 2-Cys PRX subfamily are increasingly recognized as playing a more subtle and sophisticated role as regulators of peroxide-mediated cell signaling [2]. More recently, a function as molecular chaperones has also been proposed for some of these enzymes [5,6].

Author Summary

Leishmania are insect-borne protozoan parasites that cause leishmaniasis, a spectrum of diseases with relevance in men and dogs and for which there are no adequate means of control. In order to successfully infect their mammalian hosts, *Leishmania* requires the expression of a set of key proteins. Here, we identify one mitochondrial protein of the parasite as one of such molecules. Interestingly, however, we also discovered that the reason why this enzyme is essential is not due to its well characterized peroxidase activity, which defines it as a member of the peroxiredoxin family. Instead, the importance of this protein during infection may derive from its ability to function as a chaperone, as implied here. In short, by proposing a novel, peroxidase-unrelated role for the *Leishmania* mitochondrial peroxiredoxin during mammals' infection, this work opens new perspectives regarding the physiologic function of this enzyme.

In trypanosomatids 2-Cys PRXs are present in the parasites' cytosol and single mitochondrion [1]. Cytosolic 2-Cys PRXs (or cTXNPxs) are believed to work as general antioxidant devices that minimize the oxidative insult generated by the parasites' host. In agreement with this, overexpression of cTXNPx in *L. infantum* and *T. cruzi* was found to confer resistance to H_2O_2 and peroxynitrite of exogenous origin [7–12], while down-regulation of the *T. brucei* counterpart enhanced sensitivity to bolus H_2O_2 [13]. This peroxidase activity should be particularly relevant for *Leishmania* and *T. cruzi*, which can invade and proliferate in phagocytes. Not surprisingly, cTXNPxs of these organisms were pointed out as important virulence factors [12,14–17].

Mitochondrial TXNPxs (or mTXNPxs) are, due to their location, favorably positioned to eliminate peroxides produced endogenously, namely those formed as by-products of oxidative phosphorylation, and this was argued to be their main function [7,8,11]. However, the observation that overexpression of mTXNPx confers protection towards exogenously added and macrophage-derived H_2O_2 and peroxynitrite [7,8,10,11], suggested that these enzymes might also contribute to shield trypanosomatids from the oxidative challenge induced by their hosts. This assumption was strengthened by the observation that increased mTXNPx expression is associated with virulent *T. cruzi* phenotypes [17]. Apart from functioning as general antioxidant devices, the *Leishmania donovani* mTXNPx was reported to prevent H_2O_2-induced programmed cell death [18]. One additional, peroxidase-related role suggested for mTXNPxs was regulation of kinetoplast DNA (kDNA) replication. The kDNA is a network of catenated maxi and mini DNA circles that compose the mitochondrial DNA of trypanosomatids and whose replication is initiated when the universal minicircle sequence binding protein (UMSBP) binds specific sequences on minicircle DNA [19]. UMSBP binds to the DNA when it is reduced and is released when oxidized. According to the proposed model, mTXNPx would oxidize UMSBP [20].

The present study aimed at dissecting the functional relevance of mTXNPx in *Leishmania infantum*. *Leishmania* have a digenic life cycle that includes two morphologically and physiologically distinct stages: the promastigote (an extracellular form residing in the insect vector) and the amastigote (an intracellular form living inside the mammalian host). Using a homozygous knockout *L. infantum* line unable to express mTXNPx it was found that, while redundant in promastigotes, this mitochondrial 2-Cys PRX is essential for the establishment of a successful infection in mammals. This result establishes mTXNPx as factor determinant for *Leishmania* pathogenicity. Importantly, the data gathered here indicate that the essential role played by mTXNPx is not related to its peroxidase activity. Rather, the decreased infectivity of the mutants may be explained by the ability of mTXNPx to function as a chaperone, an activity disclosed here.

Results

Generation of *mTXNPx* mutants

To study the relevance of mTXNPx during the *L. infantum* life cycle, a mutant parasite line unable to express this enzyme was produced by homologous recombination. Since *Leishmania* has a diploid genome, two successive rounds of gene targeting were required to obtain homozygous knockout mutants. The first *mTXNPx* allele was replaced by a *NEO* integration cassette, while the second was disrupted with a *HYG* construct (Figure 1A). As confirmed by Southern blot (SB) of the double transfectants, both *mTXNPx* alleles were successfully targeted, thus resulting in the generation of a Δ*mtxnpx::NEO*/Δ*mtxnpx::HYG* mutant (hereafter referred to as *mtxnpx*$^-$). The *mtxnpx*$^-$ mutant was further manipulated in order to obtain a parasite line with restored mTXNPx expression. This was achieved by integrating the *mTXNPx* ORF into the small sub-unit rRNA *locus* using the pSSU-*PHLEO*-*infantum* vector (Figure 1A), which is a modified version of pSSU-*NEO*-*infantum* [21]. The rescued knockout mutants, designated *mtxnpx*$^-$/+*mTXNPx* (Δ*mtxnpx::NEO*/Δ*mtxnpx::HYG* [pSSU-*PHLEO-infantum-mTXNPx*]), were confirmed by PCR to have the *mTXNPx* ORF and the *PHLEO* cassette correctly integrated into the ribosomal *locus* (Figure 1C). Western blot and indirect immunofluorescence analysis showed that *mtxnpx*$^-$ mutants lack mTXNPx expression and that the rescued *mtxnpx*$^-$/+*mTXNPx* parasites express mTXNPx in its correct subcellular compartment, the mitochondrion (Figures 1D and E).

mTXNPx is redundant in the insect stage of *L. infantum*

The *mtxnpx*$^-$ parasite line was generated in the promastigote stage and when grown under standard culture conditions, *i.e.* in serum supplemented RPMI medium at 25°C, it appeared morphologically normal (data not shown) and displayed growth rates similar to that of wild type promastigotes. Most notably, no defects in kinetoplast morphology and division were noticed (Figure 1E). These observations argue against the involvement of mTXNPx in kDNA replication, a function previously attributed to mitochondrial TXNPxs [20,22]. Even though these results indicate that mTXNPx is redundant in the insect form of *L. infantum*, they do not discard a relevant role for this enzyme during the amastigote stage.

mTXNPx is crucial for the long term survival of *L. infantum* in the mammalian host

The consequence of mTXNPx depletion on the survival of *L. infantum* amastigotes was assessed by inoculating BALB/c mice, an animal model for visceral leishmaniasis (VL), with equal numbers of stationary phase *mtxnpx*$^-$, wild type or *mtxnpx*$^-$/+*mTXNPx* promastigotes. At defined time points after infection, parasite loads in the liver and spleen (the organs preferentially infected by VL strains) were analyzed by the limiting dilution assay (LDA). Results in Figure 2 show that elimination of mTXNPx had a remarkable effect on the outcome of infection and that this aggravated over time. Indeed, whilst 2 weeks after infection *mtxnpx*$^-$ could still be recovered from both infected organs, at 4 and 8 weeks the *mtxnpx*$^-$ infection indexes were much lower than those of wild type parasites, being below the detection limit of the assay (2.7 log units) at 14 weeks. The impaired virulence of *mtxnpx*$^-$ was recovered in knockout parasites with restored mTXNPx expression (*mtxnpx*$^-$/

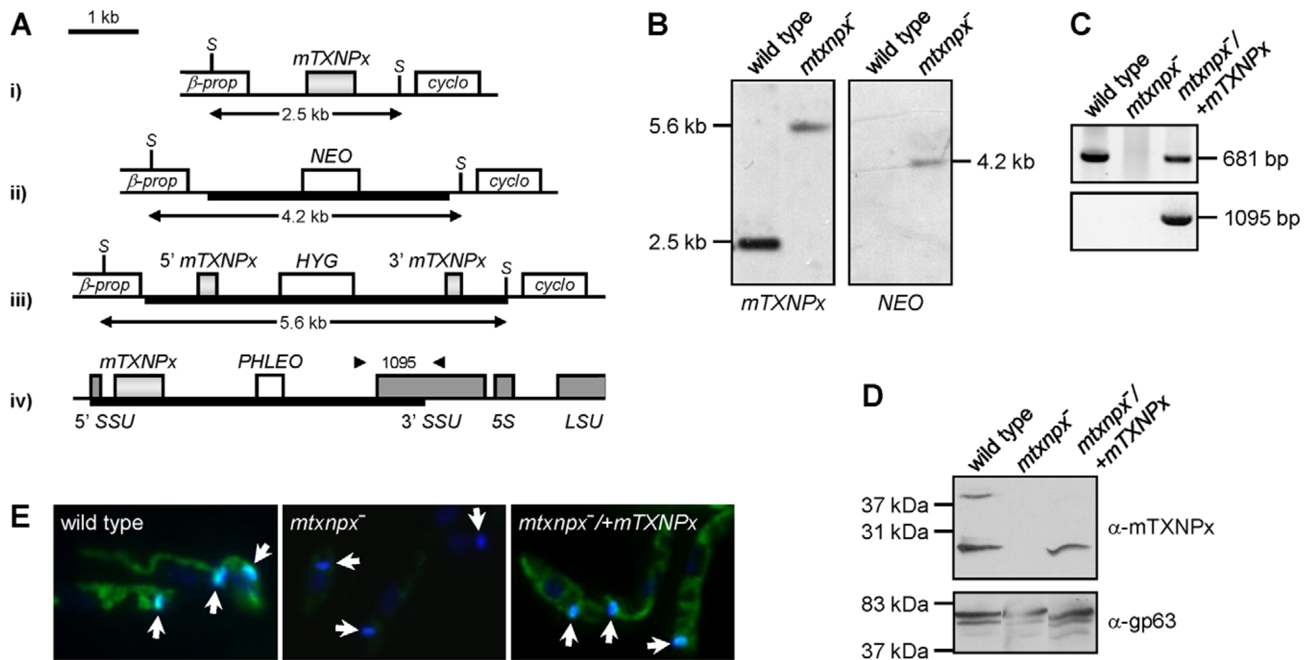

Figure 1. Generation of *mTXNPx* mutants. A. Genomic organization of the *mTXNPx locus* with its flanking β-propeller (*β-prop*) and cyclophilin (*cyclo*) genes in i) wild type and in ii) *NEO*- and iii) *HYG*- targeted alleles. The *HYG* disruption construct eliminated 144 nt of the *mTXNPx* coding sequence, leaving intact the first 292 and the last 245 nt (light grey boxes). *Sac*I (S) restriction sites are indicated. Scheme iv) represents the small subunit 18S rRNA (SSU) *locus* of *mtxnpx*⁻/+*mTXNPx* mutants with the integrated *PHLEO* construct harboring the *mTXNPx* coding sequence. Dark grey boxes represent the 5′ and 3′ coding regions of SSU, of 5S rRNA and of the large subunit 28S rRNA (LSU). Thick lines represent the integrated DNA constructs. Arrowheads show the location of primers used in PCR analysis (in C.) to diagnose for the correct integration of the *PHLEO* cassette into the ribosomal *locus*. The number in between arrowheads refers to the expected size of the corresponding PCR product. B. Southern blot analysis of *Sac*I-digested genomic DNA of wild type and *mtxnpx*⁻ mutants, hybridized with *mTXNPx* and *NEO* ORFs. In *mtxnpx*⁻, the 5.6 kb band revealed by the *mTXNPx* probe corresponds to the gene disrupted by the *HYG* cassette. C. PCR analysis of genomic DNA from wild type, *mtxnpx*⁻ and *mtxnpx*⁻/+*mTXNPx* parasites, using specific primers to amplify *mTXNPx* ORF (681 bp) or to diagnose for the correct integration of the *PHLEO* cassette into the ribosomal *locus* (1095 bp; primer location represented in A.). D. Western blot analysis of wild type, *mtxnpx*⁻ and *mtxnpx*⁻/+*mTXNPx* promastigotes, incubated with the anti-mTXNPx antibody and, upon stripping, with anti-gp63 antibody (control for loading). The band above 37 kDa in the upper panel is dimeric mTXNPx. E. Indirect immunofluorescence of parasites lines as above, incubated with anti-mTXNPx antibody (green), merged with DAPI (blue). 1000× magnification. The kDNA is indicated by arrows.
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+*mTXNPx*), confirming that it specifically results from depletion of this enzyme (Figure 2). Of notice, the parasitemia levels of mice infected with *mtxnpx*⁻/+*mTXNPx* were consistently below those produced by wild type parasites, a phenomenon that is frequently observed in complemented knockout mutants [23–25]. This is likely due to the fact that in the rescued mutants *mTXNPx* expression is not under the control of its own untranslated regions (as occurs in wild type parasites), but rather it is regulated by the rRNA promoter of the small sub-unit rRNA and by the intergenic region of the cysteine proteinase B 2.8 gene cluster [26].

In short, these results show that mTXNPx is crucial for the long term survival of *L. infantum* amastigotes in the mammalian host. The impaired capacity of mTXNPx-depleted parasites to thrive in mice is not due to their failure to invade host cells (the macrophages) or to differentiate into amastigotes, as inferred from microscopic observation of monolayers of peritoneal macrophages from C57/BL6 mice infected with *mtxnpx*⁻ parasites (Figure S1). The next question is which function, disturbed in mutant parasites, is leading to such defect in infectivity.

Depletion of mTXNPx has no impact on the parasite antioxidant capacity

The *L. infantum* mTXNPx is an efficient reductase of hydroperoxides [27] as well as of peroxynitrite (the apparent second order rate for peroxynitrite reduction by mTXNPx is $1.6\times10^{6}\ M^{-1}\ s^{-1}$, at pH 7.4 and 37°C; Romao S, Radi R and Tomás AM, unpublished results). As suggested previously [7,8,11], the most likely function for mTXNPxs is the elimination of peroxides generated as a consequence of the parasite's aerobic metabolism. Accordingly, *mtxnpx*⁻ mutants were assessed for their sensitivity to antimycin A (AA), an inhibitor of the mitochondrial electron transport chain that leads to the local production of reactive oxygen species [28]. However, the observation that AA had the same growth inhibitory effect in *mtxnpx*⁻ and wild type promastigotes (Figure 3A) suggests that mTXNPx is not critical for the elimination of peroxides generated within the mitochondrion. A different explanation for the impaired infectivity of *mtxnpx*⁻ could be their inability to deal with host-derived oxidants [11]. However, this premise found no support in the observation that susceptibility of *mtxnpx*⁻ promastigotes to H_2O_2 (added as bolus) or to the peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1) was similar to that of wild type parasites (Figures 3B and C). This hypothesis was explored further by exposing *mtxnpx*⁻ to host-derived oxidants. As shown in the NBT assay in Figure 3D (*inset*), phagocytosis of *L. infantum* promastigotes by murine peritoneal macrophages triggers the generation of superoxide anion ($O_2^{\cdot-}$), a precursor of H_2O_2. The absence of mTXNPx did not render promastigotes more sensitive to such oxidative burst, as deduced from the comparison of the infection indexes of macrophages inoculated with *mtxnpx*⁻ and control parasites (wild

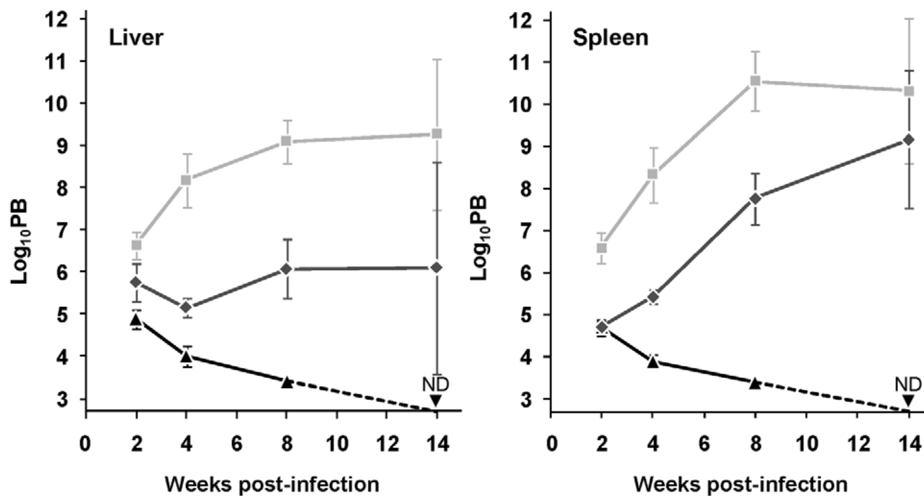

Figure 2. Depletion of mTXNPx impairs *Leishmania* virulence. Wild type (light grey squares), *mtxnpx*$^{-}$ (black triangles) and *mtxnpx*$^{-}$/+*mTXNPx* (dark grey diamonds) promastigotes were inoculated intraperitoneally into BALB/c mice. At different time points after infection, parasite burden (PB) was determined by the limiting dilution assay, as the number of parasites per gram of liver (*left*) or spleen (*right*). The Y axis indicates log_{10}PB. Data represent mean and standard error of the mean of 6 independent experiments (involving a total of 85 animals infected with *mtxnpx*$^{-}$), except for *mtxnpx*$^{-}$/+*mTXNPx*-infected mice, which refer only to 4 experiments. ND, not detected. From week 4 onward, differences between *mtxnpx*$^{-}$ and controls are statistically significant. Statistical analysis of these results is in TextS1 and Table S2.
doi:10.1371/journal.ppat.1002325.g002

type and *mtxnpx*$^{-}$/+*mTXNPx*) (Figure 3D). Additional evidence that mTXNPx is not implicated in *Leishmania* protection against host-derived oxidants was obtained in an *in vivo* infection experiment using mutant mice with impaired pro-oxidant capacity, namely B6.p47$^{phox-/-}$ and B6.RAG2$^{-/-}$ IFN-$\gamma^{-/-}$ mice. B6.p47$^{phox-/-}$ mice carry a targeted disruption of the p47 subunit of the phagocyte NADPH oxidase (phox) complex that is responsible for phagocytosis-induced production of $O_2^{\cdot -}$. B6.RAG2$^{-/-}$ IFN-$\gamma^{-/-}$ mice lack B and T cells and do not express interferon gamma (IFN-γ), *i.e.* a pro-inflammatory cytokine predominantly secreted by type-1 T cells that activates the inducible nitric oxide synthase (iNOS) and up-regulates phox, thus allowing for peroxynitrite generation in macrophages. Mutants and control mice of the same genetic background (C57BL/6) were infected with equal numbers of *mtxnpx*$^{-}$ and of control parasites (wild type and *mtxnpx*$^{-}$/+*mTXNPx*) and 5 weeks later the parasite burden was analyzed. The results, plotted in Figure 3E, show that the number of *mtxnpx*$^{-}$ parasites recovered from the organs of infected mice was either consistently below that of control parasites or undetectable irrespective of the mouse strain serving as host. In other words, even when inoculated in hosts that are unable to mount an efficient oxidative response, *mtxnpx*$^{-}$ do not recover infectivity. This observation reinforces the idea that the essential function of mTXNPx in amastigotes is not to provide protection against peroxides of exogenous origin. Additionally, it indicates that the avirulent phenotype of *mtxnpx*$^{-}$ is not due to any microbicidal component induced by the host adaptive immune system, namely by the T lymphocytes. Rather, the inability of *mtxnpx*$^{-}$ to thrive in mammalian hosts appears to be a consequence of factors inherent to the parasite.

The critical function of mTXNPx is independent of its peroxidase activity

Results in the previous section suggested that the crucial function played by mTXNPx during infection is not that of an antioxidant device. To definitively conclude about the contribution of mTXNPx peroxidase activity to *Leishmania* infectivity, a peroxidase-inactive variant of the enzyme (mTXNPxC81S) was tested for its ability to rescue the avirulent phenotype of the knockouts. Cys81 is the peroxidatic Cys of mTXNPx and its replacement by a serine should abolish peroxidase activity, as observed previously [29,30]. Loss of peroxidase activity of the C81S variant was confirmed *in vitro* using a recombinant enzyme lacking the first 26 amino acids that compose the mitochondrial targeting peptide (ΔmTXNPxC81S) to mimic the mature protein in the parasite's mitochondrion [8]. As expected from previous mutational analysis [29,30], when assayed for TXNPx activity, the ΔmTXNPxC81S mutein was unable to catalyze H_2O_2 reduction, even when present at a 10-fold higher concentration than the control wild type ΔmTXNPx enzyme (Figure 4A). Of notice, on the basis of circular dichroism spectra, substitution of the peroxidatic Cys to a Ser did not alter the secondary structure of the mutein relative to the wild type enzyme, the estimation of α-helix and β-strand contents for both enzymes being of 35% and 29%, respectively (data not shown).

To test whether expression of mTXNPxC81S could restore infectivity of mTXNPx knockouts, *mtxnpx*$^{-}$/+*mTXNPxC81S* mutants were generated by transfecting *mtxnpx*$^{-}$ with pSSU-*PHLEO-infantum-mTXNPxC81S*. Confirmation for the correct integration of the construct was obtained by PCR (Figure 4B) and the exact subcellular location verified by indirect immunofluorescence analysis (Figure 4C). The *mtxnpx*$^{-}$/+*mTXNPxC81S* transfectants were then inoculated into BALB/c mice and the parasite burden in livers and spleens evaluated at 4 and 8 weeks post infection. The results, depicted in Figure 4D, show that virulence was recovered in *mtxnpx*$^{-}$ complemented with mTXNPxC81S and that the parasite load produced by this line was similar to that of *mtxnpx*$^{-}$/+*mTXNPx*. These observations thus provide solid evidence that the essential role played by mTXNPx during the infective stage of *Leishmania* is independent of its peroxidase activity.

Is mTXNPx functioning *in vivo* as a molecular chaperone?

In addition to acting as peroxidases, 2-Cys PRXs may in some cases exhibit chaperone activity [5,6], an attribute that, to date, has never been described for any TXNPx. The possibility of mTXNPx acting as a chaperone was therefore investigated as an

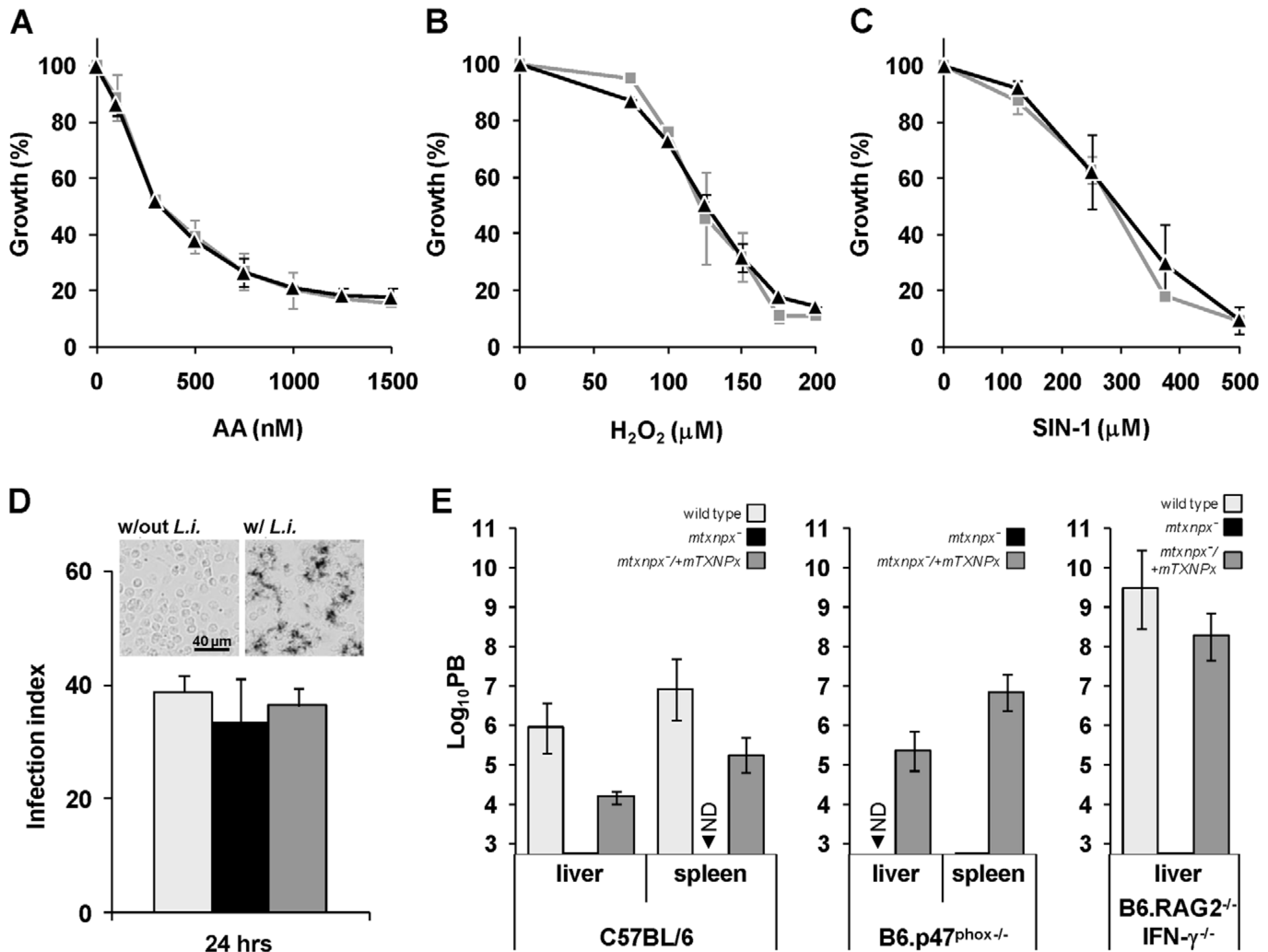

Figure 3. Depletion of mTXNPx has no impact on the antioxidant capacity of promastigotes. A. B. C. Wild type (light grey squares) and *mtxnpx*$^-$ (black triangles) promastigotes were cultured in the presence of increasing concentrations of (A) antimycin A (AA), (B) H_2O_2 or (C) the peroxynitrite donor 3-morpholinosydnonimine hydrochloride (SIN-1). Four days later cell densities were measured in a spectrophotometer at 600 nm. The data is expressed as the percentage of promastigote replication relative to control cultures without any exogenous agent. Graphs represent means and standard deviation of three or more experiments (each performed in duplicate). D. Monolayers of peritoneal macrophages from C57BL/6 mice were infected with stationary phase wild type (light grey bars), *mtxnpx*$^-$ (black bars) or *mtxnpx*$^-$*/+mTXNPx* (dark grey bars) promastigotes and 24 hrs later the infection indexes determined. The *inset* shows light microscopy photographs of macrophages incubated with NBT in the absence ("w/out *L.i.*") or presence ("w/*L.i.*") of parasites. Deposits of dark blue insoluble formazan resulting from NBT reaction with superoxide anion were visible in macrophages 30 min after contact with *L. infantum* promastigotes (*right panel*), but not in macrophages to which no parasites were added (*left panel*). Images were acquired at 100× magnification. E. Mice from different strains (C57BL/6, B6.p47$^{phox-/-}$ and B6.RAG2$^{-/-}$ IFN-$\gamma^{-/-}$) were inoculated intraperitoneally with wild type (light grey bars), *mtxnpx*$^-$ (black bars) or *mtxnpx*$^-$*/+mTXNPx* (dark grey bars) stationary phase promastigotes. Five weeks after infection parasite burden (PB) was determined in the livers and spleens of mice by the limiting dilution assay. The Y axis represents log_{10}PB. Data represent mean and standard error of the mean of 3 independent experiments involving a total of 72 animals. ND, not detected. Differences between *mtxnpx*$^-$ and controls are statistically significant. Statistical analysis of these results is in TextS1 and Table S3.
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attempt to provide an alternative function for this enzyme in *Leishmania* amastigotes.

The chaperone activity of purified recombinant ΔmTXNPx was assessed *in vitro* by testing its ability to suppress the aggregation of thermally denatured citrate synthase (CS). When incubated at 43°C citrate synthase unfolds, leading to significant aggregation that can be monitored by measuring light scattering in a spectrofluorometer [31]. Addition of ΔmTXNPx to the reaction at a 10-fold molar excess completely suppressed the thermal aggregation of CS (Figure 5A). This effect was much less pronounced when ΔmTXNPx was added at 5-fold molar excess. Importantly, the ΔmTXNPxC81S mutein was also capable of preventing CS aggregation, exhibiting the same behavior as the wild type enzyme (Figure 5A).

To gain insight into the physiological significance of the chaperone activity of mTXNPx a thermotolerance assay was conducted. The experiment consisted in monitoring cell growth of *mtxnpx*$^-$ and control parasites (wild type, *mtxnpx*$^-$*/+mTXNPx*, and *mtxnpx*$^-$*/+mTXNPxC81S*) at both 25°C and 37°C, which are the temperatures encountered by the parasite in the insect vector and in the mammalian host, respectively. The cell growth curves depicted in Figure 5B show that, while at 25°C the proliferation rates of all parasite lines were indistinguishable, at 37°C the growth rate of *mtxnpx*$^-$ was significantly lower than that of wild type parasites. The thermo-sensitive phenotype observed for *mtxnpx*$^-$ was recovered in the rescued *mtxnpx*$^-$*/+mTXNPx* and *mtxnpx*$^-$*/+mTXNPxC81S* mutants. These observations show that expression of mTXNPx

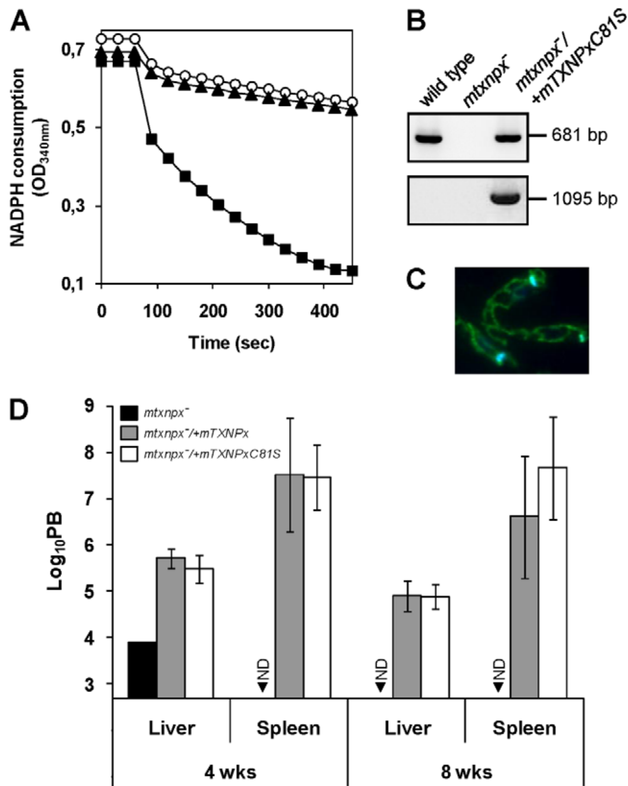

Figure 4. The peroxidase activity of mTXNPx is not a critical determinant of *L. infantum* virulence. A. Classical assay for TXNPx enzymatic activity. Reaction mixtures contained 200 μM NADPH, 0.5 U ml^{-1} *Li*TR, 50 μM TS_2, 2.5 μM *Li*TXN2 and recombinant TXNPx. ΔmTXNPx was added to a final concentration of 0.5 μM (filled squares) and ΔmTXNPxC81S to 5 μM (filled triangles). Reactions were started by addition of 70 μM H_2O_2 at 60 sec and peroxidase activity followed by monitoring NADPH consumption at 340 nm. The negative control contains no TXNPx (open circles). B. PCR analysis of genomic DNA from *mtxnpx*⁻*/+mTXNPxC81S* parasites using specific primers to amplify the *mTXNPx* ORF (681 bp) or to diagnose for the correct integration of the *PHLEO* cassette into the ribosomal *locus* (1095 bp; location of primers in Figure 1A). Controls, performed with genomic DNA from wild type and *mtxnpx*⁻ parasites, are also included. C. Indirect immunofluorescence of *mtxnpx*⁻*/+mTXNPxC81S* parasites incubated with the anti-mTXNPx antibody (green labeling), merged with DAPI (blue labeling). Parasites were photographed at 1000× magnification. D. Parasite burden (PB) in liver and spleen of BALB/c mice, determined by the limiting dilution assay, 4 and 8 weeks after infection with *mtxnpx*⁻ (black bars), *mtxnpx*⁻*/+mTXNPx* (dark grey bars) or *mtxnpx*⁻*/+mTXNPxC81S* (open bars) stationary phase promastigotes. The Y axis represents log_{10}PB. Data indicate mean and standard error of the mean of 2 independent experiments involving a total of 54 animals. ND, not detected. Differences between *mtxnpx*⁻ and controls are statistically significant. Statistical analysis of these results is in TextS1 and Table S4.
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renders promastigotes more permissive to 37°C, *i.e.* this enzyme confers thermotolerance to *L. infantum* irrespective of its peroxidase activity. It is thus reasonable to speculate that the crucial role played by mTXNPx during amastigote development in the mammalian host is that of a molecular chaperone.

Discussion

In parasites of the family Trypanosomatidae the peroxidase activity of 2-Cys PRXs (which in these organisms are designated TXNPxs) is accepted as the basis of their physiologic functions [1,3]. Data presented in this report demonstrate that this activity is not crucial in the case of mTXNPx, even though the protein itself is essential for *L. infantum* amastigote survival. Instead, experimental evidence is provided suggesting that it may be the activity of mTXNPx as a molecular chaperone, unraveled here, that is determinant for parasite viability.

The finding that a peroxidase-inactive version of mTXNPx is competent to ensure amastigote survival excludes *per se* a crucial role for the peroxidase-related functions previously attributed to mTXNPxs, namely elimination of peroxides and regulation of H_2O_2-induced apoptosis [7,8,11,18]. Accordingly, these activities must be taken over by other means. Detoxification of exogenously-derived peroxides (such as those generated by the host immune response) might be efficiently fulfilled by cytosolic 2-Cys PRXs and non-selenium glutathione peroxidase-like enzymes (nsGPX) [7,8,11,32–34]. As for reduction of peroxides of mitochondrial origin, different scenarios can be envisaged. One possibility is that mitochondrial nsGPX carries out this antioxidant function. This hypothesis requires that the substrate specificities of nsGPX and mTXNPx overlap [33–35], and implies the existence of an efficient nsGPX reductant in the mitochondrion, which is still to be found [36]. Of notice, in the particular case of H_2O_2, this could diffuse and be reduced by cytosolic peroxidases. In a different perspective, elimination of peroxides may occur via non-catalytic pathways. In this situation, low molecular weight thiols, such as trypanothione (the trypanosomatids' specific thiol), glutathione, ovothiol, free cysteines and even thiol groups exposed on protein surfaces would directly reduce peroxides [37–39]. In either circumstance, an ascorbate peroxidase, located in the intermembrane space of the *Leishmania* mitochondrion [40] (gene LinJ.34.0070 in the *L. infantum* GeneDB), may also contribute to peroxide elimination in this organelle.

A different role previously attributed to mTXNPxs is the regulation of kDNA replication. As proposed by Sela *et al.* [20], mTXNPx would affect kDNA replication by oxidizing UMSBP and, consequently, turning "off" the binding of this protein to the minicircle origin of replication. However, in this report no evidence could be found linking mTXNPx depletion to defects in replication rate or in kDNA morphology of *L. infantum* promastigotes. Even though these analyses could not be extended to amastigotes, kDNA replication is highly conserved in trypanosomatids [19], making it difficult to accept that mTXNPx would regulate kDNA replication only in this life stage. Moreover, the proposed model for kDNA replication established a fundamental role for a mitochondrial TXN as reductant of UMSBP and it was shown previously that such enzyme is not essential in *Leishmania* and does not exist in trypanosomes [36]. The mechanism underlying regulation of kDNA replication involving mTXNPxs (and a mitochondrial TXN) must, therefore, be re-evaluated.

This work has also, for the first time, uncovered that trypanosomatid 2-Cys PRXs can function as molecular chaperones and that this activity might be relevant for parasite survival *in vivo*. Several pieces of evidence point into this direction. i) Purified recombinant mTXNPx was shown to prevent the thermal aggregation of CS, a well-known chaperone substrate [31]. This activity does not depend on the peroxidatic capacity of mTXNPx, as the mTXNPxC81S mutein was also active in this assay, thus providing a rationale for the recovery of virulence observed for *mtxnpx*⁻*/+mTXNPxC81S*. This is in line with observations reported by Jang *et al.* [5]. ii) The chaperone activity of 2-Cys PRXs is known to depend on their quaternary structure and, as shown before, mTXNPx is capable of associating into decameric ring-like structures [27], which are the minimal oligomeric arrangements required for such function [41]. iii) Gain of chaperone function and loss of peroxidase activity of 2-Cys PRXs are, in most

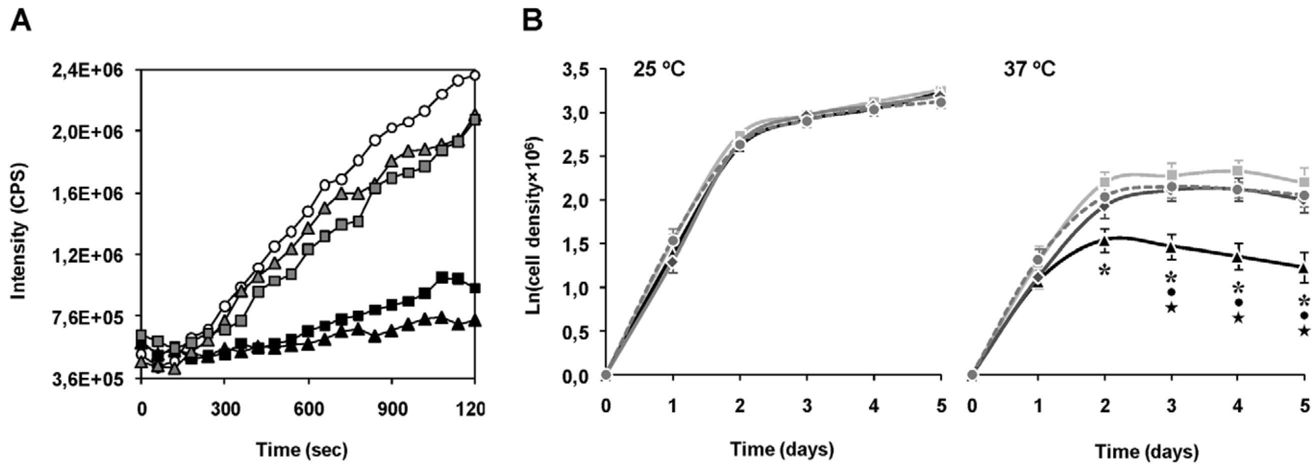

Figure 5. Evidences for the chaperone activity of mTXNPx. A. Chaperone activity of purified recombinant ΔmTXNPx and ΔmTXNPxC81S was analyzed *in vitro* by assessing the enzymes' ability to suppress the thermal aggregation of citrate synthase (CS). Aggregation of CS was induced at 43°C and monitored by measuring light scattering at 500 nm in the absence (open circles) or presence of 5:1 (grey-filled squares) or 10:1 (black squares) ratios of ΔmTXNPx monomers to CS monomers. Alternatively, the mutein ΔTXNPxC81S was added to the reaction at 5:1 (grey-filled triangles) or 10:1 (black triangles) ratios. B. Wild type (light grey squares), *mtxnpx*$^-$ (black triangles) *mtxnpx*$^-$*/+mTXNPx* (dark grey diamonds) and *mtxnpx*$^-$*/+mTXNPxC81S* (dark grey circles and dashed line) *L. infantum* promastigotes were seeded at 10^6 ml^{-1} in complete RPMI medium and incubated at 25°C (*left*) and 37°C (*right*). The Y axis represents ln (or $\log_e$) of cell densities recorded throughout 5 days. Values represent mean and standard error of the mean of 4 to 8 independent curves. Also indicated are the statistically significant differences (i.e. $p<0.05$) between *mtxnpx*$^-$ and each of the control parasite lines, *i.e.* wild type (*), *mtxnpx*$^-$*/+mTXNPx* (●) and *mtxnpx*$^-$*/+mTXNPxC81S* (★).
doi:10.1371/journal.ppat.1002325.g005

organisms, accompanied with the overoxidized form of the protein [2] and mTXNPx undergoes overoxidation when promastigotes are exposed to 37°C (Figure S2). Since *Leishmania* do not possess sulfiredoxins to enzymatically regenerate overoxidized 2-Cys PRXs [1] as occurs in higher eukaryotes, such peroxidase-inactive form of mTXNPx is likely to persist until the protein is synthesized *de novo*. iv) Additional support consubstantiating a role for mTXNPx as a chaperone came from the observation that lack of mTXNPx expression rendered promastigotes more sensitive to 37°C, the temperature encountered in the mammalian host. Such thermo-sensitive phenotype is reminiscent of some chaperone-deficient mutants [42–44]. Accepting that mTXNPx is a molecular chaperone *in vivo*, one can only speculate how this function could impact on parasite survival under the conditions met in the host. In mitochondria, molecular chaperones are associated with a multitude of functions. They participate in the biogenesis of new mitochondrial peptides by assisting their trans-membrane transport and their refolding to the native conformation inside the matrix. In addition, they are part of the mitochondrial protein quality control system that repairs damaged or misfolded proteins or mediates their removal by proteolysis [45]. Under situations of cellular stress (including elevated temperatures), the activity of mitochondrial chaperones becomes even more imperative for protein homeostasis [45]. Accordingly, it is reasonable to assume that mTXNPx functions as a molecular chaperone that ensures integrity of mitochondrial functions, its activity being particularly relevant when parasites reside in vertebrate host.

Another important outcome of this report is the identification of mTXNPx as a new factor critical for *Leishmania* infectivity. This finding may have impact on the development of new strategies to control leishmaniasis as the *mtxnpx*$^-$ parasite line produced here can be regarded as the basis of a live attenuated vaccine. In this context, the observation that *mtxnpx*$^-$ cannot give rise to a productive infection even in immunocompromised mice, suggests that this strategy would not pose major safety issues.

In conclusion, the work presented in this manuscript sheds lights on the functional relevance of mTXNPx by showing that, even though this molecule is crucial for *L. infantum* survival during infection of the vertebrate host, its peroxidase activity is superfluous. A novel peroxidase-unrelated function, as a molecular chaperone, is proposed for this enzyme, which could be critical for mitochondrial functionality under the conditions encountered by the parasite in the mammalian host. This report thus constitutes a turning point into the current state of knowledge regarding the physiologic role of peroxiredoxins in trypanosomatids.

Materials and Methods

Ethics statement

The experimental animal procedures were approved by the Local Animal Ethics Committee of Institute for Molecular and Cell Biology, University of Porto, Portugal and licensed by DGV (General Directory of Veterinary, Ministry of Agriculture, Rural Development and Fishing, Govt. of Portugal), in May 18, 2006 with reference 520/000/000/2006. All animals were handled in strict accordance with good animal practice as defined by national authorities (DGV, Law nu1005/92 from 23rd October) and European legislation EEC/86/609.

Parasite cultures

Leishmania infantum promastigostes (MHOM MA67ITMAP263) were cultured at 25°C in RPMI 1640 Glutamax medium, supplemented with 10% inactivated fetal bovine serum (FBSi), 50 U ml^{-1} penicillin, 50 μg ml^{-1} streptomycin (all from Gibco) and 25 mM hepes sodium salt pH 7.4 (Sigma).

Generation of knockout and rescue constructs

Primers used to generate the constructs are summarized in Table S1. The accuracy of all assembled constructs was verified by sequencing. To produce the *NEO* replacement construct, sections of the 5′ and 3′ non-coding sequences flanking the *mTXNPx* ORF

were PCR-amplified from a cosmid clone [8] using primers P1/P2 and P3/P4, respectively. Following digestion with the appropriate restriction enzymes, PCR products were cloned into the *Bam*HI-*Xho*I and *Kpn*I sites of the pTEX-*NEO* plasmid [46], on both sides of the neomycin phosphotransferase (*NEO*) gene. To assemble the *HYG* disruption construct, fragments containing part of the 5′ and 3′ untranslated regions and of the *mTXNPx* coding sequence were amplified by PCR from genomic DNA of the *NEO*-targeted mutants, using primers P5/P6 and P7/P8. Upon digestion with the appropriate restriction enzymes, PCR products were cloned into *Bam*HI-*Eco*RV and *Kpn*I sites of the pTEX-*HYG* plasmid, a version of pTEX-*NEO* wherein the *NEO* gene had been replaced by the hygromycin phosphotransferase (*HYG*) open reading frame. To generate the rescue construct, the *mTXNPx* ORF was introduced into the *Xma*I and the Klenow-treated *Bam*HI restriction sites of the pSSU-*NEO-infantum* plasmid [21]. This plasmid is a modified version of the pSSU-int [26], wherein the *HYG* gene had been replaced by *NEO* and the 5′ flanking sequence of the small sub-unit rRNA (18S rRNA) gene of *Leishmania mexicana* substituted by the homologous sequence of *L. infantum*. Upon cloning of the *mTXNPx* coding sequence into pSSU-*NEO-infantum*, the *NEO* ORF was replaced by the bleomycin hydrolase (*PHLEO*) gene. One additional rescue construct (pSSU-*NEO-infantum-mTXNPxC81S*), containing a mutated version of *mTXNPx*, was generated by site directed mutagenesis (see below). Before transfection of *L. infantum* promastigotes, the *NEO*, *HYG* and *PHLEO* constructs were linearized by digestion with *Hinc*II, *Bam*HI-*Sac*I, and *Nde*I-*Pme*I, respectively, and purified from agarose gels by electroelution.

Production of the mTXNPxC81S mutein

A site directed mutagenesis strategy was employed to introduce the Cys81 to Ser (C81S) mutation in the *mTXNPx* ORF cloned into pSSU-*PHLEO-infantum* (detailed above) and pET28c (detailed below). For this, the full-length plasmids were PCR-amplified with sense and antisense mutated primers (P9 and P10 in Table S1) using *Pfu* polymerase (Stratagene). Upon digestion of parental (wild type) DNA with 10 units of *Dpn*I (New England Biolabs) for 1 h at 37°C, the reaction (4 µl) was used directly to transform *Escherichia coli* DH5α strain. Mutant colonies were confirmed to carry the site-directed mutation by sequencing both strands.

Transfection of *L. infantum* and isolation of mutants

Promastigotes in the logatithmic phase of growth were electroporated at 450 V and 350–400 µF with 1 to 5 µg of DNA as described elsewhere [47]. Parasites were allowed to recover in 10 ml of culture medium without selective drugs for 24 hours. Drugs were then added to 5 ml of the liquid culture and the remaining 5 ml were pelleted and plated onto agar plates containing the same drug(s). Geneticin (G418; Sigma) was used at 15 µg ml^{-1}, hygromycin (Invitrogen) at 10 µg ml^{-1} and bleomycin (Sigma) at 17.5 µg ml^{-1}. Upon 2 to 3 weeks of growth on agar, colonies were picked up and transferred into liquid medium.

Indirect immunofluorescence assay (IFAT)

Immunofluorescence assays were performed according to Castro *et al.* [8]. Briefly, parasites were fixed with 4% paraformaldehyde (w/v) in PBS (0.1 M sodium phosphate buffer pH 7.2, 0.15 M NaCl), spotted onto polylysine-coated microscope slides, permeabilized with 0.1% (v/v) Triton X-100 and incubated with the polyclonal anti-mTXNPx antibody [8] and 4′,6-diamidino-2-phenylindole (DAPI). The secondary antibody was Alexa Fluor 488 anti-rabbit IgG (Molecular Probes). Slides were mounted in Vectashield (Vector Laboratories) and examined with an AxioImager Z1 microscope (Carl Zeiss, Germany).

Drug sensitivity assays

L. infantum promastigotes in the logarithmic phase of growth were seeded at 10^6 cells ml^{-1} in 24-well plates containing increasing concentrations (in duplicate) of either antimycin A (AA), H_2O_2 or 3-morpholinosydnonimine hydrochloride (SIN-1) (all from Sigma). Parasites were allowed to grow for 3–4 days and cell densities were measured in a spectrophotometer at 600 nm.

Animals and ethics statement

BALB/c, C57BL/6 and National Marine Research Institute (NMRI) mice were purchased from Charles River (Madrid, Spain). Mice with a targeted disruption of the p47 subunit of the NADPH-oxidase complex on a C57BL/6 background ($B6.p47^{phox-/-}$) were purchased from Taconic (Lille Skensved, Denmark). Until the day of infection with *L. infantum*, and as prophylatic treatment against bacterial infection, trimethoprim-sulfamethoxazole (Bactrim; 600 mg l^{-1}) was administered in the drinking water to $B6.p47^{phox-/-}$ mice. Mice double deficient in gamma interferon (IFN-γ) and in recombinase activating gene-2 (RAG-2) were obtained by crossing single knock out strains on C57BL/6 background. All mice were raised in specific pathogen-free conditions. Euthanasia was performed in a 20% isofluorane atmosphere and all efforts were made to minimize suffering.

Infection of monolayers of murine macrophages with promastigotes

Macrophages obtained by peritoneal lavage of C57/BL6 mice were seeded at 4×10^5 cells per well in DMEM Glutamax complemented with 10% FBSi, 50 U ml^{-1} penicillin and 50 µg ml^{-1} streptomycin (all from Gibco), and allowed to adhere for 2 hrs at 37°C, 5% CO_2. Upon washing with Hanks balanced salt solution (HBSS, Gibco), macrophages were incubated with opsonized promastigotes (in the stationary phase of growth) at a parasite:macrophage ratio of 10:1. For detection of superoxide anion ($O_2\cdot^-$), a freshly prepared solution of 1 mg ml^{-1} nitroblue tetrazolium (NBT), prepared in Dulbecco's PBS was added to macrophages. After 30 min of incubation at 37°C, cells were washed with HBSS, fixed with methanol and deposition of formazan observed with an optical microscope Olympus CX31. For determination of infection indexes, infection with promastigotes was allowed to proceed for 3 hrs, after which non-internalized parasites were removed with HBSS. Cells were cultured for additional 24 hrs and then washed, fixed with 4% paraformaldehyde (w/v) in PBS, permeabilized with 0.1% (v/v) Triton X-100, and incubated with the anti-cTXNPx1 [8] antibody and propidium idodide (PI). Secondary antibody was Alexa Fluor 488 anti-rabbit IgG (Molecular Probes). Slides were mounted in Vectashield (Vector Laboratories). Images were acquired with an Axiocam MR ver.3.0 camera (Carl Zeiss, Germany) coupled to an AxioImager Z1 microscope (Carl Zeiss, Germany). A minimum of 3000 macrophages was counted in each experiment using specifically designed software (unpublished data) and the infection index was obtained by multiplying the percentage of infected macrophages by the average number of intracellular amastigotes per macrophage.

Determination of parasite burden by the limiting dilution assay

Parasites of all lines were passaged through NMRI mice at least twice prior to infection experiments. Next, 10^8 *L. infantum*

stationary phase promastigotes, were inoculated intraperitoneally into 6- to 9-weeks old male BALB/c mice. At defined time points, mice were sacrificed and their livers and spleens excised, weighed and homogenized in Schneider's medium (Sigma) supplemented with 10% FBSi, 100 U ml^{-1} penicillin, 100 μg ml^{-1} streptomycin (all from Gibco), 5 mM hepes sodium salt pH 7.4 (Sigma), 5 μg ml^{-1} phenol-red (Sigma) and 2% sterile human urine. Homogenates were then diluted to 10 mg ml^{-1} and these cell suspensions titrated in quadruplicate across a 96-well plate in serial four-fold dilutions (four titrations per organ). After two weeks of growth at 25°C, the last dilution containing promastigotes was recorded and the number of parasites per gram of organ (parasite burden) calculated as described by Buffet *et al.* [48]. The detection limit of this method is 500 parasites g^{-1} (*i.e.* 2.7 log units).

Heterologous expression and purification of ΔmTXNPx and ΔmTXNPxC81S

To mimic the mature mitochondrial protein, truncated wild type mTXNPx (ΔmTXNPx), *i.e.* lacking the first 26 amino acids that compose the mitochondrial targeting peptide [8], was produced in the *E. coli* strain BL21 (DE3) Tuner upon transformation a pET28c (Novagen) construct containing the ΔmTXNPx gene fragment (PCR-amplified with primers P11/P12 in Table S1). To produce the mutant ΔmTXNPxC81S protein, the pET28c-Δ*mTXNPxC81S* was generated by site directed mutagenesis (detailed above) and used to transform the same *E. coli* strain. Both enzymes were expressed in bacteria as fusion proteins carrying an N-terminal six histidine tag. Upon induction for 3 hrs at 30°C in the presence of 50 μg ml^{-1} kanamycin and 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG), bacteria were pelleted, suspended in 500 mM NaCl, 20 mM Tris-HCl pH 7.6, disrupted by sonication and centrifuged at 30,000×*g* for 30 min at 4°C. The supernatant was applied to a His Bind resin (Novagen) column and the recombinant protein eluted with an imidazole gradient (5 to 1000 mM) at a flow rate of 1 ml min^{-1}. Fractions confirmed to contain the protein by SDS-PAGE were pooled, applied to PD-10 columns (Amersham) and eluted in 50 mM sodium phosphate buffer pH 8.0. For removal of the His tag, enzymes were first digested with biotinylated thrombin (Novagen), and then incubated with immobilized streptavidin for removal of the protease. Proteins were concentrated by ultrafiltration, recovered in 40 mM hepes pH 7.5 and quantified by the bicinchoninic acid (BCA) protein assay (Pierce), using bovine serum albumin (BSA) as standard.

Determination of peroxidase activity

Routine determination of TXNPx activity was performed according to Nogoceke *et al.* [4]. Briefly, reaction mixtures were prepared in a total volume of 300 μl of 50 mM Tris-HCl, 1 mM EDTA, pH 8.0, containing 200 μM NADPH, 0.5 U ml^{-1} *L. infantum* TR, 50 μM trypanothione disulfide (TS_2, Bachem), 2.5 μM *L. infantum* TXN2 [49] and varying concentrations of ΔmTXNPx or ΔmTXNPxC81S. Reactions were started by addition of 70 μM hydrogen peroxide (H_2O_2, Sigma) and NADPH consumption was followed at 340 nm. All reactions were performed at 25°C and monitored with a Shimadzu UV-2401 PC spectrophotometer (Shimadzu Corporation).

Determination of chaperone activity

The chaperone activity of ΔmTXNPx and ΔmTXNPxC81S was measured using citrate synthase (CS) from porcine heart (Sigma) as substrate, as described before [31]. Each enzyme was diluted in 40 mM hepes sodium salt pH 7.5 to reach final concentrations of 0.75 or 1.5 μM. A cocktail of protease inhibitors and 100 μM DTT were added to the reaction mixture to prevent degradation of CS and disulfide cross-linking between the peroxiredoxin and CS molecules, respectively, both of which could result in an apparent chaperone-like activity. Samples were pre-incubated at 43°C for 5 min and the reaction started by addition of CS to a final concentration of 0.15 μM. Light scattering due to CS aggregation at 43°C was monitored using a FluoroMax-4 spectrofluorometer, with excitation and emission wavelengths set to 500 nm and excitation and emission slits set to 2 nm. Data were recorded for 20 min.

Thermotolerance assays

L. infantum promastigotes of all lines, previously synchronized by 4–5 daily passages of 5×10^5 cells ml^{-1}, were seeded at 10^6 ml^{-1} and allowed to grow for 4 days at either 25°C or 37°C. Every 24 hours cell densities were determined with a Neubauer-counting chamber for growth curve determination.

Statistical analysis

Comparisons between the parasite lines were carried out using analysis of variance. When normality or homogeneity of variances was not observed the Kruskal-Wallis non parametric test was used. In this case, multiple comparisons were carried using the Mann-Whitney test, with the Bonferroni correction. In order to investigate whether the distribution of the negative versus positive organs (in the LDA) was independent of the parasite line, the Chi-square and the Fisher's exact tests were employed (see Text S1). Statistical significance was assessed for $p<0.05$. The analysis was carried out using IBM SPSS Statistics 19.

Supporting Information

Figure S1 mTXNPx depletion has no impact on the capacity of *L. infantum* to invade macrophages and differentiate into amastigotes. Fluorescence microscopy images showing monolayers of peritoneal macrophages from C57BL/6 mice 24 hrs after infection with *mtxnpx*$^-$ (*upper panels*) and with wild type *L. infantum* (*lower panels*). For parasite detection, the anti-cTXNPx1 antibody [8], which specifically recognizes *Leishmania* antigens, was used (*left*). Merging with propidium iodide (PI) is also shown (*right*). Arrows point to intracellular parasites. Images were acquired at a 200× magnification.
(PDF)

Figure S2 Susceptibility of mTXNPx to overoxidation in promastigotes exposed to 37°C. *Leishmania infantum* promastigotes grown for 4 days at either 25°C or 37°C (as in Figure 5B) were collected (1.4×10^7 cells) and lysed in the presence of 200 mM *N*-ethylmaleimide (NEM) (*left*). Alternatively, cell lysates were pre-treated with H_2O_2 (50 μM H_2O_2 for 30 min, followed by a further addition of 50 μM H_2O_2) prior to fixation with NEM (*right*). Extracts were examined by western blot with the anti-mTXNPx antibody. The dimer corresponds to the oxidized form of mTXNPx, whereas the monomer corresponds to either the reduced or the overoxidized protein. In cell lysates of parasites grown at 37°C the monomeric form of mTXNPx persists even upon H_2O_2 pre-treatment, indicating that this is the overoxidized (peroxidase inactive) form of the enzyme. This western blot is representative of two independent experiments. Protein loading was controlled by Ponceau staining.
(PDF)

Table S1 List of oligonucleotides employed to generate DNA constructs.
(PDF)

Table S2 Distribution of organs testing negative and positive in the limiting dilution assays used to generate the plot in Figure 2 of the main text.
(PDF)

Table S3 Distribution of organs testing negative and positive in limiting dilution assays used to generate the plot in Figure 3E of the main text.
(PDF)

Table S4 Distribution of organs testing negative and positive in limiting dilution assays used to generate the plot in Figure 4D of the main text.
(PDF)

Text S1 Statistical analysis of parasite burden.
(PDF)

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Author Contributions

Conceived and designed the experiments: HC AMT. Performed the experiments: HC FT SR MS TC. Analyzed the data: HC FT FFdS PO. Contributed reagents/materials/analysis tools: MF RA. Wrote the paper: HC AMT.

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