Helena Maria de Sousa Castro

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Instituto de Ciências Biomédicas de Abel Salazar
Universidade do Porto
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Orientadora: Doutora Ana M. Tomás

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Aos Meus Pais

To My Parents

Contents

Summary

In this thesis we have investigated some aspects of the hydroperoxide metabolism in the protozoan parasite *Leishmania infantum*. *Leishmania* are obligate intracellular parasites of man and dogs which, when residing inside the phagolysosomes of mammalian macrophages, are exposed to oxidants (including hydroperoxides) generated by the host immune system. How *Leishmania* are able to evade this oxidative insult and establish a successful infection has yet to be clearly defined. To adapt to the hostile environment of the phagolysosome, *Leishmania* have evolved several defense mechanisms, which include the synthesis of glycophospholipids to cover the parasites' surface, induction of heat shock protein expression and modulation of the host immune response. Enzymes capable of removing hydroperoxides are also part of the parasites' defense apparatus. *Leishmania* are reported to lack catalases and selenium-dependent glutathione peroxidases, the major hydroperoxide metabolizing enzymes found in higher eukaryotes. Instead, these parasites' main enzymatic mechanism for removing hydroperoxides is through the activity of 2-cysteine peroxiredoxins. This group of peroxidases act as general scavenging agents, capable of reducing a wide range of molecules including hydroperoxides and peroxynitrite, the latter being an immunologically important cytotoxic species generated by macrophages. One feature that distinguishes *Leishmania* peroxiredoxins from their mammalian homologues is that the parasites' enzymes are fuelled by the unique NADPH/trypanothione reductase/trypanothione redox cycle. Reducing equivalents are transferred from trypanothione to the peroxiredoxin by tryparedoxin, a thioredoxin-like oxidoreductase. This thesis describes the identification and characterization of one tryparedoxin (*Li*TXN1) and two peroxiredoxin enzymes (*Lic*TXNPx1 and *Lic*TXNPx2) localized to the cytosol of *L. infantum*. The enzymes' strategic compartmentalization along with their biochemical and functional features suggest that these molecules may be implicated in parasite protection from the host oxidative attack.

Besides being exposed to oxidants of exogenous origin, *Leishmania* have to deal with reactive oxygen species produced intracellularly as well. Like in other aerobic organisms, the mitochondrial respiratory chain constitutes the main endogenous source of oxidative stress in *Leishmania*. In these parasites elimination of mitochondrion-derived oxidants is likely afforded by a tryparedoxin/peroxiredoxin system similar to the one operating in the cytosol. This premise is supported by our findings that the *L. infantum* mitochondrion possesses at least one tryparedoxin (*Li*TXN2) and one peroxiredoxin (*Li*mTXNPx), and that these enzymes interact *in vitro* to catalyze hydroperoxide reduction. Interestingly, however, when attempting to reconstitute the entire NADPH/trypanothione reductase/trypanothione/tryparedoxin/ peroxiredoxin pathway within the *L. infantum* mitochondrion, we could not detect trypanothione reductase activity in this organelle. This observation suggests that alternative reductants, other

than trypanothione, may supply the mitochondrial tryparedoxin/peroxiredoxin system with the reducing equivalents required for its peroxidatic activity.

Apart from their importance as antioxidant devices, leishmanial tryparedoxins and peroxiredoxins are regarded as candidate targets for the development of new antiparasitic drugs. Indeed, these molecules present unique features that distinguish them from the mammalian enzymes and that may allow their specific inhibition without compromising the host survival and/or physiology. To obtain data which could be relevant for the rational design of specific enzyme inhibitors, we have performed biochemical and kinetic analysis on *L. infantum* enzymes of the tryparedoxin/peroxiredoxin systems. Furthermore, validation of tryparedoxins and peroxiredoxins as drug targets requires the demonstration that these molecules are essential for parasite survival and/or infectivity. To that end we have produced *L. infantum* mutants lacking the mitochondrial peroxiredoxin using a DNA recombination strategy. The observation that these transfectants are viable invalidates this enzyme as a drug target.

In short, the results presented in this thesis describe two independent tryparedoxin/ peroxiredoxin systems of *L. infantum*, one cytosolic and the other mitochondrial, which probably complement each other to remove oxidants of exogenous and endogenous sources. New perspectives regarding *Leishmania* hydroperoxide metabolism are thus presented, which can be explored in future investigations.

Chapter 1

General introduction

1. Leishmaniasis

1.1. The disease

Leishmaniasis is an infectious disease caused by the protozoan parasite *Leishmania* spp. It affects man and dogs and is transmitted to the vertebrate hosts through the bite of a female phlebotomine sandfly. Human leishmaniasis is expressed as different clinical manifestations, depending on the *Leishmania* species, the geographic location, and the host immune system. Accordingly, two major forms of the disease are distinguished: visceral leishmaniasis (VL) or Kala-azar and cutaneous leishmaniasis (CL), which, depending on the species, can develop into diffuse cutaneous or mucocutaneous leishmaniasis. Visceral leishmaniasis, the most severe form of the disease, is usually fatal if untreated. Unlike the other forms of leishmaniasis, VL affects internal organs, such as the liver and the spleen. It is caused by *Leishmania donovani* and *Leishmania infantum* (or *Leishmania chagasi* in the New World), the latest being the prevalent *Leishmania* species in Portugal and other Mediterranean countries. *Leishmania major* is, among other species, the causative agent of CL.

Human leishmaniasis has a significant impact on human populations. The disease, mainly affecting tropical and sub-tropical countries, has a worldwide prevalence of 12 million cases and is currently threatening 350 million people all over four continents. In 2002 the World Health Organization estimated that 59,000 deaths are caused by leishmaniasis with 1.5 to 2 million new cases occurring annually (http://www.who.int/leishmaniasis/en/). The disease has been rapidly expanding since the 1980's, and the appearance of drug-resistant *Leishmania* strains together with an enhanced risk of co-infection with HIV have largely contributed to this increase. The cases of *Leishmania*-HIV co-infection are particularly worrying to countries in the Mediterranean basin (Italy, France, Spain, Portugal), where *L. infantum* occupies the third position among HIV opportunistic parasites. The dual infection is a matter of concern not only because it favors parasite establishment in the host, but also because it accelerates the clinical course of HIV disease (Olivier *et al.*, 2003).

Canine leishmaniasis is a potentially lethal, viscerocutaneous disease with relevance in veterinary science. Infected dogs, besides being affected by the disease, constitute the main domestic reservoirs of the parasite and play a key role in transmission to humans in Brazil, China and Mediterranean countries.

1.2. *Leishmania*: the infectious agent

Kinetoplastida

Leishmania are unicellular flagellated protozoan parasites belonging to the order Kinetoplastida. Apart from *Leishmania*, two other Kinetoplastida species are pathogenic to man, namely *Trypanosoma brucei* (causing African sleeping sickness) and *Trypanosoma cruzi* (causing Chagas' disease). All three are parasites of the blood and/or of tissues of the mammalian host and are transmitted by arthropod vectors. Together, these parasites infect 30 million people worldwide and 500 million are at risk of infection. Another species of the order Kinetoplastida is *Crithidia fasciculata*, an insect parasite non-pathogenic to man that is used as a model to study the disease-causing organisms. These parasites, members of the family Trypanosomatidae, are broadly referred to as trypanosomatids.

Biology

The biochemistry of Kinetoplastida differs significantly from that of higher eukaryotes in a number of aspects. The major distinguishing feature of Kinetoplastida is a subcellular structure known as the kinetoplast; this contains a complex network of circular DNA molecules located in the single mitochondrion of these microorganisms. Another peculiar aspect of trypanosomatids is the compartmentalization of the glycolytic and other energy metabolism pathways inside a peroxisome-like organelle named glycosome (Opperdoes, 1987). Kinetoplastida molecular biology is also distinct from that of other eukaryotes (reviewed in Stiles *et al.*, 1999). Besides containing relatively few introns, Kinetoplastida genes are transcribed into large polycistronic precursor RNAs which are subsequently processed into individual messages by trans-splicing and polyadenylation. These organisms apparently lack promoters for RNA polymerase II, and gene expression is controlled by events like trans-RNA splicing, polyadenylation, mRNA half-lives, protein synthesis, and protein stability. Finally, as will be detailed later in this chapter, trypanosomatids' redox balance and antioxidant defense are dependent on a unique molecule, trypanothione. Although other peculiarities of Kinetoplastida biology could be mentioned, they are beyond the scope of this thesis.

The life cycle

At least seventeen *Leishmania* species are known to cause the distinct clinical manifestations of leishmaniasis. Nevertheless, all these species are morphologically similar and display two main developmental stages throughout their life cycle: an insect vector stage and a vertebrate host stage (Figure 1).

Inside the insect vector, the female sandfly of the genus *Lutzomyia* in the New World and of the genus *Phlebotomus* in the Old World, *Leishmania* reside in the alimentary tract and

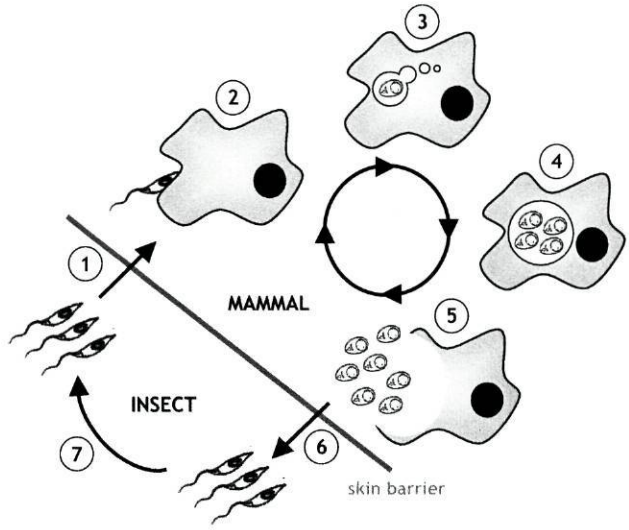

Figure 1. The *Leishmania* life cycle. *Leishmania* metacyclic promastigotes are delivered to the mammalian host through the bite of an infected sandfly (1). Promastigotes then attach to macrophages and are phagocytized (2). Inside the macrophage parasite-containing phagosomes fuse with lysosomes, forming phagolysosomes, wherein promastigotes differentiate into amastigotes (3), replicate (4), and are released from the infected macrophages, spreading the disease within the mammalian host (5). Following ingestion of the parasite by the sandfly during a bloodmeal, amastigotes undergo differentiation into promastigotes (6), which then go on to develop into the infective metacyclic stage (7). Adapted from Ponte-Sucre (2003).

exist as flagellated extracellular promastigotes with an elongated shape. The life cycle of the parasites within the sandfly includes the differentiation of promastigotes from a dividing non-infective or procyclic stage, into a non-dividing infective or metacyclic stage (reviewed in Alexander *et al.*, 1999). Metacyclic promastigotes display increased resistance to certain microbicidal mechanisms, including complement-mediated lysis, and oxygen-dependent and -independent leishmanicidal activities of their host macrophages (Sacks, 1992). Hence, metacyclic promastigotes are well adapted for infecting and surviving within the vertebrate host. This differentiation process can be mimicked *in vitro*, infectious promastigotes being predominantly found in non-dividing stationary phase cultures (Sacks and Perkins, 1985).

Infection of the mammalian host with *Leishmania* metacyclic promastigotes occurs during the blood meal of an infected sandfly. Inside the mammalian host, infective promastigotes are phagocytized by macrophages. The parasite-containing phagosome then fuses with a lysosome forming a phagolysosome, wherein promastigotes differentiate into aflagellar obligate intracellular amastigotes with a spherical shape. Normally a pathogen would be destroyed in the hostile environment of the phagolysosome, but *Leishmania* are resistant to the acidic pH, hydrolytic enzymes and oxygen and nitrogen intermediates present therein. *Leishmania* amastigotes replicate inside the phagolysosomes and, after release by an unknown mechanism, invade other macrophages, thereby propagating the infection. For *Leishmania* species causing CL the infection remains in the skin, but in the case of VL the parasites spread from the initial skin lesion into organs such as the liver, the spleen and the bone marrow. As a new insect bites

an infected vertebrate host it swallows infected macrophages and amastigotes released into the circulation. In the sandfly amastigotes transform back into promastigotes. Since amastigotes, the cellular form relevant for the mammalian disease, are difficult to obtain in sufficient number for research, it is possible to take advantage of the physiological equivalence between the axenic amastigotes and lesion-derived amastigotes (Ismaeel *et al.*, 1998). Axenic amastigotes can be obtained *in vitro* by promastigote exposure to high temperature and low pH (Zilberstein and Shapira, 1994).

1.3. Strategies to combat the disease

Current therapies and efforts

Leishmaniasis is a complex of different diseases, therefore treatment with a single approach or tool constitutes a challenging task (Ponte-Sucre, 2003; Rosenthal and Marty, 2003; Croft and Coombs, 2003). While most cases of CL heal without treatment leaving the person immune to further infection, other forms of leishmaniasis, such as VL, are extremely difficult to treat, often requiring long course administration of drugs. Also, since *Leishmania* are obligate intracellular parasites, drugs circulating in the blood may not reach the parasite easily. Although chemotherapy is the usual therapeutic approach against VL, no entirely satisfactory drugs actually exist. These usually suffer from poor efficacy, long treatment regimes, host toxicity, drug resistance, and/or impeditive costs.

Pentavalent antimonials (Glucantime® and Pentostam®) are the first line of antileishmanial drugs and have been in use since the 1920's. However, the appearance of antimonial resistance in some areas of endemicity has changed the pattern of leishmaniasis treatment. For antimony-resistant human leishmaniasis other chemotherapeutics are used, namely pentamidine and various formulations of amphotericin B. Although amphotericin B based treatments are effective, e.g. the liposomal formulation Ambisome®, their cost is prohibitive. Another efficient leishmanicidal agent is miltefosine, which has been recently approved as the first oral drug for VL in India. However, *Leishmania* develop resistance to miltefosine, at least *in vitro* (Perez-Victoria *et al.*, 2003). Immunomodulatory drugs, which enhance the host immune response against the invading parasite, are promising therapeutics in conjunction with chemotherapy (reviewed in Croft and Coombs, 2003).

Development of a vaccine to protect from *Leishmania* infection is not an easy assignment given the complex immune response developed by the host. Moreover, *Leishmania*, being intracellular parasites, are protected from the host humoral response. Although some progress in anti-*Leishmania* vaccination have been made in murine models (Coler *et al.*, 2002; Campos-Neto *et al.*, 2002; Tonui *et al.*, 2004; Aguilar-Be *et al.*, 2005), these are not entirely predictive of how effective a vaccine candidate will be in humans, making progress in this area difficult.

In short, chemotherapy, the main tool for the control of leishmaniasis, presents unsatisfactory features. It is therefore urgent to develop inexpensive, effective and rapid formulations against this disease.

Rational drug design

One popular strategy employed in the development of new antileishmanial drugs is the "rational drug design" approach. This consists on the identification of potential drug targets, their validation, by either chemical or genetic tools, and the development and testing of potential inhibitors of such molecules. Two criteria must be met for an enzyme to be validated as a drug target: (i) the enzyme must either be absent from the vertebrate host or present unique features that distinguish it from analogous molecules of the mammal, and (ii) the enzyme must be essential for parasite survival and/or infectivity. The prospect that broad-spectrum drugs with cytotoxic effects on *Leishmania* and trypanosomes might be identified has prompted many laboratories to study the biochemical pathways that are common to all Kinetoplastida. It must be mentioned, however, that the rational drug design approach is slow and costly, and that the poor resources of the developing countries, where trypanomasomiasis and leishmaniasis are mainly established, keep pharmaceutical industries away from investing in the development of new antiparasitic therapeutics.

2. The antioxidant enzymes of *Leishmania* as potential targets for antiparasitic drugs

Trypanosomatidal pathways for the elimination of reactive oxygen and nitrogen species constitute attractive targets for the development of antiparasitic drugs. In fact, not only are these parasites sensitive to oxidative and nitrosative stress, as their antioxidant enzymes present distinctive features from those of their mammalian counterparts to think of their specific inhibition without affecting the host metabolism and/or physiology.

2.1. Reactive oxygen and nitrogen intermediates

The term "reactive oxygen intermediates" (ROI) refers to a variety of highly unstable molecules and free radicals derived from molecular oxygen (O_2). The single-electron reduction of O_2 generates superoxide anion ($O_2^{\cdot-}$), a radical species which does not easily cross biological membranes (Lynch and Fridovich, 1978) and is not very reactive *per se*. Nevertheless, $O_2^{\cdot-}$ is the precursor of most ROI (Figure 2). Accordingly, dismutation of $O_2^{\cdot-}$ produces hydrogen peroxide

(H_2O_2), a freely diffusible molecule that, besides reacting with biological macromolecules, yields hydroxyl radical ($HO^{\cdot}$) via oxidation of a transition metal ion, such as Fe^{2+}. This so called "Fenton reaction" is propagated by $O_2^{\cdot-}$, which regenerates the pool of reduced metal ions available for reacting with H_2O_2 ("Haber-Weiss reaction"). Hydroxyl radical is a powerful oxidant, capable of generating cellular damage at different levels, including direct protein damage, damage to DNA, and membrane damage due to lipid peroxidation. Peroxidation of lipids yields lipid hydroperoxides, and these molecular species are also amenable to participate in Fenton reactions.

Besides yielding ROI, $O_2^{\cdot-}$ is also the precursor of peroxynitrite ($ONOO^-$), through a fast reaction with nitric oxide ($^{\cdot}NO$). Nitric oxide is produced in cells by the enzymatic activity of nitric oxide synthases (NOS), which generate $^{\cdot}NO$ and L-citrulline from L-arginine. The different nitrogen-containing species derived from $^{\cdot}NO$ are globally referred to as reactive nitrogen intermediates (RNI) (Figure 2). RNI are involved in harmful oxidation, nitration and nitrosilation reactions, $ONOO^-$ being the most cytotoxic species. Peroxynitrite reacts either directly with thiols and transition metal centers or indirectly, via its degradation products, $HO^{\cdot}$, nitrogen dioxide ($^{\cdot}NO_2$) and carbonate radical anion ($CO_3^{\cdot-}$), it initiates free radical reactions

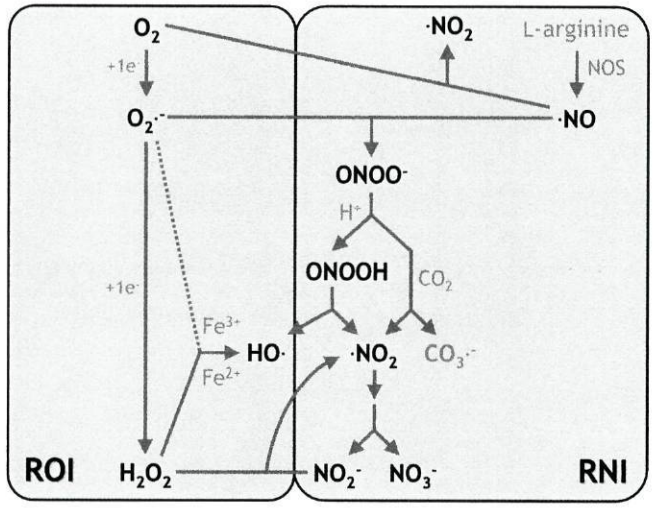

Figure 2. Routes for reactive oxygen and nitrogen intermediates generation: distinct, yet interacting, pathways. *Left panel* The generation of reactive oxygen intermediates (ROI) is initiated by the monovalent reduction of molecular oxygen (O_2) to superoxide anion ($O_2^{\cdot-}$). Superoxide anion dismutation (either spontaneous or enzymatic) yields hydrogen peroxide (H_2O_2), a molecular species that, via oxidation of a metal ion (such as Fe^{2+}), leads to hydroxyl radical ($HO^{\cdot}$) formation; in this reaction regeneration of the reduced metal ion is guaranteed by $O_2^{\cdot-}$. *Right panel* Nitric oxide ($^{\cdot}NO$) is the precursor of reactive nitrogen intermediates (RNI). Nitric oxide is derived from the nitric oxide synthase (NOS)-catalyzed oxidation of L-arginine. ROI and RNI pathways cross-talk as $^{\cdot}NO$ reacts with $O_2^{\cdot-}$ to yield peroxynitrite ($ONOO^-$). Peroxynitrite may either be protonated to peroxynitrous acid (ONOOH) or react with carbon dioxide (CO_2) and generate two radical species, nitrogen dioxide ($^{\cdot}NO_2$) and carbonate radical anion ($CO_3^{\cdot-}$). Peroxynitrous acid decomposition again yields $^{\cdot}NO_2$ and $HO^{\cdot}$. Two additional sources of $^{\cdot}NO_2$ involve ROI and RNI interactions: generation of $^{\cdot}NO_2$ either by H_2O_2 reaction with NO_2^- or by O_2 reaction with $^{\cdot}NO$. Nitrogen dioxide is the precursor of nitrite (NO_2^-) and nitrate (NO_3^-).

such as lipid peroxidation (reviewed in Augusto *et al.*, 2002). Nitrogen dioxide, alternatively derived from the reactions of $^{\cdot}NO$ with O_2 or of NO_2^- with H_2O_2, is also the precursor of nitrite (NO_2^-) and nitrate (NO_3^-).

The damage produced by ROI and RNI and the cellular mechanisms triggered in response to these species are generally designated by "oxidative stress", although the term "nitrosative stress" can be used to distinguish RNI-induced stress.

2.2. Sources of ROI and RNI in *Leishmania*

During their life cycle *Leishmania* are exposed to ROI and RNI originated both internally and by their surrounding environment. As detailed next, the major source of exogenous oxidative and nitrosative species to the parasites is the host immune response. Some drugs used clinically to treat leishmaniasis may also act as sources of exogenous oxidative stress. That is the case of the pentavalent antimonial Pentostam®, which has been reported to exacerbate the production of oxidants by macrophages (Rais *et al.*, 2000) and to interfere with the metabolism of trypanothione (Wyllie *et al.*, 2004), a low-molecular thiol which, among other functions, is responsible for the parasites' antioxidant defense. In this section of the thesis the intracellular production of oxidants is also addressed, with emphasis on the *Leishmania* mitochondrial respiratory chain.

2.2.1. The host immune response

Interaction of *Leishmania* with the host phagocytes triggers an immune response with concomitant production of harmful oxidants aimed at killing the parasite. Phagocytosis of *Leishmania* by macrophages is accompanied by a high output production of $O_2^{\cdot-}$, known as oxidative burst. This results from the activation of the host enzyme phagocyte NADPH oxidase (phox), which is usually dormant in resting cells. During phagocytosis, cytosolic and membrane phox subunits are assembled at the phagosome membrane in order to achieve a fully active enzyme capable of catalyzing the one electron reduction of O_2 to $O_2^{\cdot-}$ (reviewed in Nauseef, 2004).

The product of $O_2^{\cdot-}$ dismutation, H_2O_2, may react with chloride anion (Cl^-) and yield the highly damaging molecule, hypochlorite (OCl^-). This species is also the precursor of chloramines, a group of microbicidal oxidized halogens that result from the reaction between OCl^- and ammonia or amines. Hypochlorite generation is catalyzed by the enzyme myeloperoxidase, present in neutrophils, but absent in macrophages. Neutrophils are the first leukocytes to be recruited to the site of infection, wherein they exert a microbicidal action

through the activity of granulocytic enzymes (such as myeloperoxidase). In the case of *Leishmania* infection, however, neutrophils do not always play such protective role. Indeed, neutrophils have been reported to either favor or control parasite growth depending on the *Leishmania* species (CL versus VL causing species; Rousseau *et al.*, 2001; Ribeiro-Gomes *et al.*, 2004) and on the genotype of the host used as model (e.g. *L. major* susceptible versus *L. major* resistant mice; Ribeiro-Gomes *et al.*, 2004). The mechanisms by which neutrophils promote *L. major* growth in a susceptible mouse model are possibly by modulating of the host immune response (Tacchini-Cottier *et al.*, 2000; Ribeiro-Gomes *et al.*, 2004) and/or by serving as "Trojan horses" for the parasite to enter its definitive host cells, the macrophages (Laskay *et al.*, 2003).

Other oxidants, such as $^{\cdot}OH$ and $ONOO^{-}$, are also derived from phox-generated $O_2^{\cdot-}$. As previously mentioned, $ONOO^{-}$ generation requires the presence of both $O_2^{\cdot-}$ and $^{\cdot}NO$. Within the macrophages $^{\cdot}NO$ synthesis is driven by inducible NOS (iNOS), an enzyme that becomes fully operative upon activation by pro-inflammatory cytokines, such as γ-IFN, TNF-α, IL-1 and IL-12 (reviewed in Nathan and Hibbs, Jr., 1991).

The role of ROI and RNI in Leishmania infection control

ROI and RNI produced by macrophages in the course of infection are toxic to *Leishmania* (Murray, 1981a; Murray, 1981b; Vouldoukis *et al.*, 1995; Lemesre *et al.*, 1997; Linares *et al.*, 2001; Gantt *et al.*, 2001), and are thus used by the host as powerful weapons against invading pathogens.

Immediately upon invasion of the mammalian host, *Leishmania* are exposed to ROI, as a consequence of the macrophage phagocytic oxidative burst (Gantt *et al.*, 2001). Although phox activation enhances parasite killing by macrophages (Murray, 1981a; Gantt *et al.*, 2001), its role in *Leishmania* infection control must be carefully evaluated, as its effects may vary according to distinct leishmaniasis models. As an example, while the contribution of ROI to *L. donovani* clearance is confined to the very early stage of the intracellular infection and is dispensable to control the disease (Murray and Nathan, 1999), in the case of *L. major* the antiparasitic action of phox is prominent at latter stages of infection, being relevant for parasite clearance from the spleen (Blos *et al.*, 2003). Also, the consequence of phox activation in *Leishmania* growth control has been reported to differ with respect to a specific organ (Blos *et al.*, 2003). More recently Pham *et al.* (2005) proposed that *Leishmania* amastigotes may inhibit phox assembly as a strategy to avoid $O_2^{\cdot-}$ production by the vertebrate host.

The second line of oxidants produced by macrophages in response to *Leishmania* invasion is iNOS-derived RNI. Unlike phox, iNOS activity was undoubtedly shown to be crucial to control *Leishmania* infection (Vouldoukis *et al.*, 1995; Vouldoukis *et al.*, 1997; Murray and Nathan, 1999; Blos *et al.*, 2003). Indeed, resistance or susceptibility to *L. major* is well established to depend on the activation or the silencing of iNOS by Th1 or Th2 cytokines,

respectively (Solbach and Laskay, 2000 and references therein), and, in the case of the visceral form of the disease, resolution of the infection is also dependent on the activity of iNOS, at least in a murine model (Murray and Nathan, 1999). Still, the mechanisms by which iNOS exerts its leishmanicical activity remain controversial. While some authors state that iNOS controls *Leishmania* growth by generating cytotoxic RNI (Augusto *et al.*, 1996; Giorgio *et al.*, 1998; Linares *et al.*, 2001; Gantt *et al.*, 2001), others argue that the microbicidal effect of iNOS activation is due to the concurrent inhibition of polyamine synthesis both in the macrophage and in *Leishmania* (Iniesta *et al.*, 2001; Kropf *et al.*, 2005). In Figure 3 a schematic representation of these two iNOS-mediated leishmanicidal mechanisms is shown. As illustrated therein, iNOS-catalyzed generation of ·NO from L-arginine occurs in two steps, whereby N^G-hydroxyl-L-arginine (NOHA) is produced as intermediate. NOHA is a potent inhibitor of arginase, an enzyme that uses L-arginine to initiate the synthesis of polyamines (putrescine, spermidine and spermine). These are molecules with relevant functions during cell proliferation, differentiation and synthesis of macromolecules. Accordingly, activation of iNOS by pro-inflammatory Th1 cytokines does not only lead to ·NO production, with concomitant generation of cytotoxic RNI, but it also blocks polyamine synthesis through the arginase inhibitory action of NOHA, either mechanism having a negative impact on *Leishmania* growth. Conversely, arginase induction by

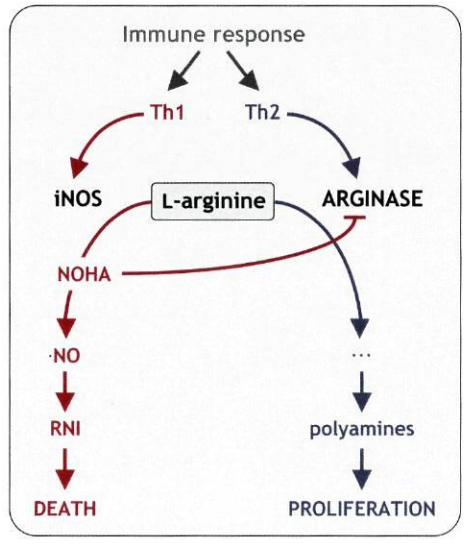

Figure 3. Proposed mechanisms for iNOS-mediated *Leishmania* growth control. In the established *Leishmania* infection model, activated murine macrophages metabolize L-arginine by two alternative pathways. In the first pathway (in blue), arginase hydrolyses L-arginine to urea and L-ornithine, the precursor of polyamines, which are molecules essential for parasite growth. In the second pathway (in red), inducible nitric oxide synthase (iNOS) catalyses the conversion of L-arginine to nitric oxide (·NO), yielding N^G-hydroxyl-L-arginine (NOHA) as intermediate. NOHA is an effective inhibitor of arginase, blocking polyamine synthesis. Furthermore, ·NO is the precursor for reactive nitrogen intermediates (RNI), molecular species that are toxic to the parasites. Accordingly, activation of iNOS by Th1 cytokines impairs *Leishmania* growth by generating nitrosative stress and/or by depleting parasites from polyamines. Contrary to this, a Th2 immune response activates arginase, thereby diverting L-arginine metabolism towards polyamine synthesis and promoting parasite growth.

Th2 cytokines (like IL-4, IL-10), drives L-arginine metabolism towards polyamine synthesis, and not RNI production, a scenario favorable for parasite survival and replication (Iniesta *et al.*, 2002; Iniesta *et al.*, 2005). Given the dual effect of iNOS activation it remains elusive whether the antileishmanial action of this enzyme is due to the generation of toxic RNI, to polyamine starvation or to both causes.

At this point, three notes must be added, regarding the role of iNOS in parasite clearance. First, in contrast to the murine model, for which there is a well-documented correlation between $^{\cdot}NO$ production and infection control (Augusto *et al.*, 1996; Giorgio *et al.*, 1998; Linares *et al.*, 2001), $^{\cdot}NO$ contribution for *Leishmania* clearance in humans is arguable. In fact, even though there is experimental evidence showing that iNOS activation impairs parasite survival within human macrophages (Gantt *et al.*, 2001), $^{\cdot}NO$ is hardly detected in these cells supernatants (Murray and Teitelbaum, 1992; Gantt *et al.*, 2001). The second point concerns the kinetics of $O_2^{\cdot-}$ and $^{\cdot}NO$ production and its implications in $ONOO^-$ generation. *In situ*, both species are produced at different time points [a short 60 min post-infection burst for $O_2^{\cdot-}$ (Pearson *et al.*, 1982), against 48-72 hours after infection for $^{\cdot}NO$ (Gantt *et al.*, 2001)], and that may prevent the generation of $ONOO^-$. However, as pointed out by Augusto *et al.* (2002), it is possible that the simultaneous production of $O_2^{\cdot-}$ and $^{\cdot}NO$ occurs within the site of infection due to the continuous invasion of macrophages (either tissue resident or newly recruited) by parasites. Although $ONOO^-$ is considered to be the main cytotoxic $^{\cdot}NO$-derived species, no direct evidence for $ONOO^-$ generation during *Leishmania* infection has ever been shown. In fact, and this relates to the third and final note, $ONOO^-$ detection in infected tissues is always performed indirectly, the most popular approach being the detection of a $ONOO^-$ nitration product, 3-nitrotyrosine. However, 3-nitrotyrosine is also generated in reactions independent of $ONOO^-$ and this compound is otherwise regarded as a marker for RNI in general (Halliwell, 1997 and references therein; Linares *et al.*, 2001).

Finally, ROI and RNI, apart from their direct leishmanicidal action, have also been reported to control the parasitic infection through modulation of the host immune response (Murray and Nathan, 1999; Bogdan *et al.*, 2000).

2.2.2. The *Leishmania* mitochondrion

Apart from the oxidative and nitrosative challenges imposed by the surrounding environment, *Leishmania* are also exposed to ROI and RNI of intracellular origin. The *Leishmania* mitochondrion constitutes the primary source of endogenously generated-ROI in the parasite. However, other organelles, such as the endoplasmic reticulum (Tu and Weissman, 2004) and the glycosomes (Boveris and Stoppani, 1977; van den *et al.*, 1992; Subramani, 1998), wherein various oxidative processes take place, should not be disregarded as important sites of

ROI production as well. The recent finding that *Leishmania* promastigotes display NOS activity (Genestra *et al.*, 2003a; Genestra *et al.*, 2003b), suggests that RNI can also be generated intracellularly.

Unlike other aerobic organisms, trypanosomatids possess one single mitochondrion with a tubular shape, extended along the parasite body. Within the *Leishmania* mitochondrion the electron transport chain contributes largely to the endogenous generation of ROI. In oxygen-dependent respiration, reducing equivalents from glucose degradation or from succinate enter the mitochondrial respiratory chain at complexes I and II, respectively. Electrons are then transported across a redox cascade that culminates in O_2 reduction to water (H_2O). Figure 4 shows the *Leishmania* respiratory chain, wherein two main differences are found with respect to mammals: (1) the existence of an alternative oxidase (cytochrome *o*, described as a *b*-type cytochrome) that drives the complex IV-independent O_2 reduction (Santhamma and Bhaduri, 1995), and (2) the presence of NADH-fumarate reductase (FR), an enzyme that regenerates succinate from fumarate (Santhamma and Bhaduri, 1995; Chen *et al.*, 2001). FR, also present in *T. cruzi* (Boveris *et al.*, 1986) and *T. brucei* (Turrens, 1987), guarantees the continuous regeneration of succinate from fumarate. Fumarate is derived from malate via a reaction that reverts one step of the Krebs' cycle. Succinate is the main electron supplier for the *Leishmania* mitochondrial chain (at complex II) and this might reflect the absence of a functional complex I able to fuel the electron transport chain with NADH-derived reducing equivalents in these

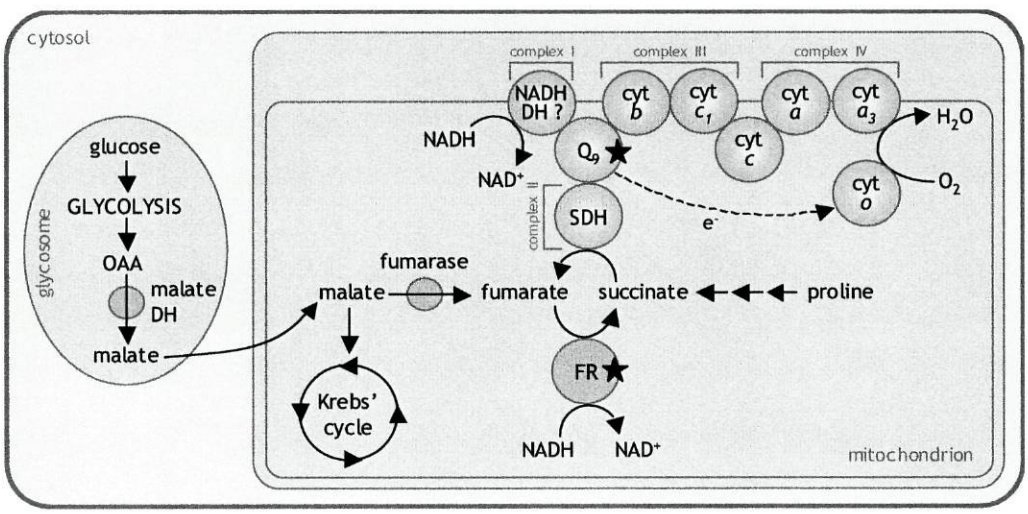

Figure 4. The *Leishmania* electron transport chain. Electrons enter the respiratory chain at complex II (succinate dehydrogenase, SDH) and possibly at a non-classical complex I (NADH dehydrogenase, NADHDH). Succinate is derived either from L-proline metabolism, one of the main energy sources of trypanosomatids, or from malate by reverting two Krebs' cycle steps: (i) malate is converted into fumarate, in a fumarase-catalysed reaction, and (ii) the unique trypanosomatidal enzyme fumarate reductase (FR) generates succinate from fumarate at expenses of NADH. Complexes I and II-derived electrons are transferred to ubiquinone Q_9 and transported to O_2 through complex III, cytochrome and complex IV. In *Leishmania* cytochrome *o* constitutes a by-pass route to O_2 reduction to water, although its specific function is unknown. In *Leishmania* the potential sites for $O_2^{\cdot-}$ generation, highlighted with a star, are the active site of FR and ubiquinone Q_9. Cyt, cytochrome; DH, dehydrogenase; OAA, oxaloacetate.

organisms. Indeed, the existence of a classical complex I in *Leishmania* remains rather elusive and controversial, and this is probably due to the different experimental conditions tested by various laboratories (Martin and Mukkada, 1979; Hart *et al.*, 1981; Santhamma and Bhaduri, 1995; Bermudez *et al.*, 1997).

The single-electron reactions occurring in the respiratory chain favor the monovalent reduction of O_2 to $O_2^{\cdot-}$ (Loschen *et al.*, 1971; Boveris *et al.*, 1972; Boveris and Chance, 1973; Loschen *et al.*, 1974; Cadenas *et al.*, 1977; Turrens, 1997). In trypanosomatids the probable sources of mitochondrial $O_2^{\cdot-}$ are the enzyme FR and ubiquinone Q_9 (Turrens, 1987; Denicola-Seoane *et al.*, 1992; Santhamma and Bhaduri, 1995). Moreover, formation of $O_2^{\cdot-}$ may be further enhanced in the presence of electron transport chain inhibitors, which cause the carriers upstream from the site of inhibition to become fully reduced and to leak electrons to O_2 (Boveris and Chance, 1973; Loschen *et al.*, 1973a; Loschen *et al.*, 1973b; Cadenas *et al.*, 1977). Superoxide anion, besides inhibiting the mitochondrial function by inactivating Fe-S centers of the electron transport chain complexes and also the Krebs' cycle enzyme aconitase (Gardner, 2002), generates H_2O_2 by the enzymatic activity of the mitochondrial superoxide dismutase (Chance *et al.*, 1979; Dufernez *et al.*, 2006; Wilkinson *et al.*, 2006). The effects of the physiological production of mitochondrial H_2O_2 remain unclear, although in higher eukaryotes this phenomenon has been implicated in cell signaling of proliferation and/or apoptosis (reviewed in Cadenas, 2004).

It is possible that, as described in higher eukaryotes (reviewed in Radi *et al.*, 2002), the *Leishmania* mitochondrion is also the place for the intracellular formation and reactions of $ONOO^-$. Nitric oxide present in mitochondria derives from the diffusion of cytosolic-produced $^{\cdot}NO$ and, at least in mammalian cells, from the enzymatic activity of a mitochondrial NOS enzyme (Giulivi *et al.*, 1998; Tatoyan and Giulivi, 1998; Ghafourifar and Cadenas, 2005). Although no obvious NOS coding sequence is annotated in trypanosomatid gene databases (http://www.genedb.org), *Leishmania* were shown to display NOS activity (Genestra *et al.*, 2003a; Genestra *et al.*, 2003b). Irrespective of the subcellular compartmentalization of such enzymatic activity (yet to be described), $^{\cdot}NO$ may easily enter the mitochondrial compartment and react with respiratory chain-derived $O_2^{\cdot-}$ to generate $ONOO^-$. In addition, since $ONOO^-$ and its protonated form (peroxynitrous acid, ONOOH) can cross biomembranes (Denicola *et al.*, 1998; Romero *et al.*, 1999; Alvarez *et al.*, 2004) and diffuse for 1-2 cell diameters (10-20 μm) (Romero *et al.*, 1999; Alvarez *et al.*, 2004), mitochondrial $ONOO^-$ may also be imported from the cytosolic compartment, or derived from an exogenous source. This phenomenon should be particularly important in the mammalian stage of *Leishmania*, which, residing inside macrophages' phagolysosomes, may become exposed to toxic amounts of $ONOO^-$ generated in response to infection. The abundance of CO_2 in mitochondria favors $ONOO^-$ decomposition into the highly reactive carbonate ($CO_3^{\cdot-}$) and nitrogen dioxide ($^{\cdot}NO_2$) radicals. Either directly or via its decomposition radicals, $ONOO^-$ may react with and inhibit critical mitochondrial

components, such as complexes I and II, and aconitase, among others (reviewed in Brown, 1999; reviewed in Radi *et al.*, 2002; Brown and Borutaite, 2004). Peroxynitrite also interferes with mitochondrial signaling of apoptosis, by promoting the opening of the permeability transition pore (Packer *et al.*, 1997) and the release of pro-apoptotic factors, such as calcium, into the cytosol (Schweizer and Richter, 1996).

2.3. *Leishmania* protection from ROI and RNI

As stated before, ROI and RNI are reactive molecules that cause damage in living organisms. In order to cope with these cytotoxic species, cells have adopted efficient mechanisms of defense. These include enzymatic and non-enzymatic systems for ROI and RNI elimination, and also mechanisms to repair oxidative and nitrosative damage. Although antioxidant defenses are widely distributed in the various cell compartments, this thesis will focus mainly on the cytosolic and on the mitochondrial enzymes, emphasizing the differences between the *Leishmania* and the mammalian host machineries for ROI and RNI detoxification.

Antioxidants: mammals versus Leishmania

Non-enzymatic antioxidant defenses of *Leishmania* include the ubiquitous heat shock proteins (Miller *et al.*, 2000) and GSH (Romao *et al.*, 1999), and the unique molecules lipophosphoglycan (LPG), trypanothione and ovothiol A (or N^1-methyl-4-mercaptohistidine). LPG is a glycolipid highly abundant at the surface of infectious metacyclic promastigotes, where it functions as a ROI scavenger (Spath *et al.*, 2003). Trypanothione and ovothiol A are low molecular weight thiols which can directly react with ROI and RNI (Spies and Steenkamp, 1994; Nogoceke *et al.*, 1997; Ariyanayagam and Fairlamb, 2001; Thomson *et al.*, 2003; Vogt and Steenkamp, 2003). Modulation of the host immune response is also part of the parasites' defense armentarium against oxidants, and this may be achieved either by prevention of phox assembly (Pham *et al.*, 2005) or by inhibition of $^{\cdot}NO$ generation (Balestieri *et al.*, 2002).

The first line of enzymatic defense against ROI is a class of enzymes called superoxide dismutases or SODs. These are metalloenzymes that catalyse the dismutation of $O_2^{\cdot-}$ to H_2O_2 and O_2 and are present in both mammals and trypanosomatids, although with a striking difference: while mammalian SODs possess either copper/zinc or manganese at their active site, depending on their cytosolic or mitochondrial matrix location, respectively (Fridovich, 1997), parasitic SODs are iron-containing enzymes (Le Trant *et al.*, 1983; Temperton *et al.*, 1996; Paramchuk *et al.*, 1997; Ismail *et al.*, 1997; Kabiri and Steverding, 2001; Plewes *et al.*, 2003). In *L. chagasi* one putative mitochondrial (Paramchuk *et al.*, 1997) and two glycosomal (Paramchuk *et al.*, 1997; Plewes *et al.*, 2003) SODs were described. Also, in *T. brucei* four iron-

SODs have been identified, which are distinctively located to the parasite cytosol, glycosome and mitochondrion (Wilkinson *et al.*, 2006; Dufernez *et al.*, 2006).

The function of SODs is to maintain $O_2^{\cdot-}$ concentration at the lowest possible level in order to avoid $^{\cdot}OH$ and $ONOO^-$ generation. However, the product of $O_2^{\cdot-}$ dismutation, H_2O_2, is also the precursor of the toxic $^{\cdot}OH$, and therefore it must be eliminated. Catalase is one of the best known H_2O_2 eliminating enzymes (rate constant for the reduction of the hydroperoxide, $k_{ROOH} \sim 10^6\ M^{-1}\ s^{-1}$) (Hillar *et al.*, 2000). This heme-containing enzyme is restricted to the peroxisomes of higher eukaryotes, but it is absent from *Leishmania* and other trypanosomatids. Equally efficient at reducing H_2O_2 ($k_{ROOH} \sim 10^8\ M^{-1}\ s^{-1}$) are selenium-containing glutathione peroxidases (GPxs) (reviewed in Brigelius-Flohe and Flohe, 2003). In addition to reducing H_2O_2, these enzymes are also active against fatty acid hydroperoxides and lipid hydroperoxides integrated into biomembranes. Upon oxidation by the hydroperoxide the pool of reduced GPx is regenerated by a small thiol, glutathione (GSH), which itself is redox-cycled by the flavoenzyme glutathione reductase (GR) at expenses of NADPH. GPxs are found in the cytosol, mitochondria and extracelullar space of mammalian cells.

In *Leishmania* no glutathione peroxidase activity was ever reported, however the *L. major* gene database (http://www.genedb.org) has four annotated GPx-like sequences. Three of these genes are identical, except in their 5' and 3' regions, and are clustered within the same genetic *locus*. An identical genomic organization is found for the GPx-like molecules of both *T. cruzi* (http://www.genedb.org) and *T. brucei* (Hillebrand *et al.*, 2003; http://www.genedb.org). While the *T. cruzi* GPxI enzyme is localized to the cytosol and glycosomes of the parasite (Wilkinson *et al.*, 2002a), the related *T. brucei* enzymes (*Tb*GPxI-III) are found in the cytosol and in the mitochondrion (Schlecker *et al.*, 2005). In addition, despite their similarity, the *T. cruzi* and *T. brucei* enzymes display different substrate specificities. Unlike *Tb*GPxIII, *Tc*GPxI does not accept H_2O_2 and is active towards fatty acid and phospholipid hydroperoxides (Wilkinson *et al.*, 2000a). A second GPx-like molecule from *T. cruzi* (*Tc*GPxII), exhibiting low similarity with the three clustered sequences, was reported to be compartmentalized in the endoplasmic reticulum and to remove lipid hydroperoxides (Wilkinson *et al.*, 2002c). A feature common to the trypanosomatidal GPx-like molecules is the fact that the conserved selenocysteine residue present in GPx of higher eukeryotes is replaced by a cysteine. This substitution determines the lower catalytical efficiency of the parasitic peroxidases in comparison to the selenium-containing homologues (Maiorino *et al.*, 1995; Sztajer *et al.*, 2001). Another difference in relation to the mammalian enzymes is that the parasites peroxidases are poorly reduced by GSH and, instead, an enzyme belonging to the family of thioredoxin-like thiol-disulfide oxidoreductases, either thioredoxin (Hillebrand *et al.*, 2003) or tryparedoxin (TXN; Wilkinson *et al.*, 2002a; Hillebrand *et al.*, 2003), is preferentially used as electron donor.

Peroxiredoxins (Prxs) are another family of H_2O_2 eliminating enzymes. These lack prosthetic groups or tightly bound metal ions and, for that reason, they are regarded as being

less efficient at reducing H_2O_2 and other hydroperoxides ($k_{ROOH} \sim 10^5\ M^{-1}\ s^{-1}$) (reviewed in Wood *et al.*, 2003). Nevertheless, some members of this family of peroxidases exhibit rate constants for ROOH reduction close to those found for catalase and selenium-containing GPxs. That is the case of the *T. brucei* peroxiredoxin ($k_{ROOH} \sim 10^7\ M^{-1}\ s^{-1}$; Budde *et al.*, 2003). Prxs usually possess two active sites, which, with few exceptions, consist of two separate cysteine residues, embedded in a Val-Cys-Pro motif. The pool of reduced Prx is maintained by proteins containing a Cys-X-X-Cys motif, like thioredoxins, tryparedoxins, glutaredoxin or AhpF (reviewed in Flohe *et al.*, 2003). Given their relatively low efficiency in reducing hydroperoxides, Prxs have been implicated in regulation of redox sensitive signaling cascades in higher eukaryotes (reviewed in Hofmann *et al.*, 2002). However, in the case of trypanosomatids, due to the absence of highly efficient heme or selenium-containing peroxidases, Prxs are probably key players in the parasitic antioxidant machinery, both at the cytosolic and mitochondrial levels.

A plant-like ascorbate-dependent peroxidase (APx), with no counterpart in mammals, was reported to also function in *T. cruzi* as antioxidant (Wilkinson *et al.*, 2002b). This hemoperoxidase is located to the same subcellular compartment as *Tc*GPxII, however, unlike this molecule, it is active towards H_2O_2. It is, therefore, likely that the enzymatic activities of both APx and GPxII molecules complement each other in order to eliminate a range of oxidants generated within the *T. cruzi* endoplasmic reticulum. Recently, an APx enzyme was also identified in *L. major* (Adak and Datta, 2005).

Finally, one last note to refer that some of the ROI-eliminating molecules may as well be involved in RNI removal, namely SODs (Quijano *et al.*, 2001), GSH (Quijano *et al.*, 1997), selenium-containing GPxs (Sies *et al.*, 1997), and Prxs (Bryk *et al.*, 2000; Dubuisson *et al.*, 2004; Jaeger *et al.*, 2004; Trujillo *et al.*, 2004;).

Trypanothione: involvement in antioxidant defense and other functions

In addition to the aforementioned discrepancies between the host and the parasite hydroperoxide-eliminating enzymes, the trypanosomatidal antioxidant system exhibits the unique feature of using trypanothione [N^1,N^8-bis(glutathionyl)spermidine] as electron supplier (Fairlamb *et al.*, 1985). Trypanothione, a thiol found only in trypanosomatids, consists of two glutathione molecules linked by a spermidine bridge. Table 1 summarizes the putative trypanothione-dependent physiological functions. As observed, trypanothione is linked to hydroperoxide removal by means of several independent redox cascades. The first pathway to be described was identified in the cytosol of *C. fasciculata* (Nogoceke *et al.*, 1997) and occurs via a thioredoxin-related molecule, tryparedoxin (TXN), and a Prx enzyme with tryparedoxin peroxidase (TXNPx) activity. This cascade was later confirmed to function in *Leishmania* (Levick *et al.*, 1998; Castro *et al.*, 2002; Flohe *et al.*, 2002; Castro *et al.*, 2004), *T. brucei*

Table I. Proposed trypanothione-dependent functions. Asc, ascorbate; APx, ascorbate peroxidase; dNTP, deoxyribonucleotides; eEFB1, eukaryotic elongation factor B1; GloI and II, glyoxalase I and II; GPx-like, non-selenium glutathione peroxidase-like enzyme; GSH, glutathione; kDNA, kinetoplast DNA; Th, thioredoxin; TXN, tryparedoxin; Prx, peroxiredoxin; RiboR, ribonucleotide reductase; UMSBP, universal minicircle sequence binding protein.

Biological function	Intermediates	References
Hydroperoxide removal	None	Nogoceke *et al.*, 1997
	TXN/Prx	Nogoceke *et al.*, 1997 Levick *et al.*, 1998 Wilkinson *et al.*, 2000b Tetaud *et al.*, 2001 Castro *et al.*, 2002 Castro *et al.*, 2004
	GSH/GPx-like	Wilkinson *et al.*, 2000a Wilkinson *et al.*, 2002c Hillebrand *et al.*, 2003
	TXN/GPx-like	Wilkinson *et al.*, 2002a Hillebrand *et al.*, 2003
	Asc/APx	Wilkinson *et al.*, 2002b
	eEFB1	Vickers *et al.*, 2004b
	Ovothiol A	Ariyanayagam and Fairlamb, 2001
Protection from nitrosative damage	None	Thomson *et al.*, 2003
	TXN/Prx	Trujillo *et al.*, 2004
	Ovothiol A	Vogt and Steenkamp, 2003
Ascorbate homeostasis	None	Krauth-Siegel *et al.*, 1996 Reckenfelderbaumer *et al.*, 2002 Wilkinson *et al.*, 2002b
Methylgyloxal removal	GloI, GloII	Irsch *et al.*, 2004 Vickers *et al.*, 2004a Sousa Silva *et al.*, 2005
Metal removal	Unknown	Mukhopadhyay *et al.*, 1996 Legare *et al.*, 1997
Xenobiotics removal	eEFB1	Vickers and Fairlamb, 2004 Vickers *et al.*, 2004b
Protein synthesis	eEFB1	Vickers and Fairlamb, 2004
dNTP synthesis	RiboR	Dormeyer *et al.*, 2001
	TXN/RiboR	Dormeyer *et al.*, 2001
	Th/RiboR	Schmidt and Krauth-Siegel, 2003
kDNA replication	(TXN/)UMSBP	Onn *et al.*, 2004

(Tetaud *et al.*, 2001) and *T. cruzi* (Guerrero *et al.*, 2000; Lopez *et al.*, 2000; Wilkinson *et al.*, 2000b). More recently, the catalytic activity of the *T. cruzi* and *T. brucei* GPx-like enzymes was also linked to trypanothione oxidation via tryparedoxin (Wilkinson *et al.*, 2002a; Hillebrand *et al.*, 2003). Alternatively, trypanothione may provide glutathione the necessary electrons for reduction of GPx-like molecules, either through a spontaneous or an enzymatic disulphide-

exchange reaction (Kelly *et al.*, 1993; Moutiez *et al.*, 1995; Moutiez *et al.*, 1997), thereby replacing the missing GR activity of trypanosomatids. This unique thiol, also responsible for ascorbate reduction (Krauth-Siegel and Ludemann, 1996; Reckenfelderbaumer and Krauth-Siegel, 2002; Wilkinson *et al.*, 2002b), acts as the source of reducing equivalents for, for instances, ascorbate-dependent peroxidases (Wilkinson *et al.*, 2002b). Reduction of the *L. major* elongation factor B1 by trypanothione may provide an alternative pathway for lipid hydroperoxide removal (Vickers *et al.*, 2004b). Additionally, trypanothione is involved in protection from nitrosative stress by means of either the direct (Thomson *et al.*, 2003) or the indirect TXN/Prx-driven (Trujillo *et al.*, 2004) elimination of $ONOO^-$. Also, in combination with ovothiol A, trypanothione promotes the non-enzymatic decomposition of nitrosothiols (Vogt and Steenkamp, 2003).

Besides fuelling ROI and RNI metabolism, trypanothione participates in other biologically relevant processes like the detoxification of methylglyoxal (Irsch and Krauth-Siegel, 2004; Vickers *et al.*, 2004a; Sousa Silva *et al.*, 2005), of toxic xenobiotics (Vickers and Fairlamb, 2004; Vickers *et al.*, 2004b) and of metals (Mukhopadhyay *et al.*, 1996; Legare *et al.*, 1997), synthesis of deoxyribonucleotides (Dormeyer *et al.*, 2001; Schmidt and Krauth-Siegel, 2003), replication of kDNA (Onn *et al.*, 2004), and, possibly, protein synthesis (Vickers and Fairlamb, 2004).

To keep the pool of reduced trypanothione at constant levels trypanosomatids rely on the activity of trypanothione reductase (TR) (Fairlamb and Cerami, 1992). TR is a NADPH-dependent flavoenzyme, homologous to the mammalian molecules GR and thioredoxin reductase. The trypanothione-dependent enzymatic complex is pivotal for trypanosomatid survival, as corroborated by distinct genetic approaches: (i) in *T. brucei* RNA interference of the enzyme responsible for trypanothione biosynthesis, trypanothione synthetase, resulted in impaired cell survival, proliferation (Comini *et al.*, 2004; Ariyanayagam *et al.*, 2005) and sensitivity to hydroperoxides (Comini *et al.*, 2004); (ii) a conditioned knockout of TR caused loss of virulence in *T. brucei* (Krieger *et al.*, 2000); (iii) attempts to disrupt both gene copies of TR in *L. donovani* failed to succeed (Dumas *et al.*, 1997; Tovar *et al.*, 1998), and (iv) mutants of *L. donovani* and *L. major* with lowered TR activity (obtained either by disruption of one TR allele or by a dominant-negative strategy) displayed decreased ability to survive intracellularly (Dumas *et al.*, 1997; Tovar *et al.*, 1998). Both the uniqueness and the essentiality of the trypanothione/TR system make it promising to control parasitic infections with specific inhibitors of trypanothione-dependent enzymes. Also, since this system is common to all trypanosomatids, it may allow the identification of broad-spectrum chemotherapeutic formulas effective against the three human pathogens.

3. Scope of this thesis

By the time this research project was initiated (in the year 2000) the TXN/Prx system was the only described trypanothione-dependent route for hydroperoxide elimination in trypanosomatids (Nogoceke *et al.*, 1997; Levick *et al.*, 1998). This observation added to the findings that trypanosomatids were sensitive to oxidative stress (reviewed in Flohe *et al.*, 1999) and that the trypanothione reductase/trypanothione redox system was essential for parasite survival (Dumas *et al.*, 1997; Tovar *et al.*, 1998), rendered trypanosomatidal TXN and Prx molecules candidate targets for the development of new chemotherapeutic drugs (Flohe *et al.*, 1999). In the face of this scenario we were prompted to investigate the TXN/Prx pathways in *L. infantum*, the resident trypanosomatid parasite in Mediterranean countries. As detailed before in this introduction, the major sources of oxidants in *Leishmania* are the host immune system and the parasite own aerobic metabolism. Accordingly, our study focused on the cytosolic and mitochondrial trypanothione/TXN/Prx systems. The goals of our research were three fold: (i) to dissect the cytosolic and the mitochondrial TXN/Prx pathways of *L. infantum*, (ii) to obtain biochemical and kinetic data on *L. infantum* TXN and Prx molecules, which could be relevant for the rational design of specific inhibitors, and (iii) to validate the players of these enzymatic pathways as drug targets.

The experimental results obtained in this thesis are organized in 5 chapters. **Chapter 2** describes the isolation and characterization of mitochondrial and cytosolic peroxiredoxins of *L. infantum*, with emphasis on their function as active peroxidases in the cell. Although the mitochondrial Prx displayed *in vitro* tryparedoxin peroxidase activity, there was no proof for a TXN operating in *Leishmania* mitochondrion. Evidence for the presence of a mitochondrial TXN was first provided by our group, as detailed in **Chapter 3**. This chapter further describes the isolation of a *Leishmania* cytosolic TXN. Still concerning the electron fuelling of the mitochondrial system, a chapter of unpublished results, **Chapter 4**, is added to this thesis, which addresses the subcellular compartmentalization of trypanothione reductase activity in *L. infantum*. Finally, a more detailed analysis of the mitochondrial peroxiredoxin is left to the last two chapters: **Chapter 5** reports on the biochemical and kinetic analysis of the enzyme, whereas in **Chapter 6** the essentiality of the mitochondrial peroxiredoxin, and therefore its validation as drug target, is dealt with.

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Chapter 2

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Original Contribution

COMPLEMENTARY ANTIOXIDANT DEFENSE BY CYTOPLASMIC AND MITOCHONDRIAL PEROXIREDOXINS IN *LEISHMANIA INFANTUM*

HELENA CASTRO,* CARLA SOUSA,* MARTA SANTOS,* ANABELA CORDEIRO-DA-SILVA,*† LEOPOLD FLOHÉ,‡ and ANA M. TOMÁS*§

*Institute for Molecular and Cell Biology, Porto, Portugal; †Faculty of Pharmacy, University of Porto, Porto, Portugal; ‡Department of Biochemistry, Technical University of Braunschweig, Braunschweig, Germany; and §Abel Salazar Institute for Biomedical Research, University of Porto, Porto, Portugal

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Abstract—In Kinetoplastida 2-Cys peroxiredoxins are the ultimate members of unique enzymatic cascades for detoxification of peroxides, which are dependent on trypanothione, a small thiol specific to these organisms. Here we report on two distinct *Leishmania infantum* peroxiredoxins, *Lic*TXNPx and *Lim*TXNPx, that may be involved in such a pathway. *Lic*TXNPx, found in the cytoplasm, is a typical 2-Cys peroxiredoxin encoded by *LicTXNPx*, a member of a multicopy gene family. *Lim*TXNPx, encoded by a single copy gene, *LimTXNPx*, is confined to the mitochondrion and is unusual in possessing an Ile-Pro-Cys motif in the distal redox center, replacing the common peroxiredoxin Val-Cys-Pro sequence, apart from an N-terminal mitochondrial leader sequence. Based on sequence and subcellular localization, the peroxiredoxins of Kinetoplastida can be separated in two distinct subfamilies. As an approach to investigate the function of both peroxiredoxins in the cell, *L. infantum* promastigotes overexpressing *Lic*TXNPx and *Lim*TXNPx were assayed for their resistance to H_2O_2 and *tert*-butyl hydroperoxide. The results show evidence that both enzymes are active as peroxidases in vivo and that they have complementary roles in parasite protection against oxidative stress. © 2002 Elsevier Science Inc.

Keywords—Peroxiredoxin, Tryparedoxin peroxidase, Antioxidant defense, Cytoplasm, Mitochondria, *Leishmania infantum*, Free radicals

INTRODUCTION

The peroxiredoxin ("peroxide-reducing") family of proteins includes a large number of molecules found in different organisms and performing distinct functions, including general cell detoxification and specific signaling in proliferation or differentiation processes [1]. In parasites, the peroxiredoxins are also present [1] and, in many of these organisms, they may be crucial to defend against oxidative stress. Indeed, due to the frequent lack or low expression of other common and more efficient antioxidant enzymes (e.g., catalase or glutathione peroxidase), removal of peroxides in parasites has been suggested to depend on the presence of peroxiredoxins [2,3]. The possibility of achieving their inhibition, immunologically [3,4] or with drugs, was consequently proposed as a potential antiparasitic strategy, even though the structural similarity of these molecules may pose a problem for chemotherapy. This last approach holds more promise for the medically important Kinetoplastida, including the life-threatening pathogens *Trypanosoma brucei*, *T. cruzi*, and *Leishmania* sp., which affect millions of people and for which better chemotherapeutics are urgently needed. In fact, while in other eukaryotes, such as the mammalian hosts of these parasites, peroxiredoxins reduce peroxides using thioredoxin as the immediate electron donor, in Kinetoplastida the peroxiredoxins up to now characterized have been shown to interact, instead, with tryparedoxin (TXN), a thioredoxin remote homologue (Fig. 1) [2,5–7]. It is possible, therefore, that the specificity of Kinetoplastida peroxiredoxins for TXN may result from unique structural features, which allow their exploitation for drug design [8].

If peroxiredoxins are to be the key enzymes for peroxide elimination in Kinetoplastida they should be

Address correspondence to: Ana M. Tomás, Institute for Molecular and Cell Biology, Rua do Campo Alegre 823, 4150-180 Porto, Portugal; Fax: +351-22-6098480; E-Mail: atomas@ibmc.up.pt.

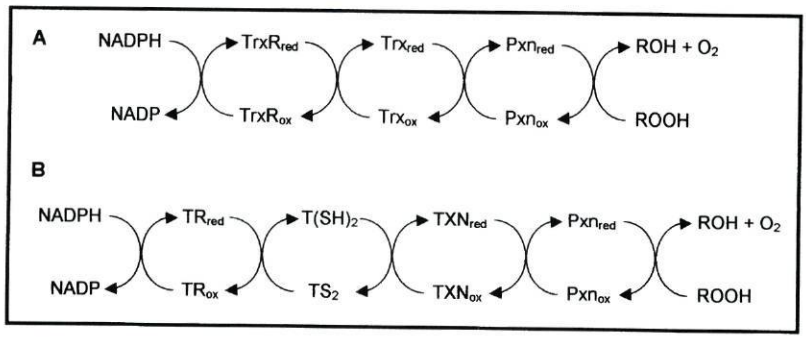

Fig. 1. Pathway for peroxide detoxification by peroxiredoxin enzymes proposed to occur (A) in eukaryotes and (B) in the cytosol of *Crithidia fasciculata* and other Kinetoplastida [2]. TrxR = thioredoxin reductase; Trx = thioredoxin; Pxn = peroxiredoxin; TR = trypanothione reductase; $T(SH)_2$ = reduced trypanothione; TS_2 = oxidized trypanothione; TXN = tryparedoxin; ox = oxidized; red = reduced; ROOH = hydroperoxide; ROH = alcohol.

present in different compartments of the parasitic cell in order to protect these from hydrogen peroxide (H_2O_2) or other peroxides. Accordingly, peroxiredoxins with distinct subcellular localizations are present in both *T. cruzi* and *T. brucei* [7,9]. In *Leishmania* sp. more than one peroxiredoxin have been described. *L. donovani* and *L. major* contain at least one of these enzymes [4,5,10] and in *L. chagasi* different isogenes are responsible for the expression of three very similar peroxiredoxins [11]. However, none of the studies performed so far reported on the cell localization or on the functional role of these peroxiredoxins. Here we show that different compartmentalization of peroxiredoxins also occurs in *Leishmania*. We describe the isolation and characterization of two peroxiredoxin genes from *L. infantum*, the Old World counterpart of *L. chagasi* [12], and present evidence that the encoded enzymes, one mitochondrial and the other cytoplasmic, can cooperate to protect the cell from peroxide-induced damage derived from different sources.

MATERIAL AND METHODS

Parasites

Promastigotes of the *L. infantum* clone MHOM/MA67ITMAP263 freshly isolated from Balb/c mice spleens were grown at 25°C in RPMI medium (Gibco-BRL, Paisley, Scotland) supplemented with 10% fetal calf serum (FCS), 2 mM L-glutamine, 50 mM Hepes sodium salt (pH 7.4), and 35 U/ml penicillin, 35 μg/ml streptomycin. To obtain exponentially and stationary phase promastigotes cells were seeded at 10^6 ml^{-1} and then harvested 1–3 and 6–8 d later, respectively [13].

Reverse transcription-PCR (RT-PCR) for amplification of peroxiredoxin sequences from L. infantum

cDNA synthesis was achieved from 1 μg of total RNA extracted from promastigotes using Superscript II RT (GibcoBRL) with random hexamers as primers. PCR to amplify peroxiredoxin transcripts was performed from 1 μl of cDNA (1/20th of the total). The sense primer was an oligonucleotide corresponding to the sequence of the *L. donovani* spliced leader 5'-gggggatccTCAGTTTCTG-$TACTTTATTG_{OH}$ (restriction site and clamp sequences in lower case). The antisense primer was a degenerated primer based on the amino acid sequence surrounding the active site of known peroxiredoxins, 5'-gggaat-tcGG(A/G)CAIAC(A/G)AAIGT(A/G)AA(A/G)TC_{OH}, where I refers to inosine. Cycling conditions were an initial step at 94°C for 2 min and 30 cycles of 94°C for 45 s, 50°C for 60 s, 72°C for 60 s, and a final step of 10 min at 72°C.

Construction and screening of a L. infantum *cosmid library*

A genomic library was constructed in the pcosTL cosmid shuttle vector using *L. infantum* DNA partially digested with *Sau*3AI, according to previously described conditions [14]. Briefly, gel eluted *Sau*3AI DNA fragments of 30 to 50 kb were dephosphorylated with calf intestinal phosphatase and ligated to the cosmid vector previously double digested with *Sma*I, to separate the two *cos* sites, and with *Bam*HI, an enzyme that generates overhanging ends compatible with those produced by *Sau*3AI. The ligation was then packaged into phage λ particles using an in vitro packaging extract (Stratagene, La Jolla, CA, USA) and competent *E. coli* DH5α infected with different aliquots of the packaging reaction mix. Three thousand clones of the library were picked and stored as individual bacterial clones into 384 well plates at −70°C under ampicillin selection (50 μg/ml). To isolate clones containing the peroxiredoxin genes of interest, the library was screened with the radiolabeled peroxiredoxin probes previously isolated by RT-PCR using standard colony hybridization techniques.

DNA sequencing

DNA was cloned into different plasmid vectors and double-stranded sequenced using the facilities at Alta Bioscience (University of Birmingham, UK) and at MWG-BIOTECH AG (Ebersberg, Germany).

DNA and RNA analysis

Genomic DNA was isolated from exponentially growing promastigotes using the proteinase K/sodium dodecyl sulfate (SDS) method [15]. Total RNA was prepared using either the guanidinium thiocyanate lysis followed by purification on a CsCl gradient [15] or the AquaPure RNA Isolation kit (BioRad, Hercules, CA, USA) according to the manufacturer's instructions. Southern and northern blots were performed using standard protocols. Membrane development and analysis of the signals were achieved with a Typhoon 8600 (Molecular Dynamics, Buckinghamshire, UK). *L. infantum* α-tubulin was used to control for loading of samples in northern blots.

Western blotting

L. infantum protein extracts, obtained by parasite solubilization in 1% (v/v) Nonidet P-40 in 0.1 M sodium phosphate, 0.15 M sodium chloride pH 7.2 (PBS) at 10^9 cells ml^{-1} in the presence of a cocktail of proteinase inhibitors, were fractionated under reducing conditions by 12% SDS/polyacrylamide gel electrophoresis (PAGE) and electroblotted onto nitrocellulose. The membranes were probed with polyclonal antibodies against purified recombinant *Lim*TXNPx (Castro et al. [15a]) raised in mice by three successive intraperitoneal injections of 25 μg of protein, purified recombinant *L. major* peroxiredoxin (thiol-specific antioxidant protein, TSA, [4]; kind gift from S. Reed) and recombinant *Lm*S3arp [16] (kind gift of A. Ouaissi). Second antibodies were peroxidase-labeled anti-mouse serum (Transduction Laboratories, Lexington, UK) and anti-rabbit $F(ab')_2$ fragment (Molecular Probes, Leiden, The Netherlands). Membranes were developed using enhanced chemiluminescence (Amersham, Buckinghamshire, UK). Protein concentrations of the parasite extracts were determined with a bicinchoninic acid protein-assay system (Pierce, Rockford, IL, USA).

Immunofluorescence assays

L. infantum promastigotes were stained with the mitochondrion-specific dye Mitotracker FM (Molecular Probes) as described previously [17], fixed with 4% paraformaldehyde (w/v) in PBS and permeabilized with 0.1% (v/v) Triton X-100 in PBS. Parasites were then incubated with the anti-*Lim*TXNPx and anti-TSA antibodies or control sera diluted in PBS, 1% (w/v) bovine serum albumin (BSA). Secondary antibodies were Alexa Fluor 568 goat anti-mouse IgG and Alexa Fluor 488 goat anti-rabbit IgG (Molecular Probes). Washed parasites were mounted in VectaShield (Vector Laboratories, Burlingame, CA, USA) and examined with an Axioskop Zeiss microscope (Göttingen, Germany).

Construction of vectors for transfection of L. infantum

The *LimTXNPx* coding sequence was amplified with high fidelity PWO polymerase (Roche, Mannheim, Germany) using the oligonucleotides 5'-cgcggatccATGCTCCGCCGTCTTCCCA$_{OH}$ and 5'-caccgctcgagTCACATGTTCTTCTCGAAAAAC$_{OH}$ (restriction site and clamp sequences in lower case; start and stop codons underlined) as forward and reverse primers and the cycling conditions 94°C for 2 min, 53°C for 30 s, 72°C for 45 s, 30 cycles at 94°C for 30 s, 58°C for 30 s, 72°C for 45 s, and a final step of 10 min at 72°C. The product was cloned into pTEX [18] to obtain pTEX-*LimTXNPx*. The *LicTXNPx* gene was amplified with the forward primer 5'-cgcggatccATGTCCTGCGGTGACGCC$_{OH}$ and the reverse primer 5'-caccgctcgagTTACTGCTTACTGAAGTACC$_{OH}$. Cycling conditions were one cycle at 94°C for 5 min, 44°C for 30 s, 72°C for 30 s, 30 cycles at 94°C for 30 s, 65°C for 30 s, 72°C for 30 s, and a final step of 10 min at 72°C. The PCR product was cloned into pTEX to obtain pTEX-*LicTXNPx*.

Transfection procedures

Transfections were done by electroporation as described [19] at 0.45 kV, 300–400 μF. Parasites were allowed to recover in culture medium for 48 h before being plated in agar selective plates containing 15 μg/ml G418 (Sigma, Steinheim, Germany). Isolated clones were grown in liquid medium under G418 selection (15–200 μg/ml G418).

Hydroperoxide sensitivity assays

To analyze the growth inhibitory effect of H_2O_2 (Sigma) and *tert*-butylhydroperoxide (*t*-bOOH) (Sigma), on wild-type and transformed parasites, cells from exponentially or, if required, stationary grown cultures were seeded at 10^6 ml^{-1} in 2 ml of growth medium in 24 well plates in the absence of G418 and allowed to recover for 24 h. Different concentrations of the hydroperoxides in parasite medium were then added to each well. Four to five days later parasite densities were determined with a hematocytometer and/or by absorbance reading at 600 nm. All promastigote lines were analyzed simultaneously and within the same number of days after parasite removal from mice spleens (a maximum of 21 d).

RESULTS

Isolation of two peroxiredoxins genes from L. infantum

The RT-PCR strategy to amplify peroxiredoxin gene fragments from *L. infantum* was based on primers designed according to conserved active site sequences of known peroxiredoxins and the spliced leader sequence of *L. donovani*. Thereby cDNA fragments of 320 and 410 bp were isolated and confirmed to belong to the peroxiredoxin family by sequencing. The complete coding sequences for the peroxiredoxin genes were obtained by screening a *L. infantum* cosmid library with the radiolabeled cDNA fragments.

The gene identified using the 320 bp cDNA fragment as a probe, *LicTXNPx* (Acc. Nr. AY058210), presents 600 nucleotides (nt) and is 99.5, 99.3, and 91.3% similar to peroxiredoxin genes recently reported by Barr and Gedamu [11] in *L. chagasi* (Acc. Nr. AF312397, AF312398, AF134161). *LicTXNPx* is also 99% and 94.7% similar to *L. donovani* and *L. major* genes previously characterized (Acc. Nr. AF225212, AF044679, and AF069386) and shown to encode proteins with tryparedoxin peroxidase (TXNPx) activity in vitro [4,5,10]. Southern blot analysis of the isolated cosmid and of genomic DNA indicated that multiple copies of *LicTXNPx* are present in the same chromosome (not shown). As shown for *L. chagasi*, this multicopy organization suggests different isogenes [11]. No obvious organelle endorsement sequence was detected in *LicTXNPx*.

The coding sequence isolated with the 410 bp cDNA fragment has 681 nt and encodes a TXNPx with an N-terminal mitochondrial targeting peptide. This gene is 96.5% similar to a noncharacterized sequence from *L. major* (Acc. Nr. AL121851) and 66.8 and 65.5% similar to peroxiredoxin genes from *T. cruzi* (Acc. Nr. AJ006226) [9] and *T. brucei* (Acc. Nr. AF196570) [7], respectively, that were shown to locate to the mitochondrion. Therefore, *L. infantum* presents a putative mitochondrial peroxiredoxin gene (*LimTXNPx*, Acc. Nr. AY058209). Southern blot analysis of genomic DNA digested with different restriction enzymes indicates that this gene is single copy (not shown).

Sequence characteristics of the predicted proteins

LicTXNPx and *LimTXNPx* are predicted to encode mature proteins of 22.136 and 22.389 kDa, with pIs of 7.72 and 5.24, respectively. To outline their peculiarities the deduced amino acid sequences were aligned with previously established TXNPx (Fig. 2). Both *Lic*TXNPx and *Lim*TXNPx are 2-Cys peroxiredoxins that share the cysteine in the N-terminal domain with several tryparedoxin peroxidases. This cysteine is embedded in a VCP motif as is typical for peroxiredoxins [1,20]. The cysteine in this position has been shown to be essential for activity in several peroxiredoxins (reviewed in [1]). It is assumed to be the residue that is oxidized by the peroxide substrate and for this purpose has to be activated by an arginine residue and a threonine [1]. As is highlighted in Fig. 2, these residues are also conserved in the sequences of *Lic*TXNPx and *Lim*TXNPx. The second VCP motif that is found in different TXNPx and thought to participate in catalysis [1] is also conserved in *Lic*TXNPx but not in *Lim*TXNPx. There is, however, a second cysteine retained near the C-terminus of *Lim*TXNPx. Its sequence context (AIPCGWKPG) is very similar to that of the mTXNPx of *T. cruzi* (VIPCNWRPG) and *T. brucei* (VIPCNWKPG) and less similar to that found in the other TXNPx (GEVCPANWKK/PG). Another characteristic feature of *Lim*TXNPx is the presence of an N-terminal extension similar in size and sequence to that of the *T. cruzi* and *T. brucei* mTXNPx. An overall comparison of the *Lic*TXNPx sequence with the *Lim*TXNPx sequence for the predicted mature protein yields an identity of 50.5%, while between *Lic*TXNPx and the homologues of *T. cruzi* and *T. brucei*, both cytoplasmic, this is of 69.3 and 71.4%. On the other hand, the identity between *Lim*TXNPx and the mTXNPx of *T. cruzi* and *T. brucei* is 71.7% and 71.2%, respectively. This reveals that *Lic*TXNPx and *Lim*TXNPx belong to two distinct peroxiredoxin subfamilies (Fig. 3) that split from each other prior to Kinetoplastida separation.

Analysis of Lic TXNPx *and* Lim TXNPx *expression in* L. infantum *promastigotes*

Expression of the *LicTXNPx* and *LimTXNPx* transcripts was analysed in exponentially and in stationary phase promastigotes, a stage that is enriched in metacyclic promastigotes, the form of the parasite that transmits the infection from the sandfly to vertebrates [22]. As shown in Fig. 4A, when the *LicTXNPx* probe was used to hybridise northern blots of these parasites, four different transcripts of 2.1, 1.7, 1.5, and 1.2 kb were evident. After correcting for loading with the α-tubulin signal the 1.5 kb mRNA was seen to be upregulated (1.5×) in stationary phase promastigotes. In contrast, the *Lim*TXNPx gene is constitutively transcribed as a 1.4 kb single product irrespective of the age of the promastigote culture (Fig. 4A).

The expression of both peroxiredoxin genes was also investigated at the protein level. An antibody directed against the TSA protein of *L. major* [4], highly homologous to *Lic*TXNPx (91% identity), was used to identify this protein in Western blots of *L. infantum* under reducing conditions. In spite of the presence of the four dif-

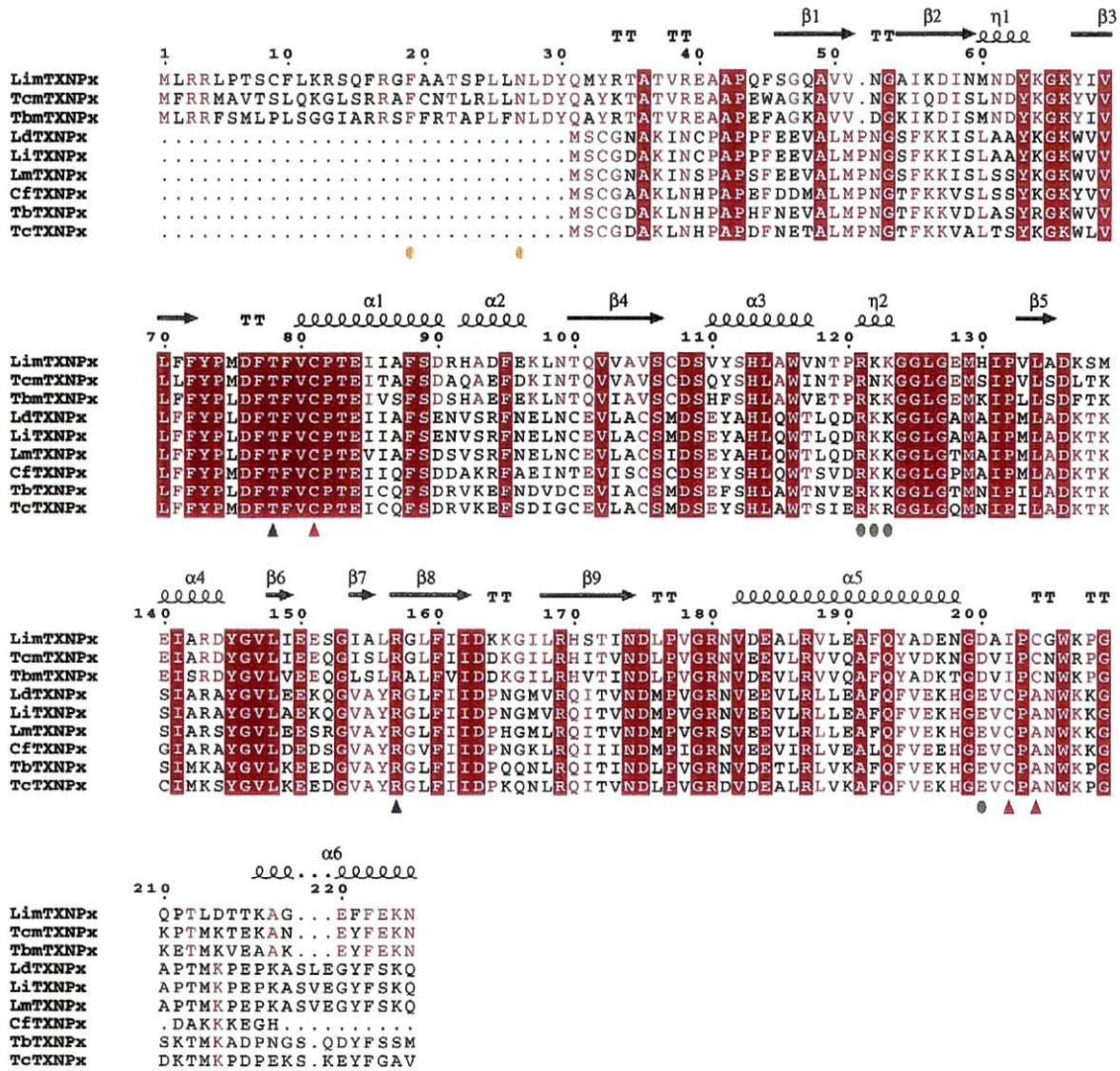

Fig. 2. Alignment of *Lim*TXNPx and *Lic*TXNPx with known tryparedoxin peroxidases. Residues conserved in all types of TXNPx are shown in red boxes, those conserved in one subfamily only are typed in red. Secondary structural elements [(β_{1-9}) = beta strands; α_{1-6} = α helices; η = 3–10 helices; TT = turns] are indicated above sequences. Red arrows mark redox-active cysteines, blue arrows mark residues implicated in the activation of C [10]; green dots highlight residues putatively interacting with TXN [10]; orange dots mark predicted cleavage sites for mitochondrial processing enzymes. *Lim*TXNPx, *L. infantum* mitochondrial TXNPx (Acc. Nr. AY058209); *Tcm*TXNPx, *T. cruzi* mitochondrial TXNPx (Acc. Nr. AJ006226); *Tbm*TXNPx, *T. brucei* mitochondrial TXNPx (Acc. Nr. AF196570); *Li*TXNPx, *L. infantum* cytoplasmic TXNPx (Acc. Nr. AY058210); *Ld*TXNPx, *L. donovani* TXNPx (Acc. Nr. AF225212); *Lm*TXNPx, *L. major* TXNPx (Acc. Nr. AF044679); *Cf*TXNPx, *C. fasciculata* TXNPx1 (Acc. Nr. AAC15095); *Tb*TXNPx, *T. brucei* TXNPx (Acc. Nr. AAG45225); *Tc*TXNPx, *T. cruzi* TXNPx (Acc. Nr. CAA09922). *Cf*TXNPx, *Tb*TXNPx and *Tc*TXNPx were also shown to be cytoplasmic [7,9].

ferentially expressed transcripts referred to above, a single and equally intense polypeptide band of 20.1 kDa was detected in both exponentially and stationary forms, indicating that the total amount of peroxiredoxin detected with this antibody remains constant along promastigote development (Fig. 4B). Western blot analysis with an antibody against recombinant *Lim*TXNPx (Castro et al., [15a]) shows that *Lim*TXNPx is expressed as a single protein product of 21.4 kDa (Fig. 4B).

Subcellular localization of Lic*TXNPx and* Lim*TXNPx*

When the anti-TSA antibody [4] was used in the immunofluorescence assays, labeling was shown through the whole parasite body, indicating that *Lic*TXNPx is cytoplasmic (Fig. 5E). No differences were observed between exponentially and stationary phase promastigotes (not shown). As suggested by the presence of a mitochondrial targeting sequence in *Lim*TXNPx, immunofluorescence analysis corroborated that this protein

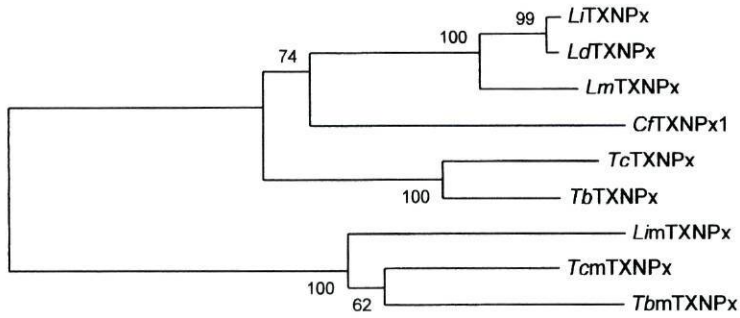

Fig. 3. Neighbor-joining tree showing different TXNPx amino acid sequences of Kinetoplastida, using the Poisson correction. Sequence names are according Fig. 2. Percentage of bootstrap replicates (500 replications) supporting the branches are shown. Trees were generated using MEGA2 [21]. 2-Cys peroxiredoxin present in the Kinetoplastida order form two subfamilies, one mitochondrial, and the other cytoplasmic, which have diverged prior to Kinetoplastida separation. This origin suggests an initial common function for each subfamily that could have been maintained along evolution.

localizes to the single mitochondrion of the parasite, an elongated structure that includes the kinetoplast (Figs. 5A –C,F). Indeed, the anti-*Lim*TXNPx antibody staining perfectly colocalizes with the Mitofluor dye, a marker for mitochondria (Figs. 5A–C). No colocalization was observed when the parasites were labeled simultaneously with the anti-*Lim*TXNPx and the anti-TSA antibodies (Figs. 5E–G), further confirming the different compartmentalization of both peroxiredoxins analyzed.

Production of parasites overexpressing Lic*TXNPx and* Lim*TXNPx*

Parasites overexpressing these proteins were produced and assayed for peroxide resistance in vivo. To this end, the expression plasmids pTEX-*Lic*TXNPx and pTEX-*Lim*TXNPx, were introduced into *L. infantum* promastigotes. pTEX transfection was used as control. Plasmid integrity and copy number in transformed parasites was evaluated by Southern blot analysis of digested genomic DNA of wild-type and G418 resistant parasites, probed with *Lic*TXNPx and *Lim*TXNPx (Figs. 6A, B) and with the *neo* resistance gene (not shown). As can be observed in Fig. 6A, *Lic*TXNPx transformed parasites contained the plasmid replicating episomally at high copy number without evidence of rearrangements. This was accompanied by an increased expression of *Lic*TXNPx (Fig. 6C). Parasites transformed with construct pTEX-*Lim*TXNPx also showed a high increase in *Lim*TXNPx copy number and in the respective protein (Figs. 6B, D). Immunofluorescence analysis of transgenic parasites showed that, when overexpressed, the peroxiredoxins maintained their cytoplasmic and mito-

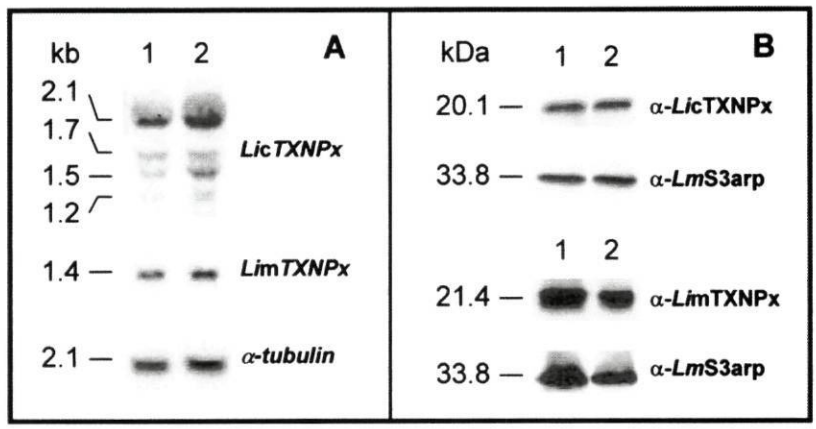

Fig. 4. Expression analysis of *LicTXNPx* and *LimTXNPx* in *L. infantum* promastigotes. (A) Northern blot analysis of 20 μg of total *L. infantum* RNA extracted from exponentially (lane 1) and stationary phase promastigotes (lane 2), hybridized with the *LicTXNPx* and *LimTXNPx* coding sequences and with a *L. infantum* α-*tubulin* probe. (B) Western blot analysis of *Lic*TXNPx and *Lim*TXNPx under reducing conditions. Twenty micrograms of total protein extracts from exponentially (lane 1) and stationary phase (lane 2) promastigotes were fractionated in a 12% SDS/PAGE gel, transferred to nitrocellulose and incubated with the anti-TSA and the anti-*Lim*TXNPx antibodies and with anti-*Lm*S3arp as a control. Equal loading was also checked by amido black staining of an equivalent set of lanes (not shown).

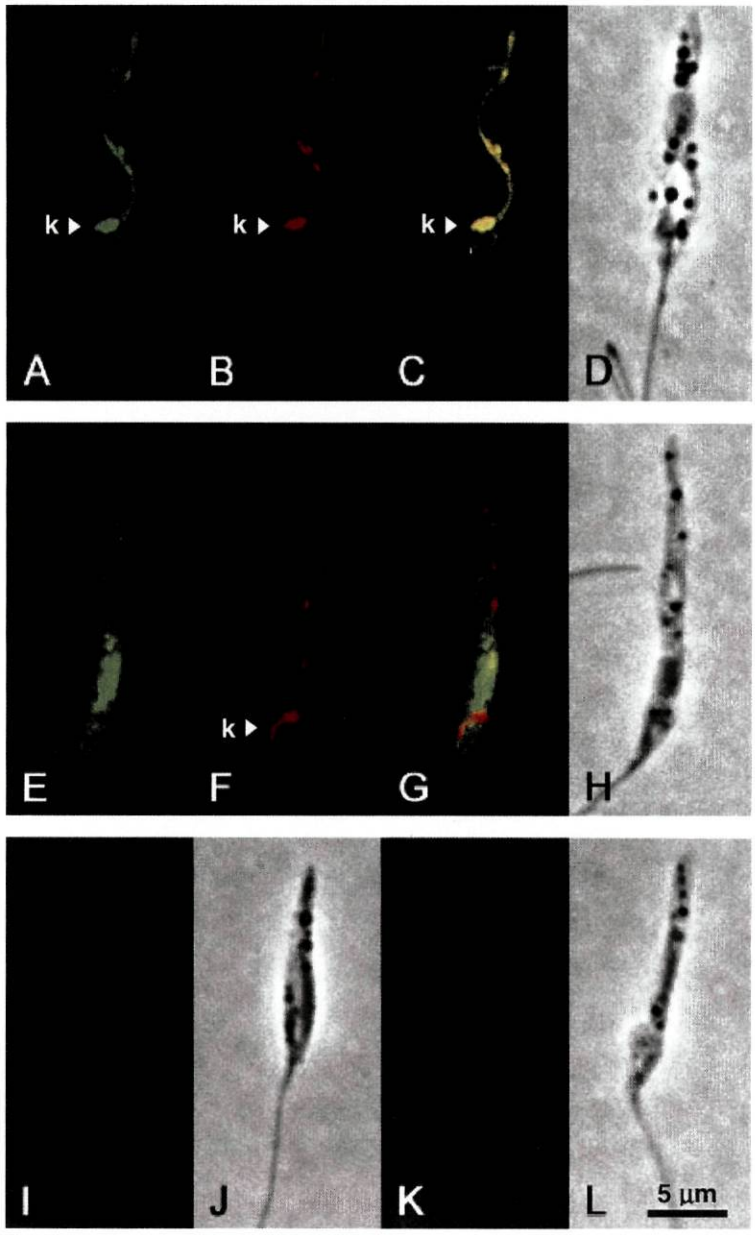

Fig. 5. Subcellular localization of *Lic*TXNPx and *Lim*TXNPx in *L. infantum* promastigotes. *L. infantum* promastigote mitochondria were stained in vivo with the Mitotracker dye (A). After fixation and permeabilization, parasites were incubated with the anti-*Lim*TXNPx (B,F) and the anti-TSA (E) antibodies, and with nonimmune serum from rabbit (I) and mice (K). Parasites were photographed at 1000× magnification. Contrast phase pictures of the preparations are also included (D,H,J,L). k = kinetoplast.

chondrial subcellular localization as no differences in the pattern of staining could be observed in relation to wild-type cells (not shown). Transgenic parasites showed no substantial alterations in their growth rate.

Phenotypic analysis of parasites overexpressing Lic*TXNPx and* Lim*TXNPx*

In vitro assays demonstrated that H_2O_2 and *t*-bOOH are substrates for recombinant *Lim*TXNPx (Castro et al., [15a]). This specificity was also observed for the *Lic*TXNPx homologue of *L. donovani* [10]. Therefore, we tested live promastigotes overexpressing *Lic*TXNPx and *Lim*TXNPx for their resistance against these peroxides when exogenously added, in comparison to wild-type and to parasites transformed with the empty expression plasmid. H_2O_2 resistance of *Leishmania* has been reported to be affected by the length of time the culture has been growing in vitro and by the stage of promastigote development [13,23]. Therefore, all parasite lines to be assayed were previously inoculated into mice for 5 d.

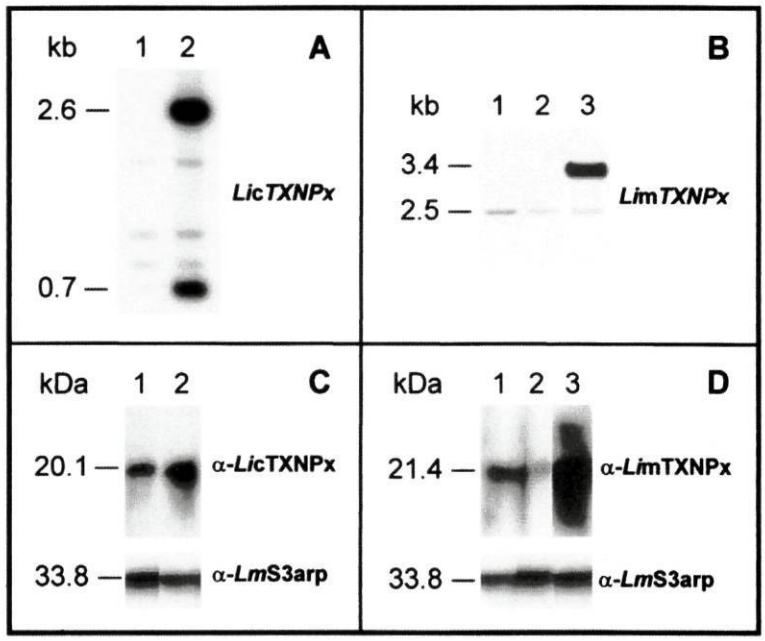

Fig. 6. Overexpression of *Lic*TXNPx and *Lim*TXNPx in transformed parasites. Southern blot analysis of *L. infantum* promastigote *Sac*I/*Kpn*I digested genomic DNA (A) of wild-type (lane 1) and pTEX-*LicTXNPx* transformed parasites (lane 2), hybridized with the *LicTXNPx* coding sequence, and (B) of wild-type parasites (lane 1) and of cells transformed with pTEX (lane 2) and with pTEX-*LimTXNPx* (lane 3), hybridized with the *LimTXNPx* coding sequence. The 2.6 and 0.7 kb bands in A indicate plasmid derived *LicTXNPx*. In B the 2.5 kb band corresponds to endogenous *LimTXNPx* and the 3.4 kb band in lane 3 to vector derived *LimTXNPx*. Western blot analysis of total protein extract (20 μg) from (C) the same parasite lines as in (A), incubated with the anti-TSA and the anti-*Lm*S3arp antibodies (to control for loading), and (D) from the same parasites as in (B), incubated with the anti-*LimTXNPx* and the anti-*Lm*S3arp antibodies. No crossreacting between the anti-*LimTXNPx* and the anti-TSA antibodies was detected (not shown).

Amastigotes were then recovered, allowed to transform to promastigotes and analyzed for peroxide resistance within the same days after isolation from mice (a maximum of 21 d). By doing this we observed that, although a small difference in the absolute levels of peroxide resistance could be observed between the experiments, the relative results between the lines were very reproducible. The slight difference observed between both control curves at the higher peroxide concentrations may be due to a small reduction in the rate of replication of plasmid-transformed parasites. As shown in Fig. 7 a different phenotype was found associated with overexpression of each peroxiredoxin studied. *L. infantum* promastigotes overexpressing *Lic*TXNPx presented an increased resistance to H_2O_2 when compared with wild-type and pTEX transformed parasites. Those parasites were also more protected against the organic hydroperoxide *t*-bOOH but not to the same extent as to H_2O_2 (Fig. 7). In contrast, overexpression of *Lim*TXNPx in promastigotes did not ensure any significant resistance to exogenously added H_2O_2, but sheltered parasites when exposed to *t*-bOOH.

DISCUSSION

To succeed as a parasite, *Leishmania* must evolve through a phlebotomine insect host as an extracellular flagellated promastigote and through a vertebrate host as a nonmotile intracellular amastigote found in macrophages. During this developmental cycle the parasite faces oxidants from external and internal sources. The oxidative burst that follows parasite internalization by macrophages [24] produces superoxide radical ($^{\bullet}O_2^-$), H_2O_2, peroxynitrite and lipoxygenase products and such detrimental oxidants might also result from defensive processes taking place in the sandfly, as occurs with some insects [25–27]. H_2O_2 has been reported to be internally produced in Kinetoplastida as a consequence of the parasite's aerobic metabolism [28–30]. It can be formed in several reactions but the most important source is the mitochondrial electron chain. Therefore, *Leishmania* survival is likely to depend on strategically localized antioxidant enzymes able to quickly eliminate these oxidants in the cell compartments where they exert their action. Previous reports have identified two iron-containing superoxide dismutases able to dismutate $^{\bullet}O_2^-$ and protect the parasite from free radical damage [31]. In this report we addressed the question of peroxide reduction in *L. infantum* and demonstrate that two distinct peroxiredoxins, one localized in the cytoplasm the other in the mitochondrion, may cooperate to preserve the parasite from peroxide-induced damage.

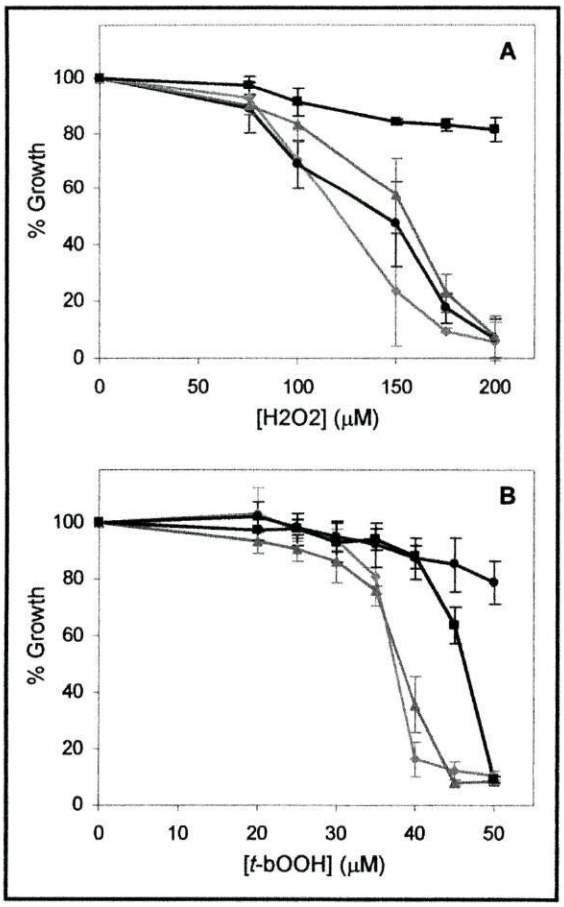

Fig. 7. Effect of hydrogen peroxide (H_2O_2) and *tert*-butyl hydroperoxide (*t*-bOOH) on replication of *L. infantum* promastigotes. Wild-type (▲), pTEX-*Lic*TXNPx transformed (■), pTEX-*Lim*TXNPx transformed (●) and pTEX transformed (◆) parasites were cultured for 5 d in medium containing H_2O_2 (A) and *t*-bOOH (B) at various concentrations. The number of promastigotes was then counted and the densities measured by spectrophotometry at 600 nm. The data are expressed as a percentage of promastigote replication in relation to control cultures without peroxide. Graphs show a representative experiment performed in triplicate. Standard deviations between the triplicates are indicated by bars.

Peroxide removal in pathogenic Kinetoplastida is believed to be largely ensured by trypanothione, TXN and TXNPx [32]. The peroxiredoxin genes cloned here, *LicTXNPx* and *LimTXNPx*, encode cytoplasmic and mitochondrial 2-Cys peroxiredoxin proteins that are homologous to previously established tryparedoxin peroxidases. In *Lic*TXNPx this homology extends along the complete molecule. *Lim*TXNPx, however, presents a number of specific characteristics. It shares with all TXNPx (e.g., *Lic*TXNPx) and with most other 2-Cys peroxiredoxins, the N-terminal conserved Cys and the residues corresponding to T49 and R128 in *Cf*TXNPx. This triad of residues was demonstrated to form one of the redox centers in *Ld*TXNPx [10], likely, the one that interacts with the peroxides. Indeed, it could not be responsible for donor substrate specificity because it is conserved in many other peroxiredoxins that use reductants other than TXN. The second redox center in *Lim*TXNPx is likely IPC and it is embedded in a sequence context distinct from cytoplasmic TXNPx molecules but is similar to that of mitochondrial peroxiredoxins of *T. brucei* and *T. cruzi* [7,9]. Emerging evidence suggests that this distal conserved cysteine represents the site of attack by specific reducing substrates in 2-Cys peroxiredoxins [10,33]. In recent models of TXNPx/TXN interactions a basic sequence stretch at positions 92–94 (RKR or more frequently RKK) and an acid residue (E) at position 171 in *Lic*TXNPx and other TXNPx have been suggested to attract TXN electrostatically. In *T. cruzi* mTXNPx the corresponding basic center is weakened (RNK); it is however fully conserved in *Lim*TXNPx and in the *T. brucei* mTXNPx. An acid residue (D) is present in the three mitochondrial TXNPx replacing E in cytoplasmic TXNPx. It is, however, shifted relative to the distal redox-active cysteine by two positions. These common denominators between the cytoplasmic and mitochondrial subfamilies may allow the mitochondrial types to function as specific tryparedoxin peroxidases ([7], Castro et al., [15a]). A specific feature of *Lim*TXNPx shared with other mitochondrial TXNPx is the presence of an N-terminal mitochondrial import peptide of 26 amino acids typical of eukaryotic organisms. This complies with previous reports that mitochondrial protein import in Kinetoplastida does not fundamentally differ from that of more evolved eukaryotes [34]. This sequence is characterized by the presence of several hydrophobic and positively charged residues, implicated in the process of targeting and transport across mitochondrial membranes, and by lack of acidic residues [35]. The N-terminal sequence of *Lim*TXNPx further suggests that processing of the mature protein requires the activity of two mitochondrial proteases [36,37]. A protein homologous to the mitochondrial processing protease, MPP, which requires an Arg residue in position -2 and in a distal position, would cleave first at position 18, leaving an octapeptide to be subsequently processed by a protein homologous to the mitochondrial intermediate peptidase (MIP). This two-step processing occurs in proteins intended to the mitochondrial matrix or to the inner membrane [35,38]. The predictions deduced from the sequence characteristics comply with the mitochondrial localization of *Lim*TXNPx here demonstrated.

All peroxiredoxins analyzed to date have been shown to display peroxidase activity in vitro [39], however, that does not necessarily imply that in vivo such peroxiredoxins function in cell defense to oxidative stress [32].

Here we demonstrate that the novel peroxiredoxins of *L. infantum* can be active as peroxidases in vivo. Indeed, an increased resistance of parasites transformed with *Lic*TXNPx and *Lim*TXNPx to at least one of the hydroperoxides tested was observed. With *Lic*TXNPx the interpretation of the results appears straightforward. Overexpression protects against H_2O_2 and *t*-bOOH added to the medium, an experimental approach meant to mimic the oxidative burst of phagocytes or analogous phenomena in the sandfly. In this respect the data mirrors the observations made with genetic disruption of trypanothione-mediated peroxide metabolism in *T. brucei*, an increased sensitivity to H_2O_2 and loss of virulence in an infection model [40]. The less pronounced protection against *t*-bOOH in comparison to H_2O_2 in *Lic*TXNPx overexpressing parasites is not easily understood. It may be tentatively attributed to a higher specific activity of *Lic*TXNPx toward H_2O_2 than toward *t*-bOOH, as shown to occur with homologous enzymes of *L. donovani* and *L. major* [5,10], or to the tendency of peroxiredoxins to become inactivated by organic hydroperoxides ([10], Castro et al., [15a]). In view of the mitochondrial localization of *Lim*TXNPx it is not surprising that overexpression of this enzyme does not induce resistance to exogenous H_2O_2 because this has little chance to reach the mitochondrion at concentrations that would not be readily detoxified by wild-type levels of *Lim*TXNPx. Unfortunately, we are not aware of any experimental design to selectively increase the hydroperoxide tone in mitochondria of Kinetoplastida to unequivocally demonstrate the role of *Lim*TXNPx. The relevance of this peroxiredoxin in mitochondrial hydroperoxide protection is, however, corroborated by the increased resistance against *t*-bOOH upon overexpression.

In conclusion, we have shown that *L. infantum* expresses at least two peroxiredoxins, a cytoplasmic, and a mitochondrial one. In their cellular context they are presumed to complement each other in protecting promastigotes against peroxide-mediated damage. Likely, therefore, these enzymes are key devices of the antioxidant armamentarium of these parasites. Preliminary results indicate that both peroxiredoxins are also expressed in amastigotes, the vertebrate stage of the parasite. If shown to be essential to amastigotes it will be important to explore unique structural features of these proteins in a chemotherapeutic perspective.

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ABBREVIATIONS

mTXNPx—mitochondrial tryparedoxin peroxidase
PCR—polymerase chain reaction
RT-PCR—reverse transcription polymerase chain reaction
t-bOOH—*tert*-butyl hydroperoxide
TSA—thiol-specific antioxidant protein
TXN—tryparedoxin
TXNPx—tryparedoxin peroxidase

Chapter 3

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MOLECULAR & BIOCHEMICAL PARASITOLOGY

Two linked genes of *Leishmania infantum* encode tryparedoxins localised to cytosol and mitochondrion☆

Helena Castro[a], Carla Sousa[a], Marta Novais[a], Marta Santos[a], Heike Budde[b], Anabela Cordeiro-da-Silva[a,c], Leopold Flohé[b], Ana M. Tomás[a,d,*]

[a] *Institute for Molecular and Cell Biology, Rua do Campo Alegre 823, 4150-180 Porto, Portugal*
[b] *Department of Biochemistry, Technical University of Braunschweig, Mascheroder, Weg 1, D-38124 Braunschweig, Germany*
[c] *Faculty of Pharmacy, University of Porto, Rua Aníbal Cunha 164, 4050-047 Porto, Portugal*
[d] *ICBAS, Abel Salazar Institute for Biomedical Research, University of Porto, Largo, Prof. Abel Salazar 2, 4099-003 Porto, Portugal*

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Abstract

Tryparedoxins are components of the hydroperoxide detoxification cascades of Kinetoplastida, where they mediate electron transfer between trypanothione and a peroxiredoxin, which reduces hydroperoxides and possibly peroxynitrite. Tryparedoxins may also be involved in DNA synthesis, by their capacity to reduce ribonucleotide reductase. Here we report on the isolation of two tryparedoxin genes from *Leishmania infantum*, *LiTXN*1 and *LiTXN*2, which share the same genetic *locus*. These genes are both single copy and code for two active tryparedoxin enzymes, *Li*TXN1 and *Li*TXN2, with different biochemical and biological features. *Li*TXN1 is located to the cytosol and is upregulated in the infectious forms of the parasite, strongly suggesting that it might play an important role during infection. *LiTXN*2 is the first mitochondrial tryparedoxin described in Kinetoplastida. Biochemical assays performed on the purified recombinant proteins have shown that *Li*TXN1 preferentially reduces the cytosolic *L. infantum* peroxiredoxins, *Lic*TXNPx1 and *Lic*TXNPx2, whereas *Li*TXN2 has a higher specific activity for a mitochondrial peroxiredoxin, *Lim*TXNPx. Kinetically, the two tryparedoxins follow a ping-pong mechanism and show no saturation. We suggest that *Li*TXN1 and *Li*TXN2 are part of two distinct antioxidant machineries, one cytosolic, the other mitochondrial, that complement each other to ensure effective defence from several sources of oxidants throughout the development of *L. infantum*.

Keywords: Tryparedoxin; Antioxidant defence; Cytosol; Mitochondria; *Leishmania infantum*

1. Introduction

Tryparedoxins [1] are a special class of oxidoreductases related to thioredoxins and found in trypanosomatids. They have acquired particular interest as potential targets for new trypanocidal agents [2–5]. In fact, although their tertiary structure resembles in many aspects that of the ubiquitous thioredoxins [4,6], they show little sequence similarity with these, are larger and possess a WCPPC signature in their active site replacing the WCG/APC motif found in thioredoxins. More important, the tryparedoxins up to now characterised have been shown to be specifically reduced by trypanothione (N^1,N^8-bis(glutathionyl)spermidine) [7,8], the small thiol that in trypanosomatids largely replaces glutathione [1,2,9,10]. It is this particular specificity, likely to result from unique structural properties of these molecules, that may render the tryparedoxins promising drug targets.

Like the thioredoxins, the tryparedoxins possess oxidoreductase activity towards disulphide bridges and, as those, they may be involved in different aspects of the parasite development some of which could be crucial to life. Evidence gathered from previously studied relatives suggests that their major role in the cell may be as components of hydroperoxide detoxification cascades transferring reducing equivalents from trypanothione to tryparedoxin peroxidase

Abbreviations: *Li*TXN1, *Leishmania infantum* tryparedoxin 1; *Li*TXN2, *L. infantum* tryparedoxin 2; TR, trypanothione reductase; $T(SH)_2$, trypanothione; *Lic*TXNPx1 and *Lic*TXNPx2, *L. infantum* cytosolic tryparedoxin peroxidase 1 and 2; *Lim*TXNPx, *L. infantum* mitochondrial tryparedoxin peroxidase

☆ *Note*: Nucleotide sequence data reported on this paper are available in GenBank under the accession numbers AY485270 (*LiTXN*1) and AY485271 (*LiTXN*2).

* Corresponding author. Tel.: +351-22-607-4956; fax: +351-22-609-8480.
E-mail address: atomas@ibmc.up.pt (A.M. Tomás).

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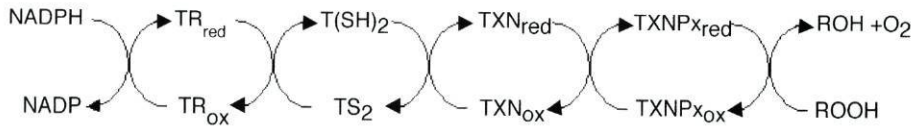


Fig. 1. Trypanothione-dependent pathway for hydroperoxide detoxification in Kinetoplastida proposed by Nogoceke et al. [1]. TR, trypanothione reductase; $T(SH)_2$, reduced trypanothione; TS_2, oxidised trypanothione; TXN, tryparedoxin; TXNPx, tryparedoxin peroxidase; ox, oxidised; red, reduced; ROOH, hydroperoxide; ROH, alcohol.

(Fig. 1) [1,3,9,11] or even to glutathione peroxidase [12,13]. However, other functions cannot be excluded. For instances, since tryparedoxin peroxidases are peroxiredoxins and these have been shown to decompose peroxynitrite in addition to hydroperoxides [14–16], it is possible that the tryparedoxins are also required for that function. In addition, in vitro studies have shown that these proteins might participate in DNA synthesis, by reducing ribonucleotide reductase [17,18]. The Kinetoplastida genomes present several homologous open reading frames (ORFs) with the potential to encode proteins containing the WCPPC motif suggesting that different tryparedoxins may be expressed by these parasites. It is however not known whether a different role in the cell is assigned to each of such molecules or, on the contrary, they can substitute for each other. Alternatively, different genes may be giving rise to proteins expressed in different cell compartments or at different timings of parasite development.

Although *Leishmania* was also likely to express tryparedoxins no such proteins had yet been characterised in this parasite. Here we analysed two related genes of *L. infantum* closely linked in the chromosome and show that they encode two different tryparedoxins with up to now undescribed features. One, cytosolic and homologous to previously known tryparedoxins of *Crithidia fasciculata*, *Trypanosoma brucei* and *Trypanosoma cruzi* [2,10,12,17] is shown to be expressed predominantly in the parasite's infective stages and may therefore be an important factor for parasite infection of the host. The other is the first mitochondrial tryparedoxin described in a Kinetoplastida organism. Its finding strongly suggests the existence of a pathway for hydroperoxide detoxification in mitochondria similar to the one described in the cytosol.

2. Material and methods

2.1. Parasites

Promastigotes of *L. infantum* clone MHOM/MA67ITMA-P263 were grown at 25 °C in RPMI medium supplemented with 10% foetal calf serum (FCS), 2 mM L-glutamine, 50 mM Hepes sodium salt (pH 7.4), 35 U ml^{-1} penicillin and 35 μg ml^{-1} streptomycin. To obtain promastigotes in different phases of growth parasites were first synchronised by five daily passages of 5×10^5 parasites ml^{-1} and then harvested at days 1 (early log, 2 to 5×10^6 cells ml^{-1}), 2 (late log, 6 to 8.5×10^6 cells ml^{-1}) and 6 (stationary, 1.5 to 2×10^7 cells ml^{-1}). Axenic amastigotes were grown at 37 °C in MAA medium supplemented with 20% FCS, 2 mM glutamax (Gibco BRL), 0.023 mM hemin as described previously [19]. Intracellular amastigotes were obtained by infecting monolayers of 2×10^5 mouse peritoneal macrophages with previously opsonised stationary phase promastigotes at a ratio of four parasites to one macrophage.

2.2. Reverse transcription–PCR (RT–PCR) and rapid amplification of mRNA 3′-end by PCR (3′-RACE)

One microgram of promastigote total RNA was reverse transcribed with Superscript II (Gibco BRL) using the AP20 sequence (5′-GGCCACGCGTCGACTAGTACTTTTTTT-$TTTTTTTTTT_{OH}$) as primer. PCR to amplify fragments of tryparedoxin transcripts was performed from 1 μl cDNA employing oligonucleotides corresponding to the *L. donovani* spliced leader, 5′-gggggatccTCAGTTTCTGTACTTTAT-TG_{OH}, and complementary to the active site of known tryparedoxins, 5′-gggaattccC(G/T)(A/G)CAIGGIGG(A/G) $CACCA_{OH}$ (restriction sites and clamp sequences in lower case, I refers to inosine). Cycling conditions were an initial denaturing step at 94 °C for 3 min, followed by 30 cycles of 94 °C, 45 s, 54 °C, 1 min and 72 °C, 1 min, finalised by a 10 min extension at 72 °C. A primer, L18, specific to one of the amplified tryparedoxin sequences (5′-cagcgcacatATGTCCGGTGTCAGCAAGC$_{OH}$) was then used in conjunction with primer AUAP21 (5′-GGCCACGCGTCGACTAGTAC$_{OH}$) to isolate the complete ORF for *LiTXN*1 (PCR conditions: 1 cycle of 94 °C, 2 min, 59 °C, 1 min, 72 °C, 1 min, 30 cycles of 94 °C, 45 s, 65 °C, 1 min, 72 °C, 1 min, and 72 °C, 10 min).

2.3. Screening of a L. infantum cosmid library

*LiTXN*2 isolation resulted from the screening of a previously constructed library [20], using the *LiTXN*1 ORF as a probe and following standard colony hybridisation techniques.

2.4. DNA sequence analysis

DNA, as PCR products or cloned into plasmid, was sequenced by MW-BIOTECH AG (Ebersberg, Germany), or using the ABI Prism® Big Dye™ Terminator Cycle Sequencing Ready Reaction Kit, version 2.0 (Applied Biosystems), in the IBMC sequencing facilities.

2.5. Expression and purification of recombinant proteins

The full-length ORF of *LiTXN1* was PCR amplified with PWO (Roche) using primers L18 and L95 (caccgctcgagTTACTCGTCTCTCCACGG$_{OH}$). The resulting PCR product was digested with *Nde*I and *Xho*I, cloned into the pET28a plasmid (Novagen) digested with the same enzymes and sequenced. Upon transformation of *E. coli* BL21, a modified *Li*TXN1 containing an amino-terminal six-histidine tag was produced. Protein used throughout this work was purified from 2 l bacteria cultures containing 50 μg ml^{-1} kanamycin and induced for 3 h at 37 °C with 0.4 mM isopropyl-B-D-thiogalactopyranoside (IPTG), following the protocol detailed before [21] except that in this case the protein was subjected to two rounds of purification on the His Bind resin (Novagen). Purified *Li*TXN1 used in enzymatic assays was dissolved in 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.15 M NaCl pH 7.2 (PBS). A similar strategy was also employed to produce recombinant *Li*TXN2. In this case induction with 0.1 mM IPTG was for 4 h at 37 °C and the protein solubilisation buffer was 0.05 M Na_2HPO_4, 0.05 M NaH_2PO_4 (phosphate buffer) pH 7.0, 0.15 M NaCl.

*Lic*TXNPx1 [20] and *Lic*TXNPx2, two tryparedoxin peroxidases of *L. infantum*, were also produced as recombinant proteins in pET28 as above. The *LicTXNPx2* gene was obtained after sequencing several *L. infantum* peroxiredoxin cDNA clones and predicts an amino acid sequence 100% identical to the *Lc*Pxn1 protein of *L. chagasi* reported by Barr and Gedamu [22], that was shown to be cytosolic [16]. Induction of the proteins was achieved with 0.1 mM IPTG for 3 h at 37 °C. The purified proteins were dissolved in phosphate buffer, 0.15 M NaCl, pH 7.2 (*Lic*TXNPx1) or pH 8.0 (*Lic*TXNPx2).

2.6. Measurement of enzymatic activity and kinetic analysis

Routinely, *Li*TXN1 and *Li*TXN2 activities were measured at 25 °C according to Nogoceke et al. [1]. Five hundred microlitres of total reaction mixture contained 300 μM NADPH, 1 U ml^{-1} *T. cruzi* trypanothione reductase (TR), 50 μM trypanothione ($T(SH)_2$), 0.5–1 μM tryparedoxin (*Li*TXN1 or *Li*TXN2), 10 μM tryparedoxin peroxidase (*Lic*TXNPx1, *Lic*TXNPx2 or *Lim*TXNPx) [20,21] and 70 μM hydrogen peroxide (H_2O_2). The reaction was started by H_2O_2 addition after a 15 min pre-incubation and was followed at 340 nm by measuring the oxidation of NADPH. The data was analysed using the UVProbe software (Shimadzu Corporation). For determination of reductant specificity trypanothione and trypanothione reductase were replaced by 3 μM *E. coli* thioredoxin and 1.5 U ml^{-1} *E. coli* thioredoxin reductase and by 100 μM glutathione and 1 U ml^{-1} yeast glutathione reductase. The kinetic studies of *Li*TXN1 and *Li*TXN2 were performed according to the basic assay described above, except that the concentration of the substrates trypanothione, *Lic*TXNPx2 and *Lim*TXNPx were varied to suitable conditions. The data obtained was analysed using the Dalziel equation for two-substrate enzyme reactions [23].

2.7. DNA and RNA isolation and analysis

Genomic DNA from *L. infantum* was prepared from exponentially grown promastigotes as described by Kelly [24]. Total RNA from the different parasite stages analysed was prepared using Trizol (Gibco BRL) according to the manufacturer instructions.

Southern and Northern blots, hybridisations and washings were done following standard protocols. All probes were ^{32}P-labelled by random priming using Klenow DNA polymerase (Gibco BRL) and membrane development and analysis of signals were achieved with a Typhoon 8600 (Molecular Dynamics).

2.8. Western blotting

L. infantum protein extracts solubilised in 1% (v/v) Nonidet P-40 [20] in the presence of a cocktail of protease inhibitors, were resolved by SDS–PAGE and electroblotted onto nitrocellulose. Membranes were probed with polyclonal antibodies against purified recombinant *Li*TXN1 produced in Wistar rats by five repeated subcutaneous injections in Freunds complete and incomplete adjuvant (first inoculum and boosts, respectively) and against recombinant *Li*TXN2 obtained in the same way. Peroxidase-labelled anti-rat immunoglobulin (Amersham) was used as secondary antibody. Membrane development was achieved using enhanced chemiluminescence (Amersham). Protein concentration in parasites was determined with a bicinchoninic acid protein-assay system (Pierce).

2.9. Indirect immunofluorescence assays (IFAT)

Immunofluorescence assays were performed essentially as described [20]. Briefly, promastigotes in the different stages of growth were fixed with 4% paraformaldehyde (w/v) in PBS, permeabilized with 0.1% (v/v) Triton X-100 and spotted onto polylysine-coated microscope slides. Amastigote infected peritoneal-derived macrophages in 8-well culture chambers (Nunc) were also treated in the same way. Parasites were then incubated with anti-recombinant *Li*TXN1 and *Li*TXN2 antibodies or with control sera. Secondary antibodies were Alexa Fluor 568 anti-rat IgG and Alexa Fluor 488 anti-rabbit IgG (Molecular Probes). Samples were mounted in VectaShield (Vector Laboratories) and examined with an Axioskop Zeiss microscope.

2.10. Digitonin fractionation of intact cells

Cell fractionation was done according to Hausler et al. [25] and Saas et al. [26]. Aliquots of 5 × 10^7 promastigotes (≈100 μg of total protein), resuspended in 1.125 ml of 25 mM Tris pH 7.5, 0.6 M sucrose, 1 mM DTT, 1 mM

EDTA and a cocktail of protease inhibitors, were permeabilized with 125 μl of prediluted digitonin (Calbiochem) to final concentrations of 0–10 mg of digitonin per mg of cellular protein. Upon incubation at 37 °C for 2 min, the samples were mixed in a Vortex and centrifuged at 12,000 × *g* at 4 °C for 10 min. Protein in supernatants was concentrated by trichloroacetic acid precipitation and supernatant and pellet fractions corresponding to 1.125×10^7 promastigotes were run in SDS–PAGE and analysed by Western blot.

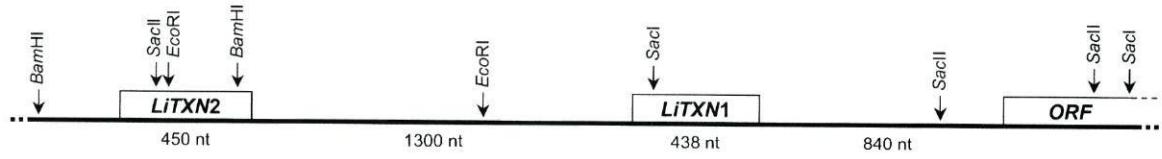
3. Results

3.1. Isolation of two tryparedoxin genes from *L. infantum*

One of the tryparedoxin genes characterised in this paper, *LiTXN*1, was isolated from *L. infantum* promastigote RNA using a RT–PCR strategy. Oligonucleotides corresponding to the *L. donovani* spliced leader and complementary to the conserved sequence encompassing the active site of *C. fasciculata* tryparedoxins were used to amplify the 5′-end of the mRNA and a primer specific to this sequence was then used in 3′-RACE to amplify its 3′-end. Once sequenced, the amplified products were found to contain an ORF 438 nucleotides (nt) long flanked by 150 nt corresponding to its 5′-untranslated region (UTR) and 542 nt of the 3′-UTR.

*LiTXN*2, the second tryparedoxin gene isolated from *L. infantum*, is 450 nt long and is found only 1300 nt upstream *LiTXN*1 (Fig. 2A). This gene was identified from sequencing a cosmid clone while attempting to know the upstream and downstream regions of *LiTXN*1. *LiTXN*2 is 64.2% homologous to *LiTXN*1. In addition, both *LiTXN*1 and *LiTXN*2 are homologous to several sequences from *C. fasciculata*, *T. brucei* and *T. cruzi* (accession numbers AAD20445, AAC61984, CAC85916 and CAA07003).

As is common for *Leishmania* genes, *LiTXN*1 and *LiTXN*2 are GC rich (55.5 and 54.4%, respectively). Since tryparedoxin proteins may occur in high amounts in the cell [1], the *LiTXN*1 and *LiTXN*2 sequences were scanned in order to see if there was a preference for the usage of codons normally associated to highly expressed genes [27,28], but this was not the case, although the level was higher in *LiTXN*2 (42% against 38% for *LiTXN*1). Southern blot analysis of genomic DNA digested with several enzymes indicates that *LiTXN*1 and *LiTXN*2 are single copy (data not shown). Recently, two new tryparedoxin-like sequences were identified in *L. infantum* (H. Castro and A. Tomás, unpublished data),



(A)

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                                                  * *  *       *
LiTXN1      MSGVSKHLGDVLKLQK-QNDMVDMSSLSGKTVFLYFSASWCPPCRGFTPK   49
LiTXN2      ...LT.FFPYSTSFL.GSATDIVLPT.A..........................  50
CfTXN1      ...LD.Y.PGIE..RR-GDGE.EVK..A..........................Q  49
CfTXN2      ...LK.FFPYSTNVL.GAAADIALP..A..........................Q  50
TcTXN1      ...LA.Y.PSTI..VS-KSGT.SPI..A..........................T  49
TbTXN1      ...LA.Y.PGATN.LS-KSGE.SLG..V..........................V  49

                                        *   *   *
LiTXN1      LVEFYEKHHNSKNFEIILASWDEEEEDFNGYYSKMPWLSIPFEKRNVVEA   99
LiTXN2      .....N..AK.....VM.I.....AD..ME..K.....AL...D.KGM.F  100
CfTXN1      .I...D.F.E.....VVFCT.....DG.A..FA.....AV..AQSEA.QK   99
CfTXN2      .ID..KA.AEK....VM.I....SA...KD..A.....AL...D.KGM.F  100
TcTXN1      .......FRE.....VV.VT..D...AY...FA.....A...SS.TEL..   99
TbTXN1      .A.......VA....VV.I....N.S..HD..G.....AL..DQ.ST.SE   99

                          **              *
LiTXN1      LTKQYKVESIPTLIGLNADTGDTVTTRARHALTQDPEGEQFPWRDE       145
LiTXN2      .KNGF...T......VE....KI......NMVEK....KE...PNVSEK   149
CfTXN1      .S.HFN.........VD..S..V......AT.VK.........K.AP      146
CfTXN2      ..TGFD.K.....V.VE..S.NII..Q..TMVVK...AKD...PNVEAKK  150
TcTXN1      .RSTFG..T...V.AV.....AV.S.KG.ER.LT....KN...S.       144
TbTXN1      .G.TFG........TI.....AIIG.Q..TRVIE..D.AN...PN       144
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(B)

Fig. 2. (A) Genomic organisation of the *locus* containing the *LiTXN*1 and *LiTXN*2 genes. (B) Sequence alignment of *Li*TXN1 and *Li*TXN2 with active tryparedoxins of other Kinetoplastida. *Cf*TXN1, *Crithidia fasciculata* TXN1, accession number AAD20445; *Cf*TXN2, *Crithidia fasciculata* TXN2, accession number AAC61984; *Tc*TXN1, *Trypanosoma cruzi* TXN1, accession number CAC85916; *Lb*TXN1, *Trypanosoma brucei* TXN1, accession number CAA07003. Residues that are common with *Li*TXN1 are represented by dots, residues that are common only with *Li*TXN2 are in bold and residues that are important for tryparedoxin activity are indicated with (*). The tryparedoxin active site is inside the box.

however these have low homology with both *LiTXN*1 and *LiTXN*2 (below 44%).

3.2. Analysis of the amino acid sequences

*LiTXN*1 and *LiTXN*2 are predicted to encode proteins with theoretical molecular weights of 16.69 and 17.18 kDa and p*I*'s of 5.24 and 6.60, respectively. Fig. 2B shows their amino acid sequences and compares them to other trypanosomatid sequences with known tryparedoxin activity. The two *L. infantum* tryparedoxin proteins share less identity to each other (57%) than when individually compared to tryparedoxins from other Kinetoplastida. *Li*TXN1 has higher identity to *Cf*TXN1 (60.3%) and *Li*TXN2 to *Cf*TXN2 (74.7%). As observed in Fig. 2B, *Li*TXN1 and *Li*TXN2 share with all tryparedoxin proteins the active site WCPPCR, positioned in the N-terminal region of the protein, as well as other residues previously shown to be important for activity [4,6,29,30]. This is the case of W39, S36, T47, Y34 and Y80 (*Li*TXN1 numbering), important to maintain the three-dimensional structure of the protein, R128, E72, I109 and P110, likely to be involved in tryparedoxin–trypanothione interaction, and D76, that along with R128 and E72 are required for the reaction with the peroxiredoxin. As observed for the other tryparedoxin sequences, *Li*TXN1 and *Li*TXN2 show little identity with human thioredoxin (around 15%).

3.3. LiTXN1 and LiTXN2 are active tryparedoxins

In order to confirm that *Li*TXN1 and *Li*TXN2 were active tryparedoxins, the proteins were produced in bacteria as N-terminal 6-histidine fusion proteins, purified by chelate chromatography and subjected to the routine test for tryparedoxin activity [1], whereby reduction of hydroperoxides by the enzymatic cascade depicted in Fig. 1 is assayed by measuring the degree of NADPH oxidation. As expected from the amino acid sequences, *Li*TXN1 and *Li*TXN2 displayed trypanothione:peroxiredoxin oxidoreductase activity, thus being essential factors in the reduction of the hydroperoxides. Indeed, no NADPH consumption was observed if these or any other of the reaction components were absent. *Li*TXN1 catalysed the reduction of *Lic*TXNPx1 and *Lic*TXNPx2, two *L. infantum* cytosolic peroxiredoxins, with similar specific activities (Table 1) and of a mitochondrial enzyme *Lim*TXNPx, although in this case the specific activity was 50% lower (Table 1). *Li*TXN2 also reacted equally well with *Lic*TXNPx1 and *Lic*TXNPx2 but, in contrast to *Li*TXN1, showed an activity slightly higher (25%) but statistically significant with the mitochondrial peroxiredoxin (Table 1). No hydroperoxide reduction was observed when trypanothione reductase and trypanothione were replaced by thioredoxin reductase and thioredoxin (not shown). However, *Li*TXN2, but not *Li*TXN1, could be reduced by glutathione but with a specific activity that was only 0.3% of that obtained with trypanothione.

Table 1
Specific activities of *Li*TXN1 and *Li*TXN2 with different *L. infantum* tryparedoxin peroxidases[a]

TXN	TXNPx	Specific activity (U mg^{-1})
*Li*TXN1	*Lic*TXNPx1	9.42 ± 0.39 (90%)
	*Lic*TXNPx2	10.52 ± 0.66 (100%)
	*Lim*TXNPx	5.26 ± 0.29 (50%)
*Li*TXN2	*Lic*TXNPx1	15.13 ± 1.05 (72%)
	*Lic*TXNPx2	15.31 ± 0.67 (73%)
	*Lim*TXNPx	21.06 ± 2.03 (100%)

[a] Means and standard deviations were calculated from six independent assays. 1 U mg^{-1} = 1 μmol NADPH reduced per minute per mg tryparedoxin. Specific activities are also indicated as a percentage of the highest specific activity measured for each TXN. Specific activities of each TXN with *Lim*TXNPx are significantly different from the values observed when *Lic*TXNPx1 or *Lic*TXNPx2 are used as oxidants ($P < 0.001$).

Kinetically, *Li*TXN1 and *Li*TXN2 behave as many oxidoreductases, that is they react with both the oxidant and the reductant in two independent steps. Indeed, double reciprocal plots of the normalised initial velocities ($[E_0]/v$) recorded at different concentrations of the peroxidases and at several fixed trypanothione concentrations yielded parallel lines (Fig. 3A and B) indicative of an enzyme substitution mechanism (ping-pong mechanism). Accordingly, the kinetic coefficients for the reactions can be determined graphically by the application of the Dalziel equation [23]:

$$\frac{[E_0]}{v} = \Phi_0 + \frac{\Phi_1}{[\text{TXNPx}]} + \frac{\Phi_2}{[\text{T(SH)}_2]} \quad (1)$$

whereby Φ_0 (the ordinate intercept in the secondary plot of Fig. 3C and D) equals $[E_0]/v_{max}$, that is, $1/k_{cat}$, Φ_1 (the slope in the primary plot, Fig. 3A and B) equals $1/k_1'$ and Φ_2 (the slope in the secondary plot, Fig. 3C and D) equals $1/k_2'$. k_1' and k_2' refer to the overall rate constants for the tryparedoxin oxidation and reduction, respectively. Table 2 shows the Φ values and kinetic constants obtained for each of the tryparedoxins analysed. As can be observed, *Li*TXN1 and *Li*TXN2 present similar kinetic behaviours when *Lic*TXNPx2 and *Lim*TXNPx are used as peroxidases, respectively (the conditions at which higher tryparedoxin specific activities were observed). In both cases, and within experimental error,

Table 2
Kinetic constants deduced according to Dalziel [23] for catalysis of *Li*TXN1 and *Li*TXN2 using $T(SH)_2$ as the reductant and *Lic*TXNPx2 and *Lim*TXNPx as the oxidants, respectively

TXN	TXNPx	Φ_0 (s)	Φ_1 (μM s)	Φ_2 (μM s)	k_{cat} (s^{-1})	k_1' (μM^{-1} s^{-1})	k_2' (μM^{-1} s^{-1})	K_{m1} (μM)	K_{m2} (μM)
*Li*TXN1	*Lic*TXNPx2	0	0.31	12.87	∝	3.22	0.08	∝	∝
*Li*TXN2	*Lim*TXNPx	0	0.51	4.32	∝	1.97	0.23	∝	∝

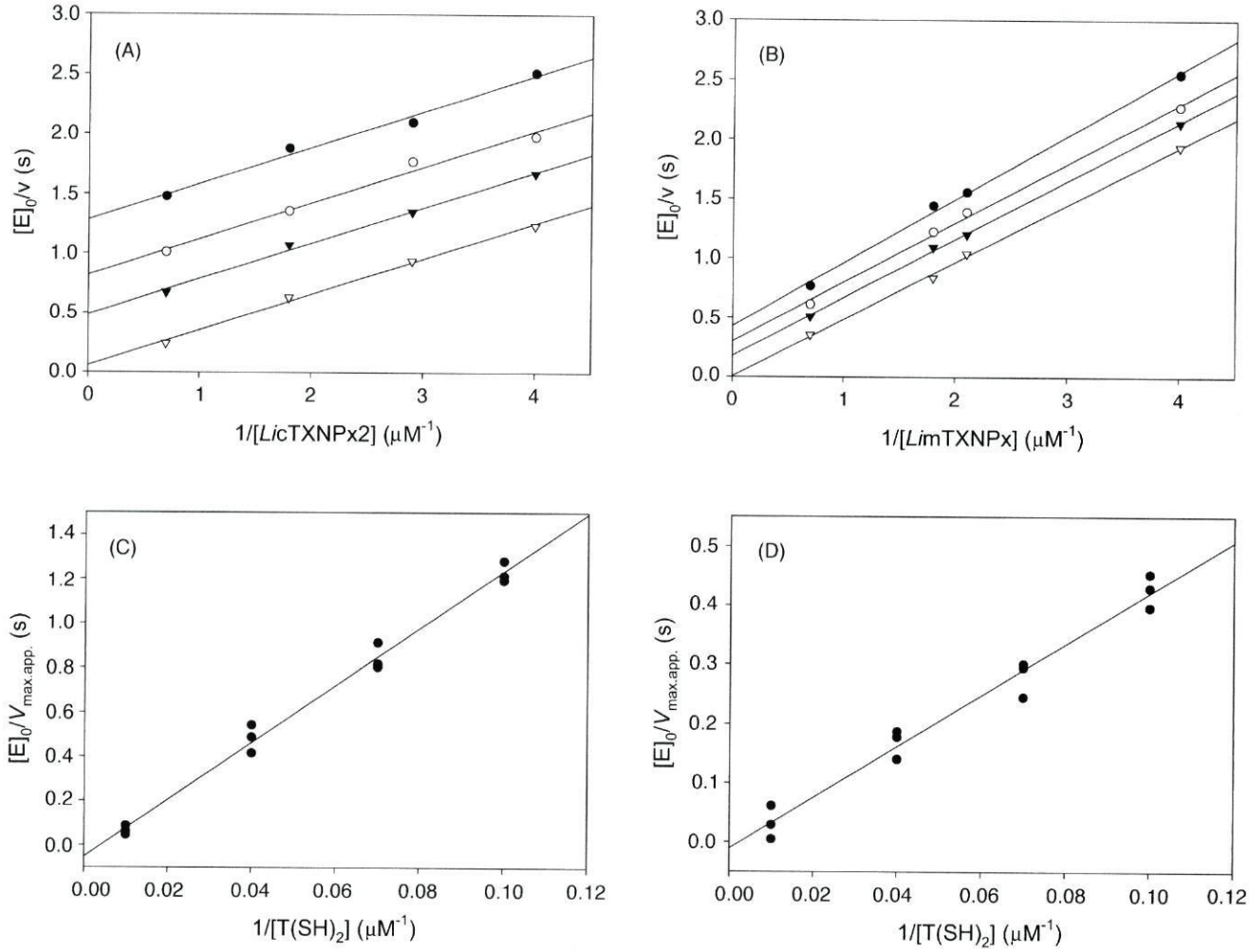

Fig. 3. Steady-state kinetic analysis of reactions between *Li*TXN1 with *Lic*TXNPx2 and $T(SH)_2$ (A, C), and of *Li*TXN2 with *Lim*TXNPx and $T(SH)_2$ (B, D). (A, B) Examples of double reciprocal primary plots showing dependency of enzyme-normalised initial velocities on TXNPx concentration at 10 μM (●), 14.7 μM (○), 27 μM (▼) and 200 μM (▽) $T(SH)_2$. (C, D) Secondary plots of ordinate intercepts of primary plots ($[E_0]/v_{max,app}$) calculated from three independent experiments plotted against reciprocal $T(SH)_2$ concentrations.

Φ_0 equals zero, which implies that no saturation kinetics is observed and that k_{cat} and K_m are infinite. The rate of enzyme–substrate complex formation, rather than its dissociation, is therefore limiting the overall reaction, and k_1' and k_2' can be defined as rate constants for the formation of the enzyme–substrate complexes. For both enzymes the reaction with $T(SH)_2$ is the rate limiting step in the tryparedoxin catalysis, as can be deduced from comparison of the k_1' and k_2' values. This added to the fact that k_2' for *Li*TXN2 has more than twice the value of k_2' for *Li*TXN1 may explain the higher specific activities measured for *Li*TXN2 in comparison to *Li*TXN1.

3.4. Expression analysis of the LiTXN1 and LiTXN2 transcripts and proteins along the L. infantum life cycle

Northern analysis was used to study the expression of the *LiTXN1*- and *LiTXN2*-specific mRNAs along the parasite life cycle. As shown in Fig. 4A, the *LiTXN1* gene is transcribed as a single RNA species of 1.3 kb in all the *L. infantum* stages. After normalising by comparison with the ethidium bromide staining it became apparent that this message increases slightly as the parasites develop from logarithmic to stationary phase promastigotes (twofold) and to (axenic) amastigotes (Fig. 4A and C). Since the genome of *Leishmania* presents other potential tryparedoxin sequences, the Northern analysis was repeated using 346 nt of the 3′-UTR of the *LiTXN1* gene as a probe because untranslated sequences are usually much less similar between themselves than coding sequences. However, the pattern observed was identical to that obtained when hybridisation was performed with the *LiTXN1* gene (Fig. 4B). In the case of *LiTXN2* the gene is transcribed as two transcripts of 1.5 and 1.3 kb, the abundance of which does not present substantial differences between developmental stages (Fig. 4D and F). Hybridisation of a similar Northern blot with 295 nt of the *LiTXN2* 3′-UTR indicates that the bands observed are likely to be derived from transcription of the same gene (Fig. 4E).

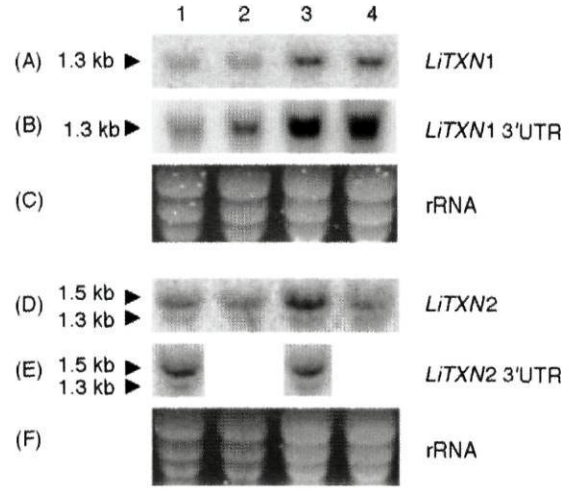

Fig. 4. Transcript analysis of *LiTXN1* and *LiTXN2* in different *L. infantum* stages. Northern blot containing 15 μg of total RNA extracted from early log (1), late log (2) and stationary phase promastigotes (3), and from axenic amastigotes (4), hybridised with the *LiTXN1* and *LiTXN2* ORFs (A, D), and with probes from the 3′-UTR *LiTXN1* and *LiTXN2* sequences (B, E). Ethidium bromide stained ribosomal RNA pictures of gels A and D are also included as a control for loading (C, F).

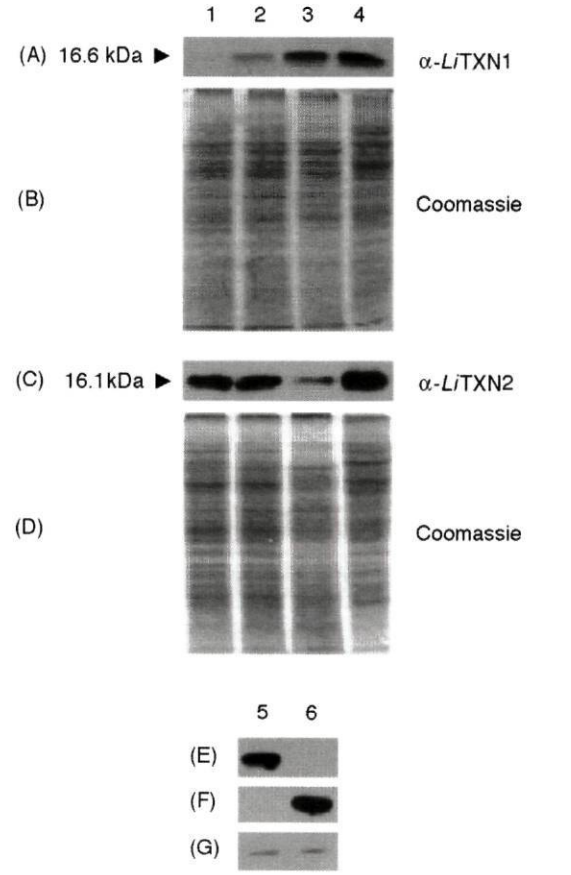

Fig. 5. Expression of *Li*TXN1 and *Li*TXN2 throughout *L. infantum* development. Western blot analysis of 20 μg (A, B) and 25 μg (C, D) of total protein extracts from early log (1), late log (2) and stationary phase promastigotes (3), and of axenic amastigotes (4), incubated with the anti-*Li*TXN1 (A) and anti-*Li*TXN2 (C) antibodies previously adsorbed (see text). Coomassie blue staining of identical gels run in parallel (B, D) are shown as a control for loading. Adsorbed anti-*Li*TXN1 and anti-*Li*TXN2 sera were tested for cross-reactivity by Western blot analysis (E, F) performed on 0.15 μg of purified recombinant *Li*TXN1 (5) and *Li*TXN2 (6). The recombinant proteins were also incubated with the anti-histidine (G) antibody to confirm that identical amounts of the protein had been loaded.

To analyse the expression of the *Li*TXN1 and *Li*TXN2 proteins in the different parasite stages polyclonal sera against the respective recombinant molecules were produced in rats. Anti-*Li*TXN1 and anti-*Li*TXN2 were then adsorbed with recombinant purified *Li*TXN2 and *Li*TXN1, respectively, in order to eliminate cross-reactivity between both sera before being used in the westerns (Fig. 5E–G). The results, presented in Fig. 5A, indicate that *Li*TXN1 is expressed along development as a single 16.6 kDa product. In addition, they clearly demonstrate that this protein is upregulated (10-fold) in the stationary phase promastigotes, which are enriched in infectious forms of *L. infantum*, suggesting a function in these parasite stages. The differences in protein abundance observed are higher than those recorded between the transcripts (see Figs. 4A, C and 5A, B). Therefore, the expression of *Li*TXN1 *L. infantum* is likely to be controlled mainly post-translationally. The mechanism and the signals required for this regulation are at present unknown. In respect to expression of *Li*TXN2, again a single band of 16.1 kDa was recognised by the anti-*Li*TXN2 sera (Fig. 5C). In this case, we noticed a decrease (four-fold) in signal intensity in stationary phase promastigotes when compared to the early and late logarithmically grown parasites. Further studies are required to draw definitive conclusions about the developmental regulation of this gene.

3.5. Localisation of LiTXN1 and LiTXN2 in L. infantum promastigotes and amastigotes

The localisation of *Li*TXN1 and *Li*TXN2 in the two parasite stages was studied by IFAT using the antibodies described above after adsorption. As shown in Fig. 6A and B *Li*TXN1 localises to the cytosol in both promastigotes and intracellular amastigotes, with the same pattern observed for the *Lic*TXNPx1/2 proteins, recognised by the anti-TSA (thiol-specific antioxidant protein) antibody [20,31]. To completely rule out the possibility of cross-hybridisation with other potential *L. infantum* tryparedoxins IFAT analysis was also performed with parasites expressing a tagged version of *Li*TXN1 by transformation with a pTEX plasmid [32] containing a fusion of the *LiTXN1* gene with the 9E10 epitope of the C-MYC protein [33]. Again the signal observed was restricted to the cytosol (not shown). Although no obvious organelle endorsement signal could be detected in the *Li*TXN2 amino acid sequence, IFAT analysis of both promastigotes and intramacrophagic amastigotes (Fig. 6A and B) demonstrates that this protein localises to the single mitochondrion of the parasite, a tubular-like structure in the interior of which can be observed the kinetoplast. This

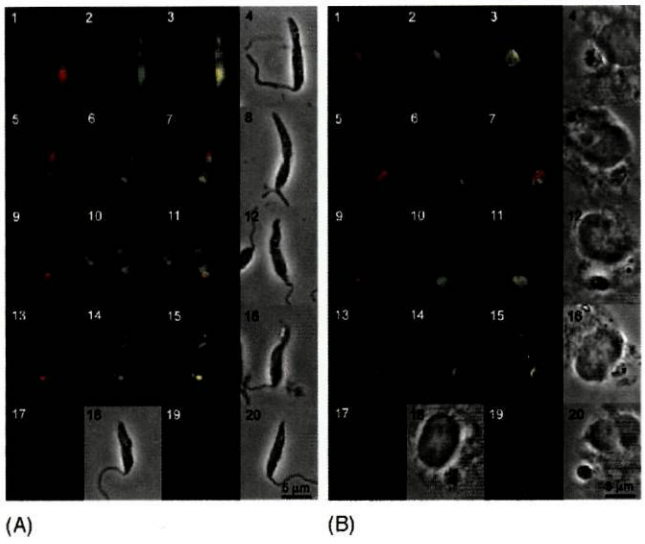

Fig. 6. Indirect immunofluorescence of *L. infantum* promastigotes (A) and intracellular amastigotes (B) showing cytosolic localization of *Li*TXN1 and mitochondrial localization of *Li*TXN2. Incubation with primary antibodies was as follows: 1, 5, previously adsorbed anti-*Li*TXN1 (see text); 2, 10, anti-TSA; 6, 14, anti-*Lim*TXNPx; 9, 13, previously adsorbed anti-*Li*TXN2; 17, 19, pre-immune serum of rats. Merging of both channels (3, 7, 11, 15) and contrast phase pictures (4, 8, 12, 16, 18, 20) are also included. Parasites were photographed at 1000× magnification.

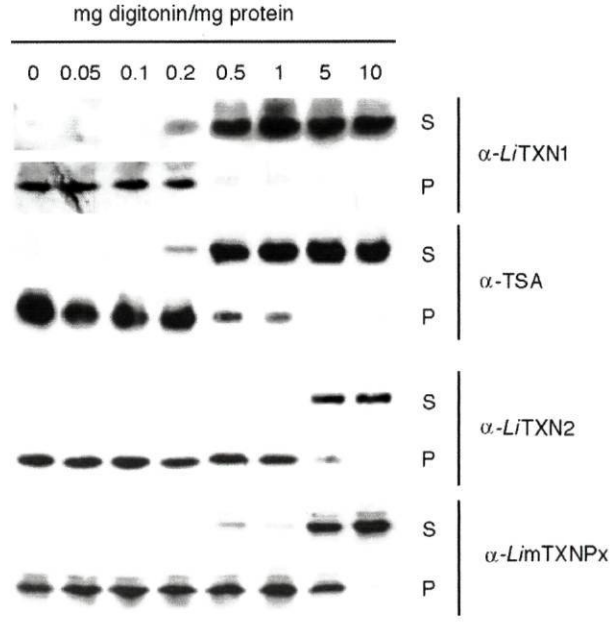

Fig. 7. Determination of *Li*TXN1 and *Li*TXN2 subcellular localization by digitonin fractionation. Supernatant (S) and pellet (P) fractions resulting from promastigote permeabilisation with increasing digitonin concentrations were analysed by Western blot using previously adsorbed anti-*Li*TXN1 and anti-*Li*TXN2 sera (see text). Anti-TSA and anti-*Lim*TXNPx were employed to detect the *Lic*TXNPx1/2 and *Lim*TXNPx proteins used as cytosolic and mitochondrial markers, respectively.

labelling completely overlays the one obtained with the antibody against the *Lim*TXNPx protein previously shown to be mitochondrial [20].

Additional evidence for the subcellular localisation of *Li*TXN1 and *Li*TXN2 was achieved by differential fractionation of parasites with digitonin as, depending on their cholesterol content, cellular membranes require increasing concentrations of this detergent to be permeabilized [34]. Intact promastigotes were therefore exposed to different digitonin concentrations and the resulting supernatant and pellet fractions were analysed by Western blotting. *Lic*TXNPx1/2 and *Lim*TXNPx were used as cytosolic and mitochondrial markers, respectively [20]. As shown in Fig. 7, *Li*TXN1 is released from the cells at relatively low digitonin concentrations (over 0.2 mg digitonin mg^{-1} cellular protein), as occurs with *Lic*TXNPx1/2. This confirms that *Li*TXN1 is located into the cytosol. On the contrary, higher digitonin concentrations (over 1 mg digitonin mg^{-1} cellular protein) are required to release both *Li*TXN2 and the mitochondrial enzyme *Lim*TXNPx, strongly suggesting that these two proteins share the same cellular localisation.

4. Discussion

Once inoculated into a vertebrate host *Leishmania* survival depends on its capacity to infect macrophages and, within these cells, differentiate and replicate as amastigotes. Then, for transmission to be accomplished, amastigotes will have to be taken up by the insect vector, transform back into promastigotes and divide before becoming infective to a new vertebrate host. To succeed this elaborate sequence of different events parasites must be equipped to deal with toxic oxidants, such as reactive oxygen and nitrogen species (ROS and RNS, respectively), that are generated as a consequence of their own aerobic metabolism and as part of the hosts antimicrobial processes [35–39]. In this paper, we describe two tryparedoxins, *Li*TXN1 and *Li*TXN2, that may be key mediators in conferring *Leishmania* resistance to ROS and RNS by being probable components of the recently discovered trypanothione-dependent hydroperoxide detoxification cascades (Fig. 1). Unlike other tryparedoxin-like sequences present in *L. infantum* (H. Castro and A. Tomás, unpublished results), *Li*TXN1 and *Li*TXN2 not only conserve all the amino acids previously implicated in interaction with both co-substrates [4,6,29,30], but they were actually shown to reduce the three previously described *Leishmania* peroxiredoxins [20,22]. In addition, we argue that *Li*TXN1 may be of particular importance to provide resistance to host-derived radicals while *Li*TXN2 could contribute to shield the parasite from endogenous produced radicals.

The leishmanicidal capacity of ROS and RNS in animal models of leishmaniasis and in man and dog macrophages has been reported earlier [39–43]. Different kinds of ROS, such as hydrogen peroxide (H_2O_2) and superoxide radical ($^{\bullet}O_2^-$) are formed during phagocytosis and RNS, like nitric oxide ($^{\bullet}NO$) and peroxynitrite ($ONOO^-$), upon macrophage activation by the immune system. Three

different observations suggest that *Li*TXN1 may be involved, although indirectly, in ensuring parasite resistance to some of those host-derived oxidants. First, the protein is upregulated in stationary-phase promastigotes, that is, parasites able to transmit infection to vertebrate hosts. This tryparedoxin is also expressed at high levels in axenic amastigotes but in this case we cannot guarantee that such level of expression completely reflects what happens in intracellular parasites. Second, *Li*TXN1 is cytosolic therefore well positioned to protect the parasite from host-derived hydrogen peroxide and peroxynitrite, which can traverse the parasite plasma membrane [44]. Finally, *Li*TXN1 was shown by biochemical assays to reduce the cytosolic *L. infantum* peroxiredoxins *Lic*TXNPx1 and *Lic*TXNPx2, two enzymes that either in this species of *Leishmania* or their homologues in *L. chagasi*, were previously shown to allow the parasite to detoxify exogenous added hydroperoxides and/or peroxynitrite [16,20]. Earlier reports have evidenced that *Leishmania* presents different antioxidant molecules, some constitutively expressed others developmentally or environmentally induced [45]. Trypanothione [46], ovothiol [47], lipophosphoglycan (LPG) [48], and heat shock protein 70 (Hsp70) [45] among others are likely to complement each other and could provide the parasite with several lines of defence towards macrophage released toxic oxidants. The data presented in this paper thus suggests that the developmentally regulated *Li*TXN1, together with the other components of trypanothione–tryparedoxin–tryparedoxin peroxidase system may be part of such parasite defence armamentarium. A definitive conclusion about the exact role(s) of *Li*TXN1 will however have to wait gene manipulation based experiments. At present we cannot exclude a contribution of this protein in shielding the parasite from endogenous produced oxidants. In the same way, although a primordial function of *Li*TXN1 in reduction of ribonucleotide reductase seems unlikely in promastigotes because the protein is barely detected in the dividing log-phase parasitic cell, it could still be involved in DNA synthesis in amastigotes.

Mitochondria are normally associated with high oxygen radical production [49,50]. In previous work, we characterized a *L. infantum* peroxiredoxin, *Lim*TXNPx, with tryparedoxin peroxidase activity and showed, in vivo, that it could guard mitochondria from hydroperoxide damage [20]. Similar peroxiredoxins are also found in *T. cruzi* [51] and *T. brucei* [52]. This suggested that Kinetoplastida could possess a hydroperoxide detoxification cascade in mitochondria similar to that described for the cytosol. However, none of the other components of such cascades were unequivocally found in those organelles and the possibility that local removal of hydroperoxides was partitioned with the cytosol was raised [51]. The finding that *Li*TXN2 locates to mitochondria and behaves as a tryparedoxin thus constitutes the first evidence for the presence of a distinct trypanothione peroxidase system in Kinetoplastida mitochondria. *Li*TXN2 and *Lim*TXNPx are likely to be functionally linked providing the mitochondrial contents defence from damage by endogenous oxidants. A remaining question regarding the hydroperoxide detoxification system in mitochondria is whether trypanothione and trypanothione reductase exist in this cell compartment. The subcellular localisation of trypanothione has never been investigated and the two previous reports regarding the distribution of trypanothione reductase may be contradictory. Indeed, while in *T. brucei* this enzyme was found restricted to the cytosol [53], in *T. cruzi* it could be detected in mitochondria [54]. This last observation, if is found true for *Leishmania*, would fit our data that implicate trypanothione as the probable reductant for *Li*TXN2. This enzyme was also shown to accept electrons from glutathione, however the specific activity was very low, and it seems unlikely that this reductant is used in vivo.

As said before, *Li*TXN1 and *Li*TXN2 are homologous to proteins described previously in other pathogenic Kinetoplastida. The kinetics of some of these enzymes have been object of previous biochemical analysis [9,11,29] and similarly to what was observed then both *Li*TXN1 and *Li*TXN2 do follow a ping-pong mechanism indicating that they react with trypanothione and the tryparedoxin peroxidases in two independent steps. In addition, the kinetic data obtained suggest that the formation of the trypanothione–tryparedoxin complex is the limiting step of the reaction between tryparedoxin and the two co-substrates. Depending on levels of substrates and system components, however, other steps may become the bottleneck of the whole pathway, as we previously demonstrated that overexpression of both the cytosolic and mitochondrial peroxiredoxins in promastigotes leads to an increased ability to cope with hydroperoxide challenge [20].

In summary we have characterized two *L. infantum* tryparedoxins related to previously identified tryparedoxins from other Kinetoplastida and likely to play different functions in the cell. If these are proved essential to the parasite they could constitute important targets for the search of new chemotherapeutics for leishmaniasis and perhaps sleeping sickness and Chagas' disease.

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Chapter 4

Unpublished results.

Subcellular distribution of trypanothione reductase activity in *Leishmania infantum* promastigotes – implications for reduction of a mitochondrial tryparedoxin

Helena Castro [a], Fernanda Gadelha [b], Ana M. Tomás [a]

[a] Institute for Molecular and Cell Biology, Porto, Portugal.
[b] Universidade Estadual de Campinas, Campinas, Brazil.

Abstract

The *Leishmania* mitochondrion is a relevant site for the generation of oxidants. Within this organelle the antioxidant function is probably supported by a tryparedoxin enzyme acting as the electron supplier for two mitochondrial peroxidases, a peroxiredoxin and a non-selenium glutathione-peroxidase like enzyme. Whether, as occurs in the cytosol of *Leishmania* and of other trypanosomatids, the NADPH/trypanothione reductase/trypanothione redox cycle provides tryparedoxin the reducing equivalents required for its oxidoreductase activity is still to be shown. As an approach to elucidate this issue we have investigated the subcellular distribution of trypanothione reductase activity in *Leishmania infantum* promastigotes using a digitonin fractionation assay. By comparing the distribution pattern of trypanothione reductase (TR) activity with that of control enzymes with known subcellular compartmentalization, we were unable to detect TR activity in the parasite mitochondrion. In the face of this result we discuss how the tryparedoxin enzyme might be reduced within this organelle.

Introduction

Trypanothione reductase (TR) is a flavoenzyme homologous to the mammalian glutathione reductase and thioredoxin reductase molecules, which displays the unique ability of reducing trypanothione at expenses of NADPH (Shames *et al.*, 1986). In trypanosomatids trypanothione is considered to be the main regulator of the intracellular redox milieu. In fact, in its reduced form, trypanothione is the electron supplier for several redox cascades, which together execute a myriad of biologically relevant functions. These include the removal of toxic species, such as ROI, RNI, methylglyoxal, xenobiotics and metals, the synthesis of DNA and proteins, and possibly the regulation of kDNA replication (e.g. Nogoceke *et al.*, 1997; Legare *et al.*, 1997; Dormeyer *et al.*, 2001; Wilkinson *et al.*, 2003; Vickers *et al.*, 2004; Vickers and Fairlamb, 2004;

Trujillo *et al.*, 2004; Onn *et al.*, 2004; Sousa Silva *et al.*, 2005). As expected from this broad usage of trypanothione, down regulation of either trypanothione synthase, the enzyme responsible for the *de novo* synthesis of trypanothione, or of TR results disastrous for trypanosomatid survival and virulence (Dumas *et al.*, 1997; Tovar *et al.*, 1998; Krieger *et al.*, 2000; Comini *et al.*, 2004; Ariyanayagam *et al.*, 2005). Although the various trypanothione-dependent routes are well established to occur *in vitro*, some of them may not operate in the parasite due, for instances, to the distinct subcellular compartmentalization of the different molecular players. For instances, as discussed next, it is unclear whether the mitochondrial pathway for removal of oxidants is trypanothione-dependent.

In trypanosomatids' mitochondria, a peroxiredoxin (Prx) (Wilkinson *et al.*, 2000; Tetaud *et al.*, 2001; Castro *et al.*, 2002b) and a non-selenium glutathione peroxidase-like molecule (Schlecker *et al.*, 2005) are likely responsible for hydroperoxide elimination. The physiological reductant for these peroxidases is possibly a thioredoxin-like oxidoreductase, known as tryparedoxin (TXN). This assumption is supported by the observations that *in vitro* both enzymes display tryparedoxin peroxidase activity (Castro *et al.*, 2002a; Hillebrand *et al.*, 2003), and by the finding that a TXN molecule is present in the *Leishmania infantum* mitochondrion (*Li*TXN2, Castro *et al.*, 2004). Still, we cannot exclude the possibility that other molecules may serve as electron suppliers for the mitochondrial peroxidases. One candidate reductant for the mitochondrial Prx, for example, could be cyclophilin, based on the finding that the human PrxII peroxidatic activity is supported by cyclophilin A (Lee *et al.*, 2001), and on the observation that trypanosomatids possess genes coding for putative mitochondrial cyclophilins (http://www.genedb.org; sequence analysis performed in PSORTII and MitoProtII internet servers).

If TXNs are the electron donors for the mitochondrial peroxidases the question arises of which is the physiological reductant for the mitochondrial TXN. In the cytosol TXNs are reduced by the TR/trypanothione system at costs of NADPH (Nogoceke *et al.*, 1997), but it remains elusive whether the same pathway operates in the mitochondrion. Indeed, the trypanothione subcellular distribution has never been addressed, and information regarding TR mitochondrial compartmentalization is rather contradictory. Meziane-Cherif *et al.* (1994) claimed that in *T. cruzi* TR, apart from having a cytosolic distribution in the cell, was also present in the mitochondrion. These authors reached this conclusion by electron microscopy analysis using antibodies against a synthetic peptide designed to a TR-specific stretch. In contrast, other groups, performing subcellular fractionation analysis of *T. brucei* and *T. cruzi*, could never find definitive proof for TR mitochondrial localization (Smith *et al.*, 1991; Wilkinson *et al.*, 2002; Schlecker *et al.*, 2005). Instead, they showed that the enzyme is mainly cytosolic and, in the case of *T. cruzi*, that a small proportion of the enzyme also elutes with the glycosomes (Wilkinson *et al.*, 2002). This last observation is consistent with the fact that the amino acid sequence for the TR of *T. cruzi* (Acc. Nr. P28593) possesses a weak SKL-like

glycosomal targeting signal (ASL; Sommer and Wang, 1994). The same feature is found for *T. brucei* (SSL; Acc. Nr. CAA44870), *L. major* (SNL; Acc. Nr. CAB89598), *L. donovani* (SNL; Acc. Nr. CAA80668); *L. infantum* (SNL; http://www.genedb.org) and *C. fasciculata* (SNL; Acc. Nr. CAA78264) enzymes. In the face of these observations and of the absence of data regarding TR subcellular distribution in *Leishmania* we find it difficult to draw a definitive conclusion about the involvement of trypanothione and TR in mitochondrial redox mechanisms. As a first approach to clarify this issue we have examined the compartmentalization of TR activity in *L. infantum* promastigotes.

Materials and methods

Reagents

All reagents were obtained from Sigma, except for digitonin and trypanothione disulfide (TS_2), which were purchased from Calbiochem and Bachem, respectively.

Parasites

Logarithmic and stationary phase *L. infantum* promastigotes were grown and collected as previously described by Castro *et al.* (2004).

Digitonin fractionation of intact cells

Cell fractionation was done as described in Castro *et al.* (2004) with minor modifications. Briefly, aliquots of 3.5×10^8 promastigotes (corresponding to approximately 700 µg of total protein) resuspended in 525 µl of 25 mM Tris pH 7.5, 0.6 M sucrose and a cocktail of protease inhibitors, were permeabilized with 175 µl of prediluted digitonin to final concentrations of 0 to 3 mg of digitonin per mg of cellular protein. Upon incubation at 37ºC for 2 min, the samples were mixed in a vortex and subsequently fractionated at 12,000 × *g* at 4ºC for 10 min. Aliquots of the supernatants were kept frozen at –80ºC until analysis of enzymatic activities.

Enzymatic assays

Enzymatic activities were determined with 40 µl of the supernatant fractions, corresponding to 2×10^7 cells. The reaction mixtures contained: (i) for TR activity, 25 mM Tris-HCl pH 7.5, 1 mM EDTA, 0.2 mM NADPH and 50 µM TS_2; (ii) for glucose-6-phosphate dehydrogenase (GPDH) activity, 25 mM KH_2PO_4/K_2HPO_4 pH 7.4, 0.6 mM $NADP^+$, 10 mM D-glucose-6-phosphate; (iii) for hexokinase (HK) activity, 0.1 M triethanolamine-HCl pH 7.6, 0.6 mM $NADP^+$, 0.64 mM ATP, 10 mM $MgCl_2$, 0.002 U glucose-6-phosphate dehydrogenase, 10 mM D-glucose; and (iv) for citrate synthase (CS) activity, 20 mM Tris pH 8.0, 0.5 mM acetyl

coenzyme A, 0.25 mM 5,5'-dithio-*bis*(2-nitrobenzoic acid) (DTNB), 1 mM oxaloacetate. Except for CS, for which the activity was monitored spectrophotometrically at 412 nm, all other enzymatic activities were determined at 340 nm.

Results and discussion

To determine the subcellular localization of TR activity in *L. infantum* a digitonin fractionation experiment was performed. Depending on the sterol content of their membranes, the various subcellular compartments of the parasite were differentially permeated with increasing detergent concentrations. Figure 1 shows two independent experiments, performed with dividing (Figure 1A) and non-dividing promastigotes (Figure 1B), wherein liberation of the cytosolic, glycosomal and mitochondrial contents was monitored by measuring the enzymatic activity of marker molecules at different digitonin concentrations. The biochemical assays were performed with a volume of cellular extract corresponding to 2×10^7 cells. At this cell number all enzymatic activities (those of TR and of control enzymes) are measurable, yet not saturated. As depicted in Figure 1, the parasite cytosolic content eluted at low digitonin concentrations (complete at 0.1-0.2 mg digitonin/mg protein), and was followed by disruption of the glycosomal compartment (complete at 1 mg digitonin/mg protein), which is more resistant to the action of the detergent. Permeation of the inner mitochondrial membrane was only achieved at higher digitonin concentrations (complete at around 1.5 mg digitonin/mg protein). A comparative analysis of TR activity at each digitonin concentration suggests that this enzymatic

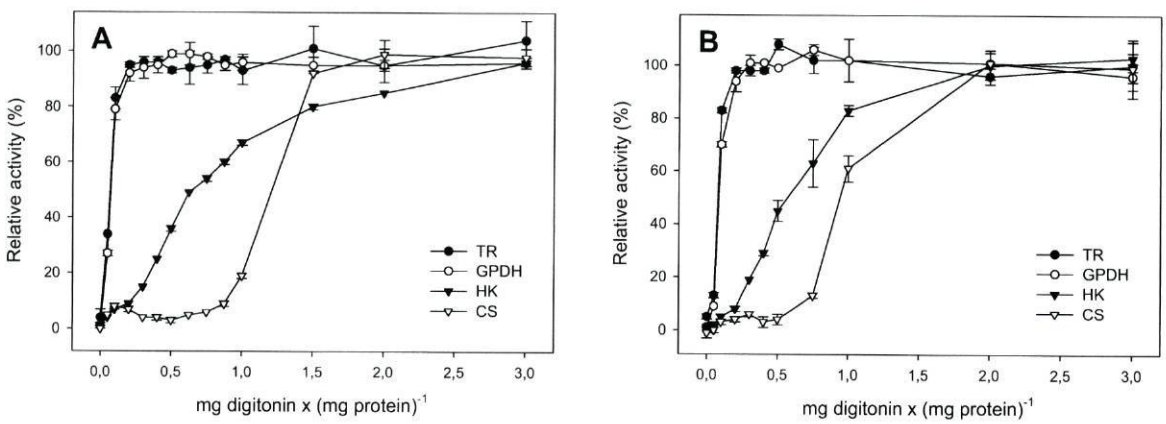

Figure 1. Digitonin titration of TR activity in *L. infantum* promastigotes. Supernantants resulting from promastigote permeabilization with 0-3 mg digitonin/mg of protein, were assayed for TR activity and for glucose-6-phosphate dehydrogenase (GPDH, cytosolic and glycosomal marker), hexokinase (HK, glycosomal marker), and citrate synthase (CS, mitochondrial marker) activities. Enzymatic activity is expressed as the percentage of total activity released after permeabilization with the highest digitonin concentration. The same experience was performed with dividing (A) and non-dividing (B) promastigotes.

activity is mostly associated with the cytosol, and possibly also with the glycosomal fraction. Indeed, the profile of activity TR is the same as that of GPDH, an enzyme previously shown to have dual location in the cytosol and glycosome (Mottram and Coombs, 1985; Heise and Opperdoes, 1999).

Assuming that the *Leishmania* mitochondrion is indeed deprived of TR activity, one may question how the TXN redox cycle is driven inside this organelle. First, it should be noted that the absence of TR activity from the mitochondrion does not preclude a role for trypanothione in mitochondrial TXN reduction. In fact, until shown otherwise, trypanothione may be present in the mitochondrion and may play a part in mitochondrial redox pathways. For that to be true a putative pool of mitochondrial trypanothione would have to be obtained from the cytosol, because trypanothione biosynthesis is likely restricted to this cell compartment. Supporting this assumption is the observation that *T.brucei*, *T. cruzi*, *L. major*, *L. infantum* and *C. fasciculata* trypanothione synthase amino acid sequences do not possess any obvious mitochondrial targeting signal (Acc. Nr. CAC87573, AAL26803, CAC83968 and AAC39132, respectively; PSORTII; MitoProtII). Once inside the mitochondrion, trypanothione would be readily oxidized by TXN (and possibly by other oxidants) and, given the apparent lack of a mitochondrial trypanothione reductase activity, it would have to return to the cytosol to complete its redox cycle. Transport of trypanothione across the outer mitochondrial membrane should pose no problem because this membrane is highly permeable to molecules with a molecular weight up to 5,000 Da (trypanothione MW ~720 Da). In contrast, the inner mitochondrial membrane is impermeable to ions and polar molecules, and transport of trypanothione (positively charged, Fairlamb and Cerami, 1992) across this lipid bilayer would require a protein carrier. Furthermore, export of oxidized trypanothione from the mitochondrial matrix would likely proceed against an electrochemical gradient, as (1) trypanothione concentration should be highest in the cytosol (where it is presumably synthesized), and (2) trypanothione has a net charge of +1 at physiologic pH, while the mitochondrial matrix is negatively charged in comparison to the cytosol. Therefore, the outward movement of trypanothione would only be possible if coupled to a thermodynamically favorable process, such as, for instances, the inward movement of reduced trypanothione. The antiport transport of reduced and oxidized trypanothione across the inner mitochondrial membrane (i.e. the simultaneous transport of both redox states of trypanothione in opposite directions) comes out as a suitable model system, and it would have no effect on the electrochemical gradient across the membrane (Figure 2A). The existence of a putative carrier driving the cytosol/mitochondrion trypanothione exchange should be investigated. First, however, the presence of trypanothione in the mitochondrial matrix of trypanosomatids must be confirmed.

In a different scenario the mitochondrion might be deprived of trypanothione and, in this case, the mitochondrial TXN would have to be reduced by an alternative molecular species. Based on the finding that the mitochondrial TXN is reduced by glutathione *in vitro* (Castro *et*

al., 2004), this thiol could be a candidate reductant for the enzyme. This premise is nevertheless improbable due to the low efficacy of GSH to reduce TXN (Gommel *et al.*, 1997; Castro *et al.*, 2004). An alternative electron supplier for the mitochondrial TXN could be lipoic acid. Lipoic acid is a low molecular weight thiol which occurs mainly as lipoamide integrated in the mitochondrial 2-oxo-ketoacid dehydrogenase multienzyme complexes (Perham, 2000). In nearly all eukaryotes the enzyme dihydrolipoamide dehydrogenase (LDH) is also part of these complexes, wherein it catalyzes the oxidation of protein-bound dihydrolipoyl residues, with concomitant regeneration of NADH from NAD^+. In addition, LDH can also catalyze the reverse reaction, i.e. the reduction of lipoamide to dihydrolipoamide at costs of NADH. In the malaria parasite *Plasmodium falciparum* the LDH/lipoamide system was shown to catalyze the *in vitro* reduction of thioredoxin, a TXN homologue (Muller, 2004). Likewise, the *Mycobacterium tuberculosis* protein AhpD, an enzyme possessing a thioredoxin-like active site (Cys-X-X-Cys),

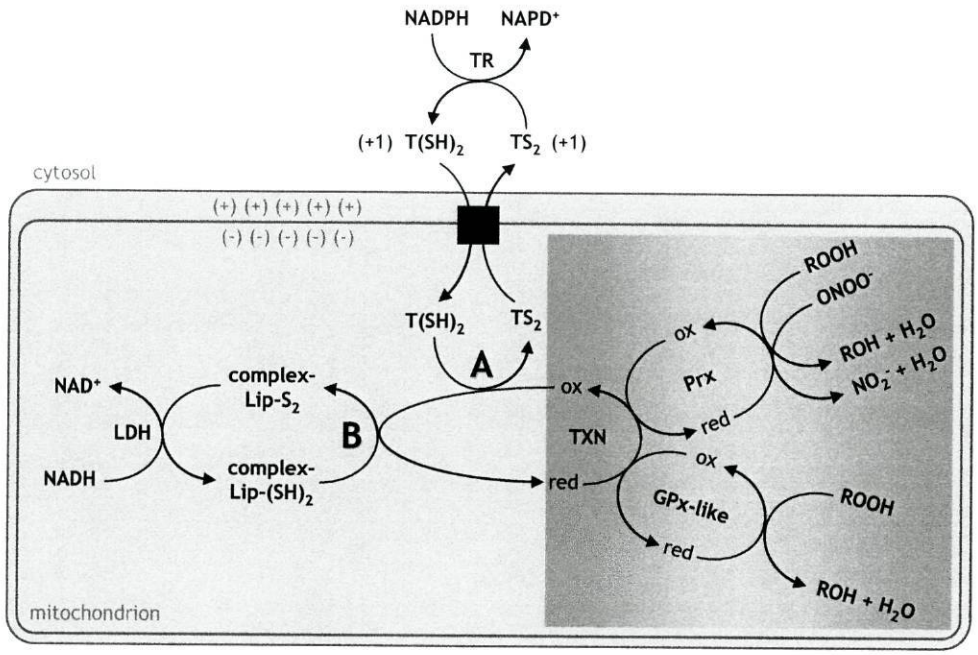

Figure 2. Hypothetical routes for TXN-dependent peroxidase reduction in the *Leishmania* mitochondrion. In the *Leishmania* mitochondrion hydroperoxide (ROOH) removal is probably achieved by a peroxiredoxin molecule (Prx) and a non-selenium glutathione peroxidase-like enzyme (GPx-like). The Prx enzyme is also possibly implicated in peroxynitrite ($ONOO^-$) detoxification within this organelle. By providing these peroxidases the reducing equivalents necessary for their activities, the mitochondrial enzyme tryparedoxin (TXN) may play a crucial role in the organelle antioxidant defense. In the cytosol of *Leishmania* TXN is reduced by the trypanothione reductase (TR)/trypanothione system. However TR activity is apparently absent from these parasites' mitochondria. Therefore we present two hypotheses for the initiation of the mitochondrial TXN redox cascades. (A) a putative antiport transport system (black box) placed at the inner mitochondrial membrane might drive the transport of dihydrotrypanothione [reduced form, $T(SH)_2$] and of trypanothione (oxidized form, TS_2) in and out of the mitochondrial matrix, respectively. The electrochemical gradient across the inner mitochondrial membrane is indicated with (+) and (-); both $T(SH)_2$ and TS_2 are positively charged (+1). Inside the mitochondrion $T(SH)_2$ would provide reducing equivalents to the TXN-peroxidase pathways; the trypanothione redox cycle would be completed in the cytosol. (B) Alternatively, TXN might be reduced by lipoic acid, covalently bound to an oxo-ketoacid dehydrogenase complex. In this system the enzyme dihydrolipoamide dehydrogenase (LDH) would regenerate the pool of dihydrolipoamide [Lip-$(SH)_2$], the reduced form of lipoamide (Lip-S_2), at expenses of NADH.

was shown also to be reduced by dihydrolipoamide (Bryk *et al.*, 2002; Jaeger *et al.*, 2004). Trypanosomatids' genomes (http://www.genedb.org) contain open reading frames coding for LDH, as well as for enzymes implicated in the biosynthesis of lipoic acid and its attachment to the multienzyme complexes, lipoic acid synthase and lipoate protein ligase, respectively (Perham, 2000). The corresponding gene products possess putative mitochondrial import sequences (PSORTII; MitoProtII), and, in the case of *T. brucei*, LDH was confirmed to have a mitochondrial compartmentalization (Schlecker *et al.*, 2005). These observations support the hypothesis that, within trypanosomatids' mitochondria, the LDH/lipoamide system might be operative, and might supply reducing equivalents to the TXN-dependent peroxidases (Figure 2B). Characterization of such redox system in the mitochondrion of *Leishmania* and other trypanosomatids merits further investigation. Finally, it should be mentioned that lipoic acid *per se* is able to reduce strong oxidants such as hydroxyl radical, hydrogen peroxide, hyochlorous acid, singlet oxygen, peroxyl radicals (for references see Trujillo and Radi, 2002), peroxynitrite (Trujillo and Radi, 2002) and peroxynitrite-derived $CO_3^{\cdot-}$ and $^{\cdot}NO_2$ radicals (Trujillo *et al.*, 2005). Therefore, and despite its low abundance as free acid in the cell, this thiol could participate in the mitochondrial antioxidant defense, possibly complementing the function of peroxidases.

In short, by establishing that in *L. infantum* TR activity occurs mostly in the cytosolic compartment, this work contributed to clarify the issue of TR compartmentalization in trypanosomatid parasites. In face of our results we propose two hypothetical routes for the redox fuelling of the mitochondrial tryparedoxin enzyme, one using trypanothione and involving a special thiol exchange system between the cytosol and the mitochondrion, and the other dependent on the LDH/lipoamide system.

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Chapter 5

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Original Contribution

SPECIFICITY AND KINETICS OF A MITOCHONDRIAL PEROXIREDOXIN OF *LEISHMANIA INFANTUM*

HELENA CASTRO,* HEIKE BUDDE,† LEOPOLD FLOHÉ,† BIRGIT HOFMANN,† HEINRICH LÜNSDORF,‡ JOSEPH WISSING,† and ANA M. TOMÁS*§

*Institute for Molecular and Cell Biology, Porto, Portugal; †Department of Biochemistry, Technical University of Braunschweig, Braunschweig, Germany; ‡National Center of Biotechnological Research (GBF), Braunschweig, Germany; and §Abel Salazar Institute for Biomedical Research, University of Porto, Porto, Portugal

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Abstract—In Kinetoplastida, comprising the medically important parasites *Trypanosoma brucei*, *T. cruzi*, and *Leishmania* species, 2-Cys peroxiredoxins described to date have been shown to catalyze reduction of peroxides by the specific thiol trypanothione using tryparedoxin, a thioredoxin-related protein, as an immediate electron donor. Here we show that a mitochondrial peroxiredoxin from *L. infantum* (*Li*mTXNPx) is also a tryparedoxin peroxidase. In an heterologous system constituted by nicotinamide adenine dinucleotide phosphate (NADPH), *T. cruzi* trypanothione reductase, trypanothione and *Crithidia fasciculata* tryparedoxin (*Cf*TXN1 and *Cf*TXN2), the recombinant enzyme purified from *Escherichia coli* as an N-terminally His-tagged protein preferentially reduces H_2O_2 and *tert*-butyl hydroperoxide and less actively cumene hydroperoxide. Linoleic acid hydroperoxide and phosphatidyl choline hydroperoxide are poor substrates in the sense that they are reduced weakly and inhibit the enzyme in a concentration- and time-dependent way. Kinetic parameters deduced for *Li*mTXNPx are a k_{cat} of 37.0 s^{-1} and K_m values of 31.9 and 9.1 μM for *Cf*TXN2 and *tert*-butyl hydroperoxide, respectively. Kinetic analysis indicates that *Li*mTXNPx does not follow the classic ping-pong mechanism described for other TXNPx ($\Phi_{1,2} = 0.8\ s{\cdot}\mu M^2$). Although the molecular mechanism underlying this finding is unknown, we propose that cooperativity between the redox centers of subunits may explain the unusual kinetic behavior observed. This hypothesis is corroborated by high-resolution electron microscopy and gel chromatography that reveal the native enzyme to preferentially exist as a homodecameric ring structure composed of five dimers. © 2002 Elsevier Science Inc.

Keywords—Peroxiredoxin, Tryparedoxin peroxidase, Mitochondria, Specificity, Kinetics, *Leishmania infantum*, Free radicals

INTRODUCTION

The term peroxiredoxin has been coined for a family of homologous proteins that is spread over all living kingdoms [1]. The representative discovered first was the "thiol-specific antioxidant protein" of yeast [2] that was later recognized to be a thioredoxin-dependent peroxidase [3]. The common functional denominator of these proteins appears to be their ability to reduce hydroperoxides at the expenses of thiol substrates [4]. In Kinetoplastida, comprising parasites of the genera *Crithidia*, *Trypanosoma*, and *Leishmania*, the peroxiredoxins so far characterized proved to catalyze the reduction of hydroperoxides by thioredoxin-related proteins called tryparedoxins (TXN) [5–11]. These "tryparedoxin peroxidases" (TXNPx) are the major, if not the only, peroxide detoxifying enzymes in Kinetoplastida. The reduction equivalents are ultimately provided by nicotinamide adenine dinucleotide phosphate (NADPH) as substrate of trypanothione reductase (TR) [12]. Reduced trypanothione [N^1,N^8-(bis)-glutathionylspermidine] reduces TXN, the substrate of TXNPx [5]. The biological relevance of this unique cascade of oxidoreductases has been corroborated by a conditioned knockout of trypanothione reductase in *T. brucei* that lead to increased sensitivity to exogenous H_2O_2 in vitro and elimination of virulence in experimental animals [13].

In *C. fasciculata* [14], *T. cruzi* [15], and *T. brucei* [10] TXN and TXNPx were shown to localize to the cytosol

Address correspondence to: Leopold Flohé, Department of Biochemistry, Technical University of Braunschweig, Mascheroder Weg 1, D-38124 Braunschweig, Germany; Fax: +49 (531) 618-1458; E-Mail: lfl@gbf.de.

by immunohistochemistry. TR was reported to be predominantly localized in the cytosol of *T. brucei* [16], but was also found associated with the mitochondrion and the kinetoplast of *T. cruzi* [17]. The cytosolic localization of the complete system appears ideal to protect the parasite against oxidative attack from outside, as expected from the phagocytic attack by the host. The reduced virulence of TR-deficient *T. brucei* [13] and of *L. donovani* with a transdominant mutation in TR [18] may therefore be due in part to an impaired cytosolic peroxide metabolism. Kinetoplastida, like higher animals [19], however, produce endogenous H_2O_2, also as byproduct of the mitochondrial energy metabolism [20–22]. This endogenous oxidative stress would not be easily balanced by any kind of cytosolic peroxidase. Therefore, it was not surprising that in *T. cruzi* and *T. brucei*, in addition to cytosolic enzymes, a mitochondrial peroxiredoxin was found [10,23]. Similarly in *L. infantum*, a human pathogen prevailing in the Mediterranean countries, two peroxiredoxin genes were identified (Castro et al., [23a]). One of them translates into a cytosolic TXNPx (*Lic*TXNPx; Acc. Nr. AY058210) and is identical to a peroxiredoxin of *L. chagasi* [24] and almost indistinguishable from those of *L. donovani* [11] and *L. major* [8]. The other gene codes for an enzyme (*Lim*TXNPx; Acc. Nr. AY058209) that reminds of the mitochondrial peroxiredoxins of *T. cruzi* and *T. brucei* and was indeed shown to localize to this organelle (Castro et al., [23a]). These proteins differ from the typical cytosolic peroxiredoxins in the sequence surrounding their second redox active cysteine and in possessing an N-terminal mitochondrial leader sequence.

Here we report on the heterologous expression, isolation and biochemical characterization of *L. infantum* mitochondrial peroxiredoxin. The specific questions addressed are: (i) is the peroxiredoxin a TXNPx? (ii) How broad is the specificity range for hydroperoxides? (iii) Is the kinetic pattern compatible with that of other peroxiredoxins? (iv) How does the native enzyme compare with related peroxiredoxins in terms of quaternary structure?

MATERIAL AND METHODS

Heterologous expression and purification of L*imTXNPx*

The complete *LimTXNPx* coding sequence (Castro et al., [23a]) was amplified with PWO polymerase (Gibco-BRL, Paisley, Scotland), using the forward primer 5'-ccgcgcacat ATGCTCCGCCGTCTTCCCA$_{OH}$ and the reverse primer 5'-caccgctcgagTCACATGTTCTTCTCGAAAAAC$_{OH}$ (restriction sites and clamp sequences indicated in lower case; start and stop codons underlined). The PCR product was cloned into the *Nde*I and *Xho*I restriction sites of the prokaryotic expression vector pET28a (Novagen, Madison, WI, USA) so that a fusion protein of *Lim*TXNPx with an N-terminal tail of six histidines was produced in *E. coli* Tuner(DE3). The transformants were grown in 3 l of medium containing 10 g/l bactotryptone, 5 g/l yeast extract, 10 g/l NaCl and 50 μg/ml kanamycin. When the culture reached an O.D.$_{600}$ of 0.6–1.0, protein expression was induced with 0.1 mM isopropyl-β-D-thiogalactopyranoside (IPTG). After 3 h of induction at 30°C, the bacteria were pelleted, resuspended in 120 ml 500 mM NaCl, 20 mM Tris-HCl pH 7.6, disrupted by sonication and centrifuged at 10,000 × *g* for 30 min at 4°C. The supernatant was applied to a His Bind resin (Novagen) column (XK 26/20; Amersham Pharmacia Biotech, Uppsala, Sweden). *Lim*TXNPx was eluted with an imidazole gradient from 5 to 1000 mM at a flow rate of 2.5 ml/min. The protein content of the collected 7.5 ml fractions was monitored by absorption at 280 nm. Fractions confirmed to contain *Lim*TXNPx by sodium dodecyl sulfate (SDS) polyacrylamide electrophoresis (PAGE) were pooled, applied to PD-10 columns (Amersham Pharmacia Biotech) and eluted with 50 mM Na_2HPO_4, 50 mM NaH_2PO_4, pH 8.0. The identity of the purified product was verified by automatized N-terminal Edman-degradation up to 30 residues with a gas-phase sequencer (PE Applied Biosystems, Weiterstadt, Germany). Further, the isolated product and tryptic digests were subjected to matrix-assisted laser desorption and ionization time-of-flight (MALDI-TOF) mass spectrometry with a Bruker Reflex MALDI-TOF mass spectrometer.

Investigation of enzymatic activities and specificity

Routine determination of tryparedoxin peroxidase activity was performed in principle according to Nogoceke et al. [5] as described in detail by Flohé et al. [25]. In brief: 0.14 μM of *Lim*TXNPx were preincubated for 15 min at 25°C with 450 μM NADPH, 1.03 U/ml TR of *T. cruzi*, 130 μM trypanothione, and 15 μM TXN (C-terminally His-tagged TXN1 of *C. fasciculata*, *Cf*TXN1H6, or the homologous TXN2, *Cf*TXN2H6, prepared according to Guerrero et al. [9] and Montemartini et al. [7], respectively) in 500 μl 50 mM Tris, pH 7.6, containing 1 mM EDTA. The reaction was started by addition of 70 μM *tert*-butyl hydroperoxide (*t*-bOOH) and the NADPH consumption was monitored continuously at 340 nm. Reactivity with other hydroperoxides was investigated accordingly. H_2O_2, *t*-bOOH and cumene hydroperoxide were from Sigma (Steinheim, Germany). Phosphatidyl choline hydroperoxide and linoleic acid hydroperoxide, both prepared by soybean lipoxygenase-catalyzed oxidation of the corresponding

lipids, were kindly provided by R. Brigelius-Flohé (Potsdam, Germany). Hydroperoxide concentrations were determined enzymatically with glutathione peroxidase (Sigma; bovine GPx-1, 0.2 mg/ml) or a phospholipid hydroperoxide glutathione peroxidase (GPx-4) preparation of rat testis as described [26,27]. The unit of TXNPx activity was defined as $\Delta\mu$mol NADPH/min [25].

Determination of glutathione peroxidase activity was investigated in an analogous test system replacing TR by bakers yeast glutathione reductase (8.0 U/ml; Sigma) and trypanothione plus TXN by 3.3 mM glutathione. In the test for thioredoxin peroxidase activity TR was replaced by thioredoxin reductase of *E. coli* (Sigma), TXN by *E. coli* thioredoxin (Sigma), and trypanothione was omitted.

Kinetics

The kinetic pattern of *Lim*TXNPx was analyzed with *Cf*TXN2H6 and *t*-bOOH as substrates. The coupled test system described above was applied but with suitable variations of substrates. Initial velocities in dependency from the hydroperoxide concentration were calculated from the progression of substrate consumption curves between 95 and 3% of the initial substrate concentration. The concentration of *Lim*TXNPx was chosen high enough to allow the reaction to proceed to completion within less than 10 min. Under these conditions the spontaneous reaction could be disregarded. The data thus obtained was further analyzed according to Dalziel [28]. Enzyme molarities were calculated per subunit (theoretical MW 27,400.41 Da) and protein concentration determined according to the Bradford reagent kit supplied by BioRad (München, Germany) calibrated with bovine serum albumin (BSA) as standard.

Estimation of the molecular size of the native enzyme

In addition to the estimation of subunit size by SDS-PAGE and, more precisely, by MALDI-TOF mass spectrometry (see above), the molecular size of the native protein was investigated as follows:

1. Particle size distributions were determined using a Proteinsolutions DynaPro 801 (Charlottesville, VA, USA) equipped with a microsampler. Prior to measurements, probes having a protein concentration of 1.3 mg/ml were centrifuged for 10 min at 14,000 $\times$ *g* and filtrated two times using a Whatman Anotop 100.1 μm filter (Whatman Intl. Inc., Maidstone, England). Forty measurement points were collected and evaluated using Dynamics V5.26.38 (Proteinsolutions).
2. The shape of the oligomeric native protein was also investigated by electron microscopy. Purified recombinant *Lim*TXNPx at a final concentration of 50 μmol/ml was analyzed by energy-filtered transmission electron microscopy at 50,000$\times$ magnification, as described by Winkler et al. [29].
3. *Lim*TXNPx was chromatographed on a Superdex 200 column (Pharmacia, Uppsala, Sweden) in 10 mM Na-phosphate pH 7.6. High molecular weight standards (Pharmacia, Uppsala, Sweden) were used for column calibration. Eluted *Lim*TXNPx was identified by activity measurements and polyacrylamide gel electrophoresis under reducing and denaturing conditions.

RESULTS

L*imTXNPx is a tryparedoxin peroxidase*

L. infantum presents a 2-Cys mitochondrial peroxiredoxin, *Lim*TXNPx (Castro et al., [23a]), that together with the mitochondrial homologues of *T. cruzi* and *T. brucei* [10,23] forms a peroxiredoxin subfamily, distinct from the cytosolic enzymes from those organisms. Apart from the N-terminal mitochondrial leader sequence, in this subfamily the second redox-active Cys, i.e., the one that interacts with the reductant [4], is not embedded in a Val-Cys-Pro (VCP) motif, as is characteristic of most 2-Cys peroxiredoxins, but instead forms an Ile-Pro-Cys (IPC) motif. Despite this difference the *T. brucei* enzyme was shown to be a TXNPx [10] and in this report we investigated whether the same was true for the *L. infantum* enzyme.

The full length *Lim*TXNPx protein was expressed in *E. coli* [Tuner(DE3)] as an N-terminally His-tagged protein. It was found primarily in the soluble fraction and could be purified in one step by chelate chromatography as judged by SDS-PAGE (Fig. 1). N-terminal Edman degradation complied with the expected sequence up to position 22 preceded by the His-tag. MALDI-TOF analysis of the product after digestion by trypsin confirmed the identity with sequence coverage of about 80%. N-terminal sequencing also proved the two minor bands migrating faster than the main product (Fig. 1) to be degradation products of *Lim*TXNPx. MALDI-TOF spectrometry of the undigested product yielded a subunit size of 27,345 Da that complies reasonably with the theoretical value of 27,400 Da. As previously observed with other peroxiredoxins [5], the peaks of the MALDI-TOF spectra are unusually broad and, apart from the subunit size, also the mass peaks of oligomeric species are seen (not shown).

When the protein thus characterized was subjected to the routine test for TXNPx activity, it clearly proved to be active (Fig. 2). Trypanothione was not oxidized by *t*-bOOH alone: the reaction required the addition of both TXN and *Lim*TXNPx (traces A and B). None of the components led to an unspecific NADPH consumption if no hydroperoxide was present (trace C) proving peroxi-

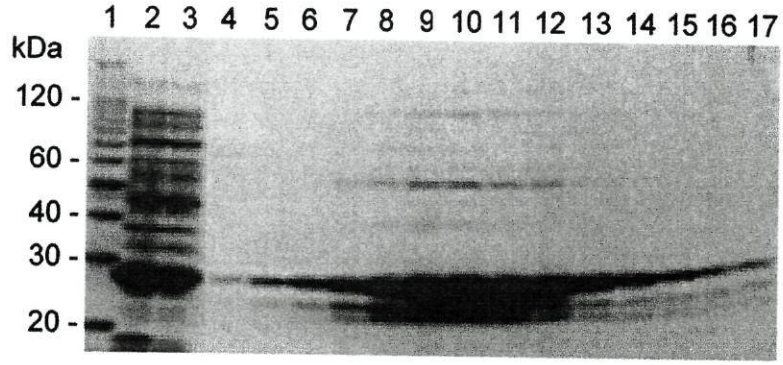

Fig. 1. Heterologous expression and purification of recombinant *Li*mTXNPx analyzed by SDS-PAGE. Lane 1: molecular weight markers; lane 2: total protein extract 3 h after induction with 0.1 mM ITPG; lane 3: soluble fraction of the transformants 3 h after induction, i.e., the supernatant of the total lysate after 30 min centrifugation at 10,000 × *g*; lanes 4–17: samples collected during Ni-chelate chromatography of the soluble fraction shown in lane 3; *Li*mTXNPx was eluted with an increasing concentration of imidazole, between 250 and 500 mM. The smaller proteins coeluting with *Li*mTXNPx are likely truncated forms thereof, as indicated by N-terminal sequencing. Also traces of dimeric and tetrameric forms are detected.

dase activity. When trypanothione, that was largely oxidized, was added last (trace D), an instant initial NADPH consumption was observed that reveals that the TR reaction was not limiting and fast enough to continuously monitor the formation of oxidized trypanothione by the peroxidase. Identical results are obtained if TR is added last, because oxidized trypanothione cannot be reduced and further accumulates in the preincubation period (trace E). In all cases the final slope indicating the TXNPx activity is the same within experimental error. The specific TXNPx activity thus determined was 5.9 ± 0.3 U/mg for *Cf*TNX1 and 42.5 ± 2.8 U/mg for *Cf*TXN2 (1 U = 1 μmol/min), i.e., the specific activity with *Cf*TXN1 is only 14% of that measured with *Cf*TXN2. Because Kinetoplastida also contain typical thioredoxins [30] analogous experiments were performed offering to the peroxiredoxin up to 5.9 μM thioredoxin of *E. coli* as reducing substrate [31]. *Li*mTXNPx proved to also catalyze the reaction between the thioredoxin and *tert*-butyl hydroperoxide, but the turnover rate amounted to 9.5% of that obtained with crithidial tryparedoxin 2 (Fig. 3). In the glutathione peroxidase assay *Li*mTXNPx proved to be practically inactive (not shown). In short, the *L. infantum* mitochondrial peroxiredoxin is indeed a tryparedoxin peroxidase with particular specificity for *Cf*TXN2.

Hydroperoxide specificity

The substrate *tert*-butyl hydroperoxide (*t*-bOOH) is conveniently used to characterize TXNPx because its spontaneous reaction with trypanothione and tryparedoxin is slow enough to be ignored in most experimental settings. It does not, however, react with all species of TXNPx as fast as, e.g., the predominant natural substrate H_2O_2 [8]. With *Li*mTXNPx, like with *Cf*TXNPx [5], almost identical activities are obtained for *t*-bOOH and H_2O_2 (Table 1). Cumene hydroperoxide, frequently chosen as model substrate for natural lipophilic hydroperoxides, is also reduced by *Li*mTXNPx at considerable rates. But naturally occurring lipid hydroperoxides such as 13-hydroperoxy octodecadienoic acid (LOOH) and soybean lipoxygenase-peroxidized phosphatidyl choline (PCOOH) are accepted with much lower rates only. This finding contrasts sharply with the specificity of *Cf*TXNPx, which appears totally promiscuous in respect to the oxidizing substrate [5]. The lipid hydroperoxides are also poor substrates of *Li*mTXNPx in a sense that they tend to inactivate the enzyme. As exemplified for PCOOH in Fig. 4 (traces B–D), the reaction rate slows down markedly long before the substrate has been consumed. This decline in substrate turnover could not be explained by coming close to any apparent K_m value. Later addition of the better substrate *t*-bOOH revealed that the enzyme has become inactivated (compare traces B and E after addition of *t*-bOOH). Linoleic acid hydroperoxide has the same deleterious effect on the enzyme (not shown). Comparison of traces B, C, and D reveals that the inactivation of *Li*mTXNPx by PCOOH depends on the enzyme concentration or on the time the enzyme is exposed to the peroxide. If the enzyme concentration is high enough to completely reduce the PCOOH (or LOOH) within a few seconds, its activity remains almost unaffected (see traces D and E). The specific activities for the lipid hydroperoxides as listed in Table 1 correspond to initial velocities measured at the highest enzyme concentrations that could technically be monitored.

Kinetics

In view of experimental difficulties in obtaining reliable initial velocities for the reduction of natural lipid hydroperoxides, the kinetic behavior of *Li*mTXNPx was only investigated with the most convenient substrate, *t*-bOOH. As an additional measure of precaution, the

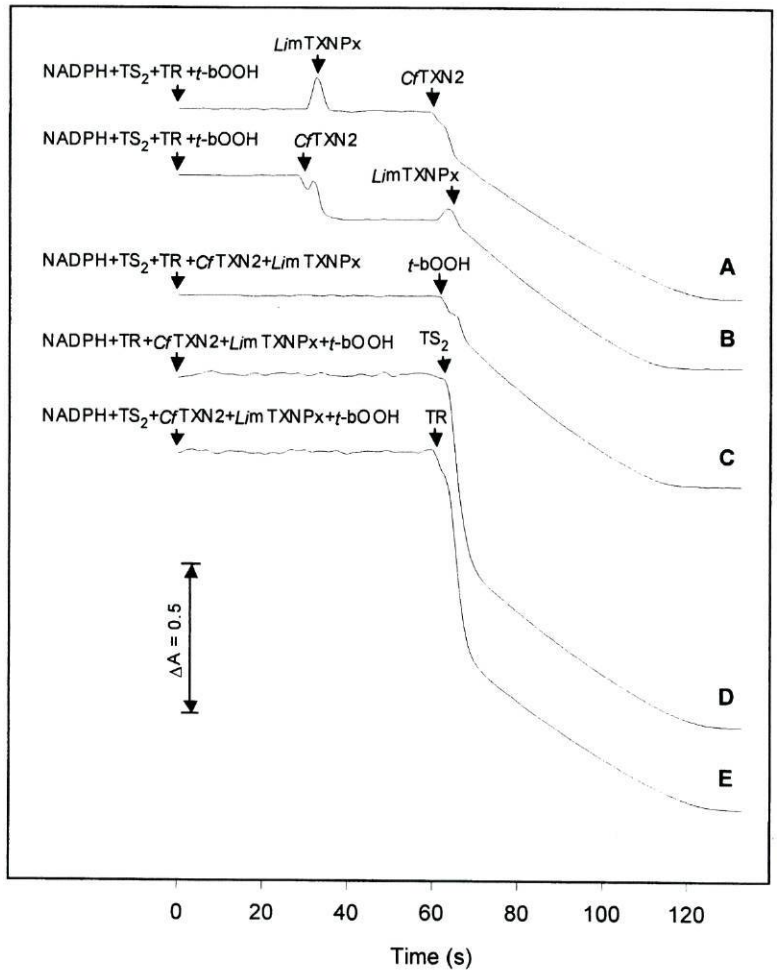

Fig. 2. Demonstration of tryparedoxin peroxidase (TXNPx) activity of *Lim*TXNPx. The components of the test system as described in Material and Methods were incubated with 0.14 μM *Lim*TXNPx at 25°C and NADPH (450 μM) consumption was recorded at 340 nm to monitor TXNPx activity. Traces A–E demonstrate that all components involved in trypanothione-dependent hydroperoxide reduction and the enzyme TR are required. Reduced trypanothione [$T(SH)_2$)] was formed in the incubation mixture from 130 μM of the oxidized form, TS_2 (not observed in traces A–C; fast NADPH consumption in traces D and E upon addition of TS_2). *Cf*TXN2, tryparedoxin 2 of *C. fasciculata* (15 μM) prepared as His-tagged protein from *E. coli*. TR = trypanothione reductase of *T. cruzi* (1.03 U/ml) also produced in *E. coli*; *t*-bOOH = *tert*-butyl hydroperoxide (70 μM).

enzyme concentration was chosen high enough to allow completion of the reaction within less than 10 min. Working at high enzyme concentration proved also to be mandatory, because *Lim*TXNPx, like other TXNPx variants [25], tends to lose activity within minutes upon dilution (not shown). In order to overcome instability problems at room temperature, the fastest possibility to generate a complete data set was chosen, i.e., a single curve progression analysis. As is demonstrated in Fig. 5A, initial velocities were read from the substrate consumption curves that correspond to 11 residual substrate concentrations giving equidistant abscissa points in the double reciprocal plot. Thereby, a balanced and unbiased statistical weight of the individual velocities over the substrate range between 95 and 3% of the starting concentration was to be guaranteed. A minimum of three data sets, as exemplified in Fig. 5A, were thus generated. The slopes of the regression lines and the ordinate intercepts of three to six independent experiments were averaged and used for the construction of secondary plots to obtain kinetic coefficients and constants (Figs. 5B and C).

According to the generally applicable algorithm proposed by Dalziel [28,32], the velocity of an enzymatic reaction involving two substrates can be described by the equation:

$$\frac{[E_0]}{v} = \Phi_0 + \frac{\Phi_1}{[A]} + \frac{\Phi_2}{[B]} + \frac{\Phi_{1,2}}{[A]\cdot[B]} \qquad (1)$$

wherein $[E_0]$ is the total enzyme concentration, v the initial velocity at pertinent substrate concentration, [A]

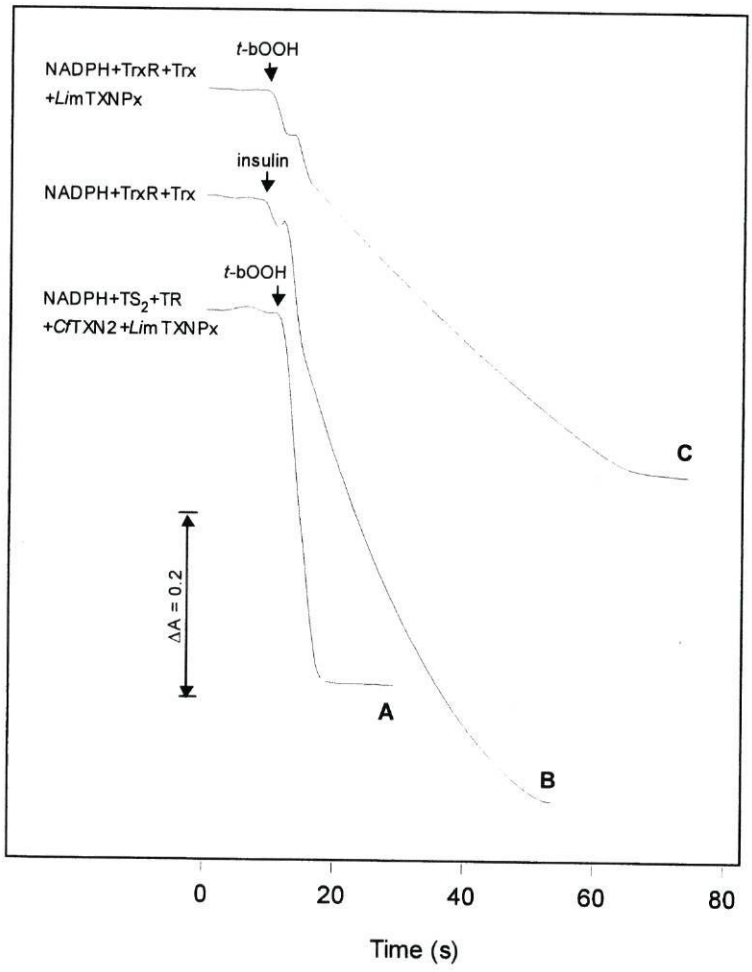

Fig. 3. Reaction of *Lim*TXNPx with thioredoxin. Trace A: tryparedoxin peroxidase activity of *Lim*TXNPx with *t*-bOOH. Trace B: insulin reduction by TrxR and Trx, as a control for the thioredoxin system [31]. Trace C: replacement of TR by TrxR and of *Cf*TXN2 plus TS_2 by Trx shows that *Lim*TXNPx works poorly with the thioredoxin system (compare traces A and C). The test system is analogous to that shown in Fig. 2. NADPH (450 μM, trace A; 300 μM traces B and C); *Cf*TXN2 (5.8 μM); *Lim*TXNPx (3.4 μM). TrxR = thioredoxin reductase of *E. coli* (8.4 U/ml); Trx = thioredoxin of *E. coli* (5.9 μM); *t*-bOOH = *tert*-butyl hydroperoxide (70 μM); insulin (218 μM).

and [B], and Φ_0, Φ_1, Φ_2, and $\Phi_{1,2}$ kinetic coefficients characterizing a particular enzyme. In analogy to the kinetic pattern observed with all peroxiredoxins so far analyzed [5,11,33–36], a ping-pong pattern, i.e., parallel lines in a primary plot like Fig. 5A, was expected for *Lim*TXNPx. The regression lines, however, consistently converged. This means that the coefficient $\Phi_{1,2}$ in Eqn. 1 is different from zero, which is highly unusual for a peroxidase [37]. Replotting of the ordinate intercepts, i.e., the reciprocal enzyme-normalized apparent maximum velocities for infinite concentrations of *t*-bOOH (= [A]) against the reciprocal concentrations of *Cf*TXN2 (= [B]) yields Φ_0 as ordinate intercepts, Φ_2 being the slope. Φ_0 is defined as the reciprocal value of the velocity at infinite concentration of both substrates, that means of k_{cat}. A defined k_{cat} is also unusual for peroxidases [37], but not uncommon for peroxiredoxins [11,34,35]. The coefficients Φ_1 and $\Phi_{1,2}$ are obtained by replotting the slopes of the primary plots against 1/[*Cf*TXN2] as ordinate intercept and slope, respectively (Fig. 5C).

Table 1. Specific activity of *Lim*TXNPx Toward Different Hydroperoxides

Substrate	Specific activity (U/mg)
H_2O_2	44.7 ± 7.1 (100%)
t-bOOH	42.5 ± 2.8 (95%)
COOH	25.4 ± 1.6 (57%)
LOOH	3.5 ± 0.6 (8%)
PCOOH	1.7 ± 0.1 (4%)

Means and standard deviations were calculated from six independent experiments, except for LOOH and PCOOH (n = 3 and 4, respectively). Specific activity is indicated as units (U) per mg of *Lim*TXNPx (1 U = 1 μmol/min). Specific activities are also given as % of the specific activity measured with H_2O_2. H_2O_2 = hydrogen hydroperoxide; *t*-bOOH = *t*-butyl hydroperoxide; COOH = cumene hydroperoxide; LOOH = linoleic acid hydroperoxide; PCOOH = phosphatidyl choline hydroperoxide.

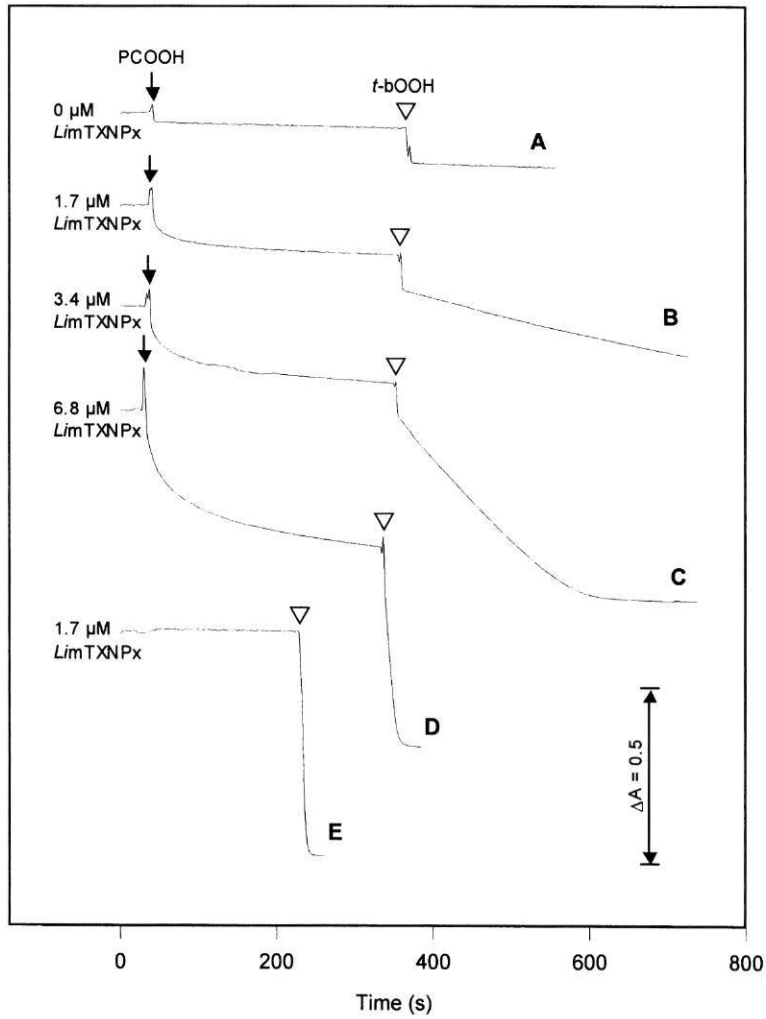

Fig. 4. Inactivation of *Lim*TXNPx by phosphatidyl choline hydroperoxide (PCOOH). Tryparedoxin peroxidase activity of *Lim*TXNPx was measured as described in Fig. 2 using 70 µM PCOOH (black arrows). To detect TXNPx activity after reaction with PCOOH, 70 µM *tert*-butyl hydroperoxide (*t*-bOOH) was added to the test tube (white arrowheads). Trace A: spontaneous reduction of PCOOH and *t*-bOOH. Traces B–D: TXNPx activity with increasing *Lim*TXNPx concentrations (1.7, 3.4, 6.8 µM). Trace E: reduction of *t*-bOOH by 1.7 µM *Lim*TXNPx without pretreatment with PCOOH.

The numeric values of the coefficients and derived constants are compiled in Table 2.

Size and shape of the native protein

Light scattering data of native *Lim*TXNPx indicated two distinct populations with a radius of 5.96 and 12.20 nm, respectively (data not shown). This observation supported the assumption that *Lim*TXNPx in its native state preferentially adopts a state of oligomerization identical to that of *Cf*TXNPx in crystals [38], but also tends to aggregate further. In the crystals the peroxiredoxin presented itself as a ring consisting of five densely packed dimers built up from inverted subunits. In order to check the validity of this interpretation, the preparation was investigated by high-resolution electron microscopy. The enzyme preparation was rather homogenous and the majority of the *Lim*TXNPx molecules were recognized as ring-like molecules, which in general appeared pentameric by the arrangement of centers of protein masses (Figs. 6A and B: dark arrowheads). Each one of the protein masses is assumed to represent a homodimer with a molecular mass of 2 × 27 kDa. Correspondingly, 5 × 2 × 27 kDa = 270 kDa are calculated for the decameric ring-like structure. The dumb-bell like structures sporadically present may be considered to be the same decamers seen from the edge. The interpretation of the pictures is supported by the recent electron microscopic investigation of the human peroxiredoxin [39] that revealed molecular shapes similar to those shown in Fig. 6. Also doublets and some triplets of rings could be detected (not shown). The latter finding is paralleled by data obtained from native gels. Here *Lim*TXNPx primar-

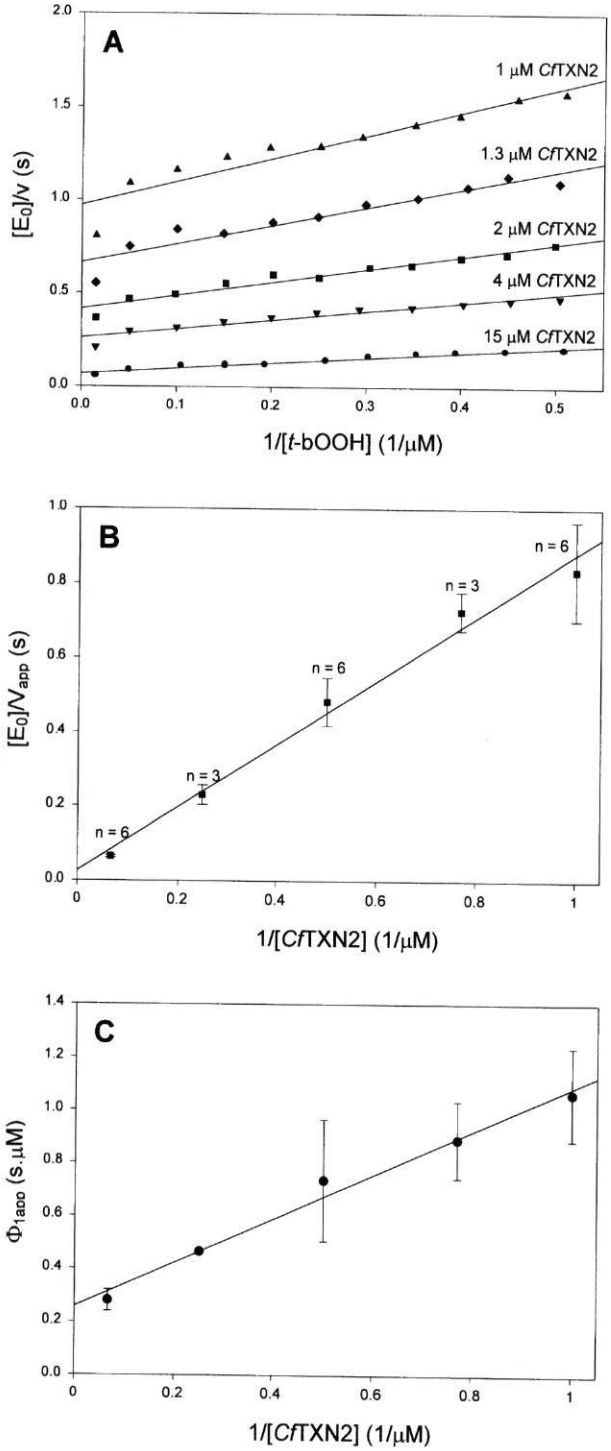

Fig. 5. Steady state kinetic analysis of *Lim*TXNPx according to Dalziel [28]. (A) Example of a double reciprocal primary plot showing dependency of enzyme-normalized initial velocities on *tert*-butyl hydroperoxide (*t*-bOOH) concentration at five concentrations of the cosubstrate CfTXN2 that were kept constant by regeneration. (B) Secondary plot of ordinate intercepts ($[E_0]/V_{app}$) of primary plots against reciprocal cosubstrate (C*f*TXN2) concentrations. Number of independent experiments are indicated for each C*f*TXN2 concentration. (C) Secondary plot of averaged slopes (Φ_{1app}) of primary plots vs. reciprocal cosubstrate (C*f*TXN2) concentrations. For evaluation of Dalziel coefficients and kinetic constants see text.

Table 2. Kinetic Constants for the *Lim*TXNPx Reaction with *Cf*TXN2 and *t*-bOOH, as Analyzed According to Dalziel [28]

ϕ_0 (s)	ϕ_1 (s · μM)	$\phi_{1.2}$ (s · μM^2)	Φ_2 (s · μM)	$K_{mCfTXN2}$ (μM)	$K_{mt\text{-}bOOH}$ (μM)
0.03	0.26	0.83	0.85	31.90	9.15

ily migrates as a diffuse band with a mol.wt. >450 kDa, which, however, tails into molecular weight regions below 270 kDa (not shown). Gel chromatography of *t*-bOOH-oxidized *Lim*TXNPx on Superdex 200 (Fig. 7) also indicates a dynamic equilibrium of aggregation/disaggregation. A major peak, with an apparent molecular weight of 319 kDa, is followed by a flat plateau and by a small peak corresponding to the monomeric state (Figs. 7A, C). All fractions collected were shown to represent *Lim*TXNPx by SDS gel electrophoresis (Fig. 7B) and activity measurements (Fig. 7A). The estimated size of the main fraction is close to the value calculated for the decamer. The front fraction likely represents higher aggregates, whereas the plateau indicates continuous depolymerization of the decamers during chroma-

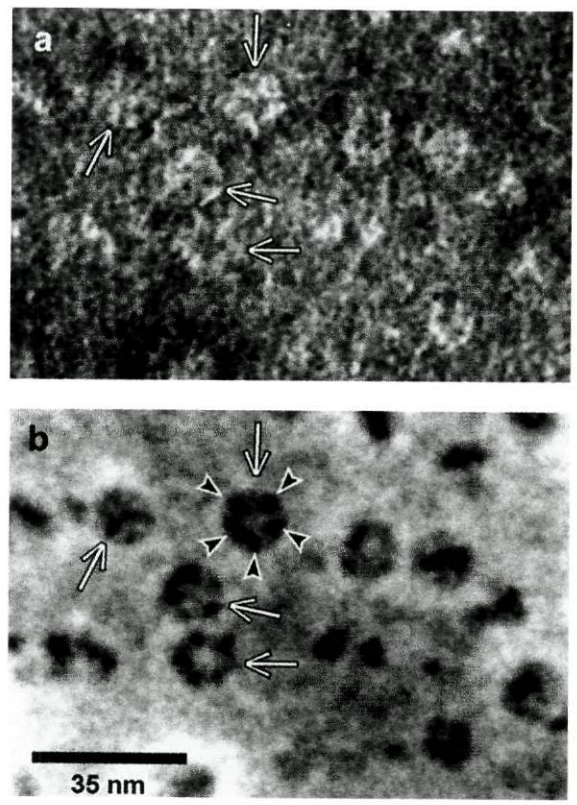

Fig. 6. Energy-filtered electron microscopy of *Lim*TXNPx. Detailed view of individual enzyme particles seen in the elastic bright-field mode (a) and in the corresponding inelastic "dark field" mode at the uranium $O_{4,5}$-edge at 115 eV (b). White arrows indicate identical molecules in (a) and (b). Dark arrowheads point to the homodimers (2 × 27 kDa) forming the decameric ring-like complex (5 × 2 × 27 kDa).

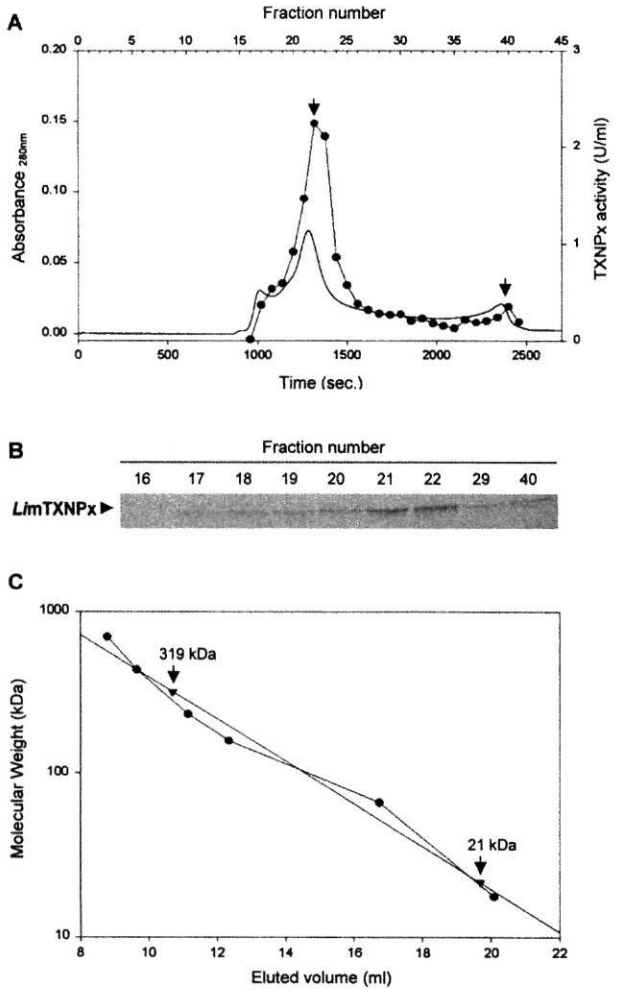

Fig. 7. Purified *Lim*TXNPx was oxidized with 140 μm *t*-bOOH and subjected to gel chromatography on Superdex 200. Eluting protein was monitored by absorption reading at 280 nm (A, solid line), checked for TXNPx activity (A, —●—) and for the presence of *Lim*TXNPx by SDS-PAGE (B). The molecular mass of the peaks in (A) was estimated by means of column calibration with tyreoglobulin, ferritin, catalase, aldolase, BSA, and myoglobin (C); the apparent weights of *Lim*TXNPx (—▲—) shown in the calibration curve correspond to the early and late peak of the elution pattern (A, arrows).

tography. The majority of the molecules, thus, appears to be present in the form of the decameric rings seen in the electron micrographs. Comparison of the ultraviolet absorption curve and activity measurements reveals that the specific activity is highest around the peak corresponding to the decamer and gradually declines with decreasing degree of polymerization.

DISCUSSION

Peroxiredoxins form a family of peroxidases that act on a variety of hydroperoxides using thiols as cosubstrates, which until now were reported to comprise glutathione, thioredoxin, tryparedoxin, or the CXXC motifs of bacterial AhpF [4]. In this report we refer to a mitochondrial peroxiredoxin of *L. infantum* (Castro et al., [23a]) that is suggested to form a novel group of TXNPxs together with the homologous molecules of *T. cruzi* and *T. brucei* [10,23]. These new peroxiredoxins share with previously characterized TXNPx the N-terminal conserved cysteine, a threonine, and an arginine that together form the redox center responsible for interaction with hydroperoxides [11,25,34,38]. They differ, however, in the second redox center that interacts with tryparedoxin. In these new TXNPx the common VCP motif is replaced by an IPC motif embedded in a sequence context also different from the cytosolic enzymes. Nevertheless, a basic center and an acidic residue suggested to be involved in tryparedoxin (TXN) specificity of cytosolic homologues [11] are also present in *Lim*TXNPx. These particularities likely contribute to the specificity of the mitochondrial tryparedoxin peroxidases.

With respect to the specificity for the hydroperoxide substrate, *Lim*TXNPx differs markedly from *Cf*TXNPx [5]. Its activity with lipid hydroperoxides is comparably weak and it is rapidly inactivated by these poor substrates. The restricted substrate specificities and sensitivities to lipid hydroperoxides cannot yet be explained, but appear not to be unique to mitochondrial TXNPx. The cytosol-type TXNPx of *L. major* [8] and *T. brucei* (Budde & Flohé, unpublished) displayed a similarly restricted specificity, and the TXNPx of *L. donovani* and *T. brucei* are equally fast inactivated by 13-hydroperoxy octodecadienoic acid [11]. In biological terms, the specificity of *Lim*TXNPx complies with its presumed role in mitochondrial H_2O_2 metabolism. Concerning the specific activity of *Lim*TXNPx towards the two crithidial tryparedoxins tested, we observed that the enzyme reacts preferentially with *Cf*TXN2. This result may indicate that in vivo the reductant for the mitochondrial peroxiredoxin is more similar to *Cf*TXN2 than to *Cf*TXN1.

The kinetic pattern obtained for *Lim*TXNPx speaks in favor of a central complex mechanism, whereby both oxidant and reductant should be bound to the active site of the enzyme before the reaction can proceed. This kinetic behavior of *Lim*TXNPx does not only conflict with kinetic data so far reported for other peroxiredoxins [5,4–36], it also appears incompatible with the reaction mechanism generally accepted for 2-Cys peroxiredoxins [3,11,36,40,41]: oxidation of the proximal cysteine by the hydroperoxide to yield a sulfenic acid, formation of a disulfide bond with the distal cysteine of an inverted second subunit, followed by an attack on the distal half-cysteine by the reducing substrate to yield a catalytic intermediate in which the reducing substrate is covalently bound to the distal cysteine via an S–S bridge, and finally regeneration of the reduced enzyme by thiol-disulfide exchange (Fig. 8). Clearly, the oxidation of

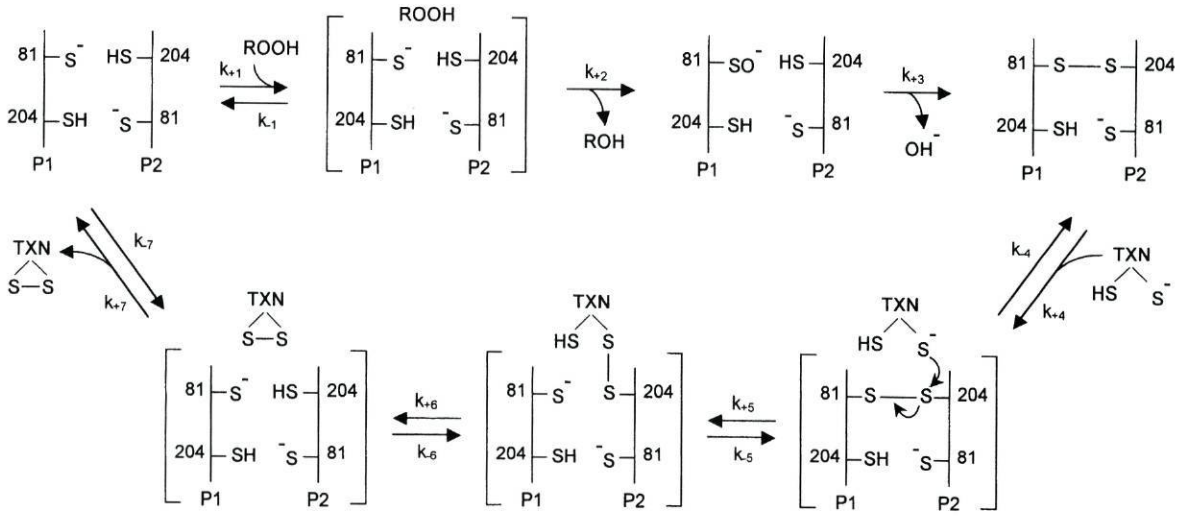

Fig. 8. Schematic representation of the classical ping-pong mechanism proposed for 2-Cys peroxiredoxins. This reaction mechanism does not conform to the kinetic pattern observed for *Li*mTXNPx. This enzyme exists as a pentameric structure of inverted dimers and we propose that cooperativity between the subunits may be responsible for the unusual kinetic behavior observed. TXN = tryparedoxin; ROOH = hydroperoxide; ROH = alcohol; P1 and P2 = first and second subunits of the dimeric protein (modified according to Hofmann et al. [4]).

these enzymes by the hydroperoxide is easily achieved without the aid of the cosubstrate and the reduction by the thiol cosubstrate does not depend on the presence of a hydroperoxide [5]. Such sequence of independent enzymatic steps meets the definition of an enzyme-substitution mechanism that should result in ping-pong kinetics, as it has been reported for peroxiredoxins [5,34–36] and consistently also for other thiol-dependent peroxidases [37]. In view of the peculiarity of the kinetic pattern observed with *Li*mTXNPx, it is difficult to interpret the Dalziel coefficients of Eqn. 1 in terms of rate constants. The approximate meaning of Φ_1 and Φ_2 is nevertheless straightforward: they are defined as the reciprocal values of the apparent rate constants k_1' ($k_1' = k_{+1} - k_{-1}$) and k_4' ($k_4' = k_{+4} - k_{-4}$) that describe the slowest hydroperoxide- and TXN-dependent step, respectively (Fig. 8). Φ_0 is the reciprocal value of $k_{cat} \cdot k_{cat}$, being substrate-independent, is likely the rate constant of one of the intramolecular reactions that follow the formation of the complex of the oxidized enzyme and the reduced TXN. $\Phi_{1,2}$ would formally be related to the formation of a ternary complex between the enzyme and both substrates that, however, is not assumed to be formed, as judged by the chemical processes involved and the emerging knowledge of the structure of these enzymes [11,38,42,43]. The meaning of $\Phi_{1,2}$ thus, cannot be deduced from conventional steady state treatment of enzymatic reactions because this relies on the assumption of simple mass low interactions of substrates with equivalent reaction centers and could lead to wrong mechanistic predictions for complex oligomeric enzymes such as the peroxiredoxins. *Cf*TXNPx [38], a mammalian thioredoxin peroxidase [39] and now the mitochondrial TXNPx of *L. infantum* were shown to be built up of ring structures composed of five inverted dimers each having two intersubunit reaction centers. This complex structure appears to essentially influence activity. The dimer of *Cf*TXNPx, being the minimum structure to be theoretically active, is devoid of activity [5]. Similarly, *Ld*TXNPx and *Li*mTXNPx lose activity upon dilution probably due to dissociation [11,25]. The importance of oligomerization on peroxiredoxin activity was also demonstrated for AhpC of *Mycobacterium tuberculosis* [44] and here we show, by gel chromatography, that the highest specific activity of *Li*mTXNPx is associated with the decameric form of the enzyme. These observations strongly suggest that not only the individual subunits but also the dimeric units interact with each other within the oligomeric complex to modulate the reactivity of the individual reaction centers. How this is being achieved at the molecular level remains elusive at present. Structures of peroxiredoxins in the reduced [38] and fully oxidized state [42], as well as redox-dependent spectral changes [44], reveal that the active sites must change appreciably during catalysis [11]. Also preliminary experiments that aimed at reacting the dead-end substrate *Cf*TXN2C44S [45] to *Li*mTXNPx showed the supramolecular aggregation state to be affected by substrate occupancy (unpublished data). It could, therefore, be envisaged that the redox state of a reaction center induces conformational changes that affect the dimer/dimer interface and ultimately facilitate or impair the substrate affinities or reactivities of remote subunits. Such model of cooperativity might provide a rational for an apparent central

complex mechanism, as suggested for *Lim*TXNPx by steady state kinetics, while, in chemical terms, the catalytic process in the individual reaction center still remains an enzyme substitution mechanism.

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ABBREVIATIONS

AhpC—alkyl hydorperoxide reductase subunit C
AhpF—alkyl hydroperoxide reductase subunit F
BSA—bovine serum albumin
COOH—cumene hydroperoxide
LOOH—linoleic acid hydroperoxide
PCOOH—phosphatidyl choline hydroperoxide
PCR—polymerase chain reaction
t-bOOH—*tert*-butyl hydroperoxide
TR—trypanothione reductase
TXN—tryparedoxin
TXNPx—tryparedoxin peroxidase

Chapter 6

Unpublished results.

Leishmania infantum mitochondrial peroxiredoxin is not essential for parasite survival

Helena Castro [a], Susana Romão [a], Carla Sousa [a], Madia Trujillo [b], Rafael Radi [b], Ana M. Tomás [a]

[a] Institute for Molecular and Cell Biology, Porto, Portugal.

[b] Facultad de Medicina, Universidad de la República, Montevideo, Uruguay.

Abstract

Leishmania infantum is a trypanosomatid with relevance as a human pathogen. Like in other aerobes, the *Leishmania* mitochondrion constitutes an important source of reactive oxygen intermediates (ROI) and possibly also of reactive nitrogen intermediates (RNI). Together these species can cause irreversible damage to mitochondrial components, but they can also regulate important cell signaling pathways. To keep the concentration of ROI and RNI at harmless levels, the mitochondrion is equipped with efficient antioxidant devices. In *L. infantum* the mitochondrial peroxiredoxin, *Li*mTXNPx, presumably participates in ROI and RNI removal from this organelle. This assumption is supported by the early finding that *Li*mTXNPx displays peroxidase activity, and by the evidence presented in this chapter that peroxynitrite is a substrate for the purified recombinant enzyme. Whichever role(s) *Li*mTXNPx plays in the cell, we demonstrate that this enzyme is not essential for parasite survival. Using a DNA recombination strategy, we have produced *L. infantum* mutants lacking *Li*mTXNPx. Within these transfectants *Li*mTXNPx depletion had impact neither on promastigote proliferation, susceptibility to exogenously added hydrogen peroxide and *tert*-butylhydroperoxide, nor on the parasite ability to complete its life cycle within a mammalian host. These observations suggest that *Li*mTXNPx functions may be compensated by alternative antioxidant devices and/or repair mechanisms.

Introduction

In all aerobic organisms mitochondria are the major source of reactive oxygen intermediates (ROI) (reviewed in Turrens, 2003) and also relevant sites for generation of reactive nitrogen intermediates (RNI) (reviewed in Radi *et al.*, 2002). Within this organelle, superoxide anion ($O_2^{\cdot-}$), derived from the univalent reduction of molecular oxygen by electrons leaking from the mitochondrial electron transport chain (Loschen *et al.*, 1971; Boveris *et al.*,

1972; Boveris and Chance, 1973; Loschen *et al.*, 1974; Cadenas *et al.*, 1977; Turrens, 1997), is the precursor for other cytotoxic species, namely hydrogen peroxide (H_2O_2), hydroxyl radical ($^{\cdot}OH$) and peroxynitrite ($ONOO^-$). Mitochondrial H_2O_2 results from $O_2^{\cdot-}$ dismutation either spontaneously or by the enzymatic activity of superoxide dismutases. The transition metal-catalyzed reaction involving H_2O_2 and $O_2^{\cdot-}$ generates $^{\cdot}OH$, one of the strongest oxidants in nature. Superoxide anion additionally reacts with nitric oxide ($^{\cdot}NO$) to yield $ONOO^-$. Nitric oxide present in mitochondria results both from the passive diffusion of cytosol-derived $^{\cdot}NO$ and from the activity of a mitochondrial nitric oxide synthase (NOS) (Giulivi *et al.*, 1998; Tatoyan and Giulivi, 1998; Ghafourifar and Cadenas, 2005). The high concentration of carbon dioxide (CO_2) in this organelle favors the fast decomposition of $ONOO^-$ into two highly reactive species, carbonate anion ($CO_3^{\cdot-}$) and nitrogen dioxide ($^{\cdot}NO_2$) radicals.

ROI and RNI are pro-oxidants which, within mitochondria, exert their toxic action by reacting with and inhibiting critical components, such as aconitase (Gardner, 2002), complex I (Brown and Borutaite, 2004) cytochrome *c* oxidase, and creatine kinase among others (reviewed in Radi *et al.*, 2002). However, H_2O_2 and $^{\cdot}NO$, two uncharged and diffusible species, also participate in the signaling of cell regulatory pathways which command differentiation, proliferation and death (reviewed in Cadenas, 2004). The mitochondrial pathway for apoptotic death, for instances, is controlled by redox reactions involving H_2O_2 (e.g. Clement and Pervaiz, 1999; Takeyama *et al.*, 2002; Le Bras *et al.*, 2005). Hydrogen peroxide and $^{\cdot}NO$ signal either for proliferation or apoptosis (two opposite biological actions) depending on their intracellular concentrations (Antunes and Cadenas, 2001; Cadenas, 2004). Control of regulatory pathways by these species is achieved through the modulation of critical regulatory kinases (Boyd and Cadenas, 2002). One additional role of $^{\cdot}NO$ in cell signaling is the regulation of cellular respiration, hence of intramitochondrial H_2O_2 release, by inhibiting complexes III and IV of the electron transport chain (reviewed in Brown, 1999). Within mitochondria $ONOO^-$ was also reported to promote apoptosis by inducing the opening of the permeability transition pore (Packer *et al.*, 1997) and the release of pro-apoptotic signals (such as calcium, Schweizer and Richter, 1996) to the cytosol.

To keep the concentration of ROI and RNI at harmless levels, mitochondria are equipped with efficient antioxidant machineries. As a first line of defense against $O_2^{\cdot-}$, these organelles possess two SODs, a Mn-SOD and a Cu,Zn-SOD, localized on both sides of the inner mitochondrial membrane (Okado-Matsumoto and Fridovich, 2001). Although removal of $O_2^{\cdot-}$ by SODs prevents $^{\cdot}OH$ and $ONOO^-$ generation, the activity of this class of enzymes yields another oxidant, H_2O_2. Within mitochondria hydroperoxide elimination is accomplished by selenium-containing glutathione peroxidases (GPxs) (Cadenas, 2004) and by peroxiredoxin (Prx) enzymes (Chang *et al.*, 2004). Regarding protection from mitochondrial RNI, this is afforded by $ONOO^-$ scavengers, namely cytochrome *c* oxidase, glutathione, ubiquinol and NADH (Quijano *et al.*, 1997; Pearce *et al.*, 1999; Schopfer *et al.*, 2000). GPxs and Prxs may

also participate in $ONOO^-$ reduction (Sies *et al.*, 1997; Bryk *et al.*, 2000; Trujillo *et al.*, 2004; Jaeger *et al.*, 2004; Dubuisson *et al.*, 2004).

Trypanosomatids are parasitic protozoa within the order Kinetoplastida, which comprise three human pathogens, the *Trypanosoma brucei* complex (African sleeping sickness), *Trypanosoma cruzi* (Chagas' disease), and *Leishmania* spp. (Leishmaniasis), together threatening 500 million people worldwide. Within these organisms mitochondria are important sources of ROI (Boveris and Stoppani, 1977; Turrens, 1987; Denicola-Seoane *et al.*, 1992; Santhamma and Bhaduri, 1995) and probable sites for RNI reactions as well. Trypanosomatids' mitochondria lack the highly efficient seleno-containing GPxs present in higher eukaryotes and, instead, their hydroperoxide eliminating function is likely ensured by Prxs (Wilkinson *et al.*, 2000; Tetaud *et al.*, 2001; Castro *et al.*, 2002b). Mitochondrial Prxs may also constitute important lines of defense against RNI, based on the observation that their cytosolic homologues display $ONOO^-$ reductase activity (Trujillo *et al.*, 2004). Trypanosomatidal Prxs exhibit the distinctive feature of being specifically reduced by tryparedoxin (TXN). This thioredoxin homologue has been recently found in the *Leishmania* mitochondrion (Castro *et al.*, 2004). At least *in vitro*, the mitochondrial TXN/Prx redox pathway is fuelled by reducing equivalents derived from NADPH, via the enzyme trypanothione reductase (TR) and the unique thiol trypanothione [N^1,N^8-bis(glutathionyl)spermidine] (Castro *et al.*, 2004). Recently, additional mitochondrial antioxidant devices have been found in other trypanosomatids, which likely cooperate with mitochondrial Prxs to eliminate ROI. These include two iron-SODs (Wilkinson *et al.*, 2006; Dufernez *et al.*, 2006) and a non-selenium glutathione peroxidase-like molecule (Schlecker *et al.*, 2005).

The trypanosomatidal enzymes of the Prx-dependent systems for hydroperoxide elimination are regarded as candidate targets for the development of new chemotherapeutic drugs, due to some of their distinctive features (Flohe *et al.*, 1999). In this chapter we report on the disruption of a mitochondrial Prx enzyme of *L. infantum* (*Li*mTXNPx; Castro *et al.*, 2002a; Castro *et al.*, 2002b), performed by homologous recombination to address its essentiality for parasite survival. We observed that *Li*mTXNPx is not vital, thereby invalidating this molecule as a drug target. Furthermore, *Li*mTXNPx abrogation produced no impact on parasite proliferation, resistance to exogenous hydroperoxides and ability to transform into viable amastigotes. It is possible that alternative antioxidant devices or efficient repair mechanisms take over the function(s) of the mitochondrial Prx in parasites not expressing this enzyme.

Material and Methods

Leishmania infantum cultures

Wild-type and transfected *Leishmania infantum* promastigostes (MHOM MA67ITMAP263) were cultured at 25ºC in RPMI 1640 medium (GibcoBRL) containing 10% inactivated fetal calf serum, 50 mM Hepes sodium salt (pH 7.4), 2 mM L-glutamine, 35 U ml^{-1} penicillin, 35 µg ml^{-1} streptomycin and, in the case of transfectants, the appropriate concentration of the selective drug. To obtain promastigotes in different phases of growth, parasites were cultured at 5×10^5 cells ml^{-1} during 5 consecutive days. *Leishmania* were then harvested at days 1 (early logarithmic, $2\text{-}5\times10^6$ cells ml^{-1}), 2 (late logarithmic, $6\text{-}8.5\times10^6$ cells ml^{-1}) and 6 (stationary, $1.5\text{-}2\times10^7$ cells ml^{-1}). Axenic amastigotes were grown at 37ºC in MAA medium supplemented with 20% foetal calf serum, 2mM glutamax (Gibco BRL), 0.023 mM hemin as described previously (Lemesre *et al.*, 1997).

DNA constructs

To produce the *NEO* disruption construct, two fragments of the *LimTXNPx* gene *locus* were PCR amplified from a cosmid clone (Castro *et al.*, 2002b) and cloned into the pTEX NEO vector (Kelly *et al.*, 1992) on both sides of the neomycin phosphotransferase gene (*NEO*). The oligonucleotide primers used to amplify the 5' and 3' flanking regions of the *LimTXNPx* gene were 5'-caccggatggCTTCGATCAAGTTAACCGCC-3' and 5'-caccgctcgagAGACGGCGGA-GCATCGTGT-3', and 5'-gcggggtaccATGTCTTTCACCTATACACATG-3' and 5'-acggggtac-cTGTTTGATCTGTCGACTGGG-3', which incorporate *Bam*HI, *Xho*I and *Kpn*I restriction sites (underlined). The *Bam*HI-*Xho*I and *Kpn*I digested PCR products were then cloned into the corresponding restriction sites of pTEX NEO. To assemble the HYG disruption construct, *LimTXNPx* 5' and 3' non-coding sequences were re-amplified by PCR using as template the genomic DNA of the NEO targeted mutants. The oligonucleotide primers used were 5'-cgcgGATCCGGGTGGCAGTATC-3' and 5'-cggaggatatcGCTTCTCA-AAGTCGGCGT-3', and 5'-gcggggtaccGTGTGCTGATCGAGGAAT-3' and 5'-gcggggtaccGAGCTCAAAAGCTC-GCAT-3', containing *Bam*HI, *Eco*RV, *Kpn*I and *Sac*I restriction sites (underlined). Following digestion with the appropriate restriction enzymes, the PCR products were cloned into *Bam*HI-*Eco*RV and *Kpn*I sites of pTEX HYG plasmid, a version of pTEX NEO where the *NEO* gene was replaced by the hygromycin phosphotransferase (*HYG*) open reading frame (ORF). Before transfection of *L. infantum* promastigotes, the NEO and HYG constructs were linearized by digestion with *Hinc*II and *Bam*HI-*Sac*I, respectively, and purified by electroelution.

Transfections procedures and isolation of LimTXNPx targeted mutants

Transfections were done by electroporation according to Beverley and Clayton (1993), at 0.45 kV, 300-400 µF. Parasites were allowed to recover in 10 ml of culture medium without

selective drugs for 24 hours. Drugs were then added to 2 ml of the liquid culture, at 7.5 and 10 μg ml^{-1} G418 (Sigma) and/or hygromycin (GibcoBRL), while the remaining 8 ml were pelleted and plated on agar plates containing the same concentration of the drug(s). Individual clones of the transfectants growing in the liquid culture were isolated by 24 serial two-fold dilutions ranging from 12.5 to 0.006 cells ml^{-1} in 96-well plates.

DNA manipulations

Total genomic DNA from *Leishmania* was prepared as described by Kelly (1993), digested with *Sac*I, resolved on 0.7% agarose gels and transferred to nylon membranes. Southern blot hybridisations were performed following standard procedures.

Western blotting

Preparation of *L. infantum* protein extracts, protein quantification and western blotting (WB) procedures were performed as described previously (Castro *et al.*, 2002b). Primary antibodies were polyclonal antibodies against purified recombinant *Li*mTXNPx (Castro *et al.*, 2002a) raised in rabbit (Eurogentec, Belgium), and against purified recombinant *Lic*TXNPx2 (Castro *et al.*, 2004) produced in rat by five successive subcutaneous injections in Freunds adjuvant. Secondary antibodies were peroxidase anti-rabbit $F(ab')_2$ fragment (Molecular Probes) and anti-rat immunoglobulin (Amersham).

Indirect immunofluorescence assay (IFAT)

Immunofluorescence assays were performed according to Castro *et al.* (2002b). Briefly, recombinant parasites were fixed with 4% paraformaldehyde (w/v) in 0.1 M Na_2HPO_4, 0.1 M NaH_2PO_4, 0.15 M NaCl pH 7.2 (PBS), permeabilized with 0.1% (v/v) Triton X-100, spotted onto polylysine-coated microscope slides and incubated with anti-*Li*mTXNPx and anti-*Li*TXN1 antibodies (Castro *et al.*, 2004). Secondary antibodies were Alexa Fluor 488 anti-rabbit IgG and Alexa Fluor 568 anti-rat IgG (Molecular Probes). Slides were mounted in VectaShield (Vector Laboratoires) and examined with an Axioskop Zeiss microscope.

Growth curve determination

Wild-type and transfected *L. infantum* promastigotes, previously synchronized by 4-5 daily passages of 5×10^5 cells ml^{-1}, were cultured in 24-well plates, at 1×10^6 cells ml^{-1} and allowed to grow for 6 days. Every 24 hours cell densities were determined spectrophotometrically at 600 nm.

Hydroperoxide sensitivity assays

L. infantum promastigotes in the late logarithmic phase of growth were seeded at 1×10^6 cells ml^{-1}, in 24-well plates containing a range of hydroperoxide concentrations (in duplicate).

The compounds tested were H_2O_2 (Sigma) or *tert*-butylhydroperoxide (*t*-bOOH, Sigma). The parasites were allowed to grow for 3 days and cell densities were measured in a spectrophotometer at 600 nm. Drug sensitivity was expressed as the hydroperoxide concentration that inhibited parasite growth by 50% (IC_{50}).

Amastigote viability

Adult male BALB/c mice were inoculated intraperitoneally with 10^8 *L. infantum* wild-type and *Li*mTXNPx knockout stationary phase promastigotes (2 mice per each parasite line). Eleven days after infection, mice were sacrificed and their spleen excised, weighed and homogenized in 10 ml of *Leishmania* growth medium. Cell pellets were collected by centrifugation at 1,200 rpm, for 10 min, at 4ºC, and diluted to 10 mg/ml in growth medium. The volume of the cell suspension corresponding to 1 mg of tissue was then titrated across a 96-well plate, in serial two-fold dilutions ranging from 1:1 to 1:128 (four titrations per parasite line). After one week growing at 25ºC, the last dilution containing promastigotes was recorded and the number of parasites per gram of spleen (parasite burden) was calculated as described by Buffet *et al.* (1995).

Kinetics of peroxynitrite decomposition

The reaction of peroxynitrite with reduced *Li*mTXNPx was performed according to Trujillo *et al.* (2004), following an initial rate approach. The recombinant *Li*mTXNPx enzyme used in the assays was obtained as described previously (Castro *et al.*, 2002a).

Results

1. *Li*mTXNPx is expressed along the *Leishmania* life cycle

Leishmania have a digenic life cycle that alternates between a flagellated extracellular insect stage (promastigote) and an aflagellar obligate intracellular mammalian stage (amastigote). *Li*mTXNPx expression in dividing non-infective (logarithmic) and non-dividing infective (stationary) promastigotes, and in axenic amastigotes was analyzed by WB. The results, shown in Figure 1, confirm that *Li*mTXNPx is expressed along the *L. infantum* development (Castro *et al.*, 2004). Although a slight decrease in *Li*mTXNPx expression was observed in non-dividing metacyclic promastigotes, additional studies must be conducted to conclude about the regulation of this protein throughout the parasite life cycle.

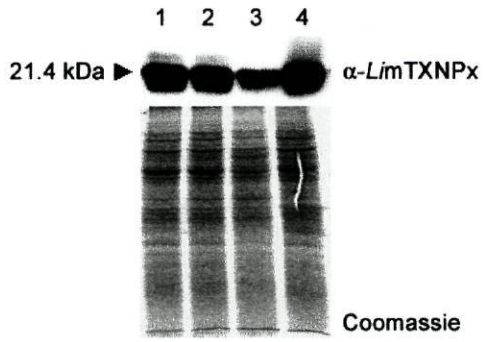

Figure 1. Expression of *Li*mTXNPx along the *L. infantum* life cycle. Western blot analysis of 20 μg of total protein extracts from early log (1), late log (2) and stationary phase (3) promastigotes, and from axenic amastigotes (4), incubated with the anti-*Li*mTXNPx antibody. An identical gel, run in parallel, was stained with Coomassie blue as a control for loading.

2. Peroxynitrite removal by recombinant *Li*mTXNPx

In order to test whether, as described for other Prxs (Bryk *et al.*, 2000; Trujillo *et al.*, 2004; Jaeger *et al.*, 2004; Dubuisson *et al.*, 2004), *Li*mTXNPx reduces $ONOO^-$, we performed stopped-flow experiments following peroxynitrite decomposition in the presence of the reduced purified recombinant enzyme. Using an initial rate approach, an apparent second order rate of 1.4×10^6 M^{-1} s^{-1} at pH 7.4 and 37°C was estimated for $ONOO^-$ reduction, which is within the range of highly reactive protein thiols (Trujillo *et al.*, 2004 and references therein). This value also fits with the second order rate constant for *t*-bOOH reduction by recombinant *Li*mTXNPx, 3.8×10^6 M^{-1} s^{-1}, calculated previously (Castro *et al.*, 2002a).

3. Depletion and disruption of the *LimTXNPx* ORFs

To determine whether the mitochondrial Prx is crucial for *Leishmania* survival, parasites unable to express *Li*mTXNPx were produced using a gene targeting strategy. For this purpose two integration cassettes, NEO and HYG, were constructed aimed at replacing one *LimTXNPx* allele and disrupting the other.

The first *LimTXNPx* allele was targeted with the NEO construct. Genomic DNAs isolated from colonies growing on G418 containing agar plates, were *Sac*I digested and analyzed by Southern blot (SB). Figure 2B depicts the SB analysis of one single-targeted colony, *LimTXNPx*+/-15. As shown, integration of the NEO cassette into the *LimTXNPx locus*, replaced one of the wild-type 2.5 kb *Sac*I fragments with a new 4.2 kb band, while leaving the other 2.5 kb fragment intact (Figure 2A, 2B). This observation confirmed that integration of the NEO cassette occurred as expected. Western blot analysis of *LimTXNPx*+/-15 revealed that *Li*mTXNPx expression is decreased in this clone in comparison to wild-type promastigotes (Figure 3A).

In order to replace the second *LimTXNPx* allele, *LimTXNPx*+/-15 was subjected to a second round of transfection using the HYG disruption construct. While a few parasites could be observed in the culture medium (but not in the mock transformed), they failed to thrive in G418 and hygromycin containing media and replication was only possible upon removal of the drugs from the liquid cultures. When analysed for *Li*mTXNPx expression by IFAT, these cell cultures revealed a mixed population of parasites either labelling or not labelling for the mitochondrial Prx (Figure 4). In order to isolate the transfectants depleted of *Li*mTXNPx, these cell cultures were serially diluted. The resulting individual clones were then screened for *Li*mTXNPx expression by IFAT and two clones not labeling for *Li*mTXNPx, clones 9 and 12, could be isolated.

The genomic organization of clones 9 and 12 was analyzed by SB (Figure 2C). If integration of the HYG cassette had occurred as planned, two *Sac*I fragments of 4.2 and 5.6 kb, corresponding to the targeted NEO and HYG alleles, respectively, should hybridize to the 5' and 3' flanking regions of *LimTXNPx* (Figure 2A). However, only one band was detected by SB analysis. This band also hybridized to the hygromycin phosphotransferase ORF, suggesting that the HYG construct had probably replaced both *LimTXNPx* and *NEO* alleles in the

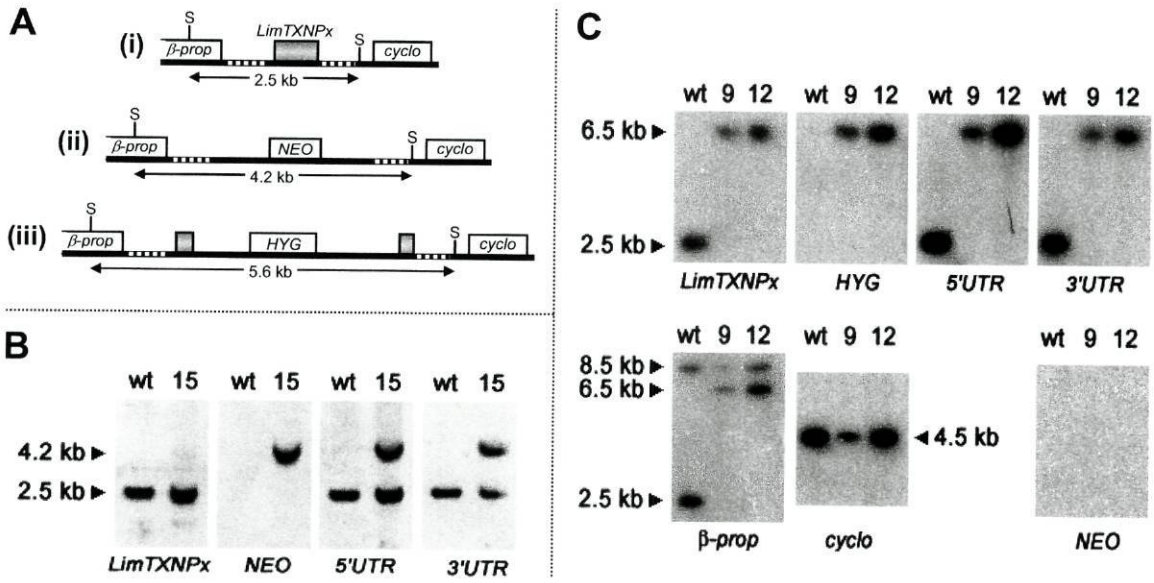

Figure 2. Depletion and disruption of *L. infantum LimTXNPx* alleles. (A) (i) Genomic organization of *L. infantum locus* containing the *LimTXNPx* (grey box), β-propeller (*β-prop*) and cyclophilin (*cyclo*) genes, and its *Sac*I restriction sites (S). Five prime and 3' flanking regions of *LimTXNPx* gene are indicated with a dashed line. (ii) Upon the first round of integration with the NEO construct, one of the wild-type *Sac*I fragments should increase from 2.5 kb to 4.2 kb. (iii) The second round of transfection with the HYG cassette should replace the remaining 2.5 kb *Sac*I fragment with a band of 5.6 kb. The 5' and 3' regions of the *LimTXNPx* coding sequence (grey boxes) are kept in the HYG-targeted allele. (B,C) Southern blot analysis of *Sac*I digested genomic DNA of wild-type parasites (wt), and of *LimTXNPx* single (clone 15) and double targeted mutants (clones 9 and 12), hybridized to *LimTXNPx*, neomycin phosphotransferase (*NEO*), hygromycin phosphotransferase (*HYG*), β-propeller (*β-prop*) and cyclophilin (*cyclo*) coding sequences, and to 5' and 3' flanking regions of the *LimTXNPx* gene (*5'UTR* and *3'UTR*, respectively).

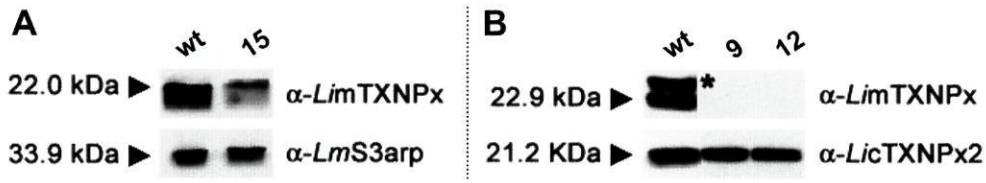

Figure 3. Western blot analysis of *L. infantum* transfectants. Twenty micrograms of total protein extracts from wild-type (wt), single (15) and double targeted mutants (9 and 12) were transferred onto nitrocellulose membranes and incubated with the anti-*Li*mTXNPx, and with the anti-*Lm*S3arp and the anti-*Lic*TXNPx2 antibodies to control for loading. The band indicated by * possibly corresponds to the oxidized form of *Li*mTXNPx.

LimTXNPx+/-15 transfectant. Consistent with this we observed that the neomycin phosphotransferase ORF was missing from clones 9 and 12. This finding explains why these transfectants were unable to grow in the presence of G418. One possible cause for this could be that parasites suffered some genomic rearrangement, the nature of which we do not know. One intriguing point is that the targeted *Sac*I fragments seem to be 6.5 kb and not 5.6 kb, as expected for the correct integration of the HYG construct. Finally, the integrity of *LimTXNPx* neighboring genes was checked by hybridizing the blots to β-propeller and cyclophilin ORFs, and found unaltered. Additional evidence supporting that *LimTXNPx*-/-9 and 12 mutants are deprived of *Li*mTXNPx, came from WB analysis of these transfectants (Figure 3B).

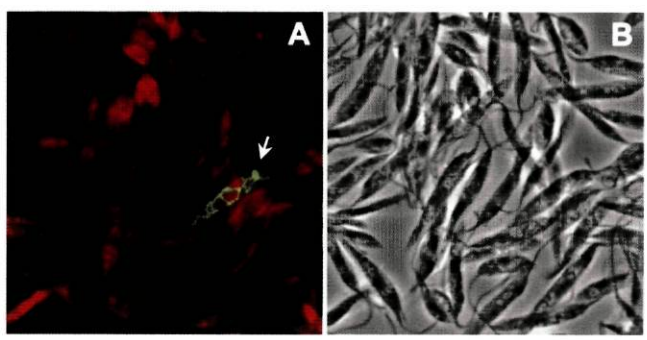

Figure 4. Identification of *L. infantum* promastigotes depleted of *Li*mTXNPx by IFAT analysis. (A) *L. infantum* transfectants were fixed, permeabilized and incubated with the anti-*Li*mTXNPx (green labeling) and anti-*Li*TXN1 antibodies (red labeling). This cell culture contains *LimTXNPx* null mutants, which do not stain for *Li*mTXNPx, and also few *LimTXNPx*+/-parasites, staining for *Li*mTXNPx (indicated with an arrow). Parasites were photographed at 1000× magnification. Phase contrast picture is also included (B). To isolate *L. infantum* knockouts for *LimTXNPx* this cell culture was cloned by limiting dilution.

4. Phenotypic characterization of *Li*mTXNPx null mutants

To look at the consequences of the loss of *Li*mTXNPx expression on *L. infantum* we performed a phenotypic analysis of the mutants, comparing them to the wild-type strain.

Cell proliferation

The effect of *Li*mTXNPx disruption on promastigote growth rate was evaluated by monitoring the cell culture density of one of the *Li*mTXNPx null mutants (clone 12) every 24 hours, for 6 days. As shown in Figure 5, disruption of the mitochondrial Prx produced no impact on the promastigote growth curve, indicating that this enzyme is not crucial for the control of cell proliferation or growth arrest. The growth curve of parasites carrying the pTEX NEO *LimTXNPx* episome, previously shown to overexpress *Li*mTXNPx (Castro *et al.*, 2002b), was also determined and again no deviations from the growth curve of wild-type cells were detected (Figure 5).

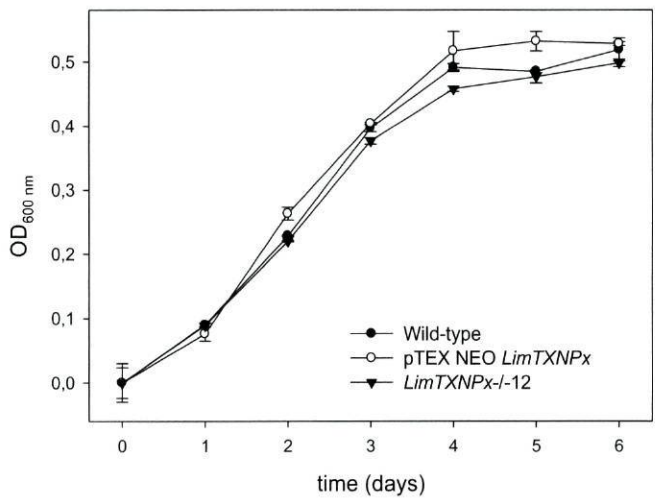

Figure 5. Effect of *Li*mTXNPx expression levels on *L. infantum* promastigote proliferation. Cell growth was monitored daily for 6 days, by spectrophotometric measurement of cell density at an optical density of 600 nm. Proliferation of *Li*mTXNPx overexpressing parasites (○) and of *LimTXNPx*-/-12 (▼) was compared to that of wild-type promastigotes (●). A representative experiment out of two assays is shown. For each clone, two cultures were set up and monitored simultaneously, and the resulting means and standard deviations are represented in this plot.

Promastigote susceptibility to exogenous hydroperoxides

Since *Li*mTXNPx is an active peroxidase (Castro *et al.*, 2002a; Castro *et al.*, 2002b), it was of interest to investigate whether *Li*mTXNPx disruption conferred parasites increased sensitivity to agents causing oxidative stress. To this end we tested promastigotes in the logarithmic phase of growth for their ability to survive the direct addition of H_2O_2 or *t*-bOOH. As shown in Table 1, the IC_{50} for both H_2O_2 and *t*-bOOH did not differ between *LimTXNPx*-/-12 and wild-type parasites. In contrast, *LimTXNPx*-/-9 displayed a slight increase in sensitivity to H_2O_2, but not to *t*-bOOH. However, since the observation regarding this clone refers to one isolated experience, this should be confirmed in the future.

Table 1. Effect of *Lim*TXNPx disruption on *L. infantum* promastigote susceptibility to exogenous hydroperoxides. The IC_{50} for each hydroperoxide was determined as described in Material and Methods. The data are the means from duplicates within the same experiment followed by the corresponding standard deviations. H_2O_2, hydrogen peroxide; *t*-bOOH, *t*-butyl hydroperoxide.

	IC_{50} (μM)	
Cell line	**H_2O_2**	***t*-bOOH**
Wild-type	141.0 ± 12.1	26.4 ± 0.2
LimTXNPx-/-9	115.3 ± 5.2	26.1 ± 0.2
LimTXNPx-/- 12	125.2 ± 5.6	24.2 ± 1.0

Amastigote viability

To assess the consequence of *Lim*TXNPx depletion on the ability of *L. infantum* to survive intracellularly, BALB/c mice were infected with *LimTXNPx*-/-9 and 12, and also with wild-type promastigotes. Eleven days later spleens were removed from mice, amastigotes were allowed to revert into promastigotes and parasite burden was determined for the different parasite strains. As shown in Figure 6, no differences were found between the wild-type strain and both *LimTXNPx* knockout clones. This experiment, however, was conducted with few animals and therefore it is not possible to compare infectivity between wild-type and mutant parasites. In the same way, the finding that *Lim*TXNPx does not seem relevant for amastigote survival within the first 11 days of infection does not preclude a role for this enzyme at later stages of infection. From this preliminary experiment we can safely conclude that (i) *L. infantum* amastigotes are able to survive without *Lim*TXNPx, and (ii) that the mitochondrial Prx is not necessary for the promastigote differentiation into amastigotes and vice versa.

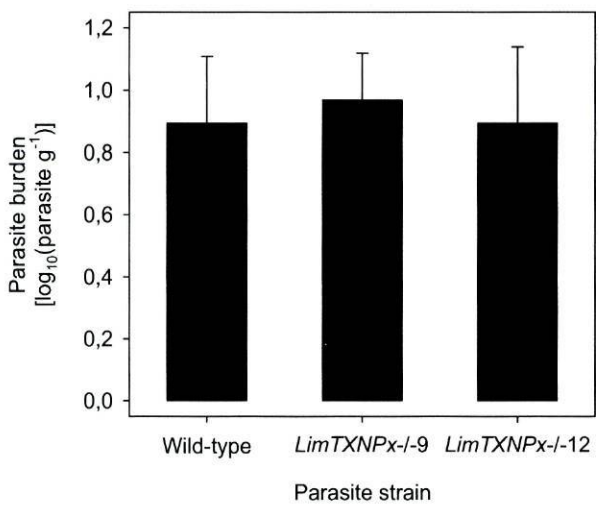

Figure 6. Effect of *Lim*TXNPx disruption on the *L. infantum* ability to infect BALB/c mice. Animals were inoculated with 10^8 promastigotes in the stationary phase of growth and parasite burden was determined eleven days post-infection. Data represent means ± standard deviation for two animals per each parasite strain.

Discussion

Peroxiredoxins constitute a ubiquitous family of enzymes with moderate peroxidase activity (Flohe *et al.*, 2003). Within the mitochondrion, the most obvious function for a Prx is likely to protect the organelle from aerobic metabolism-derived oxidants. In higher eukaryotes, however, mitochondrial Prxs are also implicated in regulation of peroxide-mediated signaling pathways, such as cell proliferation and programmed cell death (PCD) (Nonn *et al.*, 2003b; Chang *et al.*, 2004). In trypanosomatid's mitochondria, lacking the more efficient selenium-containing GPx enzymes of higher eukaryores, Prx functions remain rather elusive. Trypanosomatidal Prxs are part of unique redox pathways (Hofmann *et al.*, 2002), and for this reason they are amenable for selective inhibition with chemotherapeutic drugs. Here we report on the disruption of the mitochondrial Prx from the trypanosomatid parasite *L. infantum*, as a strategy to infer the importance of this enzyme for cell survival and also to understand its physiological role within the parasite.

In this work *Li*mTXNPx knockout mutants were generated by homologous recombination of both gene alleles of *L. infantum*. Although targeting of the second *LimTXNPx* ORF was followed by unexpected recombination events, these did not affect the *LimTXNPx* neighbouring genes and the resulting mutant parasites were deprived of *Li*mTXNPx, as unequivocally demonstrated by IFAT (Figure 4), WB (Figure 3) and also by PCR (data not shown). The *Li*mTXNPx disruption mutants were viable and indistinguishable from the wild-type strain. Indeed, enzyme depletion produced no obvious morphological changes in promastigotes, and it did not influence promastigote growth and completion of the parasite life cycle in an *in vivo* infection model. Together, these observations indicate that *Li*mTXNPx is not essential for *L. infantum*. This finding is in line with the observation by Wilkinson *et al.* (2003) that down regulation of the mitochondrial Prx is not detrimental for survival of the bloodstream form of *T. brucei*.

In *Leishmania* the mitochondrial Prx was previously shown to guard promastigotes from the direct addition of *t*-bOOH (Castro *et al.*, 2002b; Lin *et al.*, 2005). These observations prompted us to investigate the consequences of *Li*mTXNPx disruption on parasite survival under conditions of oxidative stress. To increase hydroperoxide levels in *Li*mTXNPx knockouts, parasites were treated with a bolus of H_2O_2 and of *t*-bOOH. *Li*mTXNPx depletion, however, did not influence promastigote sensitivity to either of these hydroperoxides in comparison to the wild-type strain. In the case of H_2O_2 interpretation of the results is straightforward. Previous data have shown that overexpression of the mitochondrial Prx does not protect *L. infantum* and *Leishmania amazonensis* promastigotes from the direct addition of H_2O_2 (Castro *et al.*, 2002b; Lin *et al.*, 2005), suggesting that H_2O_2 is possibly detoxified by cytosolic peroxidases and/or commits cells to death pathways before reaching the mitochondrion. Accordingly, abrogation of *Li*mTXNPx was not expected to influence parasite susceptibility to H_2O_2 of exogenous origin,

as we indeed observed (Table 1). In contrast to H_2O_2, the cytotoxic effects of *t*-bOOH can be circumvented by upregulation of the leishmanial mitochondrial Prx (Castro *et al.*, 2002b; Lin *et al.*, 2005). Therefore, our finding that *Lim*TXNPx depletion does not affect promastigote sensitivity to *t*-bOOH suggests that compensatory mechanisms might have been activated in the *Lim*TXNPx null mutants. Expression of alternative proteins in response to Prx depletion has been previously reported in *Saccharomyces cerevisiae* (Wong *et al.*, 2002) and *Xanthomonas campestris* (Charoenlap *et al.*, 2005). In the case of *LimTXNPx* knockouts such compensatory molecules, yet to be identified, may include other peroxidases, namely a putative mitochondrial non-selenium glutathione peroxidase-like enzyme (http://www.genedb.org) homologous to the one described in *T. brucei* (Schlecker *et al.*, 2005), and/or molecules involved in the repair of oxidative damage, such as heat shock proteins (Searle *et al.*, 1993; Miller *et al.*, 2000). The identification and characterization of such alternative antioxidants should be further investigated as these may be of interest for drug chemotherapy.

One important question still awaiting elucidation concerns the ability of *Lim*TXNPx knockouts to deal with oxidative stress generated inside the mitochondrion. Production of $O_2^{\cdot-}$, the precursor of other ROI, can be induced intramitochondrially by treating parasites with specific inhibitors of the electron transport chain, such as antimycin A (Mehta and Shaha, 2004). In these conditions, the impact of *Lim*TXNPx expression levels on the parasite ability to remove mitochondrial hydroperoxides can be evaluated.

Mitochondria are major sites for generation of H_2O_2, a metabolite increasingly recognized as a messenger for cell proliferation and death (reviewed in Cadenas, 2004). Mitochondrial Prxs, given their strategic subcellular compartmentalization and their modest peroxidatic activity, are considered to be critical regulators of the H_2O_2 intracellular concentration and are thus regarded as important mediators of the cell physiological state. As an example of such regulatory phenomena, overexpression of the mitochondrial Prx (Prx-3) in mouse thymoma cells causes growth retardation (Nonn *et al.*, 2003a), whereas down regulation of the same enzyme sensitizes HeLa cells to programmed cell death (PCD) (Chang *et al.*, 2004). Whether, as occurs in higher eukaryotes, trypanosomatidal mitochondrial Prxs are implicated in regulation of cell proliferation and cell death has never been addressed. Although the involvement of *Lim*TXNPx in signaling of cellular states requires a detailed investigation, from our preliminary observations it appears that this enzyme is dispensable for *L. infantum* growth control. In fact, neither *Lim*TXNPx overexpression nor its abrogation produced any effect on *L. infantum* promastigote growth rate (Figure 5). The hypothesis that an alternative peroxidase could replace the regulatory function of *Lim*TXNPx in *LimTXNPx* knockouts seems improbable. Indeed, regulation of cell signaling pathways by Prxs is usually attributed to two features unique to this family of enzymes, which allow the fine control of H_2O_2 steady state concentrations: (i) the moderate peroxidatic activity of Prxs (Hofmann *et al.*, 2002), and (ii) the Prx sensitivity to inactivation by H_2O_2 (Wood *et al.*, 2003).

This study also provides evidence that recombinant *Li*mTXNPx displays peroxynitrite reductase activity *in vitro*. Although this activity has been described for other Prxs (Bryk *et al.*, 2000; Trujillo *et al.*, 2004; Jaeger *et al.*, 2004; Dubuisson *et al.*, 2004), it may not be a general feature of this family of enzymes (Comtois *et al.*, 2003). The reduced *Li*mTXNPx enzyme removes $ONOO^-$ at a high rate, comparable to that of cytosolic trypanosomatidal Prxs (Trujillo *et al.*, 2004). The fast reduction of $ONOO^-$ by *Li*mTXNPx suggests that the enzyme possibly interferes with $ONOO^-$-mediated damage. It may, therefore, be that the *Li*mTXNPx peroxynitrite reductase activity reflects a defined physiological role for this enzyme, namely protection of the mitochondrion from the cytotoxic effects of $ONOO^-$, a function previously attributed to the mammalian mitochondrial enzyme Prx-3 in rat neuronal cells (Hattori *et al.*, 2003). In *Leishmania* $ONOO^-$ may be generated intramitochondrially by the reaction between $O_2^{\cdot-}$ and cytosol imported-$^{\cdot}NO$ [which can be derived either from the host immune response (Nathan and Hibbs, Jr., 1991)) or from the parasite own NOS activity (Genestra *et al.*, 2003)]. Alternatively, since $ONOO^-$ and its protonated form, ONOOH, can cross biomembranes (Denicola *et al.*, 1998; Romero *et al.*, 1999; Alvarez *et al.*, 2004) these species can be formed outside the mitochondrion and then diffuse to its interior (by yet unknown mechanisms). The possibility that *Li*mTXNPx might be implicated in $ONOO^-$ removal within the parasite could be addressed by determining the impact of *Li*mTXNPx expression levels on promastigote susceptibility to donors of $^{\cdot}NO$ and $O_2^{\cdot-}$, such as SIN-1 (Feelisch *et al.*, 1989).

In further support of the idea that *Li*mTXNPx is not essential for *L. infantum*, we have observed that enzyme depletion had no impact on the parasite ability to establish an early infection in BALB/c mice. This finding, indicating that the *Li*mTXNPx peroxidatic activity is dispensable for the parasite to evade the oxidative burst mounted by the host, is consistent with the observation that *Li*mTXNPx disruption has no impact on promastigote susceptibility to exogenously added H_2O_2 and *t*-bOOH. Besides ROI, other insults are produced by the host in response to infection, namely the generation of RNI. However, the infection experiment as we performed it does not allow us to infer about a potential role of *Li*mTXNPx in parasite protection against host-derived RNI. In fact, at least in the case of *L. amazonensis* infection, BALB/c mice do not generate significant amounts of RNI within the first 11 days of infection, the time point at which we determined parasite burden (Giorgio *et al.*, 1998; Linares *et al.*, 2001). To further examine the effect of *Li*mTXNPx disruption on *L. infantum* ability to resist the host immune response, the course of the parasitic infection should be monitored for a longer period. Alternatively, the use of another mouse strain, with a different kinetics of RNI production (e.g. C57Bl/6; Giorgio *et al.*, 1998; Linares *et al.*, 2001) should be considered.

Trypanosomatidal Prxs display unique features that distinguish them from the mammalian homologues, and for that reason they could be potential targets for chemotherapeutic drugs (Flohe *et al.*, 1999). The results presented in this chapter, however, by demonstrating that

L. infantum mitochondrial Prx is not crucial for promastigote survival and ability to invade a mammalian host, invalidate this molecule as a drug target.

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Chapter 7

General discussion

This thesis addresses the problem of hydroperoxide elimination in the trypanosomatid parasite *Leishmania infantum*. *Leishmania* are sensitive to hydroperoxide challenge, but they are capable of surviving the oxidative attack mounted by the host macrophages, and to succeed as intracellular parasites. Furthermore, *Leishmania* have the ability to cope with oxidative species produced endogenously, mainly as a consequence of their own aerobic metabolism. Starting an analysis of the pathways for hydroperoxide removal within these parasites was, therefore, one of the aims of this work.

One additional interest in this issue came from the fact that the *Leishmania* hydroperoxide metabolism is regarded as a potential target for antiparasitic drugs (Flohe *et al.*, 1999). In *Leishmania* and other trypanosomatids peroxidases are distinctively fuelled by the trypanothione reductase (TR)/trypanothione system, which replaces the glutathione reductase/glutathione system present in other organisms. The findings that TR and trypanothione are crucial for trypanosomatid survival and infectivity (Dumas *et al.*, 1997; Tovar *et al.*, 1998a; Tovar *et al.*, 1998b; Krieger *et al.*, 2000; Comini *et al.*, 2004; Ariyanayagam *et al.*, 2005) suggest that parasitic infections might be controlled with specific inhibitors of trypanothione-dependent enzymes. TR, however, is not a suitable drug target, as its activity has to be reduced by 95% before any deleterious effects are observed (Krieger *et al.*, 2000).

One trypanothione-dependent pathway for hydroperoxide elimination utilizes 2-cysteine peroxiredoxins. Peroxiredoxins (Prxs) constitute a family of peroxidases with moderate activity, that use redox active cysteines (instead of prosthetic groups) to reduce hydroperoxides and also peroxynitrite (reviewed in Rhee *et al.*, 2005). In trypanosomatids, lacking heme- and selenium-containing peroxidases, 2-Cys Prxs are regarded as physiologically relevant antioxidant devices. Trypanosomatidal Prxs, despite their high sequence homology with the mammalian homologues, display the unique feature of being reduced by trypanothione via an intermediate enzyme, tryparedoxin. Tryparedoxins (TXNs) belong to the large family of thioredoxin-like thiol-disulfide oxidoreductases, which include thioredoxins, glutaredoxins, protein disulfide isomerases and the bacterial protein DsbA. Tryparedoxins possess a distinctive signature in their active site, WCPPC, are specifically reduced by trypanothione, and, when compared with mammalian thioredoxins, display unusual structural details (Alphey *et al.*, 1999). These features may render Prxs and TXNs amenable for selective inhibition with antiparasitic drugs.

In this context, the research carried out during the course of this thesis has focused on the characterization of two TXN/Prx systems, one cytosolic and the other mitochondrial, of *L. infantum*, the prevalent *Leishmania* species in Portugal.

1. The *Leishmania infantum* cytosolic TXN/Prx system

During their life cycle *Leishmania* are exposed to hydroperoxide insult of exogenous origin. This challenge is particularly relevant during phagocytosis of *Leishmania* by the host macrophages, whereby a burst of oxidative species is triggered (Gantt *et al.*, 2001). In addition to producing ROI, macrophages also generate RNI in response to *Leishmania* invasion (Augusto *et al.*, 1996; Giorgio *et al.*, 1998; Linares *et al.*, 2001) and probably during the course of infection. Although the role played by ROI and RNI in *Leishmania* infection control is not fully elucidated, it is clear that, at least *in vitro*, these species are toxic to the parasite (Murray, 1981a; Murray, 1981b; Vouldoukis *et al.*, 1995; Lemesre *et al.*, 1997; Linares *et al.*, 2001; Gantt *et al.*, 2001). The presence of fully operative antioxidant apparatus able to shield the parasite from host-derived ROI and RNI should thus be pivotal for *Leishmania* survival and establishment of a successful infection.

The cytosolic TXN/Prx pathway, given its localization, is possibly a crucial element for *Leishmania* evasion from oxidants produced by the host immune system. In *L. infantum* we have isolated three enzymes which are part of the cytosolic TXN/Prx system. These include one TXN molecule, *Li*TXN1 (Castro *et al.*, 2004), and two Prx enzymes, *Lic*TXNPx1 and *Lic*TXNPx2 (Castro *et al.*, 2002b; Castro *et al.*, 2004).

*Lic*TXNPx1 and *Lic*TXNPx2 are nearly identical enzymes, differing only at their carboxyl termini. In *L. chagasi*, the New World counterpart of *L. infantum*, Barr and Gedamu (2001) observed that the mRNA corresponding to the *Lic*TXNPx1 gene is upregulated in dividing promastigotes, whereas *Lic*TXNPx2 mRNA is preferentially expressed in metacyclic promastigotes and amastigotes. The authors proposed this differential expression to reflect distinct physiological functions and, indeed, they reported that each enzyme displayed specific substrate preferences (Barr and Gedamu, 2003). In conflict with these results, we observed that both *L. infantum* cytosolic Prxs indiscriminately use H_2O_2 and *t*-bOOH as substrates (unpublished results). Apart from their peroxidatic activity, both *Lic*TXNPx1 and *Lic*XNPx2 also reduce $ONOO^-$ in a trypanothione/TXN-dependent fashion (Susana Romão, Madia Trujillo, Rafael Radi, Ana M. Tomás, to be published). Consistent with the *in vitro* activities, we showed that, when overexpressed, *Lic*TXNPx1 guarded *Leishmania* promastigotes from the direct addition of H_2O_2 and *t*-bOOH (Castro *et al.*, 2002b). These results were recently corroborated by another group working with *L. amazonensis*, who additionally implicated the cytosolic Prx in promastigote resistance to nitroprusside (Lin *et al.*, 2005), a NO donor in cellular systems. Evidence that cytosolic Prxs may play a protective role during invasion of the mammalian host came from the observation that in *L. chagasi* overexpression of the *Lic*TXNPx2 homologue enhanced parasite intracellular survival within macrophages (Barr and Gedamu, 2003).

Together, these observations strongly support a role for cytosolic Prxs in *Leishmania* shielding from ROI and RNI generated by the host. Furthermore, the presumed abundance of

Prxs in trypanosomatids (about 5% of the total soluble protein content in *C. fasciculata*; Nogoceke *et al.*, 1997) makes these enzymes relevant targets for hydroperoxide and $ONOO^-$ reactivity. It must be stressed, however, that *Leishmania* possess or may induce alternative mechanisms, whose roles in parasite evasion from the host immune response may complement or overlap with those of Prxs. These include heat shock proteins (Miller *et al.*, 2000), ovothiols (Spies and Steenkamp, 1994), and non-selenium glutathione peroxidase-like (GPx-like) enzymes (http://www.genedb.org; Schlecker *et al.*, 2005). In particular, the major cell surface glycoconjugate of *Leishmania*, lipophosphoglycan (LPG), has been implicated in parasite resistance to the hostile environment of the phagolysosome through a myriad of functions which include scavenging of ROI (Spath *et al.*, 2003) and modulation of the macrophage immune response (Proudfoot *et al.*, 1996; Piedrafita *et al.*, 1999). Also, *Leishmania* amastigotes may circumvent the host respiratory burst by inhibiting phox assembly (Pham *et al.*, 2005). The contribution of cytosolic Prxs for parasite survival, antioxidant defense and pathogenecity thus awaits confirmation by gene targeting of *LicTXNPx1* and *LicTXNPx2* mediated by homologous recombination (the RNA interference tool is not available for *Leishmania* and the antisense technology is not always effective). Unfortunately, disruption of *Lic*TXNPx1 and *Lic*TXNPx2 may not be easily achieved because the corresponding ORFs are organized in one genetic cluster containing repetitions of each Prx gene interspaced by an additional gene (http://www.genedb.org). Using RNA interference technology Wilkinson *et al.* (2003) demonstrated that, for the bloodstream form of *T. brucei*, down-regulation of the cytosolic Prx impaired parasite growth and hypersensitized cells to H_2O_2.

Within the cytosol of *L. infantum* the hydroperoxide and $ONOO^-$ reductase activities of *Lic*TXNPx1 and *Lic*TXNPx2 are likely to be catalysed by the TXN enzyme *Li*TXN1 (Castro *et al.*, 2004; Susana Romão, Madia Trujillo, Rafael Radi, Ana M. Tomás, to be published). *Li*TXN1 upregulation in metacyclic infective promastigotes indicates that this enzyme might be involved in parasite protection from host-derived oxidants. *Li*TXN1 antioxidant function may also result from its putative ability to reduce GPx-like enzymes, as described for its homologues in *T. brucei* (Hillebrand *et al.*, 2003) and *T. cruzi* (Wilkinson *et al.*, 2002). Whether *Li*TXN1 catalyses the synthesis of deoxyribonucleotides, by reducing the enzyme ribonucleotide reductase, an activity previously attributed to the *T. brucei* enzyme (Dormeyer *et al.*, 2001), remains to be shown. It is nevertheless unlikely that this would be the main function of *Li*TXN1 based on the observation that in dividing promastigotes expression of the enzyme is downregulated.

In order to infer the function and essentiality of *Li*TXN1, our laboratory is currently producing *Li*TXN1 knockouts by homologous recombination. The first *LiTXN1* allele has been successfully disrupted, but attempts to replace the second allele are resulting in abnormal DNA rearrangements, such as formation of amplicons, without loss of the *LiTXN1* gene (Susana Romão, Ana M. Tomás, unpublished results). Although these results await confirmation, they

suggest that *Li*TXN1 may be essential for *Leishmania* survival. At least for the bloodstream form of *T. brucei*, the homologous enzyme, *Tb*TXN, was shown to be important for parasite growth (Wilkinson *et al.*, 2003) and survival (Marcelo Comini, personal communication).

In summary, *L. infantum* protection from host-derived ROI and RNI is probably afforded by the cytosolic Prxs *Lic*TXNPx1 and *Lic*TXNPx2. Furthermore, these enzymes may also play an important part in detoxification of hydroperoxides of endogenous origin (e.g. produced in the parasite endoplasmic reticulum or glycosomes). Both Prxs are likely to be reduced by the trypanothione redox cycle using *Li*TXN1 as intermediate. Whether *Lic*TXNPx1 and *Lic*TXNPx2 are pivotal for parasite survival and infectivity, or whether their function can be replaced by alternative antioxidant devices, awaits elucidation by gene manipulation based experiments. Preliminary data indicate that *Li*TXN1 is crucial for *L. infantum* survival, probably reflecting an essential role for at least one of its putative physiological oxidants, *Lic*TXNPx1 and *Lic*TXNPx2, GPx-like enzymes and/or ribonucleotide reductase.

2. The *Leishmania infantum* mitochondrial TXN/Prx system

The *Leishmania* mitochondrion is an important site for the generation of ROI and possibly of RNI as well. In this organelle a TXN/Prx system similar to that found operating in the cytosol appears to function in the elimination of hydroperoxides and $ONOO^-$, as suggested by the presence of a TXN enzyme, *Li*TXN2 (Castro *et al.*, 2004), and one Prx molecule, *Lim*TXNPx (Castro *et al.*, 2002b), within this organelle.

*Lim*TXNPx is a functional peroxidase, as demonstrated by hydroperoxide resistance assays using promastigotes expressing increased levels of the enzyme (Castro *et al.*, 2002b). Besides reducing hydroperoxides, purified recombinant *Lim*TXNPx also accepts $ONOO^-$ as substrate (Chapter 6). Despite its apparent function as an antioxidant, *Lim*TXNPx depletion was found to have no impact on *L. infantum* survival (Chapter 6). One possible reason for this could be that alternative antioxidant mechanisms operate in the parasite mitochondrion which might compensate for the missing mitochondrial Prx. Candidate substitutes for *Lim*TXNPx could include the mitochondrial heat shock protein HSP70 (Searle *et al.*, 1993), and/or putative mitochondrial GPx-like enzymes (http://www.genedb.org). The presence of one GPx-like molecule in the mitochondrion of *T. brucei* was recently reported by Schlecker *et al.* (2005).

In *L. infantum* the mitochondrial TXN, *Li*TXN2, may be linked to hydroperoxide and peroxynitrite removal via reduction of *Lim*TXNPx, an activity documented to occur *in vitro* (Castro *et al.*, 2004; Chapter 6), and possibly through interaction with putative mitochondrial GPx-like molecules. However, other biological functions may be suggested for *Li*TXN2, namely kinetoplast DNA (kDNA) replication. Kinetoplast DNA, the unique trypanosomatid

form of mitochondrial DNA, consists of a network of double stranded DNA mini and maxicircles organized into a compact disk structure. Minicircle replication is dependent upon the redox state of the universal minicircle sequence binding protein (UMSBP) and the *C. fasciculata* TXNs, *Cf*TXNI and *Cf*TXNII, have recently been shown to catalyse UMSBP reduction *in vitro*, thereby initiating kDNA replication (Onn *et al.*, 2004). Since *Li*TXN2 is the only mitochondrial TXN described to date, it may be that this protein is necessary for replication of kDNA. *Li*TXN2 can thus be envisioned as the reductant for various mitochondrial enzymes, each playing complementary or distinct functions. Identification and characterization of the *Li*TXN2 molecular partners is important to elucidate the functions of this protein.

While in the cytosol of trypanosomatids the TXN/Prx system is catalytically reduced by the TR/trypanothione redox cycle at expenses of NADPH, it remains an enigma how this system is fuelled in the mitochondrion as no TR activity could be detected in this organelle (Smith *et al.*, 1991; Wilkinson *et al.*, 2002; Schlecker *et al.*, 2005; Chapter 4). Although we cannot exclude the hypothesis that reduced trypanothione may be transported across the inner mitochondrial membrane and operate inside the mitochondrion, it seems more likely that an alternative molecular species provides reducing equivalents to the mitochondrial TXN/Prx couple. Based on previous reports on *Plasmodium falciparum* (Muller, 2004) and *Mycobacterium tuberculosis* (Bryk *et al.*, 2002; Jaeger *et al.*, 2004), we have proposed such reductant to be lipoamide (Chapter 4).

3. Shapes and kinetics of peroxiredoxins

The kinetic pattern followed by most peroxidases, Prxs included, conforms to a ping-pong mechanism (Hofmann *et al.*, 2002), whereby oxidation of the enzyme by the hydroperoxide substrate occurs independently of the reaction with the reductant. Accordingly, in a steady state kinetic analysis the kinetic pattern of a Prx should comply with the Dalziel equation for a two-substrate reaction $[E_0]/v = \Phi_0 + \Phi_1/[A] + \Phi_2/[B] + \Phi_{1,2}/[A][B]$ (Dalziel, 1957), wherein the term $\Phi_{1,2}$, describing the formation of a central complex, should equal zero. When investigating the kinetics of *Li*mTXNPx, however, deviations from the expected pattern were observed (Castro *et al.*, 2002a). The $\Phi_{1,2}$ value obtained for *Li*mTXNPx kinetics deviated from zero, suggesting the formation of a ternary complex between the enzyme and both substrates. Also, in the double reciprocal primary Dalziel plot, the enzyme showed non-linear slopes, reflecting lower than expected rates at high hydroperoxide concentrations and low TXN levels. Although this kinetic pattern was rather atypical for a Prx, it was later found to apply also to the cytosolic Prxs of *L. infantum*, *Lic*TXNPx1 (Budde, 2003), and of *T. brucei*, *Tb*TXNPx (Budde *et al.*, 2003). Furthermore, when Budde *et al.* (2003) reanalysed the original kinetics for other Prxs they

detected the same deviations from the generally accepted ping-pong mechanism, indicating that this kinetic behavior might be a general characteristic for this family of enzymes.

Based on the observations that 2-Cys Prxs tend to form decameric structures (Alphey *et al.*, 2000; Castro *et al.*, 2002a), we have proposed a cooperativity model to explain the *Li*mTXNPx kinetics. According to this model, the redox state of a reaction centre would induce conformational changes in its vicinity, with consequences on the reactivity of the remote subunits. This hypothesis was subsequently confirmed by Budde *et al.* (2003) for the *T. brucei* enzyme *Tb*TXNPx. These authors have additionally proposed such cooperativity to be negative, meaning that oxidation of one reaction centre would negatively affect the other subunits. This hypothesis could provide a rational for the unusual trypanosomatidal Prxs kinetics, while, in chemical terms, the catalytical process in the individual reaction centre would remain a simple ping-pong mechanism.

One feature of Prxs that may influence enzyme kinetics is the redox-sensitive oligomerization. According to the generally accepted mechanism for typical 2-Cys Prxs, the proximal Cys of an enzyme subunit attacks the hydroperoxide and is oxidized to a sulfenic acid (Cys-SOH). This is subsequently attacked by the distal Cys of an inverted subunit to form a stable intersubunit disulfide bond, which is then reduced by a disulfide oxidoreductase. The reduced form of the enzyme is stabilized in the decameric state, whereas the disulfide-bonded forms exist predominantly as dimers (reviewed in Wood *et al.*, 2003b). For the disulfide bond to be formed, the Prx structure has to suffer significant conformational changes, known as "local unfolding". If local unfolding is favorable, as occurs for most prokaryotic Prxs, then the catalytic cycle proceeds normally and the enzymes are said to be "hydroperoxide robust". On the contrary, if the local unfolding is unfavorable or occurs too slowly, and if the hydroperoxide concentration is high enough, then the disulfide bond is not formed and the sulfenic acid Cys may be overoxidized to a sulfinic acid (Cys-SO_2H), which, again, favors the decameric state. These Prxs, usually found in eukaryotes, are known as "hydroperoxide sensitive Prxs" (detailed in Wood *et al.*, 2002 and in Wood *et al.*, 2003a). For some time it was thought that the sulfinic acid intermediate was a dead-end product. However, the observations that overoxidized Prxs can be regenerated (Woo *et al.*, 2003), either by sulphiredoxins (Biteau *et al.*, 2003; Chang *et al.*, 2004) or by sestrins (Budanov *et al.*, 2004) have changed this concept.

Although it has never been addressed whether trypanosomatidal Prxs are robust or susceptible to overoxidation, their amino acid sequences possess the Gly-Gly-Leu-Gly and the Tyr-Phe motifs typical of hydroperoxide sensitive Prxs (Wood *et al.*, 2003a). Furthermore, the unusual kinetics observed for the parasitic Prxs (low reaction rates at high hydroperoxide concentrations), may be a consequence of these enzymes sensitivity to hydroperoxide inactivation.

The finding that the peroxidatic activity of Prxs is impaired under strong oxidative stress, challenges the knowledge that these molecules function as general antioxidants. For the Prxs of

higher eukaryotes some hypotheses have been proposed to explain these rather contradictory concepts. Rabiloud *et al.* (2002), for example, suggested that the ratio of active to inactive Prx may play a role in mammalian cell sensitivity to apoptosis induced by TNF-α. Wood *et al.* (2003a), incorporating the knowledge that Prxs are involved in regulation of redox signaling pathways, suggested that Prx inactivation by H_2O_2 would be part of a biochemical mechanism to limit H_2O_2 elimination, thereby allowing this molecule to act as a messenger. More recently, Jang *et al.* (2004) demonstrated that upon oxidative challenge by H_2O_2 the yeast Prxs cPrxI and cPrxII aggregated into high molecular weight complexes, subsequently switching from a peroxidase activity to a chaperone function.

In short, the kinetic behavior of *Li*mTXNPx, originally rated as atypical, was, in the meantime, found to apply for other Prxs and also possibly to comply with the concept that eukaryotic Prxs are sensitive to hydroperoxide inactivation. Still, the physiological implications of such behavior are yet to be fully understood.

4. The enzymes of the TXN/Prx systems: suitable drug targets?

Apart from their importance as antioxidant devices, TXNs and Prxs are regarded as candidate targets for the development of antiparasitic drugs. Their validation as drug targets depends on three main requisites, which include divergence from homologous host molecules, essentiality for parasite survival and/or infectivity, and availability of structural and mechanistic data. The Prx and TXN usefulness for drug design is discussed next.

In trypanosomatids, lacking more efficient cofactor-containing peroxidases, Prxs have been regarded as relevant antioxidant devices. However, the finding that other peroxidases, such as GPx-like and ascorbate peroxidase enzymes, also operate in these cells has raised some doubts as to what extent Prxs are essential. At least in the case of *L. infantum* and *T. brucei*, mitochondrial Prx functions are dispensable for parasite survival (Wilkinson *et al.*, 2003; Chapter 6). In contrast, the cytosolic Prx of *T. brucei* was found essential for viability of the bloodstream form of the parasite and for protection against exogenously added H_2O_2 (Wilkinson *et al.*, 2003). However, Prxs present some limitations as drug targets: (i) they are probably highly abundant in trypanosomatids (Nogoceke *et al.*, 1997), making it difficult to maintain the required high drug concentration of a specific inhibitor, even if irreversible, within the cell; (ii) their three-dimensional structure is highly conserved among different organisms, whereby specific inhibition of the parasite enzymes may not be possible. Accordingly, the Prx peroxidatic function may more easily be prevented by inhibiting their electron suppliers, the TXNs.

TXNs are considerably distant from their mammalian homologues, the thioredoxins, and this may allow their specific inhibition with drugs. The TXN molecular weight exceeds that of thioredoxins by 50%, they possess a unique signature in their active site (WCPPC), they share low homology with typical thioredoxins, they exhibit distinctive structural features, and they uniquely accept trypanothione as their reductant. Furthermore, TXNs interact *in vitro* with a variety of cellular components other than Prxs, namely GPx-like molecules (Wilkinson *et al.*, 2002; Hillebrand *et al.*, 2003), ribonucleotide reductase (Dormeyer *et al.*, 2001) and UMSBP (Onn *et al.*, 2004), making it possible that their inhibition leads to parasite death by affecting different physiological functions. In *T. brucei* the cytosolic TXN was shown essential in the bloodstream form (Marcelo Comini, personal communication) and that may also hold true for *L. infantum* (Susana Romão, Ana M. Tomás, unpublished results). The relevance of the mitochondrial TXN for parasite survival has never been addressed. However, since no other oxidoreductase was ever described in the mitochondria of these parasites it is possible that such enzyme is critical for trypanosomatid viability.

Finally, and in what refers to the third requisite referred to above, the biochemical and kinetic data obtained in this work for the enzymes *Li*TXN1, *Li*TXN2 and *Li*mTXNPx has contributed to a better definition of their mode of action, and is useful for the development of reliable test routines for inhibitors that may be developed in the path for the rational design of drugs against trypanosomatids.

5. Final remarks

This thesis describes the identification and characterization of two distinct TXN/Prx systems of *L. infantum* with distinct subcellular compartmentalizations, one cytosolic and the other mitochondrial. By employing biochemical and genetic techniques, we have inferred the possible physiological functions of such systems, which are likely the elimination of hydroperoxides and of peroxynitrite generated by the host immune response and/or by the parasite own metabolism. Kinetic analysis was performed on some of the enzymatic components of the Prx-based systems, which elucidated the mechanism of action of such molecules. Accordingly, *Li*TXN1 and *Li*TXN2 were shown to react via a typical ping-pong mechanism, whereas *Li*mTXNPx was found to display an unusual kinetic behavior, possibly a consequence of its oligomeric nature. Finally, the possibility of using some of the enzymes of the TXN/Prx systems as targets for drugs has also been addressed. In this regard, and employing a gene disruption strategy, we have found the mitochondrial Prx, *Li*mTXNPx, not to be valid as a drug target.

The work documented in this thesis thus contributed to the knowledge on the metabolic routes for hydroperoxide elimination in *Leishmania*. New perspectives regarding these enzymatic pathways were opened, which can be explored in future investigations.

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Resumo

Nesta tese são investigados alguns aspectos do metabolismo de hidroperóxidos do protozoário *Leishmania infantum*. *Leishmania* são parasitas intracelulares obrigatórios do homem e do cão que, quando a residir no interior dos fagolisossomas dos macrófagos de mamíferos, estão expostos a espécies oxidantes (entre as quais hidroperóxidos) produzidas pelo sistema imune do hospedeiro. A forma como as *Leishmania* conseguem evadir este ataque oxidativo e estabelecer uma infecção não está totalmente elucidado. Para fazer face ao ambiente hostil do fagolisossoma, as *Leishmania* desenvolveram vários mecanismos de defesa, tais como o revestimento da sua superfície com glicofosfolípidos, a indução da expressão de proteínas de choque térmico ("heat shock proteins") e a modulação da resposta imune do hospedeiro. Enzimas com actividade de remoção de hidroperóxidos fazem ainda parte deste aparato de defesa dos parasitas. As *Leishmania* não possuem catalases nem glutationa-peroxidases dependentes de selénio, enzimas que nos eucariotas superiores são responsáveis pela eliminação de hidroperóxidos. Em vez disso, o principal mecanismo enzimático de redução de hidroperóxidos nestes parasitas é através da actividade de peroxiredoxinas com dois resíduos de cisteína activos. Este grupo de enzimas actua como um agente geral de eliminação de espécies oxidantes, reduzindo uma vasta gama de moléculas, incluindo hidroperóxidos e peroxinitrito, sendo esta última uma espécie citotóxica produzida pela resposta imune do hospedeiro. Uma característica que distingue as peroxirredoxinas das *Leishmania* dos seus homólogos de mamíferos é o facto das peroxirredoxinas dos parasitas serem reduzidas pelo ciclo de oxido-redução de NADPH/tripanotiona redutase/tripanotiona. A transferência de electrões entre a tripanotiona e a peroxirredoxina é mediada pela triparredoxina, uma oxidorredutase da família das tiorredoxinas. Esta tese descreve a identificação e a caracterização de uma triparredoxina (*Li*TXN1) e de duas peroxirredoxinas (*Lic*TXNPx1 e *Lic*TXNPx2) citosólicas de *L. infantum*. A distribuição estratégica destas enzimas pelo citosol do parasita, bem como as suas propriedades bioquímicas e funcionais sugerem que estas moléculas podem estar envolvidas na protecção do parasita contra o ataque oxidativo do hospedeiro.

Para além da exposição a oxidantes produzidos exogenamente, as *Leishmania* têm também que lidar com espécies reactivas de oxigénio produzidas no seu interior. Tal como acontece noutros organismos aeróbicos, a cadeia respiratória mitocondrial constitui a principal fonte endógena de stress oxidativo em *Leishmania*. É possível que nestes parasitas a eliminação de hidroperóxidos mitocondriais dependa de um sistema triparredoxina/peroxirredoxina semelhante ao que opera no citosol. Esta hipótese é sustentada pelas nossas observações de que a mitocôndria de *L. infantum* possui uma triparredoxina (*Li*TXN2) e uma peroxirredoxina (*Li*mTXNPx) e de que estas enzimas interagem *in vitro* para catalizar a redução de

hidroperóxidos. Curiosamente, quando tentámos reconstituir toda a cascata enzimática de NADPH/tripanotiona redutase/tripanotiona/triparredoxina/peroxirredoxina na mitocôndria de *L. infantum*, não detectámos actividade de tripanotiona redutase neste organelo. Esta observação sugere que outras espécies redutoras, que não a tripanotiona, poderão fornecer ao sistema mitocondrial de triparredoxina/peroxirredoxina os electrões necessários à sua actividade peroxidática.

Além da sua importância como antioxidantes, as triparredoxinas e as peroxirredoxinas de *Leishmania*, são consideradas potenciais alvos para novas drogas antiparasitárias. De facto, estas moléculas apresentam características únicas que as distinguem das enzimas dos mamíferos e que poderão permitir a sua inibição específica sem comprometer a sobrevivência e/ou fisiologia do hospedeiro. De modo a obter informações relevantes para o desenho racional de inibidores específicos de triparredoxinas e de peroxirredoxinas de *L. infantum*, estas enzimas foram estudadas do ponto de vista bioquímico e cinético. A validação das triparredoxinas e das peroxirredoxinas como alvos de droga necessita ainda da demonstração de que estas moléculas são essenciais para a sobrevivência e/ou capacidade de estabelecer infecção do parasita. Para este fim, foram produzidos, recorrendo a uma estratégia de DNA recombinante, mutantes de *L. infantum* incapazes de expressar a peroxirredoxina mitocondrial. A observação de que estes transfectantes são viáveis invalida esta enzima como alvo de droga.

Em suma, os resultados apresentados nesta tese descrevem dois sistemas triparredoxina/ peroxirredoxina de *L. infantum* com localizações subcelulares distintas, um citoplasmático e o outro mitocondrial, cujas actividades peroxidáticas possivelmente se complementam para garantir a eliminação de espécies oxidantes de origem exógena e endógena. São assim apresentados novos dados relativos ao metabolismo de hidroperóxidos de *Leishmania* que abrem portas a futuras investigações.

Résumé

Dans cette thèse nous avons étudié certains aspects du métabolisme des hydroperoxydes du protozoaire *Leishmania infantum*. *Leishmania* sont des parasites intracellulaires obligés des hommes et des chiens qui, quand résident à l'intérieur des phagolysosomes des macrophages de mammifères, sont exposé aux oxydants (en particulier aux hydroperoxydes) produits par le système immunitaire de l'hôte. La façon dont *Leishmania* sont capables d'éviter cet attaque oxydative n'est complètement élucidée. Pour s'adapter à l'environnement hostile du phagolysosome, *Leishmania* ont développé plusieurs mécanismes de défense qui comprennent le revêtement de la surface des parasites avec des glycophospholipides, l'induction de l'expression de protéines de choc thermique ("heat shock proteins") et la modulation de la réponse immunitaire de l'hôte. Des enzymes capables de supprimer les hydroperoxydes font aussi partie de l'équipement de défense des parasites. *Leishmania* ne possèdent pas des catalases ni des glutatione-peroxydases dépendantes de sélénium, des enzymes que, chez les eucaryotes supérieurs, sont responsables pour l'élimination des hydroperoxydes. Au lieu de ça, le principal mécanisme enzymatique de réduction des hydroperoxydes dans ces parasites est en travers de la activité des peroxyredoxines avec deux résidus de cysteíne actives. Ce group d'enzymes opère comme un agent général d'élimination des espèces oxydantes, qui réduit une vaste variété des molécules, comme des hydroperoxydes et du peroxynitrite, cette dernière soient une espèce cytotoxique produite pour la réponse immune de l'hôte. Une caractéristique qui distingue les peroxyredoxines de *Leishmania* de leurs homologues mammifères est le fait que les enzymes du parasite sont réduites par le cycle oxydoréducteur de NADPH/trypanotione réductase/ trypanotione. Le transfert des électrons de la trypanotione vers la peroxyredoxine est engendré par tryparedoxine, une oxydoréductase apparentée à la thioredoxine. Cette thèse décrit l'identification et la caractérisation d'une tryparedoxine (*Li*TXN1) et de deux peroxyredoxines (*Lic*TXNPx1 et *Lic*TXNPx2) cytosoliques de *L. infantum*. La localisation stratégique de ces enzymes, aussi bien que leurs caractéristiques biochimiques et fonctionnelles, suggèrent que ces molécules peuvent être impliquées dans la protection du parasite contre l'attaque oxydative de l'hôte.

Au-delà d'être exposée aux oxydants d'origine exogène, *Leishmania* ont également à combattre les espèces réactives d'oxygène produites intérieurement. Comme chez autres organismes aérobiens, la chaîne respiratoire mitochondriale constitue la principale source endogène de stress oxydatif chez *Leishmania*. Il est possible que, chez ces parasites, l'élimination des hydropéroxides mitochondriales dépende d'un système tryparedoxine/ peroxyredoxine pareil à celui qui opère dans le cytosol. Cette hypothèse est soutenue par nos observations de que la mitochondrie de *L. infantum* possède une tryparedoxine (*Li*TXN2) et une

peroxyrédoxine (*Li*mTXNPx) et en plus que ces enzymes interagissent *in vitro* pour catalyser la réduction de hydroperoxydes. Curieusement, quand nous avons essayé de reconstituer la voie intégrale de NADPH/trypanotione réductase/trypanotione/tryparedoxine/peroxyredoxine à l'intérieur de la mitochondrie de *L. infantum*, nous n'avons pas détectée d'activité de trypanotione réductase dans cette organelle. Cette observation suggère que des réducteurs alternatifs à la trypanotione peuvent fournir au système tryparedoxine/peroxyredoxine mitochondrial les électrons nécessaires à son activité péroxydatique.

Les tryparedoxines et les peroxyredoxines de *Leishmania*, outre leur importance comme antioxydants, sont considérés potentiels cibles pour nouvelles drogues antiparasitaires. En effet, ces molécules présentent des caractéristiques uniques qui les distinguent de leurs homologues des mammifères et qui pourraient permettre leur inhibition spécifique sans compromettre la survie et/ou la physiologie de l'hôte. Pour obtenir des données utiles à une modélisation rationnelle d'inhibiteurs spécifiques des tryparedoxines et des peroxyredoxines de *L. infantum*, nous avons réalisé une analyse biochimique et cinétique de ces enzymes. De plus, la validation de tryparedoxines et de peroxyredoxines comme cibles de drogues nécessite la démonstration que ces molécules sont essentielles à la survie et/ou à l'infectiosité du parasite. Dans ce but, nous avons produit des mutants de *L. infantum* sans peroxyredoxine mitochondriale en utilisant une stratégie de recombinaison de l'ADN. Nous avons observé que ces transfectants sont viables, un résultat que invalide cette enzyme comme cible de drogue.

En conclusion, les résultats présentés dans cette thèse décrivent deux systèmes tryparedoxine/peroxyredoxine de *L. infantum* avec des localisations subcellulaires distinctes, un cytosolique et l'autre mitochondrial, dont activités peroxydatiques possiblement se complémentent pour enlever les oxydants issus des sources exogène et endogène. Les nouvelles perspectives présentées concernant le métabolisme de hydroperoxydes de *Leishmania* pourront être plus largement explorées dans de futures recherches.

List of publications Lista de publicações

Under the Portuguese law N.º 388/70, article N.º 8, it is stated that the following publications, in which I have actively participated, are part of this thesis:

Ao abrigo do artigo 8º do decreto-lei nº 388/70 fazem parte desta tese as seguintes publicações, nas quais participei activamente na recolha e estudo do material nelas incluídas e na redacção dos textos, com a colaboração dos co-autores:

Castro,H., Sousa,C., Novais,M., Santos,M., Budde,H., Cordeiro-da-Silva,A., Flohe,L., and Tomas,A.M. (2004). Two linked genes of Leishmania infantum encode tryparedoxins localised to cytosol and mitochondrion. *Mol. Biochem. Parasitol.* **136:**137-147.

Castro,H., Sousa,C., Santos,M., Cordeiro-da-Silva,A., Flohe,L., and Tomas,A.M. (2002). Complementary antioxidant defense by cytoplasmic and mitochondrial peroxiredoxins in Leishmania infantum. *Free Radic. Biol. Med.* **33:**1552-1562.

Castro,H., Budde,H., Flohe,L., Hofmann,B., Lunsdorf,H., Wissing,J., and Tomas,A.M. (2002). Specificity and kinetics of a mitochondrial peroxiredoxin of Leishmania infantum. *Free Radic. Biol. Med.* **33:**1563-1574.

Other publications:

Outras publicações:

Cordeiro-da-Silva,A., Cardoso,L., Araujo,N., **Castro,H.**, Tomas,A., Rodrigues,M., Cabral,M., Vergnes,B., Sereno,D., and Ouaissi,A. (2003). Identification of antibodies to Leishmania silent information regulatory 2 (SIR2) protein homologue during canine natural infections: pathological implications. *Immunol. Lett.* **86:**155-162.

Flohe,L., Budde,H., Bruns,K., **Castro,H.**, Clos,J., Hofmann,B., Kansal-Kalavar,S., Krumme,D., Menge,U., Plank-Schumacher,K., Sztajer,H., Wissing,J., Wylegalla,C., and Hecht,H.J. (2002). Tryparedoxin peroxidase of Leishmania donovani: molecular cloning, heterologous expression, specificity, and catalytic mechanism. *Arch. Biochem. Biophys.* **397:**324-335.

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Appendix

1. Abbreviations

3'-RACE	rapid amplification of mRNA 3'-end
∞	infinite
Φ_0	reciprocal value of the velocity at infinite substrate concentrations
Φ_1 or Φ_2	reciprocal values of the rate constants k_1' and k_2', respectively
$\Phi_{1,2}$	kinetic coefficient characterizing a central complex mechanism
Φ_{1app}	interpolated Φ_1 values
[A] or [B]	substrate concentration
$[E_0]$	total enzyme concentration
γ-IFN	gamma interferon
Acc. Nr.	Accession number
AhpC	alkyl hydroperoxide reductase subunit C
AhpF	alkyl hydroperoxide reductase subunit F
APx	ascorbate peroxidase
Asc	ascorbate
ATP	adenosine triphosphate
bp	base pair
BSA	bovine serum albumin
cDNA	complementary DNA
Cf	*Crithidia fasciculata*
CL	cutaneous leishmaniasis
COOH	cumene hydroperoxide
C-terminal	carboxy-terminal
cTXNPx	cytosolic TXNPx
Cys	cysteine
Da	Dalton
DNA	deoxyribonucleic acid
dNTP	deoxyribonucleotide
DTT	dithiothreitol
EDTA	ethylene-diamide-tetraacetic acid
eEFB1	eukaryotic elongation factor B1
eV	electron Volt
FCS	foetal calf serum
FR	fumarate reductase
G	gravitation
G418	neomycin
Glo	glyoxalase
GPx	glutathione peroxidase

GPx-like	non-selenium glutathione peroxidase-like
GR	glutathione reductase
GSH	glutathione
Hepes	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HSP	heat shock protein
HYG	hygromycin phosphotransferase gene
IC_{50}	inhibitory concentration
IFAT	indirect immunofluorescence assay
IgG	immunoglobulin G
IL	interleukin
iNOS	inducible nitric oxide synthase
IPTG	isopropyl-β-D-thiogalactopyranoside
k_1' or k_2'	rate constants
kb	kilobase pairs
k_{cat}	rate constant at infinite substrate concentrations
K_m	Michaelis Menten constant
kDNA	kinetoplast DNA
k_{ROOH}	rate constant for the reduction of the hydroperoxide
Ld	*Leishmania donovani*
LDH	lipoamide dehydrogenase
Li	*Leishmania infantum*
LimTXNPx+/-	single knockout for *LimTXNPx*
LimTXNPx-/-	double knockout for *LimTXNPx*
Lm	*Leishmania major*
log	logarithmic
LOOH	linoleic acid hydroperoxide
LPG	lipophosphoglycan
MALDI-TOF	matrix-assisted laser desertion and ionization time-of-flight
min	minute
mRNA	messenger RNA
mTXNPx	mitochondrial TXNPx
MW	molecular weight
NADH	nicotinamide adenine dinucleotide, reduced state
NADPH	nicotinamide adenine dinucleotide phosphate, reduced state
neo	neomycin or G418
NEO	neomycin phosphotranferase gene
NOHA	N^G-hydroxyl-L-arginine
NOS	nitric oxide synthase
nt	nucleotide(s)
N-terminal	amino-terminal
OD	optical density
ORF	open reading frame
ox	oxidized
PAGE	polyacrilamide gel electrophoresis
PCD	programmed cell death
PCOOH	phosphatidyl choline hydroperoxide
PCR	polymerase chain reaction

phox	NADPH phagocyte oxidase
pI	isoelectric point
Prx	peroxiredoxin
red	reduced
RiboR	ribonucleotide reductase
RNAi	RNA interference
RNA	ribonucleic acid
RNI	reactive nitrogen intermediates
RNS	reactive nitrogen species
ROI	reactive oxygen intermediates
ROS	reactive oxygen species
ROH	alcohol
ROOH	hydroperoxide
rpm	rounds per minute
rRNA	ribosomal RNA
RT	room temperature
RT-PCR	reverse transcription PCR
SB	southern blot
SDS	sodium dodecyl sulfate
SOD	superoxide dismutase
Taq	Thermophilus aquaticus
Tb	*Trypanosoma brucei*
t-bOOH	*tert*-butyl hydroperoxide
Tc	*Trypanosoma cruzi*
TNF-α	tumor necrosis factor alpha
TR	trypanothione reductase
TS_2	trypanothione (oxidized form)
TSA	thiol-specific anti-oxidant protein
$T(SH)_2$	dihydrotrypanothione (reduced form)
TXN	tryparedoxin
TXNPx	tryparedoxn peroxidase
U	unit
UMSBP	universal minicircle sequence binding protein
UTR	untranslated region
v	initial velocity
v/v	volume per volume
$V_{max\ app}$ or V_{aap}	apparent maximum velocity
VL	visceral leishmaniasis
WB	Western blot
WHO	World Health Organization
wt	wild-type
w/v	weight per volume

2. Abbreviations of nucleotides and amino acids

Base	Abbreviation
Adenosine	A
Cytidine	C
Guanosine	G
Thymidine	T
Inosine	I

Amino acid	Three letter code	One letter code
Alanine	Ala	A
Arginine	Arg	R
Asparagine	Asn	N
Aspartic acid	Asp	D
Cysteine	Cys	C
Glutamine	Gln	Q
Glutamic acid	Glu	E
Glycine	Gly	G
Histidine	His	H
Isoleucine	Ile	I
Leucine	Leu	L
Lysine	Lys	K
Methionine	Met	M
Phenylalanine	Phe	F
Proline	Pro	P
Serine	Ser	S
Threonine	Thr	T
Tryptophan	Trp	W
Tyrosine	Tyr	Y
Valine	Val	V