Chaperone activation and client binding of a 2-cysteine peroxiredoxin

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Many 2-Cys-peroxiredoxins (2-Cys-Prxs) are dual-function proteins, either acting as peroxidases under non-stress conditions or as chaperones during stress. The mechanism by which 2-Cys-Prxs switch functions remains to be defined. Our work focuses on \textit{Leishmania infantum} mitochondrial 2-Cys-Prx, whose reduced, decameric subpopulation adopts chaperone function during heat shock, an activity that facilitates the transition from insects to warm-blooded host environments. Here, we have solved the cryo-EM structure of mTXNPx in complex with a thermally unfolded client protein, and revealed that the flexible N-termini of mTXNPx form a well-resolved central belt that contacts and encapsulates the unstructured client protein in the center of the decamer ring. In vivo and in vitro cross-linking studies provide further support for these interactions, and demonstrate that mTXNPx decamers undergo temperature-dependent structural rearrangements specifically at the dimer-dimer interfaces. These structural changes appear crucial for exposing chaperone-client binding sites that are buried in the peroxidase-active protein.
Peroxiredoxins are ubiquitous, highly abundant proteins found in every biological kingdom. Best known for their ability to detoxify a variety of different peroxides, peroxiredoxins act as general antioxidants, sophisticated regulators of peroxide-dependent cell signaling pathways and thiol oxidases. The catalytic activity of 2-Cys-peroxiredoxins (from hereon abbreviated as Prx), which comprise the Prx1 family, is mediated by the active site peroxidatic cysteine, which reacts with peroxide and related oxidants, and undergoes reversible sulfenic acid formation. Attack by a second cysteine that is located in the other subunit of the Prx-dimer leads to the formation of a disulfide bond, which is typically resolved by the thioredoxin system to enable another catalytic cycle.

Peroxiredoxins have long been known to undergo major reversible changes in their quaternary structure during redox cycling. The basic structural unit of Prx is a homo-dimer, in which two subunits are organized in a head-to-tail orientation, stabilized through the antiparallel arrangement of two β-strands (i.e., B-type or β-sheet based interface). In the reduced state, most Prx1-family members associate into donut-shaped ring-like decamers. The active site cysteine-containing Cp-loop-helix adopts a closed conformation through an elaborate network of electrostatic interactions, thereby exposing critical aromatic amino acids that pack tightly against the other dimer, stabilizing the dimer-dimer interface (i.e., A-type or alternate interface). Upon oxidation of the active site cysteine, the Cp-loop-helix transitions into a more open conformation (i.e., unfolded state), which leads to the dissociation into oxidized dimers. Overoxidation of the active site cysteine to sulfonic acid has been shown to cause the formation of even higher molecular weight oligomeric structures, including filamentous or spherical structures.

These higher oligomeric structures were reported to take on a peroxidase-independent second function as molecular chaperones, which protect cells against stress-induced protein unfolding, and serve as an integrated member of the eukaryotic proteostasis network during distinct stress conditions. Other conditions that trigger the functional switch from a peroxidase to a chaperone through changes in the oligomeric status include exposure to low pH or phosphorylation events in the Cp-loop-helix.

We recently reported that the mitochondrial 2-Cys Prx of Leishmania infantum (mTXNPx, Prx1m) also adopts two functions, as a peroxidase and as a molecular chaperone. However, in contrast to previous studies with cytosolic Prx from yeast or mammalian cells, we found that neither overoxidation of the active site cysteine nor the formation of higher oligomeric structures were necessary to convert the peroxidase into a chaperone. In fact, our data revealed that reduced mTXNPx decamers alone serve as an effective chaperone reservoir when exposed to physiologically relevant heat shock conditions. Once activated by elevated temperatures, reduced mTXNPx decamers protect a range of proteins from heat-induced aggregation. In vitro and in vivo, two separate studies came to similar conclusions and showed that both plant C2C-Prx1 as well as mitochondrial Prx from the anaerobic archaeon Thermococcus kodakaraensis serve as molecular chaperones specifically under heat shock conditions. Upon return to non-stress heat conditions, mTXNPx then transfers its client proteins to ATP-dependent chaperones for proper refolding, suggesting that mTXNPx acts as chaperone holdase. Expression studies using an mttxnpx Δ deletion strain of L. infantum confirmed the physiological significance of this chaperone activity. In contrast to mutant parasites expressing wild-type mTXNPx, strains that express a chaperone-inactive but peroxidase-active variant of mTXNPx were unable to deal with the extensive protein unfolding that they experience when they are forced to adjust to the body temperature of mammals. As a result, these strains failed to propagate in mammalian hosts.

To obtain insights into the structural basis and mechanism of mTXNPx chaperone activity, we have now determined the cryo-EM structures of reduced, heat-activated mTXNPx with and without a bound model client protein to 3.7 and 2.9 Å resolution, respectively. We resolved most of the missing N-terminal residues of mTXNPx, and discovered that the N terminus forms a well-resolved central belt surrounding an unstructured and highly flexible client protein in the center of the ring. Our in vivo and in vitro cross-linking studies revealed additional nearby residues that are potentially involved in client binding, and provide new insights into the mechanism by which heat-induced structural rearrangements trigger the activation of mTXNPx chaperone function.

Results

Cryo-EM structure of mTXNPx-red: luciferase complexes. In our previous work, we used negative-stain EM to visualize complexes formed between reduced mTXNPxs decamers and thermally unfolded luciferase. These studies revealed additional density in the center of the decameric ring structure of mTXNPx-red in the presence of the client protein but not in its absence. However, the structures lacked the resolution necessary for detailed analysis. Therefore, we sought to determine 3D structures of mTXNPx-red by cryo-EM in order to identify the structural basis for mTXNPx-red-client interactions. Cryo-EM datasets were collected for heat-activated mTXNPx-red in the presence or absence of the luciferase model client. 2D class averages revealed well-defined features of the decamer ring and a globular, central density that was present only after incubation with thermally unfolded luciferase. In side views of the structures solved in the presence of luciferase, the ring appeared slightly wider in the center (arrow, Fig. 1a). This new density did not extend beyond the plane of ring, suggesting that the bound luciferase was either restricted to the region surrounded by the decamer or was too flexible to resolve. Both the architecture and the diameter of the mTXNPx-red decameric ring appeared similar with or without bound luciferase, thus indicating that it is unlikely that major conformational rearrangements occur upon client binding. Notably, the dataset for mTXNPx-red incubated with luciferase contained a mixture of both luciferase bound and luciferase free mTXNPx-red decamers. By comparison of the top-view 2D averages, we determined that ~60% of mTXNPx-red was luciferase-bound under these conditions. 

We determined 3D reconstructions of mTXNPx-red incubated with and without luciferase, which yielded structures at 3.7 and 2.9 Å estimated resolution, respectively (Fig. 1b, c; Supplementary Figure 1C and Table 1). In the final sharpened map, additional central density corresponding to the bound luciferase client was not present at threshold values of >2.0 sigma. However, this density was clearly present in the unsharpened map and in 2D projections of the reconstruction (Fig. 1d and Supplementary Figure 1D). The overall size of the central density attributed to luciferase was consistent with a single 63 kDa luciferase molecule. This conclusion was supported by analytical ultracentrifugation experiments (Supplemental Methods), which showed that one molecule of luciferase is bound per decamer (Supplementary Figure 1E). Angular distribution analysis confirmed that while the top-view orientation of the ring was prefered, additional
orientations, including side-views were also present (Supplementary Figure 1F). Local resolution analysis identified uniform higher resolution (~3.6 Å) for the core of the mTXNPx ring and lower resolution regions for the inner face of the ring (i.e., 4.4 Å), indicating structural flexibility in proximity to the bound client (Supplementary Figure 1G). A homology model from Leishmania braziliensis (pdb: 4KB3), a close homolog of L. infantum, was used for model building and the final model showed good agreement with the cryo-EM density for the core regions of the decamer (Supplementary Figure 1H).

Based on our previous negative-stain EM analysis, we proposed that the N-terminal residues, which are present following cleavage of the mitochondrial targeting sequence (aa 1–27), serve as flexible extensions that likely contact the client in the interior of the decamer ring. However, the first eight residues (aa 28–35) of the N-terminus are not resolved in the dimeric and decameric X-ray crystal structures of L. braziliensis Prx1m. Moreover, no other structures of peroxiredoxins have N-termini resolved that extend into the interior of the ring, similar to what we previously observed. Remarkably, in the cryo-EM structure of client-bound mTXNPxred, we identified an additional inner ring of density that extends from position T35 and encapsulates the client density (Fig. 1b, d). Residues Q31-T35 of mTXNPx were modeled into this density and identified to extend from each peroxiredoxin monomer at the dimer interface to form the inner ring. This inner ring is distinct from the larger decamer structure and consistent with the spoke-like structures that we previously observed.

**Table 1 Summary of atomic modeling statistics and validation scores**

|                      | mTXNPx-apo | mTXNPx-Luciferase |
|----------------------|------------|-------------------|
| Amino acid residuesa | 35–199     | 31–204            |
| RMS deviations       |            |                   |
| Bond lengths (Å)     | 0.007      | 0.009             |
| Bond angles (°)      | 0.954      | 0.984             |
| Ramachandran         |            |                   |
| Favored (%)          | 94.05      | 92.44             |
| Allowed (%)          | 5.95       | 7.56              |
| Outliers (%)         | 0          | 0                 |
| Molprobity score     | 1.72       | 1.85              |
| EMRinger score        | 2.55       | 3.23              |

*aMitochondrial signal sequence: aa1-27*
Although we were unable to build a model for this entire region, this inner ring of density is consistent with the eight N-terminal amino acids of the mature peroxiredoxin that were not previously resolved. This N-terminal ring was not observed in the cryo-EM reconstruction of apo mTXNPyred at an equivalent density threshold, indicating that the presence of client likely stabilizes these residues (Fig. 1c).

Based on the position of the N-terminal extensions, residues Y33 and R34 appear to contact the bound client along with additional residues in the N-terminal strand of the chaperone that were unable to be modeled (Fig. 1e). For these reconstructions, D5 symmetry was imposed due to the known symmetry of the mTXNPyred decamer. When refinements were performed without imposing symmetry (C1), the density projections remained present, indicating that all subunits likely make bona fide contact with the client, although some variability is observed (Supplementary Figure 1I). In an attempt to better define the client interactions and improve the resolution of the luciferase density, focused refinement was performed using a mask to exclude the outer ring of the decamer (Supplementary Figure 1I). However, density for the bound luciferase did not improve for either symmetrized or unsymmetrized reconstructions. Thus, we conclude that the luciferase client is likely bound heterogeneously and in an unstructured state that, as previously shown, is competent for re-folding.

In the mTXNPyred reconstruction in the absence of luciferase, density corresponding to residues R34 and T35 was present as well (Fig. 1b, c). However, we found that these projections were less prominent than in the client-bound complex. The position of this density is identical in both maps, suggesting that these positions do not rearrange, but instead become more ordered upon client binding. In summary, these structures reveal high-resolution views of a chaperone-active peroxiredoxin in complex with a bound client. We found that the N-terminal extensions of mTXNPyred become ordered upon client binding, and directly contact and encapsulate the client. This likely stabilizes the chaperone in an unstructured state that is competent for re-folding in the presence of the DnaK/DnaJ/GrpE system.

mTXNPy’s N-terminus is necessary for client binding in vitro. The cryo-EM structure of mTXNPyred in complex with luciferase suggested that residues in the N-terminal extensions of mTXNPy participate in client binding. To directly determine the extent to which the N-terminal residues of mTXNPy influence its chaperone function, we engineered and purified N-terminal truncation mutants, lacking either the first 5, 8 or 10 residues of the mature protein (Supplementary Figure 2A). We were unable to cleave the N-terminal histidine tag from the ∆5mTXNPy or ∆10mTXNPy proteins but successfully removed the His-tag from the ∆5mTXNPy variant. After cleavage, this mutant variant lacks the five N-terminal aa N_{20}LDY_{33} from the mature protein but still contains the N-terminal 3-aa scar sequence (i.e., Gly-Ser-His), which is a remnant of the thrombin cleavage and present in all of our purified mTXNPy proteins (Supplementary Figure 2A).

In vitro activity assays revealed that this N-terminally truncated mutant variant showed near wild-type peroxidase activity at 30 °C (Supplementary Figure 2B). In contrast, ∆5mTXNPyred was at least 70% less active as a molecular chaperone in our in vitro aggregation assays as compared to wt-mTXNPy (Fig. 2a). Whereas a 20-fold molar excess of wt mTXNPyred over luciferase completely blocked aggregation, a 40-fold molar excess of the ∆5mTXNPyred variant was necessary to at least partially suppressed luciferase aggregation. These results agreed well with our cryo-EM data and further illustrated the importance of mTXNPy’s N-terminal extensions in chaperone function.

Two other residues, i.e., Y33 and R34, also appeared to be involved in client interactions according to our cryo-EM structure but were not covered by our 5-aa truncation. To directly test their role in in vitro client binding, we substituted the respective residues individually with Ala, purified the two mutant variants (mTXNPyY33A, mTXNPyR34A) as before, and tested for their chaperone activity. We found that while replacing the bulky hydrophobic tyrosine with the smaller alanine slightly decreased the chaperone activity of mTXNPy (Fig. 2b, left panel), substituting the charged R34 with Ala significantly increased the ability of mTXNPy to prevent luciferase aggregation (Fig. 2b, right panel). These mutations only mildly (Y33A) or not significantly (R34A) altered the peroxidase activity at 30 °C (Supplementary Figure 2B). Finally, we tested the chaperone activity of the two L. infantum cytosolic peroxiredoxins isoforms, which are highly homologous to the mitochondrial protein but lack the N-terminal 4 aa sequence “NDY” and harbor “CG” instead of “YR” at positions 33/34 (Supplementary Figure 2C). As shown in Supplementary Figure 2D, neither one of the two cytosolic homologs showed any detectable activity in the in vitro chaperone assay. In summary, these results revealed that truncating the N-terminal extensions and/or altering the hydrophobicity of residues facing inside of the decameric ring, greatly affects client binding, supporting the conclusion that the flexible N-termini play a crucial role in the chaperone function of *Leishmania* mTXNPy.

![Fig. 2](https://example.com/fig2.png)

**Fig. 2** N-terminal extensions of mTXNPy are crucial for in vitro chaperone activity. Influence of reduced ∆5mTXNPy (a) or mTXNPyY33A/mTXNPyR34A (b) on the thermal aggregation of luciferase. Native luciferase (0.1μM) was incubated in the absence or presence of different ratios of mTXNPy at 42 °C. Light scattering (expressed in arbitrary units, A.U.) was monitored at 360 nm. All reactions were carried out in the presence of 0.2 mM DTT to maintain reduction of mTXNPy. Purified wt mTXNPy was used as positive control.
Incorporation of an unnatural amino acid for in vivo cross-linking. To independently evaluate mTXNPs\textsubscript{red}-client-interaction sites in the cellular context, and test whether in vivo clients engage in interactions that involve residues within and/or beyond the inner ring structure, we utilized an in vivo cross-linking technique that was originally developed by Schultz et al.\textsuperscript{20,21}. By applying site-specific mutagenesis to mTXNPs, we individually substituted each phenylalanine or tyrosine codon in the mTXNP with a TAG amber stop codon. Upon co-transformation of the mutant plasmids together with the aminoacyl-tRNA synthetase/ suppressor tRNA pair into \textit{E. coli}, and addition of the unnatural, UV-cross-linkable amino acid \textit{p}-benzoyl-\textit{L}-phenylalanine (Bpa) to the growth media, these mutant variants incorporate Bpa and hence contain potential cross-linking sites at 19 distinct sites that are distributed throughout the amino acid sequence of mTXNPs (Fig. S3A, Supplementary Figure 3A). We reasoned that mTXNPs would, in general, be likely to tolerate these substitutions since Bpa is a structural analogue of Phe/Tyr. Once activated by UV, Bpa acts as zero-length cross-linker, which interacts irreversibly with other residues but only if they are in very close proximity to each other.

Upon co-transformation of the plasmids, we tested for in vivo Bpa incorporation into mTXNPs by growing the cells in the presence or absence of exogenously-added Bpa at 30 °C for 3 h. Next, we analyzed the expression level of full-length mTXNPs by western blot analysis using anti-mTXNP antibodies. We found that the majority of our \textit{E. coli} strains expressed some measurable amounts of full-length mTXNPs in the presence of Bpa but not in its absence (Supplementary Figure 3B). The expression levels of Y63Bpa, F192Bpa, and F194Bpa were reproducibly low. The F221Bpa variant, whose Bpa substitution is located only 5 aa from the C-terminus of mTXNP, was also detectable in the absence of Bpa, making it impossible to determine whether this variant, and by extension the F222Bpa variant, contained Bpa or not.

In vitro studies have shown that mTXNPs is only activatable as chaperone when present in the reduced form.\textsuperscript{14} To assess the in vivo oxidation status of mTXNPs when expressed in the cytosol of \textit{E. coli} under both non-stress and stress conditions, we conducted in vivo thiol-trapping experiments using N-ethylmaleimide (NEM) followed by SDS-PAGE of the extracts under non-reducing conditions.\textsuperscript{4} This approach was based on previous results that revealed that under these conditions, reduced mTXNPs migrates in the monomeric form while oxidized mTXNPs migrates as a disulfide-linked dimer.\textsuperscript{22} As shown in Supplementary Figure 3C, the majority of NEM-labeled mTXNPs migrated in its monomeric form (i.e., reduced form), and no change in its oxidation status was observed upon exposure to 45 °C. We obtained very similar results with our NEM-labeled mTXNP-Bpa expressing strains, indicating that they were present in their normal form.

In all, we concluded that mTXNPs-Bpa expressing strains most likely represent cross-linked complexes between select mTXNP-Bpa variants and in vivo client proteins (mTXNP-X) (Fig. 3b).

A comparison of the cross-linking patterns and efficiencies of different mTXNP mutant variants at 30 °C and 45 °C revealed that the Bpa-variants fit into three different categories (Fig. 3b; summary of all cross-linking results can be found in Supplementary Table 1). Group 1 Bpa variants (F45, Y67, F71, F72, Y73, F77, F79, F88, and F95) reproducibly showed a significant increase in the amount of higher-migrating bands upon incubation at 45 °C as compared to what was found at 30 °C (Fig. S3, indicated by red bars). This suggests that these residues are the most important for temperature-dependent in vivo client binding. Group 2 Bpa variants formed irreversible cross-links with other proteins upon UV-exposure independent of the incubation temperature (Fig. 3b, indicated by magenta bars). Surprisingly, the only variants that behaved in this manner were those that contained Bpa substitutions in the two N-terminal tyrosine residues (Y30, Y33), which contact therally unfolding luciferase in vitro according to our cryo-EM model. Group 3 Bpa variants (Y63, Y111, Y145, F160, and F192) did not reproducibly show any higher migrating smear or formed only a small number of discrete higher-migrating bands (Fig. 3b, indicated by blue bar), suggesting that these residues do not usually come in direct contact with in vivo clients. One Bpa-variant (Y194) showed inconclusive cross-linking results (Supplementary Table 1) and was disregarded for further analysis.

Dimer–dimer interface serves as transient interaction site. To visualize which region(s) of mTXNP in addition to the N-terminus might be involved in in vivo client binding, we mapped...
**Fig. 3** UV-cross-linking of mTXNPx to client proteins in vivo. 

**a** Bpa substitutions in mTXNPx. Phe and Tyr residues (indicated in red) were individually mutated to allow for the site-specific incorporation of the photo-activatable amino acid Bpa. The Cp-loop-helix, which contains the peroxidative Cys81 and which is folded when mTXNPx is reduced and decameric, is underlined in red. All regions (regions I–IV) involved in dimer–dimer interactions are underlined in blue. The mitochondrial targeting sequence is underlined in black. The targeting sequence is not present in the in vivo constructs.

**b** In vivo cross-linking of mTXNPx-Bpa variants. E. coli expressing either wild-type mTXNPx or the mTXNPx-Bpa variants were grown at 30 °C and either kept at 30 °C or shifted to 45 °C for 30 min. Afterwards, aliquots of the cells were either left untreated (−) or were exposed (+) to UV light for 10 min to induce cross-linking. Subsequently, bacteria were lysed and cell extracts were analyzed by western blot using anti-mTXNPx antibodies. Two different exposure times are shown. The two most prominent bands corresponded to monomeric (mTXNPx) and dimeric mTXNPx-mTXNPx, respectively. Additional bands, seen as higher-migrating smear, are suggestive of mTXNPx-E. coli protein cross-links (mTXNPx-X). A representative experiment is shown (results from all experiments conducted are summarized in Supplementary Table 1). Based on the extent of higher migrating smear (i.e., cross-links) upon incubation at 30 °C and/or 45 °C, the residues were classified into three groups: Group 1: Bpa-variants that cross-link more extensively upon incubation at 45 °C compared to 30 °C (red); Group 2: Bpa-variants that show equal cross-linking at 45 °C and 30 °C (magenta); Group 3; Bpa-variants that either do not show any reproducible higher migrating smear, or show only a small number of discrete higher migrating bands (blue). Bpa-194, Bpa-221, and Bpa-222 variants either produced inconsistent results or were not reliably produced in vivo.
our Bpa variants onto the solved cryo-EM structure of *L. infantum* mTXNPx (Fig. 4a). For simplicity, we only show one dimeric unit of the decamer (monomers shaded in light blue or pink, respectively) encompassing the B-type monomer–monomer interface, and one neighboring subunit on each side (shaded in grey) to illustrate contacts in the A-type dimer–dimer interfaces. We applied the same color scheme we had used in Fig. 3. As expected, the two Group 2 residues, i.e., Y30, Y33 face the interior of the decameric ring (Fig. 4a, magenta) while most of the non-cross-linking Group 3 residues were buried. Three of them (i.e., Y145, Y160, and Y192) clustered in the monomer–monomer interface, explaining their ability to crosslink mTXNPx dimers but not client proteins (Fig. 4a, blue). To our surprise, however, most of our Group 1 residues, which formed higher molecular weight cross-links specifically upon shift to elevated temperatures, were also not surface accessible according to our cryo-EM structure (Fig. 4b, red and grey dots) form part of the dimer-dimer interface. Different views of the selected mTXNPx dimer are presented. Amino acids that, when replaced by Bpa, reproducibly produce higher migrating smear upon UV-cross-linking either after shift to 45 °C (Group 1, red), at both 30 °C and 45 °C (Group 2, magenta) or no shift (Group 3, blue) are shown. Close-up view of the dimer-dimer interface and location of Group 1 residues. Group 1 residues F77 and F79 (red and grey dots) form part of the dimer-dimer interface.

**Fig. 4** Mapping of in vivo cross-linking sites onto quaternary structure of mTXNPx. a Quaternary structure of reduced *L. infantum* mTXNPxred. For simplicity, only one of the five dimers that are present in the decamer (subunit A in pink and subunit B in blue) as well as one adjacent subunit on each side (grey) are shown. Different views of the selected mTXNPx dimer are presented. Amino acids that, when replaced by Bpa, reproducibly produce higher migrating smear upon UV-cross-linking either after shift to 45 °C (Group 1, red), at both 30 °C and 45 °C (Group 2, magenta) or no shift (Group 3, blue) are shown. b Close-up view of the dimer-dimer interface and location of Group 1 residues. Group 1 residues F77 and F79 (red and grey dots) form part of the dimer-dimer interface. As expected, the two Group 2 residues, i.e., Y30, Y33 face the interior of the decameric ring (Fig. 4a, magenta) while most of the non-cross-linking Group 3 residues were buried. Three of them (i.e., Y145, Y160, and Y192) clustered in the monomer–monomer interface, explaining their ability to crosslink mTXNPx dimers but not client proteins (Fig. 4a, blue). To our surprise, however, most of our Group 1 residues, which formed higher molecular weight cross-links specifically upon shift to elevated temperatures, were also not surface accessible according to our cryo-EM structure (Fig. 4b, red and grey dots) form part of the dimer-dimer interface.

In vitro cross-linking experiments support in vivo results. To independently validate these additional interaction sites and to potentially gain more insights into conformational rearrangements that mTXNPxred undergoes upon heat-induced activation and/or client binding, we conducted in vitro cross-linking experiments. We used two different primary amine-reactive cross-linkers, DiSuccinimidylSubarate (DSS) and DiSuccinimidylAdipate (DSA) followed by a tryptic digestion and MS/MS analysis. We incubated decameric mTXNPxred with thermally unfolded luciferase at 42 °C as before, separated insoluble proteins by centrifugation, and cross-linked the soluble proteins using either DSS or DSA (see Supplementary Figure 4A for cross-linking scheme). As a control, we used oxidized dimeric
mTXNPxred, which neither functions as a heat-shock activated chaperone nor undergoes conformational rearrangements upon incubation at heat shock temperatures in vitro\textsuperscript{14}. As expected, we found that only the presence of mTXNPxred was able to protect luciferase against thermal aggregation and to maintain the protein in its soluble form (Supplementary Figure 4A, lane 9). Upon cross-linking with DSS or DSA, we observed specific higher molecular weight complexes, suggestive of mTXNPxred–luciferase complexes (Supplementary Figure 4A). We excised and extracted these higher molecular-weight complexes from the gel, digested the proteins with trypsin and analyzed the peptides by MS/MS analysis (Supplementary Data 1). The experiments were designed so that data sets contain information about \textit{intra}-protein cross-links, i.e. cross-links within each of the two proteins, as well as \textit{inter}-protein cross-links that connect amine-containing residues in mTXNPxred with those of luciferase.

Analysis of \textit{intra}-protein cross-links revealed the expected cross-links between physically close lysine residues in either mTXNPxred or luciferase (Fig. 5a, Supplementary Figure 4B, purple lines; Supplementary Data 1). In luciferase, we also detected a number of cross-links that could not be explained by proximity (Supplementary Figure 4B, right panel). These longer-range cross-links (>20 residues apart in the primary sequence) support the notion that luciferase is in a partially unfolded state when bound to mTXNPxred. Analysis of the \textit{inter}-protein cross-links between mTXNPxred and luciferase revealed that the vast majority of cross-links involves residues in the N-terminus of mTXNPxred and different regions in luciferase (Fig. 5a, cyan lines; Supplementary Data 1). These findings were in excellent agreement with our previous results. In addition, however, we also identified three residues to cross-link with luciferase that were not located in the N-terminus. Although their respective Kojak scores were below our arbitrary cut-off of 2, the fact that we detected 10 different peptides involving K165, seven different peptides involving K123 and three different peptides involving K137 makes us reasonably confident that these residues in mTXNPx are actually involved in cross-linking with luciferase as well. All three residues are found in close proximity to our previously identified in vivo interaction sites (Fig. 5b, indicated in orange). While both K123 and K137 are located immediately adjacent to F77/F79 in the A-type interface, K165 localizes near Y67, another Group 1 residue (Fig. 5b). These results support our conclusion that client interactions not only engage the N-terminal extensions of mTXNPxred but also involve other regions, including residues close to or part of the dimer–dimer interface.

**Fig. 5** In vitro cross-linking of mTXNPxred-luciferase complexes. \textbf{a} \textit{Intra}-protein (purple lines) and \textit{inter}-protein (cyan lines) cross-link maps using xiNET (Supplementary Data 1). The mTXNPx sequence including the mitochondrial targeting sequence was used for the map. \textbf{b} Mapping of the in vitro cross-linking sites between mTXNPxred and luciferase (shown in orange) and the in vivo cross-linking sites between mTXNPx-Bpa variants and cellular client proteins (red: Group 1 cross-links; pink: Group 2 cross-links, Fig. 4a) onto the quaternary structure of \textit{L. infantum} TXNPxred. For simplicity, only one of the five dimers that are present in the decamer (subunit A in pink and subunit B in blue) as well as one adjacent subunit on each side (grey) are shown.

**Elevated temperatures destabilize the dimer–dimer interface.**

Both in vivo and in vitro cross-linking experiments implicated the dimer–dimer interface of mTXNPxred in client interactions. We therefore wondered whether temperature-induced activation of decameric mTXNPxred could lead to the destabilization of the dimer–dimer interface, causing potentially-buried hydrophobic regions to turn into (transient) binding sites for unfolding proteins. Upon closer inspection of the decameric structure of mTXNPxred, we identified two lysine residues (K122/K123), which are positioned in very close proximity (12.1–12.9 Å) to the two corresponding lysine residues of the neighboring dimer (Supplementary Figure 5A). We now reasoned that any significant rearrangement of the decamer might alter the extent to which these lysine residues become cross-linked by amine-reactive cross-linker (CL). We therefore decided to conduct quantitative cross-linking (qCL) experiments using the isotopically coded forms of either DSS (DSS\textsuperscript{12C\textsubscript{6}/13C\textsubscript{6}}) or DSA (DSA\textsuperscript{13C\textsubscript{6}/13C\textsubscript{6}}). As a proof-of-concept experiment, we first cross-linked dimeric mTXNPxred with heavy-labeled CL and decameric mTXNPxred with light-labeled CL at 30 °C, and mixed the samples 1:1. After tryptic digest and MS/MS analysis, we then calculated the mass spectrum signal intensity ratios of the heavy to light forms (i.e., H/L-ratios) focusing on inter-protein cross-linked peptide pairs containing the lysines of interest, i.e., K122/K123. By using DSS as CL, we identified two different inter-protein K123-K123 peptide pairs, which both revealed negative log2-ratios (i.e., −2.09 and −1.71) (Table 2). These results indicated that the light-labeled cross-link (i.e., the decameric form) is favored (a complete list of all qCL results can be found in Supplementary Data 2). Very similar results were obtained when we conducted the same experiments with the isotopically coded forms of DSA (Supplementary Table 2). These results confirmed that inter-protein cross-links between K123 residues of neighboring dimers are favored when mTXNPx is decameric and disfavored when mTXNPx is dimeric. To now test how shifting reduced mTXNPx decamers from 30 to 42 °C affects the cross-linking behavior of these neighboring residues, we incubated mTXNPxred in the presence of either the isotopically heavy or the light form of the cross-linker at 30 °C or 42 °C and mixed the samples according to the flow chart outlined in Supplementary Figure 5B. In one set-up, for instance, we combined mTXNPxred that was incubated at 42 °C and cross-linked with heavy DSS with a sample containing mTXNPxred that was incubated at 30 °C and cross-linked with light DSS (Table 2). As before, we quantified the relative changes in inter-protein cross-links after tryptic digest and mass spectrometric analysis. We identified three different K123–K122/K123 inter-protein peptides, all of which gave negative H/L log2 ratios (−1.55 to −2.57), indicating that the light-labeled cross-links (i.e., 30 °C incubation) are favored (Table 2).
These results strongly suggested that incubation of decameric mTXNPx\textsubscript{red} at heat shock temperatures weakens the dimer–dimer interface and may potentially lead to the dissociation of mTXNPx\textsubscript{red} into reduced dimers. Again, we obtained very similar results with DSA as crosslinker (Supplementary Table 2) and equivalent results when we tested other combinations (Supplementary Figure 5B, Supplementary Data 2). It is of note that in addition to the observed changes in inter-protein cross-links, we also found at least two intra-protein cross-linked peptides that were specifically underrepresented in heat-treated mTXNPx\textsubscript{red} compared to mTXNPx\textsubscript{red} that was incubated and cross-linked at 30 °C (Supplementary Data 2). These results suggested that structural changes occur also within the mTXNPx-dimers upon heat-induced activation that lead to altered cross-linking behavior.

To directly test whether mTXNPx\textsubscript{red} decamers dissociate at heat shock temperatures, we conducted in vitro cross-linking experiments with glutaraldehyde (GA)\textsuperscript{26}. We determined cross-linking efficiencies at 30 °C under which the majority of the mTXNPx\textsubscript{red} molecules cross-linked in the decameric form while the remainder cross-linked in the dimeric form (Fig. 6a). When we conducted the same incubation and cross-linking at 42 °C, the overall cross-linking efficiency did not change. However, upon shift to 42 °C, less than 15% of mTXNPx\textsubscript{red} molecules cross-linked as decamers while the remaining 85% of molecules cross-linked as dimers (Fig. 6a, b). Subsequent cooling of the sample from 42 to 4 °C followed by cross-linking at 30 °C re-established the decameric cross-link, indicating that the dissociation is reversible (Fig. 6a, b). Presence of luciferase increased the cross-linking yield of the decamers upon shift to 42 °C, suggesting that client-binding stabilizes the decamer (Fig. 6a, b). We obtained very similar results when we applied mTXNPx\textsubscript{red} or mTXNPx\textsubscript{red}-luciferase complexes to negative-stain EM grids immediately after incubating the peptides that were specifically cross-linked to mTXNPx\textsubscript{red} (Supplementary Figure 5B, Supplementary Data 2). It is of note that the structural information regarding the position and involvement of the N-termini in parasitic Prx, due to their intrinsic flexibility, and the fact that no additional structural information was available to directly tie the N-termini to client interactions. In the current study, therefore, we used a range of independent techniques, including cryo-EM structure analysis of mTXNPx-client complexes, mutational studies as well as in vivo and in vitro cross-linking, to identify client-interaction sites and gain insights into the structural rearrangements that trigger the switch from peroxidase to chaperone function in mTXNPx.

We have now obtained what, to our knowledge, represents the first high-resolution cryo-EM structure of the mTXNPx chaperone-client complex. Our approach has not only allowed us to solve the structure of much of the previously missing N-terminus of mTXNPx but revealed that the N-terminal residues form a belt-like structure, which apparently stabilizes the unfolded client protein. Our mutational analysis and cross-linking studies supported these results, identifying the N-terminal residues of mTXNPx to be important for in vitro chaperone activity, as well as for coming into sufficiently close contact with both in vivo and in vitro client proteins to promote site-specific cross-linking. Similar flexible extensions have been found in several other chaperones, particularly the sHsps, where they are also thought to play a dominant role in client recognition and/or binding\textsuperscript{28,29}.

Current chaperone models suggest that flexible, intrinsically disordered extensions mediate the initial, potentially low-affinity and long-range interactions with client proteins, and that these interactions are then enforced and strengthened by hydrophobic interactions\textsuperscript{30,31}. Our observation that the two N-terminal residues Y30 and Y33 cross-link with potential in vivo clients even at 30 °C supports the idea of the N-termini playing a role in low-affinity interactions. In addition, we identified several other residues that cross-linked with client proteins. To our surprise,
however, according to our cryo-EM structure, most of these residues appeared to be inaccessible in the substrate-free structure of mTXNPx, either shielded by loop regions or directly involved in the hydrophobic dimer–dimer interface that stabilizes the decamer. These results implied that activation of mTXNPx’s chaperone function involves substantial conformational rearrangements that at least transiently expose buried hydrophobic sites for binding to client proteins. This conclusion was consistent with our previous negative-stain EM studies, where side-view projections of mTXNPx-luciferase complexes revealed client-associated density specifically protruding from only one side of the decameric ring. Similarly, our cross-linking studies revealed cross-links between mTXNPx and client proteins that specifically engage residues at only one of the two mTXNPx surfaces that are perpendicular to the central ring opening (Fig. 4). At this point, we do not know why our cryo-EM approach was not capable of capturing these additional mTXNPx-client interactions, or why we were unable to observe any major conformational rearrangements in the client-bound mTXNPx that were predicted based on previous structural analyzes.

One region in mTXNPx that reproducibly cross-linked with clients both in vivo or in vitro involved residues in the highly
conserved active site Cp-loop-helix, whose folding state has been previously shown to play a major role in regulating the oligomeric state and functional activity of Prx. The Cp-loop-helix, which is folded in reduced, decameric Prx, has been shown to locally unfold not only upon oxidation but also in response to either low pH, overoxidation of the peroxidatic cysteine Cp, or in response to phosphorylation. Each of these events (apart from oxidation) has also been shown to trigger the conversion of 2-Cys-Prx into the chaperone-active form. Partial unfolding of the Cp-loop-helix in response to oxidation disrupts the dimer–dimer interface, thereby promoting the dissociation of 2-Cys-Prx into the respective dimers. Although stress-induced unfolding of the Cp-loop-helix was shown to lead to the formation of higher oligomeric states (i.e., spherical arrays of decamers, filaments), it is entirely possible that it too triggers initial dissociation into dimers, which subsequently re-associate into higher oligomeric states. We now propose that mTXNPx might sense elevated temperatures directly through the folding status of the Cp-helix, which would trigger rearrangements in the dimer–dimer interface and could potentially explain how otherwise bound residues become accessible for client interactions. Our in vitro qCL and negative-stain EM experiments support this model, and showed that client-free mTXNPx dissociates into reduced dimers upon incubation at elevated temperatures. The presence of client proteins either prevented the dissociation or caused rapid re-association of the dimers. Given that the stability of the Cp-loop-helix together with specific structural features in the C-terminus appear to define which members of the 2-Cys-Prx family are sensitive towards hyperoxidation and which ones are not (for a recent review see ref. 32), we hypothesize that some members of the 2-Cys-Prx families, such as mTXNPx, have evolved features to directly use the folding status of this helix to sense elevated temperatures. It remains to be demonstrated whether destabilization of the Cp-helix in mTXNPx or other 2-Cys-Prx is sufficient to turn these proteins into chaperones without the need for any other cues.

Two-Cys-Prxs such as mTXNPx share many intriguing features with members of the sHsp family—like 2-Cys-Prx, sHsps form high molecular-weight oligomers of various sizes, use dimers as their basic structural units, and undergo stress (i.e., heat)-induced dissociation into their dimeric subunits (e.g., Hsp26)24,25. We now postulate that, like sHsps, mTXNPx dissociates into reduced dimers upon incubation at elevated temperatures. The presence of client proteins either prevented the dissociation or caused rapid re-association of the dimers. Given that the stability of the Cp-loop-helix together with specific structural features in the C-terminus appear to define which members of the 2-Cys-Prx family are sensitive towards hyperoxidation and which ones are not (for a recent review see ref. 32), we hypothesize that some members of the 2-Cys-Prx families, such as mTXNPx, have evolved features to directly use the folding status of this helix to sense elevated temperatures. It remains to be demonstrated whether destabilization of the Cp-helix in mTXNPx or other 2-Cys-Prx is sufficient to turn these proteins into chaperones without the need for any other cues.

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E. coli. The accuracy of all constructs was verified by sequencing. The sequence encoding 5mTXNXp was PCR-amplified from pET28c6His-ThR-TmTXNXp using M13 primers and the primer pairs 5′-ccgccaga-ACGATGTAC-GAGTAGT-GACAGCATTC-CC-3′ and 5′-caagcttggqTACATGTTCCTTCCTCAGAACAAC-3′. The PCR fragment was subsequently digested with NdeI and Xhol for cloning into pET28c. Heterologous expression and purification of wild-type mTXNXp and mutants from E. coli BL21(DES), as well as removal of the histidine tags with thrombin, were performed following previously described protocols.8,14,15 Processed proteins were kept in 40 mM Hepes pH 7.5 and quantified by either A280 measurements14 or the bicinchoninic acid (BCA) protein assay, using bovine serum albumin (BSA) as standard.

Chaperone activity assays. The chaperone activity of purified recombinant, histidine-tag free mTXNXp (wild type and mutants) was measured by testing the effects on thermal aggregation of luciferase.14 Briefly, mTXNXp was diluted into a cuvette containing 200 µM DTT in 40 mM Hepes pH 7.5. After a pre-incubation period of 5 min at 42 °C, 0.1 µM luciferase (Promega) was added to the reaction mixture and light scattering was measured under constant stirring using a fluorescence spectrophotometer Fluoromax-4 (Horiba) Vmax 360 nm; slit widths, 2.5 nm. The light scattering signal of thermal luciferase in the absence of any chaperones was measured as control.

Plasmid construction for in vivo expression. To express mTXNXp without the mitochondrial import sequence or any additional tags, pET28c6His-ThR-TmTXNXp25 was PCR-amplified using Phusion polymerase and primers 5′-ccgccaga-ACGATGTAC-GAGTAGT-GACAGCATTC-CC-3′ and 5′-aagtttaaagcataatattttct-3′. The PCR product was digested with NdeI and subsequently re-ligated to originate a plasmid encoding mTXNXp devoid of any tag—pET28c TmTXNXp. The plasmid was further sequenced to ensure that no mutations on mTXNXp ORF had occurred and that the N-terminal 6-His-ThR tag had been successfully removed.

Production of mTXNXp—p-benzoyl-L-phenylalanine variants. For the construction of mTXNXp-Bpa variants, we used a site-directed mutagenesis approach and substituted each phenylalanine or tyrosine codon in the sequence with an amber stop codon (TAG) using the primers listed in Supplementary Table 3. Following Pdnl digestion of the parental DNA for 1 h at 37°C and desalting, the PCR product was transformed into E. coli (XL1-Blue strain). Mutant colonies were sequenced and confirmed to carry the desired mutation.

In vivo Bpa-mediated photo-cross-linking. Bpa-mediated cross-linking was performed as previously described.37 Briefly, E. coli cells (BL21 strain) were co-transformed with a plasmid encoding the respective mTXNXp-Bpa variant (pET28cTmTXNXp-Bpa) and a plasmid encoding the orthogonal aminosulfonyl-riboflavin synthetase/riboflavin pair (pEVOL) necessary for Bpa incorporation.32 Cells were then plated in the presence of appropriate selective drugs and incubated overnight at 30 °C. Colonies were scraped off the plates and resuspended in LMB medium (OD600 = 0.4), which contained a combination of antibiotics with or without 1 mM Bpa (Bachem AG, Bubendorf, Switzerland). Cells were then grown at 37°C for 1 h. After induction with 1 mM Bpa, cells were cooled to 30°C and expression of mTXNXp-Bpa variants and the tRNA synthetase/riboflavin pair was induced with 10 µM IPTG and 0.1% L-arabinose, respectively. Cells were grown for 3 h at 30°C, after which they were harvested and resuspended in PBS buffer using a volume of 100 µl per OD600 of 1.0. Each sample was then subjected to a 30-min incubation at either 30°C (control) or 45°C (heat shock), transferred onto ice and exposed to a 10-min UV irradiation (366 nm) using a lamp distance of 2.5 cm. Cells were subsequently lysed using three cycles of sonication and two cycles of freeze-thaw. Total cell extracts were then supplemented with 5X SDS sample-loading buffer before analysis by SDS-PAGE. Immunoblotting using polyclonal anti-mTXNXp antibodies was used to visualize mTXNXp and its cross-linking products.

In vitro cross-linking for LC-MS/MS analysis. Purified mTXNXp36 in either oxidized (mTXNXpox) or reduced forms (mTXNXpox at 30 °C, mTXNXpred at 42 °C, or mTXNXpox at 42 °C; or mTXNXpred at 42 °C) were prepared, split into two equal volumes, and cross-linked using a final concentration of 0.1 M of the bis-acrylamid-light reactive and iso-topically-heavy forms of the cross-linking reagent, separately resulting in a total of eight cross-linking sample preparations per cross-linking reagent (DSA or DSS). These eight preparations were then combined in equal amounts according to the scheme described in Supplementary Figure 5B, ultimately yielding 16 unique combined samples (six forward-label and a corresponding six reverse-label samples) per cross-linking reagent. Combined samples were subsequently digested in-solution with trypsin and prepared for LC-MS/MS analysis as described.

LC-MS/MS analysis. Mass spectrometric analysis was performed using a nano-HPLC system (Easy-nLC II, ThermoFisher Scientific), coupled to the ESI-source of an LTQ Orbitrap Velos Pro (ThermoFisher Scientific). Samples were injected onto a 100 µm ID, 360 µm OD, 10 mm length trapping column packed with Magic C18 (Bucker-Michrom), 100 Å, 5 µm pore size (prepared in-house) and desalted with washing with Solvent A (2% acetonitrile: 98% water, 0.1% formic acid (FA)). Peptides were separated with a 90-min gradient (mTXNXp + luciferase experiments: 0–90 min: 5–30% solvent B (90% acetonitrile, 10% water, 0.1% FA), 90–92 min: 30–100% B; 92–100 min: 100% B; mTXNXp cDNA experiments: 0–90 min: 5–40% B, 92–90 min: 100–300% B, 92–100% 100% B), on a 75 µm ID, 360 µm OD, 150 mm length analytical column packed with Magic C18AQ 100 Å, 5 µm pore size (prepared in-house), with IntegraFrit (New Objective Inc.) and equilibrated with solvent A. MS data were acquired using a Top 8 data dependent method in which precursors ions with charge states ≥2 and a minimum signal to noise ratio of at least 3:1 were selected for fragmentation. MS and MS/MS events used 60,000 and 15,000 resolution FTMS scans, respectively, with a scan range of 400–2000 m/z in the MS. For MS/MS, CID collision energy was set to 35% and wideband activation was enabled.

Cross-linking LC-MS/MS data analysis. Cross-linking data was searched using Kojak (v.1.5.5)38 against a concatenated target-decoy (reverse) sequence databases. All search output peptide-spectrum matches (PSMs) are included in Supplementary Data 1, 2. All cross-links that are discussed further in this text were required to be the single highest scoring PSM for their respective MS2 scan (i.e., Unique Score ID = “Unique”) and to either have a score of 2 or greater (corresponding to an expected value of 13.8%) or to be included in multiple confident results for a set of at least 1 and have spectra manually inspected. Quantitation was performed using Xiq (v.0.2.31) parameters (expected_shift = 0 and time_window = 1). The difference in cross-linking yield between experiments was expressed as “fold-change” and was calculated as the binary logarithm of the observed cross-link / Batch H/L Median as reported in the “temperate.csv” Xiq output. The fold-change for forward labeled experiments was calculated as the binary logarithm of the observed cross-link H/L median as reported by Xiq. For the reverse-labeled experiments this was calculated as the negative binary logarithm of the observed cross-link H/L ratio (log2(1/H/L)). Cross-link map shown in Fig. 5a and Supplementary Figure 4B were generated using xlinkIntegrate interpretation of the in vitro LC-MS/MS cross-linking results, the identified cross-links were mapped onto the whole-chain model of luciferase (pdb: 1LCI). The MS datasets, search parameters and search outputs have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository36. The accession number is: PXD010281.

Oligomerization state of mTXNXpred in vitro. To determine the oligomerization state of chaperone-activated mTXNXpred (2 μM) either alone or in complex with 0.2 μM of luciferase at 30°C or 42°C, proteins were incubated with 40 mM GA for 10 min at the respective temperatures. Cross-linking was stopped by transferring 42 μl of each sample into a tube containing 8 μl of 1 M Tris-HCl, pH 8.0. The cross-linked species were separated by SDS-PAGE under reducing conditions and visualized by silver staining. The bands were quantified using the ImageJ software.

Negative-stain EM. Reaction mixtures of mTXNXpox or mTXNXpred (10 μM) either alone or in complex with 1 μM of luciferase at 30°C or 42°C were either immediately or after incubation at 4°C applied onto thin carbon layered 400-mesh copper grids (Pelco) as described, negatively stained with 0.75% uranyl formate (pH 5.5–6.0) and imaged at room temperature using a Tecnai T12 Microscope (FEI) equipped with a LaB6 filament operated at 120 kV. Images were collected at 69444× magnification with a 2.2 Å/pixel spacing on a 4 k × 4 k CCD camera (Gatan). Particle images were selected EMAN2 software eboxer on micrographs with similar stain thickness to estimate the number of decamers present in each micrograph.

Data availability. The MS datasets, search parameters and search outputs have been deposited to the ProteomeXchange Consortium via the PRIDE partner repository. The accession number is: PXD010281. The structures have been deposited in the protein data base; mTXNXp...
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with bound client: 6E9F, EMD-8946, and apo mTXNPx: 6E9G, EMD-8947. Other data
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