Calcium and Magnesium Ions Modulate the Oligomeric State and Function of Mitochondrial 2-Cys Peroxiredoxins in *Leishmania* Parasites

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**ABSTRACT**

*Leishmania* parasites have evolved a number of strategies to cope with the harsh environmental changes during mammalian infection. One of these mechanisms involves the functional gain that allowed mitochondrial 2-Cys peroxiredoxins to act as molecular chaperones when forming decamers. This function was demonstrated to be critical for the parasite infectivity in mammals and its activation was considered to be controlled exclusively by the enzyme redox state under physiological conditions. Herein, we revealed that magnesium and calcium ions play a major role in modulating the ability of these enzymes to act as molecular chaperones, surpassing the redox effect. These ions are directly involved in the mitochondrial metabolism and now also integrate a novel mechanism to stabilize the decameric form of 2-Cys peroxiredoxins in *Leishmania* mitochondrion. Moreover, we demonstrated that a constitutively dimeric Prx1m mutant impairs *Leishmania's* survival under heat stress, supporting the central role of chaperone function of Prx1m for *Leishmania* parasites during the transition from insect to mammalian hosts.

The *Leishmania* parasites, causative agents of human and canine leishmaniasis, are exposed to different growth conditions during their life cycle due to the migration from the insect to the mammalian host environment. Among the differences are the elevation of temperature, exposure to oxidants produced by the macrophages, pH acidification and lower availability of oxygen and nutrients (1). In this context, parasite survival as well as the establishment of a successful intracellular infection rely on the development of adaptive mechanisms to hostile conditions. In *Leishmania infantum*, for instance, one such mechanisms involves a mitochondrial 2-Cys...
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Peroxiredoxin (LiPrx1m) (2), also named tryparedoxin peroxidase, which allows the parasite to cope with heat stress during the transition from the insect (25 ºC) to the mammalian host (37 ºC) (2,3).

Prx1 subfamily members are peroxide-scavenging enzymes that display a 2-Cys catalytic mechanism and can assume distinct oligomeric states (dimers, decamers and higher-order oligomers) (4). A dual function of peroxidase and molecular chaperone has been reported for several Prx1 enzymes and seems to be modulated by changes in quaternary structure (5-7). Factors such as pH (8-10), ionic strength (8,11,12), protein concentration (3,12,13) and protein redox state (14,15) can affect the dimer-decamer equilibrium of Prx1 members, but how these factors modulate the peroxidase and chaperone activities is still poorly understood. Overoxidation of peroxidasic cysteine, in particular, has been demonstrated to shut down the peroxidase function and to enhance the chaperone activity by stabilizing oligomers larger than decamers (5,6). However, this functional switch appears to be relevant for only some members of Prx1 subfamily (16).

In *Leishmania*, the mitochondrial Prx1 can act as molecular chaperone and peroxidase, but only its role as chaperone is crucial for the parasite infectivity in mammals (2,3). Nonetheless, *in vitro* studies showed that the peroxidase catalytic cycle can modulate the chaperone reservoir of *LiPrx1m*, favoring chaperone-active decamers when reactive cysteine is reduced (C\textsubscript{P}-SH) and chaperone-inactive dimers when C\textsubscript{P} is oxidized into C\textsubscript{P}S-SC\textsubscript{R} (3). On the other hand, the enzyme from *Leishmania* mitochondrion (2) seems to be resistant to the over-oxidation mechanism that inactivates the peroxidase function, stabilizes high-order oligomers, and enhances the chaperone activity of fungal (5) and mammalian (6) 2-Cys Prxs.

Recently, we have demonstrated that pH variations also affect the dimer-decamer equilibrium of *Leishmania braziliensis* Prx1m (*LbPrx1m*), a close orthologue of the *L. infantum* enzyme, indicating that the chaperone function of these proteins might not be exclusively modulated by their redox state (9). A pH shift from 8.0 to 7.0 – commonly observed in mitochondria of nutrient-deprived cells (17) – is sufficient to stabilize oxidized decamers of *LbPrx1m* (9). Furthermore, it is unclear whether other factors from the mitochondrial environment affect the dimer-decamer interconversion of *Leishmania* Prx1m and its dual function. For 2-Cys Prxs from distant-related species, it has been demonstrated that, at least *in vitro*, such equilibrium can be affected by ionic strength variations (11,18) and post-translational modifications (19,20).

**RESULTS**

### Ca\textsuperscript{2+}/Mg\textsuperscript{2+} ions induce *LbPrx1m* decamerization

Analytical size-exclusion chromatography (aSEC) and small-angle X-ray scattering (SAXS) experiments at physiological pH revealed that oxidized *LbPrx1m* assumes a dimeric structure in presence of chelating agents, whereas it assembles into decamers in the absence of chelators (Fig. 1a-c).

**Post-treatment of Ca\textsuperscript{2+}-decamers with EDTA induced the disassembly into dimers, showing that the cation effect is a reversible process (Table 1).** Comparing several divalent cations, only Mg\textsuperscript{2+} and Ca\textsuperscript{2+} showed similar effects on *LbPrx1m* oligomerization (Table 2).
indicating a specific role for these ions in stabilizing \textit{LbPrx1m} decamers. The hypothesis that the \textit{Ca}^{2+}/\textit{Mg}^{2+} effect would be due to ionic strength variations in the medium was discarded, since \textit{LbPrx1m} presents the same SEC elution profile in presence or absence of 150 mM NaCl (data not shown). As the effects of \textit{Mg}^{2+} and \textit{Ca}^{2+} were indistinguishable from each other, and because \textit{LbPrx1m} eluted as dimers when incubated with EGTA (Fig. 1a), which chelates \textit{Ca}^{2+} with higher selectivity (\textit{> 10^5}) (24) over \textit{Mg}^{2+}, some of the \textit{in vitro} and \textit{in silico} assays described here were performed only with \textit{Ca}^{2+}.

To study the effect of increasing concentrations of \textit{Ca}^{2+} and \textit{Mg}^{2+} in the dimer-decamer equilibrium of \textit{LbPrx1m}, we monitored changes in the oligomerization state of oxidized samples titrated with \textit{CaCl}_2 or \textit{MgCl}_2 by measuring the anisotropy of intrinsic protein fluorescence (Fig. 1d). Based on these data, we estimated a \textit{K}_{d2} near 3 mM for both ions, indicating a low-affinity system. During these assays, we noticed that the cation effect was dependent on a critical protein concentration (~ 80 \text{\mu M}), below which oxidized \textit{LbPrx1m} dimers became less sensitive to the cation presence (data not shown). However, after the formation of cation-stabilized decamers, protein dilution to levels below the critical concentration did not induce decamer disassembly, indicating that cation binding to \textit{LbPrx1m} involves the formation of “transitional” decamers followed by the binding of \textit{Ca}^{2+} or \textit{Mg}^{2+} to yield stable cation-decamer complexes.

As a first approach to evaluate the physiological relevance of the \textit{Ca}^{2+}/\textit{Mg}^{2+} effect in the quaternary structure of \textit{LbPrx1m}, we performed aSEC assays in two conditions: one simulating mitochondrial basal concentrations of free \textit{Mg}^{2+} and \textit{Ca}^{2+} ions (25,26) and other mimicking a \textit{Ca}^{2+} increase to levels already reported for the \textit{L. braziliensis} mitochondrial (27). Basal concentrations of \textit{Mg}^{2+}/\textit{Ca}^{2+} were sufficient to maintain most of the reduced enzyme in the decameric form, indicating that physiological levels of \textit{Mg}^{2+}/\textit{Ca}^{2+} stabilize reduced decamers (Fig. 1e and f). We next exposed the cation-stabilized reduced decamers to a low concentration of \textit{H}_2\text{O}_2 and evaluated the aSEC profile of the oxidized (S-S bonded) enzyme. In basal concentrations of \textit{Mg}^{2+}/\textit{Ca}^{2+}, most of the decamers dissociate into dimers upon \textit{C}P oxidation/resolution (Fig. 1e and f), which correlates with the low affinity of these cations to the oxidized enzyme (Fig. 1d). However, in the condition simulating a calcium overload, almost half of the population remained decameric, indicating that supraphysiological \textit{Ca}^{2+} concentration already reported for \textit{Leishmania} mitochondrion can increase the level of oxidized decamers (Fig. 1e).

\textit{Prx1m} decamer stabilization by \textit{Ca}^{2+}/\textit{Mg}^{2+} is redox independent and a unique feature of the mitochondrial \textit{Prx1} from \textit{Leishmania} parasites — To further investigate how the redox state affects the oligomerization of \textit{LbPrx1m}, the hydrodynamic behavior of oxidized and reduced proteins was assessed under chelating conditions or in the presence of \textit{Ca}^{2+} (Fig. 2). aSEC results indicate that, when the cation is absent, the dimer-decamer equilibrium becomes more responsive to the protein redox state: the oxidized enzyme remains dimeric, regardless the protein concentration (Fig. 2a), whereas the reduced enzyme gets into a dimer-decamer equilibrium that is shifted to the decamer by increasing protein concentrations (Fig. 2b). In contrast, 25 mM \textit{CaCl}_2 stabilizes a major population of oxidized and reduced decamers even when they are diluted to low protein concentrations (Fig. 2c and d), supporting that the \textit{Ca}^{2+} effect surpasses the redox state in stabilizing \textit{LbPrx1m} decamers.

Unlike to \textit{LbPrx1m}, the cytoplasmic 2-Cys \textit{Prx} from the same pathogen, as well as from two evolutionary distant organisms, were not as dependent as \textit{LbPrx1m} on the decamer-stabilizing effect of \textit{Ca}^{2+}, eluting mainly as decamers regardless the presence of this ion or EDTA (Fig. 3). These findings indicate that the high sensitivity of dimer-decamer equilibrium to \textit{Ca}^{2+}/\textit{Mg}^{2+} ions is a unique feature of \textit{LbPrx1m} and possibly of other mitochondrial orthologues from \textit{Leishmania} spp, according to comparative structural and sequence analyses described below.
Structural basis for the cation-dependent mechanism of decamer stabilization —

Despite extensive efforts, we were unable to crystallize \textit{LbPrx1m} in complex with \(\text{Ca}^{2+}\). Crystals of \textit{LbPrx1m} decamers, pre-stabilized with \(\text{CaCl}_2\), were only observed in acidic conditions (pH 4.4), known to enhance the decamer stability in a cation-independent manner (9) and to decrease \(\text{Ca}^{2+}\)-binding affinity to proteins (28,29). Thus, to determine the \(\text{Ca}^{2+}/\text{Mg}^{2+}\)-binding site in \textit{LbPrx1m}, we analyzed the crystal structure of the pH-stabilized decamer (PDB accession code: 4KB3 (9)) and used \textit{in silico} approaches combined with site-directed mutagenesis to validate the predicted site.

Like other members of the AhpC/Prx1 subfamily (30), \textit{LbPrx1m} decamer is formed when five dimers bind to each other via the A-type interface (9). Since the formation of this interface depends on conformational changes of the region I (residues 75-79) preceding the \(\text{CP}\)-loop (residues 80-84) (9), we hypothesized that the decamer-stabilizing effect of \(\text{Ca}^{2+}/\text{Mg}^{2+}\) might be related to their binding to the A-type interface and concomitant stabilization of the region I in a conformation that favors the decameric assembly.

To test this hypothesis, we searched in the A-type interface of \textit{LbPrx1m} decamer (9) for negatively charged cavities in which a positive ion such as \(\text{Ca}^{2+}\) could bind and maintain the closed conformation of region I required for decamer stabilization (9). As expected, we found a site in which \(\text{Ca}^{2+}\) can be coordinated by residues from both interfacing subunits, assuming a distorted trigonal bipyramidal geometry according to molecular dynamics simulations (Fig. 4a). This site includes the main-chain of Asp76, a residue from region I, and is duplicated at each A-type interface, implying a stoichiometry of 10 cations per decamer.

We next evaluated the \(\text{Ca}^{2+}\) effect on the aSEC profile of mutants lacking one of the side chains predicted to coordinate this cation. These side chains belong to the residues Asp108, Ser109 and Ser112, located at the region II (residues 107-120) of the A-type interface (Fig. 4). The mutants D108A and S109A eluted essentially as dimers in presence of \(\text{Ca}^{2+}\) at pH 7.5, whereas mutant S112A showed a concentration-dependent behavior, eluting as decamer at 86 \(\mu\text{M}\) and as dimer at 13 \(\mu\text{M}\) (Fig. 4c and d). These results indicate that residues Asp108 and Ser109 are essential for the \(\text{Ca}^{2+}\)-dependent stabilization of \textit{LbPrx1m} decamer, while Ser112 plays a facultative role in this mechanism.

Intriguingly, the cation-binding site identified in \textit{LbPrx1m} is highly conserved in some 2-Cys Prxs whose decamers remain stable without \(\text{Ca}^{2+}/\text{Mg}^{2+}\) ions, such as the cytoplasmic tryparedoxin peroxidase from \textit{T. cruzi} (\textit{TcPrx1a}), whose decamer was crystallized in presence of EDTA (31), and the human \textit{Prx2} (\textit{HsPrx2}) (Fig. 4b). This finding prompted us to search for other structural elements that possibly account for the \(\text{Ca}^{2+}/\text{Mg}^{2+}\) effect on \textit{LbPrx1m}.

A comparison of the A-type interfaces of \textit{LbPrx1m}, \textit{TcPrx1a} and \textit{HsPrx2} crystallographic decamers (Fig. 5a-c) showed that residues Cys107 and Met139 are exclusive of 2-Cys Prxs whose decamers remain stable without \(\text{Ca}^{2+}/\text{Mg}^{2+}\) ions, such as the cytoplasmic tryparedoxin peroxidase from \textit{T. cruzi} (9). To test this hypothesis, we mutated the corresponding Lys137 (Fig. 5c). Thus, we hypothesized that the attraction of Asp108 side-chain by a residue bulkier than Cys107 (Met78), which seems to favor the interaction between Asp79 side-chain and a water molecule that occupies the predicted cation-binding site (Fig. 5b). The same water-mediated link occurs in \textit{HsPrx2}, which lacks the corresponding Lys137 (Fig. 5c). To test this hypothesis, we mutated Cys107 (region II) to a methionine and Met139 (region III) to a lysine, thus mimicking the \textit{TcPrx1a} Asp79 (equivalent to Asp108) microenvironment, in which Met78 blocks the access of the lysine to the aspartate (Fig. 5b). As envisioned, this double mutation rendered \textit{LbPrx1m} decamer stabilization \(\text{Ca}^{2+}\)-independent (Fig. 4c), demonstrating that changes in the regions II and III of the A-type interface
EDTA and next supplemented with Ca\textsuperscript{2+} decreased the peroxidase activity of purified reducing hydrogen peroxide. Importantly, the observation that Ca\textsuperscript{2+} ions did not affect the peroxidase activity of LbPrx1m (Fig. 6a). The increment of Ca\textsuperscript{2+} in purified LbPrx1m did not alter its catalytic activity (Fig. 6a), an effect that can be ascribed to divalent cations from E. coli that remained bound to LbPrx1m decamers during the purification process, as indicated by aSEC analysis of untreated samples (results not shown). The observed effects of EDTA and Ca\textsuperscript{2+} cannot be attributed to interference with the other molecules of the trypanothione cascade, because these additives did not alter the peroxidase activity of the cytoprotective Prx1 from L. infantum (LiPrx1a) (Fig. 6b).

Ca\textsuperscript{2+}/Mg\textsuperscript{2+} ions enhance the peroxidase activity of LbPrx1m — The dimer-decamer switch seems to play a role during the peroxidase catalytic cycle of AhpC/Prx1 subfamily members, influencing the enzymatic efficiency (15,32). We thus used the trypanothione-dependent enzyme cascade from Leishmania to evaluate, in vitro, how the decamer-stabilizing effect of Ca\textsuperscript{2+}/Mg\textsuperscript{2+} influences the capability of LbPrx1m in reducing hydrogen peroxide. The chelation of metal ions drastically decreased the peroxidase activity of purified LbPrx1m (Fig. 6a). In samples pre-treated with EDTA and next supplemented with Ca\textsuperscript{2+} or Mg\textsuperscript{2+}, the catalytic activity was recovered, showing that these divalent cations play a role in the peroxidase function of LbPrx1m (Fig. 6a). The increment of Ca\textsuperscript{2+} in purified LbPrx1m did not alter its catalytic activity (Fig. 6a), an effect that can be ascribed to divalent cations from E. coli that remained bound to LbPrx1m decamers during the purification process, as indicated by aSEC analysis of untreated samples (results not shown). The observed effects of EDTA and Ca\textsuperscript{2+} cannot be attributed to interference with the other molecules of the trypanothione cascade, because these additives did not alter the peroxidase activity of the cytoprotective Prx1 from L. infantum (LiPrx1a) (Fig. 6b).

Importantly, the observation that Ca\textsuperscript{2+} ions did not affect the peroxidase activity of LiPrx1a correlates with the fact that Ca\textsuperscript{2+}/Mg\textsuperscript{2+} ions are not required to stabilize the decameric structure of its ortholog in L. braziliensis (Fig. 3), corroborating the specific effect of Ca\textsuperscript{2+}/Mg\textsuperscript{2+} on the peroxidase activity of the Leishmania mitochondrial enzyme.

To demonstrate that the Ca\textsuperscript{2+} effect on the peroxidase activity is due to its binding at the A-type interface and consequent decamer stabilization, we compared the peroxidase activity of Ca\textsuperscript{2+}-binding site mutants with that of the wild-type (wt) protein or the mutant H113A (Fig. 6a-c), which preserves the dimeric structure but is unable to decamerize in response to pH (9), redox and cation stimuli (Fig. 7a). Mutants D108A and S109A displayed only residual activity compared to the wt enzyme in the presence of Ca\textsuperscript{2+} (Fig. 6c). As expected, the mutation S112A was not as efficient as the D108A and S109A substitutions in decreasing the peroxidase activity of LbPrx1m, which correlates with the facultative role of Ser112 in Ca\textsuperscript{2+} binding. The effects of D108A and S109A mutations on the peroxidase activity were equivalent to that caused by the decamer-disrupting H113A substitution, further supporting that Ca\textsuperscript{2+}/Mg\textsuperscript{2+} ions enhance the LbPrx1m peroxidase activity by stabilizing its decameric form.

Reduced and oxidized cation-stabilized decamers possess the required conformation to perform chaperone function — Our finding that Ca\textsuperscript{2+}/Mg\textsuperscript{2+} ions stabilize oxidized decamers of LbPrx1m led us to investigate whether these decamers are able to suppress luciferase thermal aggregation similarly to those reduced (3). Under non-reducing conditions, metal chelation inhibited the chaperone activity of LbPrx1m, indicating a role for Ca\textsuperscript{2+}/Mg\textsuperscript{2+} in activating the chaperone function of oxidized LbPrx1m (Fig. 6d). When cation-free samples were supplemented with CaCl\textsubscript{2} or MgCl\textsubscript{2}, the chaperone activity was recovered, reaching levels significantly higher than that of untreated samples in the case of Ca\textsuperscript{2+} (Fig. 6d). Although LbPrx1m reduction also rescued the chaperone activity of cation-free LbPrx1m, the average activity of reduced decamers was further stimulated by CaCl\textsubscript{2} supplementation (Fig. 6d), which is in agreement with aSEC data showing the better performance of Ca\textsuperscript{2+} than protein reduction in stabilizing decamers (Fig. 2).

To demonstrate that protection against luciferase aggregation depends on the formation of decamers and to evaluate the effect of Ca\textsuperscript{2+}/Mg\textsuperscript{2+}-binding site mutations on chaperone function, we measured chaperone activity of LbPrx1m mutants H113A, D108A, S109A and S112A. The H113A substitution – which prevents the decameric assembly of dimers – completely abolished the chaperone activity of oxidized LbPrx1m and drastically decreased that of the reduced protein, demonstrating that decamer formation is a pre-
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requisite for chaperone activity (Fig. 6e and f). Unexpectedly, from the four Ca\(^{2+}\)-binding site mutations, only D108A significantly decreased the chaperone activity in both non-reducing and reducing conditions (Fig. 6e and f). However, the mutant D108A was more active than H113A, which prompted us to investigate whether the high temperature of the chaperone assay could favor the formation of D108A decamers. In agreement with the chaperone activity data, mutant D108A entered in a dimer-decamer equilibrium at 42 °C (Fig. 7b and e), whereas mutant H113A remained in the dimeric state at this temperature (Fig. 7a and d). Under the same conditions, the wt protein was decameric, indicating that decamer formation is necessary and sufficient to trigger the chaperone function of LbPrx1m (Fig. 7c and f).

Based on studies of reduced decamers, it has been proposed that chaperone function of Leishmania Prx1m is activated by temperature increase, which induces conformational rearrangements that expose hydrophobic regions (3). As our data showed that oxidized decamers also display chaperone activity, we investigated whether their behavior upon temperature increase supports the model in which thermo-induced conformational changes activate the chaperone function of Leishmania Prx1m. For this purpose, circular dichroism (CD) spectra of wt LbPrx1m were collected under reducing and non-reducing conditions at 25 and 42 °C, in the presence of Ca\(^{2+}\). Interestingly, at 25 °C, the CD spectra of reduced and oxidized decamers were virtually identical, showing that the conformational differences between their respective fully-folded and locally-unfolded active sites are undetectable by this technique (Fig. 8a and b). Upon a temperature increase from 25 to 42 °C, the CD spectrum of reduced decamers showed a decrease in the absolute values at 193 and 222 nm and presented a shift of the minimum at 208 nm toward smaller wavelengths, indicating the loss of α-helical structures (Fig. 8a). In agreement with this result, DSF data showed the exposure of hydrophobic surfaces in reduced decamers heated up to 42 °C (Fig. 8c). However, no significant changes in CD spectra or exposure of hydrophobic regions in oxidized decamers in the same temperature range (Fig. 8b and c). This enhanced thermostability of oxidized species correlates with the presence of the disulfide bond linking C\(_P\) - located in the loop connecting strand β3 to helix α2 - and C\(_R\), placed at the C-terminal extension downstream the helix α6. Since this is the only disulfide bond present in oxidized LbPrx1m (9), the loss of structure induced by CD and DSF analyses of reduced decamers likely reflects, among other events, the unfolding of helices α2 and/or α6, which is favored when C\(_P\) and C\(_R\) are reduced.

Considering that the helices α2 and α6 are located at the external surface of the decamer, i.e. far from the luciferase-binding site (3), their unfolding might not interfere with the chaperone function of reduced decamers (Fig. 9). Supporting this hypothesis, the Ca\(^{2+}\)-stabilized reduced species preserved the decameric structure at 42 °C (data not shown) and were as effective as those oxidized in suppressing the thermal-aggregation of luciferase in vitro (Fig. 6d). Together, these analyses suggest that the thermo-induced conformational changes observed for the reduced decamer might not be required to activate the chaperone function of Leishmania Prx1m.

For comparison purposes, we also performed CD and DSF analyses for the wt LbPrx1m in presence of EDTA, a condition that favors the dimeric state, and the mutant H113A, which is fully dimeric in solution regardless its redox state (Fig. 8d-i). These experiments confirmed that the mutant H113A is properly folded (Fig. 8g and h) and revealed that the C\(_P\)-S-SC\(_R\) disulfide bond has a higher impact in thermostability than variations in the oligomeric state, leading to an increase of at least 12 °C in the melting temperature of both dimers and decamers (Fig. 8c, f and i).

Preventing Prx1m decamer formation impairs Leishmania survival at 37 °C — Previous work has shown that L. infantum parasites devoid of LiPrx1m are thermo-sensitive when exposed to 37 °C, a phenotype that is partially reverted upon reintroduction of the enzyme.
(2). To investigate phenotypic implications of Prx1m mutants unable of decamerizing or with lower tendency to form Ca\(^{2+}\)/Mg\(^{2+}\)-stabilized decamers, L. infantum knockout for LbPrx1m (prx1m) were transfected with wt LbPrx1m and the corresponding H113A and D108A muteins. Using indirect immunofluorescence (Fig. 10a), Western-blot (Fig. 10b), and PCR (data not shown) analyses, it was verified that knockout parasites lack the expression of LbPrx1m, whereas those transfected, expressed the wt protein and mutants into the mitochondrion.

As expected, at 25 °C, all parasites have the same growth rates (Fig. 10c). However, at 37 °C, the H113A dimers were incapable to rescue the thermo-sensitive phenotype of Prx1m promastigotes, demonstrating that the decameric state of Prx1m is crucial for Leishmania survival in temperatures akin to that encountered in the mammalian host (Fig. 10d and e). When exposed to 37 °C, knockout parasites expressing the D108A mutein, which is less prone to form Ca\(^{2+}\)/Mg\(^{2+}\)-stabilized decamers, presented a similar behavior to those complemented with the wt protein (Fig. 10d and e). This result is likely explained by the fact that the chaperone activity of this mutant retains a residual response to Ca\(^{2+}\) (Fig. 10f). Furthermore, mutant D108A is susceptible to the influence of medium acidification, which stabilizes a subpopulation of D108A decamers as dimers in vitro (Fig. 7b) and probably counterbalances the lower responsiveness of D108A decamers to Ca\(^{2+}\)/Mg\(^{2+}\) in vivo. Interestingly, the chaperone activity of the mutant D108A in vitro is irresponsive to DTT treatment even in cation-free conditions (Fig. 10f), indicating that decamer stabilizing factors other than protein reduction support the chaperone function of this mutein in vivo.

**DISCUSSION**

Ca\(^{2+}\)/Mg\(^{2+}\) ions stabilize decamers and activate the dual-function of mitochondrial 2-Cys Prxs from Leishmania parasites — In this work, we revealed that Ca\(^{2+}\) and Mg\(^{2+}\) ions affect the quaternary structure and the dual-function of mitochondrial 2-Cys peroxiredoxins from Leishmania parasites. Our data showed that these divalent cations stabilize LbPrx1m decamers and thereby stimulate the peroxidase and chaperone activities. The mechanism involves the binding of Ca\(^{2+}\) or Mg\(^{2+}\) at transitional A-type interfaces, stabilizing dimer-dimer interactions. Although the K\(_{1/2}\) estimated for Ca\(^{2+}\) and Mg\(^{2+}\) binding was near 3 mM for the oxidized enzyme, our analyses suggest that this affinity can be enhanced by protein reduction, which stabilizes transitional decamers and favors the formation of cation-bound decamers. Supporting this hypothesis, air-oxidized samples treated with EDTA and then incubated with 1 mM Ca\(^{2+}\) behave essentially as dimers (Fig. 1d); however, when these EDTA-treated samples are reduced with DTT, incubated with similar amount of cation and then re-oxidized, the enzyme remain about 50% in the decameric form (Fig. 1 e-f). In other words, the amount of cation-bound oxidized decamers increases when these complexes are formed under reducing conditions prior enzyme oxidation. In summary, our data indicate that physiological concentrations of free Ca\(^{2+}\)/Mg\(^{2+}\) stabilize reduced decamers, stimulating the peroxidase function and contributing to maintain a basal pool of chaperone-active LbPrx1m at the alkaline environment of mitochondria (pH 7.5-8.0) (33). When mitochondrial Ca\(^{2+}\) uptake is stimulated (23), the level of oxidized decamers increase, contributing to enlarge the chaperone reservoir of Prx1m in Leishmania.

The closed conformation of Region I is required to stabilize the fully-folded conformation of reduced C\(_{P}\)-loop — According to literature data, the decameric structure of 2-Cys Prxs contributes to stabilize the fully-folded conformation of the active site, allowing the optimal orientation of substrate and the activation of the catalytic C\(_{P}\) to reduce the peroxide oxygen (15,32). Our data support that, for C\(_{P}\)-loop to adopt the fully-folded conformation, the adjacent region I might be stabilized in a closed conformation via interactions involving residue Asp76 (Fig.
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Disruption of such interactions by point mutations severely decrease the peroxidase activity of \( LbPrx1m \) (Fig. 6c). While the link between Asp76 and His113 side-chains is intramolecular and direct, those involving Asp76 main-chain requires decamer assembly and are mediated by \( Ca^{2+} \) or \( Mg^{2+} \) ions, in the case of \( LbPrx1m \) (Fig. 11). Our data revealed that \( Mg^{2+} \) or \( Ca^{2+} \) ions increase the peroxidase activity by binding at the A-type interface of \( LbPrx1m \), thus holding the Asp76 main-chain with the assistance of Asp108, Ser109, and optionally Ser112 side-chains. Noticeably, mutations at positions 108 and 109 displayed a higher impact on peroxidase activity compared to EDTA treatment, suggesting that, in the absence of divalent cations, a solvent molecule could link this Ser/Asp cluster, allowing a suboptimal activity. Indeed, in several members of AhpC/Prx1 subfamily, from bacteria to mammals, a water molecule plays the role of linking the highly conserved Ser/Asp cluster at the A-type interface (Fig. 12).

By converting \( LbPrx1m \) into a cation-independent 2-Cys Prx, we provided strong evidences that the microenvironment of Asp108 selects \( LbPrx1m \) as a \( Ca^{2+}/Mg^{2+} \)-sensitive Prx1. According to our model, \( LbPrx1m \) requires \( Ca^{2+}/Mg^{2+} \) to surpass the electrostatic attraction caused by Lys137 on Asp108 and to restore the solvent-mediated link that holds the main-chain of Asp76 and contributes to maintain the fully-folded conformation of C\( \beta \)-loop, which is mandatory for substrate binding and catalysis. Comparative sequence analysis suggests that the high \( Ca^{2+}/Mg^{2+} \) sensitivity observed for \( LbPRx1m \) extends exclusively to mitochondrial 2-Cys Prxs from \( Leishmania \) species (Fig. 12).

\( Ca^{2+}/Mg^{2+} \) ions compose a redundant molecular system that supports the chaperone function in vivo — Our studies demonstrated the similar efficiency of oxidized and reduced cation-stabilized decamers in preventing luciferase aggregation under heat stress conditions. This finding suggests that the \( Leishmania \) reservoir of Prx1m chaperones is not only formed by reduced proteins, as previously envisaged (3), but can also comprise those oxidized (S-S bonded). As the S-S bonded species represent about half of the Prx1m population present in the parasite (2), their capability to form chaperone-active decamers might be of great relevance for \( Leishmania \) virulence, considering that the chaperone function of Prx1m is crucial for the parasite survival in the mammalian host (3). Moreover, the resistance of \( Leishmania \) Prx1m to \( C_{\beta} \) overoxidation (2) and the lack of the sulfiredoxin in these parasites further support the importance of an alternative mechanism to modulate the chaperone activity of Prx1m in \( Leishmania \) species.

When \( LbPrx1m \) dimers fail to respond to the three components of the decamer-stabilizing system (pH, redox state and \( Ca^{2+}/Mg^{2+} \)), they lose their capacity to rescue the temperature-sensitive phenotype of \( prx1m \) promastigotes, as indicated by our studies with mutant H113A. Based on our results, we suggest that the residue His113 is not directly involved in cation binding, but is necessary for the formation of a transitional A-type interface that is then stabilized by cation binding, His113 protonation (9), or, less effectively, by \( C_{\beta} \) reduction. The crystal structure of oxidized \( LbPrx1m \) dimers show that the region I preceding the \( C_{\beta} \)-loop is highly flexible and can transit between an open and a closed conformation, which favors dimers and decamers, respectively (9). The pre-requisite to form transitional A-type interfaces likely involves the H-bond between His113 and Asp76 when region I accesses the closed conformation. Since this interaction is insufficient to lock region I in the closed conformation, decamer stabilization requires a second stimulus.

According to our model (Fig. 13), in basal concentrations of enzyme, \( Mg^{2+} \) and \( Ca^{2+} \), most of Prx1m enzymes are decameric when reduced and dimeric when oxidized. During heat shock - which stimulates \( Ca^{2+} \) uptake in \( Leishmania \) promastigotes (34) - higher levels of \( Ca^{2+} \) at the mitochondrion (27) feed the chaperone reservoir with oxidized decamers. Besides stimulating mitochondrial \( Ca^{2+} \) uptake, cytosolic \( Ca^{2+} \) elevations can also lead to mitochondrial pH decrease of about 0.2
pH units (35), providing an extra stimulus to enlarge the reservoir of Prx1m decamers (9). At pH 7, for example, we detected in vitro a cooperative effect between pH and Ca\(^{2+}\) to stabilize oxidized decamers (data not shown).

Together, our studies showed that basal concentrations of Mg\(^{2+}/Ca\(^{2+}\) ions support the dual function of mitochondrial Prx1 from *Leishmania* and revealed a molecular mechanism that might help to explain why calcium uptake is crucial for *Leishmania* thermotolerance and differentiation in the mammalian host (34). Furthermore, we demonstrated that the decameric structure – independently from its redox state – is both necessary and sufficient for the protective effect of Prx1m against heat stress in *Leishmania*, a vital attribute for the establishment of a successful infection in the mammalian host (2,3). This finding implies that the search for compounds that prevent Prx1m decamerization represents the best strategy to inhibit the crucial chaperone function of this attractive therapeutic target (36). Zhao and coworkers already identified chaperone inhibitors for the human Prx I, demonstrating the feasibility of such approach (37).

**EXPERIMENTAL PROCEDURES**

*Molecular cloning and site-directed mutagenesis* — The *Lb*Prx1m gene (RefSeq: XM_001562186.1) was cloned into a pET28a-His-TEV vector as described in (38). The human gene *PRX2* (RefSeq: NM_005809.5) was amplified by PCR and cloned into the pET28a vector between NdeI and SalI restriction sites. The gene *TSA1* from *S. cerevisiae* was cloned into the pET15b vector as described in (39). The pET28a construct containing the DNA sequence of *Lb*Prx1a gene (RefSeq: XM_001563506.1) between the NdeI and SalI restriction sites was purchased from GenScript (Piscataway, NJ). All *Lb*Prx1m mutants were produced using the QuikChange™ site-directed mutagenesis kit (Stratagene).

**Protein expression and purification** — The protein *Lb*Prx1m and corresponding muteins were expressed and purified as described previously (9). *Lb*Prx1a and *Hs*Prx2 were produced in *Escherichia coli* BL21(DE3)ΔSlyD cells containing the plasmid pRARE2 whereas TSA1 was produced in BL21(DE3) cells. After the cell culture reached OD\(_{600}\)~ 0.6 in LB medium, protein expression was induced with 0.5 mM IPTG at 30 °C for 4 hours at 200 rpm (*Hs*Prx2 and *Lb*Prx1a) or with 1 mM IPTG at 37 °C for 3 hours at 200 rpm (TSA1). Protein extraction and affinity purification followed that described for *Lb*Prx1m (9). All purified proteins were dialyzed against the buffer used in the analytical size-exclusion chromatography step and concentrated using Amicon Ultra devices (Millipore). The oxidized (S-S bonded) state of purified proteins was confirmed by SDS-PAGE analysis under non-reducing conditions, whereas the reduced state of proteins treated with DTT was confirmed by quantification of free thiol groups using DTNB as previously described (9).

**Analytical size-exclusion chromatography (aSEC)** — A total of 2 mL of *Lb*Prx1m at 130 µM, *Lb*Prx1a at 43 µM, yeast TSA1 at 43 µM and *Hs*Prx2 at 130 µM were loaded onto a HiLoad 16/600 Superdex 200 column (GE Healthcare), pre-equilibrated with 25 mM Tris-HCl (pH 7.5) containing 25 mM CaCl\(_2\), 25 mM MgCl\(_2\), 5 mM EDTA or 5 mM EGTA. aSEC experiments for the *Lb*Prx1m mutants D108A, S109A, S112A at 86 µM (500 µL input) were carried out using the same column pre-equilibrated with buffer T-Ca (25 mM Tris-HCl, 150 mM NaCl, 25 mM CaCl\(_2\), pH 7.5). The same assay was performed using the mutant C107M/M139K at 86 µM (500 µL input) in buffer T-EDTA (25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA, pH 7.5). Additionally, wt *Lb*Prx1m was cleaved with TEV protease to remove the His-tag, pre-incubated at 94 µM with or without 10 mM DTT in buffer T-Ca or T-EDTA, and divided into three samples of different protein concentrations (94, 23 and 9 µM) that were loaded (250 µL) onto a Superdex 200 10/300
GL column (GE Healthcare) pre-equilibrated with the sample buffer.

aSEC assays mimicking in vivo conditions (3,25-27,40,41) were performed with TEV-cleaved LbPrx1m at 100 µM (200 µL input), in buffer T-EDTA plus the following additives: (I) 5.7 mM MgCl$_2$ (free Mg$^{2+}$ = 0.7 mM) and 200 nM CaCl$_2$; and (II) the same described in condition (I), but with 90 µM CaCl$_2$. These assays were carried out in a Superdex 200 10/300 GL column (GE Healthcare). Samples were pretreated and eluted in presence of 2 mM DTT or pretreated with 2 mM DTT, re-oxidized, and eluted in buffer without DTT. To obtain re-oxidized samples, DTT was removed as described in 4 and the concentrated protein was incubated with H$_2$O$_2$ in 1:1 (protein: H$_2$O$_2$) molar ratio prior injection. The redox state of samples was analyzed by non-reducing SDS-PAGE.

aSEC assays of LbPrx1m wt and S112A mutant at 13 µM (200 µL input) were performed in buffer T-Ca using a Superdex 200 10/300 GL column (GE Healthcare). Additional aSECs comparing wt LbPrx1m and H113A and D108A mutants at 48 µM (250 µL input) were performed at pH 4.0, as described in Morais et al., 2015 (9); in buffer T-EDTA plus 2 mM DTT; or in buffer T-Ca. For comparative purposes, the molar concentrations estimated for all analyzed proteins refer to monomers. Columns were calibrated using the gel filtration calibration kits LMW and HMW (GE Healthcare).

Dynamic Light Scattering (DLS) — DLS measurements were performed on a Dynapro Molecular Sizing instrument at 25 or 42 ºC. Protein samples at 100 µM were previously centrifuged for 20 min at 20,000 x g. Data were collected with intervals of 10 s with at least 100 acquisitions. The diffusion coefficient (D) was determined from the analysis of measured time-dependent fluctuations in the scattering intensity and used to calculate the hydrodynamic radius ($R_H$) of the protein according to the Stokes-Einstein equation. Data analysis was performed using the software Dynamics V6.3.40.

Small Angle X-ray Scattering (SAXS) — SAXS data were collected at the D02A/SAXS2 beamline (Brazilian Synchrotron Light Laboratory, Campinas, Brazil). The radiation wavelength was set to 1.48 Å and a 165 mm MarCCD detector was used to record the scattering patterns. The sample-to-detector distance was set to 1534.5 mm to give a scattering vector-range from 0.25 to 2.5 nm$^{-1}$. Protein samples at 108 µM were prepared in 25 mM Tris-HCl (pH 7.5) with 5 mM EDTA or 25 mM CaCl$_2$. Frames with exposure time of 600 s were recorded and buffer baselines were collected under identical conditions. Background scattering was subtracted from the protein scattering pattern, which was then normalized and corrected. Experimental data fitting and evaluation of the pair-distance distribution function $P(r)$ were performed using the program GNOM(42). The low-resolution envelopes were determined using ab initio modeling as implemented in the program DAMMIN (43). An averaged model was generated using the package DAMAVER (44) The low-resolution model and the crystal structure were superimposed using the program SUPCOMB (45).

Fluorescence anisotropy measurements — Fluorescence anisotropy data were collected in a PC-1 fluorimeter (ISS) coupled to a thermal bath at 25 ºC using an excitation wavelength of 280 nm (46). Samples of LbPrx1m at 80 µM were pre-incubated in buffer at pH 7.5 containing 25 mM Tris-HCl, 150 mM NaCl, 5 mM EDTA and increasing amounts of CaCl$_2$ or MgCl$_2$. For data acquisition, samples were diluted in buffer 25 mM Tris-HCl, 150 mM NaCl, pH 7.5 to a final protein concentration of 2 µM. Free cation concentrations were defined by subtracting the added Ca(Mg)Cl$_2$ concentration from the EDTA concentration of each sample. The mean data of three independent experiments were fitted to a non-linear regression to estimate the constant $K_{1/2}$ using GraphPad Prism v.6.0.

Molecular dynamics simulations — The most favorable geometric coordination of Ca$^{2+}$ by the residues Ser109$_A$, Ser112$_A$, Asp76$_A$ and Asp108$_A$ of the LbPrx1m
decamer (PDB accession code: 4KB3) was evaluated using molecular dynamics simulations. The system was submitted to an explicit solvent simulation with water density of 1 g cm⁻³ and neutralized using 0.9 % NaCl solvent mass fraction on 298 K. The protonation states for ionizable groups were set according to pH 7.0 using an empirical equation, derived from experimental data, that considers electrostatic potential, hydrogen bonds and accessible surface area (47). The calcium coordination sphere considers electrostatic potential, hydrogen bonds and accessible surface area (47). The calcium coordination sphere of the cation parameters - on the program YAMBER3 force field (48) - which includes simulation was carried out for 10 ns using the YAMBER3 force field (48) - which includes the cation parameters - on the program YASARA. The calcium coordination sphere for every 25-ps snapshot was analyzed using a customized script implemented in FindGeo (49).

Enzymatic assays — NADPH consumption (ε340 nm = 6220 M⁻¹ cm⁻¹) by Leishmania trypanothione system was monitored at 340 nm in a spectrophotometer Shimatzu UV-2401 (Shimatsu Corporation) with temperature set to 25°C. The reactions were carried out with 280 µM NADPH, 0.4 µM trypanothione reductase LiTR (50), 75 µM trypanothione (Bachem), 4 µM of tryparedoxin LiTXN1 (50) and 4.5 µM of LbPrx1m (wt or mutants) or 0.4 µM LiPrx1a (50) in buffer 50 mM Tris-HCl (pH 7.5), LiTR and trypanothione were used in excess. The Prx enzyme was either untreated or pretreated with 5 mM EDTA or 5 mM EDTA followed by the addition of 25 mM CaCl₂ or MgCl₂. The others components were previously incubated at 25 °C for 15 min. Treated Prx samples were diluted in the reaction medium and the reaction was started with the addition of 70 µM H₂O₂. All experiments were performed with TEV-cleaved LbPrx1m in triplicate. Relative activities were calculated as mean values considering untreated samples as reference for the cation and EDTA treatments or the wt activity of LbPrx1m in presence of CaCl₂ as reference for the muteins assayed in the same condition.

Chaperone activity assays — To investigate the chaperone activity of reduced and oxidized LbPrx1m (wt or mutants), 100 nM luciferase (Promega) was incubated in 40 mM HEPES (pH 7.5) at 42 °C with the molar ratio of 1:10 (Luciferase:LbPrx1m). LbPrx1m was pretreated with 20 mM CaCl₂ or MgCl₂; 5 mM EDTA; or 5 mM EDTA plus 25 mM CaCl₂ or MgCl₂. The reduced samples were incubated with 2 mM DTT (final concentration). The reactions were kept at 42 °C and luciferase aggregation was monitored in a Fluoromax-4 spectrofluorometer (Horiba) for 900 seconds using the wavelength of 360 nm for excitation and emission. Relative activities were calculated as mean values considering the activity of untreated samples as reference for those treated with additives (Ca²⁺, EDTA and DTT) or the activity of wt protein as reference for the muteins assayed in the same condition, unless stated otherwise. To exclude the effect that some additives have on luciferase aggregation, relative activities were calculated according to the formula (N₆₇₄₄₋₅)/(N₆₇₄₄₋₅), in which N₆₇₄₄, T, N₈₀ and R refer to light scattering values recorded at 900 s of N₆₇₄₄, negative control reaction of the test condition (luciferase + additives); T, test condition (N₆₇₄₄ + wt or mutant LbPrx1m); N₈₀, negative control reaction of the reference condition (luciferase + additives) and R, reference condition (N₆₇₄₄ + wt LbPrx1m). All assays were performed with TEV-cleaved LbPrx1m in triplicate.

Circular dichroism (CD) — CD measurements were acquired at 25 or 42 °C on a JASCO J-815 spectropolarimeter equipped with a Peltier temperature controller (Jasco Analytical Instruments). TEV-cleaved LbPrx1m samples (wt and H113A mutant) at 80 µM were pretreated with 5 mM EDTA plus (or not) 25 mM CaCl₂, and diluted to the final concentration of 2 µM in 10 mM sodium phosphate (pH 7.5), with or without 2 mM DTT. Far-UV CD spectra were recorded between 190 and 260 nm at a speed of 50 nm / min with a total of 16 accumulations. CD data were buffer subtracted and normalized to molar residual ellipticity allowing the comparison between different treatments.

Differential scanning fluorimetry (DSF) — DSF assays were performed in triplicate using a Real Time PCR machine 7300 (Applied
Biosystems). Samples of TEV-cleaved LbPrx1m (wt and H113A mutant) were pre-treated as described in the above section and diluted to a final concentration of 2 µM in buffer 20 mM HEPES (pH 7.5), 150 mM NaCl, with or without 2 mM DTT, containing 5× of the fluorescent dye SYPRO Orange (Invitrogen Molecular Probes). The 96-well plates were heated from 25 to 95 ºC, increasing 1 ºC per cycle, and the fluorescence emission was measured at 580 nm. The DSF melting curves were analyzed using the GraphPad Prism software v. 6.0.

**Generation of L. infantum transfectants** — To construct the pSSU-PHLEO-infantum-LbPrx1m plasmids, a DNA fragment corresponding to the mitochondrial targeting sequence of Prx1m was PCR-amplified with PfuTurbo from genomic DNA of L. amazonensis with primers P1 and P2 (Supplementary Table 3) and cloned into the BamHI and XbaI sites of pSSU-PHLEO-infantum-LiPrx1m vector (2). The resulting plasmid was subsequently digested with StuI and NheI, and ligated to the rest of the LbPrx1m ORF (either wt or mutated versions of the gene), obtained by PCR amplification with PfuTurbo and primers P3 and P4 from the plasmids pET28a-His-TEV-LbPrx1m wt or mutants. The accuracy of all constructs was verified by DNA sequencing at GATC Biotech (Germany). Before transfection of L. infantum, pSSU-PHLEO-infantum-LbPrx1m constructs were linearized by digestion with NdeI and PmeI and purified from agarose gels.

**Transfection of L. infantum and isolation of mutants** — Transfections were carried out on L. infantum promastigotes (MHOM MA67ITMAP263) missing both Prx1m alleles (i.e. Prx1m null mutants or Prx1m-), previously produced (2). Parasites were grown to the logarithmic phase and electroporated at 450 V and 350–400 µF with 5 µg of DNA as described elsewhere (51). Transfectants were allowed to recover in culture medium without selective drug for 24 hours, prior to being plated onto agar plates containing 17.5 µg mL⁻¹ bleomycin (Sigma-Aldrich). Upon 1 to 2 weeks of growth on agar, colonies were picked up, transferred to liquid medium and analyzed by PCR, western blot and indirect immunofluorescence to confirm LbPrx1m expression in the transfectants, according to previously described procedures (2,52).

**Thermotolerance assays** — L. infantum promastigotes, previously synchronized by 3 to 4 daily changes of culture medium, were seeded at 10⁶ cells mL⁻¹ in 24 well plates containing RPMI 1640 Glutamax medium, supplemented with 10% inactivated fetal bovine serum (FBSi), 50 U mL⁻¹ penicillin, 50 mg mL⁻¹ streptomycin (all from Gibco) and 20 mM HEPES sodium salt (pH 7.4) (Sigma). Parasites were allowed to grow for 4 days at either 25 or 37 ºC. Every 24 hours, cell densities were determined with a Neubauer-counting chamber for growth curve determination. Two independent clones were analyzed for each transfectant.

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**Conflict of interest**

The authors declare that they have no conflicts of interest with the contents of this article.

**Footnotes**

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Regulatory Mechanisms of Mitochondrial 2-Cys Peroxiredoxins

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2The abbreviations used are: aSEC, analytical size-exclusion chromatography; CD, circular dichroism; c, cytoplasmic; Cp, peroxidatic cysteine; Cr, resolving cysteine; DLS, dynamic light scattering; DSF, differential scanning fluorimetry; FF, fully folded; LbPrx1m, mitochondrial 2-Cys peroxiredoxin from Leishmania braziliensis; LiPrx1a, cytosolic 2-Cys peroxiredoxin from Leishmania infantum; LiPrx1m, mitochondrial 2-Cys peroxiredoxin from Leishmania infantum; LiTR, trypanothione reductase from L. infantum; LiTXN1, cytosolic tryparedoxin from Leishmania infantum; LU, locally unfolded; m, mitochondrial; Prx, peroxiredoxin; SAXS, small angle X-ray scattering; SEC, size-exclusion chromatography; TXNPx, tryparedoxin peroxidase; Trx, thioredoxin; TrxR, thioredoxin reductase; Tc, Trypanosoma cruzi; wt, wild-type.

Author contributions
MABM, POG, TACBS, HC, RVH, PSLO, LESN, AMT and MTM conceived and designed the experiments. MABM, POG, TACBS, HC and RVH performed the experiments. MABM, POG, TACBS, HC, RVH, PSLO, LESN, AMT and MTM analyzed the data. MABM, POG and MTM wrote the paper. MABM, POG, TACBS, HC, RVH, PSLO, LESN, AMT and MTM revised the paper.

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### Tables

#### Table 1. DLS analysis of *LbPrx1m* samples pre-treated with CaCl$_2$ (I) and then incubated during 10 min with EDTA (II) to illustrate the reversibility of the decamerization process. Note that the hydrodynamic radius ($R_H$) of the protein decreases upon the addition of EDTA in samples pre-treated with CaCl$_2$. Pd = polydispersity.

| Condition        | $R_H$ (nm) | Pd (%) | Mass (%) |
|------------------|------------|--------|----------|
| I (25 mM CaCl$_2$) | 6.5        | 9.7    | 83.6     |
| II (I+50 mM EDTA) | 3.1        | 11.9   | 99.6     |

#### Table 2. DLS analysis of *LbPrx1m* pretreated with 20 mM of several divalent cations in 25 mM Tris-HCl pH 7.5 and 5 mM EDTA. Note that only the additives CaCl$_2$ and MgCl$_2$ led to a monodisperse protein size distribution (Pd < 20 %) in which most of the enzyme population ($\geq$ 80 % mass) displayed an average $R_H$ (~ 6 nm) compatible with the decameric structure ($R_G = 5$ nm). Zn$^{2+}$, in particular, induced the formation of larger aggregates besides the species of $R_H$ ~ 9 nm, probably due to non-specific interactions with the enzyme.

| Salt     | $R_H$ (nm) | Pd (%) | Mass (%) |
|----------|------------|--------|----------|
| CaCl$_2$ | 5.9        | 8.0    | 90       |
| MgCl$_2$ | 5.6        | 10.1   | 80       |
| NiCl$_2$ | 6.0        | 30.9   | 55       |
| FeCl$_2$ | 4.6        | 29.3   | 50       |
| MnCl$_2$ | 4.6        | 86.4   | 25       |
| ZnCl$_2$ | 9.2        | 11.9   | 10       |
FIGURE LEGENDS

Fig 1. Ca\(^{2+}\) and Mg\(^{2+}\) ions induce the decamerization of LbPrx1m. (a) aSEC chromatograms of oxidized LbPrx1m at 130 µM (2 mL) in a HiLoad Superdex 200 16/600 column pre-equilibrated with Tris buffer (pH 7.5) containing 25 mM CaCl\(_2\), 25 mM MgCl\(_2\), 5 mM EDTA, or 5 mM EGTA. (b) Experimental SAXS curves (open circles with error bars) and theoretical scattering profiles (lines) computed from the P(r) function (inset) of oxidized LbPrx1m (108 µM) in Tris buffer (pH 7.5) containing 25 mM CaCl\(_2\) (blue) or 5 mM EDTA (pink). (c) Radius of gyration (R\(_G\)) for LbPrx1m incubated with EDTA or CaCl\(_2\), calculated independently from SAXS curves (graph in b), P(r) function (inset in b) or crystallographic data. R\(_G\) (Crystal) was calculated from the scattering profiles computed for the crystal structures (PDB accession codes 4KB3 and 4KCE) using Crysol. The crystal structures of dimer (pink) and decamer (blue) are fitted into the respective envelopes calculated from SAXS data. (d) Fluorescence anisotropy data of oxidized LbPrx1m pre-incubated at 80 µM with increasing concentrations of MgCl\(_2\) or CaCl\(_2\). The lines represent the non-linear data fitting (Boltzmann sigmoid function) used to estimate the K\(_{1/2}\), i.e. the Mg\(^{2+}\) or Ca\(^{2+}\) concentration in which 50% of dimers are converted to decamers. All measurements were performed in triplicate. (e) aSEC chromatograms of LbPrx1m at 100 µM (200 µL) in Tris buffer (pH 7.5) containing 700 µM MgCl\(_2\) and 200 nM CaCl\(_2\) (solid lines) or 90 µM CaCl\(_2\) (dashed lines). The protein was incubated with 2 mM DTT (blue lines) or submitted to DTT treatment followed by DTT removal and protein oxidation using 100 µM H\(_2\)O\(_2\) (orange lines). (f) Non-reducing SDS-PAGE confirming the reduced state (SH monomers) of DTT-treated samples and the oxidized state (S-S dimers) of samples pre-treated with DTT and then oxidized with H\(_2\)O\(_2\).

Fig 2. Ca\(^{2+}\) stabilizes both oxidized and reduced LbPrx1m decamers. aSEC chromatograms of LbPrx1m at different concentrations in Tris buffer (pH 7.5) containing 5 mM EDTA (a), 5 mM EDTA with 10 mM DTT (b), 25 mM CaCl\(_2\) (c), or 25 mM CaCl\(_2\) with 10 mM DTT (d). Roman numerals represent the protein concentration of the input samples (250 µL): I (94 µM), II (23 µM) and III (9 µM). Numbers above the graphic represent the molecular weight (kDa) of standard proteins used for column calibration. For this assay, the His-tag was removed using TEV protease to show that the untagged protein behaves similar to His-tagged samples upon CaCl\(_2\) and EDTA treatments (see Fig. 1a). Note that the Ca\(^{2+}\) stabilized decamers, pre-formed at 94 µM protein, did not dissociate upon protein dilution, even in the absence of DTT. Samples not treated with DTT are air-oxidized (S-S bonded).

Fig 3. Decamer stabilization of cytoplasmic 2-Cys Prx from Leishmania and some distantly related homologues is Ca\(^{2+}\) independent. SEC chromatograms of air-oxidized (a) LbPrx1m at 130 µM, (b) cytoplasmic Prx1 from L. braziliensis (LbPrx1a) at 43 µM, (c) human Prx2 at 130 µM and (d) yeast TSA1 at 43 µM loaded (2 mL) into a HiLoad Superdex 200 16/600 column pre-equilibrated with Tris buffer (pH 7.5) containing 25 mM CaCl\(_2\) or 5 mM EDTA.

Fig 4. Structural basis for the Ca\(^{2+}\)/Mg\(^{2+}\)-dependent decamerization of LbPrx1m. (a) A magnified view of the A-type interface from LbPrx1m crystal structure (PDB accession code: 4KB3) after molecular dynamics simulations in presence of Ca\(^{2+}\) (green sphere). Interfacing subunits are colored in orange and violet. For clarity purposes, we named the A-type interface regions according to the nomenclature proposed by Wood and coworkers (15) (regions I-III) and showed as sticks the residues involved in the cation-dependent mechanism of decamer stabilization: Asp76 (region I); Cys107, Asp108, Ser109 and Ser112 (region II); Lys137 and Met139 (region III). (b) Sequence alignment of A-type interface regions from LbPrx1m and two cation-independent 2-Cys Prxs, highlighting the residues predicted to play a role in cation binding.
(red box) and those predicted to determine the cation dependency of decamer stabilization (green box). (c) aSEC chromatograms of air-oxidized \textit{LbPrx1m} D108A, S109A, S112A and C107M/M139K mutants at 86 \textmu M (500 \textmu L) in Tris buffer (pH 7.5) containing 25 mM CaCl\textsubscript{2} (red lines) or 5 mM EDTA (green line). The single mutant C107M was unstable in solution and thus it was not included in our analyses. (d) aSEC chromatogram of air-oxidized \textit{LbPrx1m} wt and S112A mutant at 13 \textmu M (200 \textmu L). Note that wt protein eluted as decamer, whereas the S112A mutant eluted mainly as dimer under the same conditions. SECs were carried in 25 mM Tris-HCl pH 7.5 containing 25 mM CaCl\textsubscript{2}.

**Fig 5.** Structural comparison of the A-type interface between \textit{LbPrx1m} (a), \textit{TcPrx1a} (PDB accession code: 4LLR) (b) and human \textit{Prx2} (PDB accession code: 1QMV) (c) reveals the molecular determinants for calcium specificity in \textit{LbPrx1m}. Green or gray dashed lines indicate interatomic distances labeled in Å and red spheres represent water molecules. Note, in panel (b), the long distance between Asp79 and Lys108 side-chains, indicating they are not interacting in \textit{TcPrx1a} structure. Below each panel there is a schematic representation of the A-type interface of \textit{LbPrx1m}, \textit{TcPrx1a} and human \textit{Prx2} highlighting the changes observed in the regions II and III (grey box), which determine the Ca\textsuperscript{2+} dependence of \textit{LbPrx1m} decamerization at physiological pH.

**Fig 6.** Ca\textsuperscript{2+} and Mg\textsuperscript{2+} ions enhance both peroxidase and chaperone activities of \textit{LbPrx1m}. (a) Relative peroxidase activity of \textit{LbPrx1m} untreated, pretreated with 5 mM EDTA only, pretreated with 5 mM EDTA followed by addition of 25 mM CaCl\textsubscript{2} or MgCl\textsubscript{2}, and pretreated with 20 mM CaCl\textsubscript{2} or MgCl\textsubscript{2} alone. (b) Relative peroxidase activity of \textit{LbPrx1a} prepared as described for \textit{LbPrx1m}. The absolute peroxidase activity values (\textmu mol NADPH.min\textsuperscript{-1}.mg\textsuperscript{-1}) were 0.18 ± 0.04 (for untreated \textit{LbPrx1m}) and 5.12 ± 1.19 (for untreated \textit{LbPrx1a}). (e) Comparison between relative peroxidase activity of \textit{wt LbPrx1m} and mutants pretreated with 5 mM EDTA followed by addition of 25 mM CaCl\textsubscript{2}. (d) Relative chaperone activity of air-oxidized \textit{LbPrx1m} untreated and pretreated with 5 mM EDTA only or 5 mM EDTA followed by addition of 25 mM CaCl\textsubscript{2} or MgCl\textsubscript{2}. The same treatments were carried out with the protein reduced using 2 mM DTT. Comparison between relative chaperone activity of \textit{wt LbPrx1m} and mutants pretreated with 5 mM EDTA followed by addition of 25 mM CaCl\textsubscript{2} without DTT (air-oxidized) (e) or with 2 mM DTT (reduced) (f). *p<0.1, **p<0.05, ***p<0.01. All experiments were performed in triplicate.

**Fig 7.** The influence of low pH, CaCl\textsubscript{2}, DTT or temperature on the hydrodynamic behavior of the mutants H113A and D108A, and \textit{wt LbPrx1m}. SECs of H113A (a), D108A (b) and \textit{wt LbPrx1m} (c) were carried out with proteins at 48 \textmu M (250 \textmu L) in MMT buffer pH 4.0 and 10 mM EDTA (dash-dotted line); 25 mM Tris-HCl pH 7.5 and 25 mM CaCl\textsubscript{2} (solid line); or 25 mM Tris-HCl pH 7.5, 5 mM EDTA containing 2 mM DTT (dotted line). Chromatograms of mutant H113A and \textit{LbPrx1m} at pH 4.0 as well as \textit{LbPrx1m} at pH 7.5 plus 2 mM DTT were previously shown in (9) and are represented here for comparison purposes. DLS analysis at 25 and 42 °C of H113A (d), D108A (e) and \textit{wt LbPrx1m} (f) were performed with protein samples at 100 \textmu M pretreated with 5 mM EDTA, followed by addition of 25 mM CaCl\textsubscript{2} in Tris buffer (pH 7.5). Vertical gray lines represent the mean radius estimated for \textit{wt LbPrx1m} decamers. Samples not treated with DTT are air-oxidized (S-S bonded).

**Fig 8.** Biophysical analyses of \textit{LbPrx1m} dimers and decamers. Circular dichroism spectra of \textit{wt LbPrx1m} and mutant H113A pretreated with 5 mM EDTA, followed by addition of 25 mM CaCl\textsubscript{2} and 2 mM DTT (a, g), only 25 mM CaCl\textsubscript{2} (S-S bonded) (b, h), or without further additives (air-oxidized) (e) at 25 °C (green) and 42 °C (pink). DSF data of reduced (+DTT) or air-oxidized (-DTT) \textit{LbPrx1m} (c and f) as well as mutant H113A (i) treated as described for the CD experiments. In these assays, \textit{wt LbPrx1m} data in presence of Ca\textsuperscript{2+} reflect...
the CD and DSF profiles of decamers while those of wt *LbPrx1m* in presence of EDTA or those of the mutant H113A represent the behavior of dimers. The calculated melting temperatures are color coded according to the respective curves. Note, at panel (e), that the dashed line indicates the temperature of the chaperone assay, in which reduced decamers expose hydrophobic patches, whereas those oxidized remain almost completely folded.

**Fig 9.** The disulfide bond between C\(_P\) and C\(_R\) probably suppresses the unfolding of helices α2 and/or α6 at 42 °C. (a) Cartoon representation of *LbPrx1m* crystallographic decamer highlighting the helices α2 (pink) and α6 (violet). (b) Magnified view of the region boxed in panel a, showing the disulfide bond between C\(_P\) (grey carbon atoms) and C\(_R\) (green carbon atoms). The C-terminal extension harboring C\(_R\) is colored in green.

**Fig 10.** *Leishmania* growth at 37 °C depends on *Prx1m* decamerization. (a) Indirect immunofluorescence of *L. infantum* (WT), *L. infantum* knockout for *LiPrx1m* (*prx1m*), and *prx1m* complemented with *LbPrx1m*H113A (clones 5 and 8), *LbPrx1m*D108A (clones 6 and 7) and wt *LbPrx1m* (clones 1 and 4). Parasites were incubated with anti-*Prx1m* antibody (green), merged with DAPI (blue). n = nucleus and k = kinetoplast. (b) Western blot using anti-*LiPrx1m* antibody (53) of *L. infantum* WT, *prx1m*, and the transfectants. A total of 20 µg of protein extracts were loaded per lane. *Leishmania* growth curves at 25 °C (c) and 37 °C (d) for WT, *prx1m* and transfectants. The experiments were performed in duplicate for *L. infantum* WT and *prx1m*, and triplicate for the transfectants. (e) Statistical analysis of *Leishmania* relative growth at 37 °C considering the day 4 represented in (d), *p*<0.1, **p**<0.05, ***p***<0.01. (f) *In vitro* chaperone activity of mutant D108A in presence of 5 mM EDTA or 5 mM EDTA followed by addition of 25 mM CaCl\(_2\), with or without 2 mM DTT, in Tris buffer pH 7.5 (*p*<0.1). All experiments were performed in triplicate. Relative activities were calculated with respect to the activity of wt protein in presence of Ca\(^{2+}\) or Ca\(^{2+}\) plus DTT, according to the absence or presence of DTT in the tested condition. Samples not treated with DTT are air-oxidized (S-S bonded).

**Fig 11.** Stabilization of region I in the closed conformation is required to maintain the fully folded conformation of C\(_P\)-loop upon C\(_P\) reduction. 3D alignment of the loop-helix active-site motif (region I + C\(_P\)-loop) of *LbPrx1m* oxidized dimer (shades of orange; PDB accession 4KCE), *LbPrx1m* oxidized decamer (shades of orange; PDB accession 4KB3) after molecular dynamics simulations with Ca\(^{2+}\)-bound (green sphere) at the A-type interface (grey surface), and reduced decamer *TcPrx1a* (shades of pink; PDB accession 4LLR). Note that region I adopts an open conformation in dimers, but it assumes a closed conformation in the oxidized and reduced decamers. According to our data, stabilization of region I in the closed conformation requires at least an H-bond between Asp76 and His113 as well as Ca\(^{2+}\) (green sphere), Mg\(^{2+}\) or (less efficiently) water molecule at the A-type interface. The establishment of such interactions is crucial to maintain the fully-folded (FF) form of the reduced C\(_P\)-loop. In the *LbPrx1m* dimer, the high entropy of the region I - trapped in an open conformation in chain B, but disordered in chain A - might propagate to the adjacent C\(_P\)-loop, hampering the stabilization of FF conformation required for substrate binding and catalysis.

**Fig 12.** Stabilization of region I in the closed conformation is mediated by Ca\(^{2+}\)/Mg\(^{2+}\) in mitochondrial Prx1 from *Leishmania* species and by water molecule in other AhpC/Prx1 subfamily members. (a) Sequence alignment of regions I, II and III from the A-type interface, as well as the C\(_P\)-loop, from mitochondrial (Prx1m) and cytoplasmic (Prx1a) 2-Cys Prxs from trypanosomatids with known crystallographic decamers. Note that residues involved in cation binding in *LbPrx1m* are highly conserved in the AhpC/Prx1 subfamily (red boxes). However, only the mitochondrial enzymes from *Leishmania* (black box) conserve the residues Cys107, Lys137 and Met139 (green boxes) that render decamer stabilization of *LbPrx1m* highly sensitive
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to Ca\textsuperscript{2+} and Mg\textsuperscript{2+} ions (green circle). In other 2-Cys Prxs, Lys137 is replaced by neutral polar or hydrophobic residues, or kept away from Asp108 by residues bulkier than Cys107. In most analyzed structures of AhpC/Prx1 subfamily members (blue circles), a water molecule mediates the link between Asp76 main chain and Asp108, Ser109 and eventually Ser112 side-chains. In few cases, solvent molecules are not observed in the crystallographic structures due to the low resolution of data (open circles). \(Lb = L.\) braziliensis; \(Li = L.\) infantum; \(Lp = L.\) panamensis; \(Ld = L.\) donovani; \(La = L.\) amazonensis; \(Lm = L.\) major; \(Tv = T.\) vivax; \(Tb = T.\) brucei; \(Tc = T.\) cruzi; \(Tco = T.\) congolense; \(Ac = Ancylostoma\) ceylanicum; \(Hs = H.\) sapiens; \(Bt = Bos\) taurus; \(Pe = Pseudoschiaena\) crocea; \(Mm = Mus\) musculus; \(Sm = Schistosoma\) mansoni; \(Pv = Plasmodium\) vivax; \(Sc = Saccharomyces\) cerevisiae; \(Hp = Helicobacter\) pylori; \(Mt = Mycobacterium\) tuberculosis; \(Se = Salmonella\) enterica. (b) 3D alignment of cation-independent 2-Cys Prxs showing the highly conserved water that links the Asp/Ser cluster at the A-type interface. Note the high conservation at positions 108 and 109 in contrast with the higher variability at positions 112 (facultative role in cation or water coordination) and 76 (main-chain involved in cation/water binding). PDB accession codes are between parentheses.

Fig 13. Physiological role of Mg\textsuperscript{2+}/Ca\textsuperscript{2+}, pH and redox state in the maintenance of the chaperone reservoir of mitochondrial Prx1 in Leishmania. During the peroxidase cycle, Leishmania Prx1m transits between dimeric (chaperone-inactive) and decameric (chaperone-active) forms. This oligomeric shift is regulated by conformational changes of two regions from the loop-helix active-site motif: the C\(\alpha\)-loop (LU \(\Leftrightarrow\) FF), which is redox-sensitive, and the region I (open \(\Leftrightarrow\) closed), which is Ca\textsuperscript{2+}/Mg\textsuperscript{2+}/pH-sensitive. When dimers are reduced (SH), they tend to form weak decamers, in which free Mg\textsuperscript{2+} and Ca\textsuperscript{2+} ions bind to enhance its peroxidase and chaperone functions. In basal conditions, after the peroxidation (I) and resolution (II) steps, oxidized (S-S) decamers tend to release Mg\textsuperscript{2+} and Ca\textsuperscript{2+} ions and dissociate into dimers, a process that involves an intermediate state, named here transitional A-type interface. However, under heat-stress conditions, two stimuli can boost the chaperone reservoir of Prx1m, mainly by stabilizing oxidized decamers: small pH decreases (red arrows) and Ca\textsuperscript{2+} overload (green arrows). Since our data cannot discriminate what is the preferable substrate of TXN (Prx1m dimers or decamers), we labeled the C\(\alpha\) reduction step with a question mark. The same is applicable for the conformational state proposed for the active site in reduced dimers and reduced weak decamers. For clarity purposes, the lockers illustrating the conformational state of region I are only showed for a pair of dimers at the decamers.
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FIGURE 1

a) Abs at 280 nm (mAU) vs. Elution volume (mL)

b) q (nm²) (arbitrary units) vs. q (nm²)

FIGURE 2

a) Abs at 280 nm (mAU) vs. Elution volume (mL)

b) Abs at 280 nm (mAU) vs. Elution volume (mL)

c) Abs at 280 nm (mAU) vs. Elution volume (mL)

d) Abs at 280 nm (mAU) vs. Elution volume (mL)
FIGURE 3

(a) LbPrx1m
(b) LbPrx1a
(c) HsPrx2
(d) ScTSA1
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FIGURE 4

a

b

| I   | C-loop | II | III |
|-----|--------|----|-----|
| LbPrx1m | MEPT/VCFPT... | 75 | 119 | 137 |
| TcPrx1a | MEPT/VCFPT... | 84 | 119 | 137 |
| HsPrx2 | MEPT/VCFPT... | 107 | 119 | 137 |

A-type

C

decamer  dimer

Abs. at 280 nm (mAU)

Elution volume (mL)

30

25

20

15

10

5

50

40

30

20

10

0

60

50

40

30

20

10

0

FIGURE 5

a LbPrx1m

b TcPrx1a

c HaPrx2

hydrophobic or neutral residues
positively charged residues

Ca\(^{2+}\)/Mg\(^{2+}\)
electrostatic attraction of D108 side chain

Ser/Asp cluster cross-bridged by Ca\(^{2+}\)/Mg\(^{2+}\) or water

water
FIGURE 6

a) **LbPx1m**
- Relative peroxidase activity
- No treatment, EDTA, EDTA-Ca^2+, Ca^2+, EDTA-Mg^2+, Mg^2+

b) **LPx1a**
- Relative peroxidase activity
- No treatment, EDTA, EDTA-Ca^2+, Ca^2+, EDTA-Mg^2+, Mg^2+

c) **LdPx1m**
- Relative peroxidase activity
- LbPx1m, H15A, D108A, S109A, S112A

d) **LbPx1m**
- Relative chaperone activity
- No treatment, EDTA, EDTA-ODT, EDTA-Ca^2+, EDTA-Mg^2+, EDTA-Mg^2+/ODT

e) Relative chaperone activity
- LbPx1m, H15A, D108A, S109A, S112A
- - DTT

f) **LbPx1m**
- Relative chaperone activity
- No treatment, EDTA, EDTA-ODT, EDTA-Ca^2+, EDTA-Mg^2+, EDTA-Mg^2+/ODT
- + DTT

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FIGURE 7

a) H113A
   - pH 4.0
   - pH 7.5 + CaCl₂
   - pH 7.5 + DTT

b) D108A
   - pH 4.0
   - pH 7.5 + CaCl₂
   - pH 7.5 + DTT

c) LbPtx1m
   - pH 4.0
   - pH 7.5 + CaCl₂
   - pH 7.5 + DTT

d) H113A + Ca²⁺
   - 25°C
   - 42°C

e) D108A + Ca²⁺
   - 25°C
   - 42°C

f) LbPtx1m + Ca²⁺
   - 25°C
   - 42°C

FIGURE 8

a) LbPtx1m + Ca²⁺ + DTT
   - 25°C
   - 42°C

b) LbPtx1m + Ca²⁺
   - 25°C
   - 42°C

C) Unfolded fraction (%)
   - 38°C
   - 53°C

D) Unfolded fraction (%)
   - 44°C
   - 56°C

E) Unfolded fraction (%)
   - 44°C
   - 62°C

F) Unfolded fraction (%)
   - 44°C
   - 62°C

G) H113A + Ca²⁺ + DTT
   - 25°C
   - 42°C

H) H113A + Ca²⁺
   - 25°C
   - 42°C

I) H113A + Ca²⁺
   - 44°C
   - 62°C
FIGURE 9

(a) 

(b)
### FIGURE 12

#### a

|       | I         | II          | III         |
|-------|-----------|-------------|-------------|
| LpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| LiPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| lpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| lpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| lpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| lpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| lpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| lpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| lpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |
| lpPrx1m | MFTFCPETD...LSEYHSLAWNT...KRM |             |             |

#### b

![Image of a diagram](http://www.jbc.org/Downloaded from)
FIGURE 13

Regulatory Mechanisms of Mitochondrial 2-Cys Peroxiredoxins
Calcium and Magnesium Ions Modulate the Oligomeric State and Function of Mitochondrial 2-Cys Peroxiredoxins in Leishmania Parasites

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