

Thioredoxin 2, an Oxidative Stress-induced Protein, Contains a High Affinity Zinc Binding Site*

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Two thioredoxins have been described in *Escherichia coli*, TrxA and Trx2. Both thioredoxins are capable of reducing disulfide bonds using a conserved pair of cysteine residues present in a WCGPC motif. A number of unique structural and regulatory features distinguish the Trx2 subfamily from the much larger TrxA family. The Trx2 subfamily has an additional N-terminal domain of ± 30 residues, which contains two additional conserved CXXC motifs. Moreover, the gene coding for Trx2 is under control of the oxidative stress transcription factor OxyR in *E. coli*. This suggests that Trx2 may play a role in the cellular defense against oxidative stress. We show here that Trx2 contains zinc in a 1:1 stoichiometry, making it the first identified zinc-binding thioredoxin. The zinc atom is coordinated by the four cysteines of the two N-terminal CXXC motifs. The zinc center of Trx2 binds zinc with a very high affinity (K_a of $>10^{18} \text{ M}^{-1}$). We show that *in vitro* oxidation of the zinc binding cysteines by H_2O_2 releases the zinc and induces a conformational change. The zinc-free protein conserves its reductase activity. Altogether, our results suggest that the zinc center might play the role of a redox switch, changing a yet to be identified activity.

Thioredoxins are small redox proteins present in many eukaryotic and prokaryotic cells. All thioredoxins share a similar three-dimensional structure and possess a conserved WCGPC catalytic motif. The main function of thioredoxins is to reduce disulfide bonds formed between cysteine residues of cytoplasmically localized proteins. These disulfides can occur either as part of a catalytic cycle (e.g. ribonucleotide reductase) or be caused by reactive oxygen species (1). In the course of the disulfide exchange reaction, the two catalytic cysteine residues of the WCGPC motif of thioredoxins are oxidized. They are then converted back to the reduced form by thioredoxin reductase, which draws electrons from NADPH (2).

Two thioredoxins have been described in *Escherichia coli* (1, 3). The first one, TrxA, has been identified 40 years ago and is well characterized. It is a 12 kDa protein, which serves as

electron donor for 3'-phosphoadenosyl sulfate (PAPS)¹ reductase, ribonucleotide reductase, methionine sulfoxide reductase, and the membrane protein DsbD (2, 4). It is also an essential subunit of the bacteriophage T7 DNA polymerase. Although the role of TrxA is thought to be central in controlling the redox equilibrium of the cytoplasm, *trxA*⁻ strains do not have any apparent growth phenotype (1, 5).

The second thioredoxin, Trx2, which is encoded by the gene *trx2*, has been discovered recently and is only poorly characterized (6). It shares a 28% sequence identity with TrxA. *In vitro*, Trx2 has been shown to be a functional thioredoxin and is an electron donor for ribonucleotide reductase (6) and PAPS reductase (7). *In vivo*, Trx2 can efficiently reduce ribonucleotide reductase (8) but requires overexpression to reduce DsbD (9) and PAPS reductase (8). For the majority of activities tested so far, Trx2 seems equivalent to TrxA.

Trx2 has two striking characteristics that distinguish it from TrxA and suggest that it has a specific role to play. Trx2 contains an additional N-terminal domain of 32 residues. Interestingly, two CXXC motifs are present in this domain. A CXXC motif is present at the active site of thioredoxin-related proteins where it serves in thio-disulfide exchange. However, most of these CXXC motifs, even in very divergent thioredoxin-like proteins, also share a residue upstream the first cysteine; this residue is lacking in both CXXC motifs found in the N terminus of Trx2, leading to consider other possible roles for these CXXC motifs. CXXC are also found in metal-binding proteins including those that interact with copper, cadmium, iron, or zinc (10). These metals may play either structural or catalytic roles. For instance, in the case of the *E. coli* chaperone Hsp33, two adjacent CXC and CXXC motifs have been identified to coordinate zinc (11). Here, the zinc binding motif plays the role of a redox switch, leading to changes in protein conformation and activity upon exposure of Hsp33 to oxidative stress conditions. Under normal conditions, Hsp33 binds zinc via 4 reduced cysteine residues present in a CXC(27–32)CXXC motif (12). The reduced protein is inactive. Active oxygen species like hydroxyl radicals oxidize the zinc binding cysteines. This leads to zinc release and conformational changes, which results in the dimerization of Hsp33 and the activation of its chaperone function (12, 13).

The second interesting feature of Trx2 is its transcriptional regulation. The expression of Trx2 is under the control of the oxidative stress transcription factor OxyR. Whereas Trx2 has been shown to be 5-fold less abundant than TrxA under normal conditions (6), its concentration increases about 20-fold upon

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¹ The abbreviations used are: PAPS, 3'-phosphoadenosyl sulfate; PAR, 4-(2-pyridylazo)resorcinol; DTT, dithiothreitol; PMPS, *p*-hydroxymercuriphenylsulfonic acid; AMS, 4-acetamido-4'-maleimidylstiebene-2-2'-disulfonate.

exposure of cells to oxidative stress (9). Under these conditions, Trx2 becomes even more abundant than TrxA. OxyR is a transcription factor that is activated during oxidative stress and induces the expression of several antioxidant genes including peroxidases (e.g. AhpCF, KatG, Tpx) and reducing proteins such as glutaredoxin 1 and glutathione reductase (14, 15). The control of Trx2 expression by OxyR suggests that Trx2 plays a specific role in response to oxidative stress, although *trxC*⁻ strains do not show altered sensitivity to hydrogen peroxide (9).

Here, we show that Trx2 contains an N-terminal zinc center, which is constituted by the four cysteines of the two additional CXXC motifs. Trx2 binds zinc with a very high affinity. Exposure of Trx2 to oxidative stress leads to the reversible oxidation of the zinc center and to the release of zinc. We show that this oxidation induces major conformational changes in the protein.

EXPERIMENTAL PROCEDURES

Cloning—*E. coli* genomic DNA was amplified with *Pfu* polymerase (Invitrogen). The PCR-amplified product was purified and cloned into a pET11a plasmid. The resulting plasmid pJFC67 was verified by sequence analysis and then introduced into BL21(DE3)pLysS cells.

Expression and Purification of E. coli Trx2—BL21(DE3) harboring the plasmid pJFC67 were grown aerobically in 1 liter of LB medium at 37 °C until an OD₆₀₀ of 0.8 was reached. Isopropylthiogalactoside (0.5 mM) and ZnCl₂ (0.8 mM) were added and shaking was continued for 5 h to allow protein expression. Cultures were then centrifuged at 5,000 rpm and 4 °C for 20 min. From this point on, all steps were performed at 4 °C. Bacteria were resuspended in 25 ml of buffer A (25 mM sodium phosphate, pH 8.0), and cells were disrupted by 2 passes through a French Press cell at 1200 PSI. The cell lysate was then centrifuged for 40 min (20,000 × *g* at 4 °C) and the resulting supernatant (≈25 ml) was diluted 3-fold with buffer A and applied onto a Q-Sepharose column (1.6 × 10 cm). The column was washed with 100 ml of buffer A, and proteins were eluted with a NaCl gradient (0–400 mM in 250 ml of buffer A). The fractions containing Trx2 were pooled, concentrated by ultrafiltration in an Amicon cell (YM-10 membrane), and loaded onto a PD10 column (Amersham Biosciences) to perform a buffer exchange with buffer B (20 mM Hepes, pH 7.0). The protein was then loaded onto a DEAE-Sepharose column (1.6 × 10 cm) equilibrated with buffer B. The column was washed with buffer B, and the protein was eluted with a NaCl gradient (0–400 mM in 250 ml of buffer B). The fractions containing Trx2 were pooled and concentrated by ultrafiltration in an Amicon cell (YM-10 membrane). The Trx2 preparation was further purified by gel filtration using a Superdex 75 (Amersham Biosciences) column (1.6 × 50 cm) at a flow rate of 0.5 ml/min. The column was equilibrated in buffer B containing 200 mM KCl. The fractions containing Trx2 were pooled, concentrated in an Amicon cell (YM-10 membrane), and desalted using a PD10 column equilibrated with buffer B. The pure protein was stored in buffer B at –80 °C. The concentration of Trx2 was calculated using an extinction coefficient ϵ_{280} of 17,060 M⁻¹ cm⁻¹.

Metal Analysis—For the determination of the metal content in Trx2, 2 different preparations of Trx2 (50 μM each) were separated from exogenous metals by gel filtration using a PD10 column. The PD10 columns were equilibrated with metal-free H₂O prepared by incubation with Chelex 100 resin (BioRad). The samples were then analyzed using a Finnigan Element inductively coupled plasma-high resolution mass spectrometer (ICP-HRMS), located in the Keck Elemental Geochemistry Laboratory of the Department of Geological Sciences at the University of Michigan. The sample was concentrated 2-fold by ultrafiltration using a centricon (Millipore). The metal content of the protein-containing retentate was compared with that of the flow-through. Zinc, nickel, iron, cobalt, and manganese concentrations were measured. As additional controls, two buffer samples were analyzed using the same procedure.

PAR-PMPS Assay—Zinc concentrations were determined using the zinc-complexing reagent PAR (4-(2-pyridylazo)resorcinol). PAR binds zinc to form a Zn(PAR)₂ complex, which absorbs strongly at 500 nm ($\epsilon = 66,000$ M⁻¹ cm⁻¹ in Hepes, pH 7.0). To determine the amount of zinc bound to the protein, *p*-hydroxymercuriphenylsulfonic acid (PMPS) was added to release zinc from the zinc center and allow the formation of the Zn(PAR)₂ complex. PMPS forms mercaptide bonds with thiols using the following reaction mechanism R-HgOH + P-SH → R-Hg-S-P + H₂O where the R stands for the phenylsulfonic acid group of PMPS and the P stands for a protein. This reaction mechanism proceeds as a substi-

tution reaction whereby the hydroxyl anion is displaced by the thiol-containing protein to form a mercuric sulfide derivative of the protein (R-Hg-S-P). If the thiols on the protein are involved in coordinating a metal such as zinc, this covalent modification leads to the release of the zinc. To determine the number of cysteines involved in zinc binding, defined amounts of PMPS were added and the changes in A₅₀₀ were recorded according to Jakob *et al.* (12). The *K_a* of zinc binding was determined using competition experiments as previously described (12).

Preparation of the Three Different Redox Forms of Trx2—Freshly purified Trx2 was saturated with zinc and the 6 cysteines were in the reduced form (Trx2_{6cysred}) as determined using 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB) as described (2). However, following purification, a slow oxidation of the two catalytic cysteines was observed over time. When needed, these two cysteines were reduced by incubation of Trx2 with 5 mM DTT for 30 min at 25 °C. 500 μl of the protein solution was then loaded onto a gel filtration column (NAP5, Amersham Biosciences) and Trx2 was eluted with 700 μl of the appropriate buffer.

To prepare a completely oxidized Trx2 (Trx2_{6cysox}), the protein was incubated with a 40-fold excess of hydrogen peroxide at 43 °C for 2 h. Excess H₂O₂ was removed by gel filtration. The thiol content of the preparation was measured using DTNB.

To prepare zinc-saturated Trx2 with both catalytic cysteines oxidized (Trx2_{2cysox}), the protein was incubated with a 10-fold excess of GSSG for 10 min at 4 °C and gel filtrated as explained above. After the gel filtration, the zinc content was determined using the PAR-PMPS assay using HPLC. The oxidized (Trx2_{2cysox}) and reduced forms of Trx2 (Trx2_{6cysred}) were separated on a C18 column (Phenomenex Prime-sphere, 250 × 4.60 mm) using a linear aqueous 39–51% acetonitrile gradient in 0.1% trifluoroacetic acid over 24 min at a flow rate of 1 ml/min. Absorbance was recorded at 214 and 280 nm.

The zinc center of Trx2 is very stable and freshly purified Trx2 was saturated with zinc. However, after multiple freezing/thawing cycles, a slow decrease of the zinc content of the purified protein could be observed. When required, zinc-saturated Trx2 was prepared by incubating 50 μM Trx2 with 200 μM Zn²⁺ and 2 mM DTT at 25 °C for 15 min. The protein was then purified away from the unbound zinc by gel filtration.

Preparation of Reduced, Zinc-free Trx2—Zinc-free Trx2 was prepared by incubating Trx2 (50 μM) with 2 mM hydrogen peroxide at 43 °C. After 2 h, 5 mM EDTA was added, and the preparation was applied onto a gel filtration column (NAP-5 column) equilibrated with 50 mM potassium phosphate, pH 7.0. The zinc-free oxidized protein was then reduced with 2 mM DTT and loaded onto a second NAP-5 column equilibrated with the same buffer. Both buffer and DTT solution had been depleted from their metals by incubation with Chelex resin. After this treatment, Trx2 was reduced and contained less than 5% zinc.

Proteolytic Digestion—The protease sensitivity of reduced Trx2 (Trx2_{6cysred}) was compared with that of oxidized Trx2 (Trx2_{6cysox}) by incubating 35 μM of each protein preparation with 3 μg of trypsin at 23 °C in 100 μl of 25 mM Hepes, pH 7.5. At different time points, aliquots were taken and the tryptic digestion was stopped by the addition of SDS-PAGE loading buffer containing 1 mM phenylmethylsulfonyl fluoride. The samples were then applied on SDS-PAGE under reducing conditions.

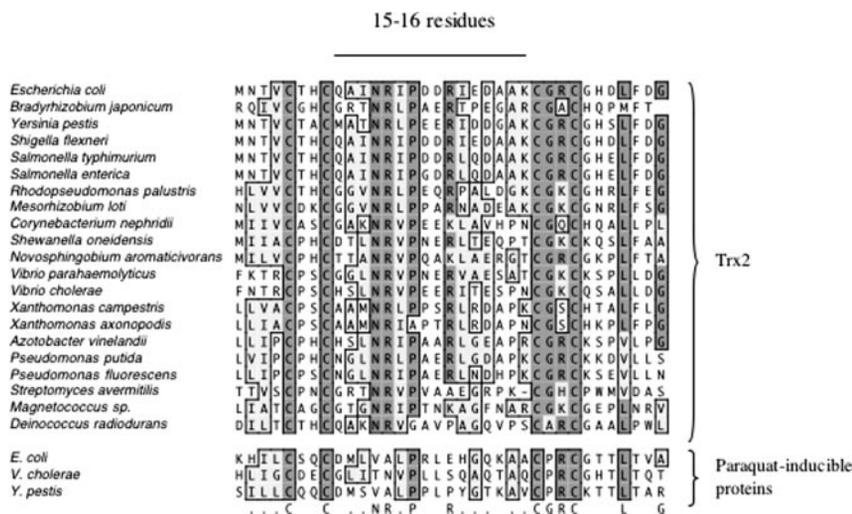
CD Analysis—Far UV circular dichroism spectra were recorded using a JASCO J-810 spectropolarimeter. 30 μM of Trx2_{6cysred} or Trx2_{6cysox} in 20 mM sodium phosphate buffer, pH 8.0 was used (1-mm cuvettes).

TrxB-mediated Reduction of Trx2—For the reduction of oxidized Trx2 (Trx2_{6cysox}) by TrxB, 10 to 20 μM of Trx2 were incubated at 30 °C with 50 μM NADPH and 10 nM TrxB in 20 mM sodium phosphate buffer, pH 8. The reduction was followed by monitoring the absorbance at 340 nm using a Beckman DU600 spectrophotometer. At different time points, aliquots were taken for AMS trapping.

AMS Trapping—AMS is a reagent that covalently reacts with free thiol groups, adding a 490 Da group. This leads to a major mobility shift respective of modified protein in SDS-PAGE gels. For AMS trapping, samples containing 25–75 μg of Trx2 were precipitated with 5% ice-cold trichloroacetic acid and centrifuged at 16,000 × *g* for 15 min. The pellets were washed with acetone, dried, and resuspended in 50 mM Tris-HCl, pH 7.5, 10 mM AMS, 0.1% SDS, and 10 mM EDTA. Samples were analyzed by SDS-PAGE under non-reducing conditions.

Insulin Reduction Assay—Zinc-free or zinc-containing reduced Trx2 (1 μM) was incubated with 130 μM insulin and 0.6 mM DTT in 100 mM potassium phosphate, pH 7.0 at 25 °C. Precipitation of reduced insulin was monitored at 650 nm. Before the reaction, all reagents were depleted of their metals by incubation with Chelex resin (Bio-Rad).

FIG. 1. Trx2 belongs to a new subclass of thioredoxins. Top, the N-terminal domain of *E. coli* Trx2 is aligned with the N-terminal domain of Trx2 homologues from 20 other bacterial genomes. Bottom, the N-terminal domain of a family of paraquat-inducible membrane proteins. The alignment is shown for 3 members of this family (from *E. coli*, *Yersinia pestis*, and *Vibrio cholerae*).



RESULTS

Sequence Analysis—One of the most striking differences between TrxA and Trx2 is the presence of an N-terminal domain containing two additional CXXC motifs arranged in the sequence CTHCX₁₆CGRC. These 2 cysteine motifs are present in addition to the CGPC active site motif that is conserved in all thioredoxins. To clearly distinguish between these three different CXXC motifs, we designated the N-terminal CTHC motif as CXXC_{n1}, CGRC as CXXC_{n2} and the active site CGPC motif as CXXC_{active}.

At the time of the identification of Trx2 (6), the two additional cysteine motifs CXXC_{n1} and CXXC_{n2} were only found in one other thioredoxin from *Corynebacterium nephridii*. However, only three bacterial genomes had been completely sequenced at that time, which prevented a more general sequence analysis. We used the BLAST algorithm to search for Trx2 homologues in finished and unfinished eukaryotic and prokaryotic genomes (www.ncbi.nlm.nih.gov/sutils/genom_table.cgi). We found more than 20 other bacterial thioredoxins that contain a homologous amino-terminal domain (Fig. 1). In all these thioredoxins, the two additional CXXC_{n1} and CXXC_{n2} motifs are strictly conserved, strongly suggesting that these motifs are important for Trx2 function and/or regulation. In addition to these four cysteine residues, an asparagine found at position 12 of the *E. coli* Trx2 and an arginine found at position 13 are also absolutely conserved in Trx2 homologues. Noteworthy, the N-terminal domain of Trx2 including the CXXC_{n1} and CXXC_{n2} sequences is homologous to the N-terminal domain of the PqiA family of paraquat-inducible proteins (pfam04403.2). This is a family of unknown function membrane proteins, whose expression is under SoxR control (16). In analogy to OxyR, which is activated by oxidative stress, SoxR is a transcription factor that is activated under superoxide stress conditions (15). Apart from the N-terminal domain, paraquat-inducible proteins share no homology with thioredoxins.

Trx2 Purification and Metal Analysis—The conservation of the two CXXC_{n1,n2} motifs suggested that they might play a role in Trx2 function, possibly either by conferring thiol-disulfide exchange reactions or by coordinating metals. To help determine the role of these conserved cysteines, we purified Trx2 to homogeneity using classical chromatography techniques. We decided against using His-tagged Trx2 because histidines are known to bind a number of different metals, which could interfere with the meaningful determination of the metal content of Trx2. After two anion exchange chromatography steps and one size exclusion chromatography, Trx2 was >95% pure (not shown). The average yield was ≈10 mg of pure Trx2 from 1 liter

of culture. At the end of the purification, all cysteines of the protein were in the reduced state as determined using DTNB as described (2).

To study the metal content of the protein, Trx2 (50 μM) was first separated from free metals by gel filtration against metal-free buffer and was then analyzed by inductively coupled plasma-high resolution mass spectrometry (ICP-HRMS). We found that zinc is present in the protein in a 1:1 molar stoichiometry (1.03 zinc/protein). No other metals were detected in the protein preparation.

The Four Cysteines Present in the N-terminal Domain of Trx2 Coordinate Zinc—Cysteines and histidines are frequently involved in zinc coordination. Trx2 contains 6 cysteine residues, 4 in the N-terminal domain and 2 in the thioredoxin domain, all of which are conserved. In addition, Trx2 contains two histidine residues, which both are located in the N-terminal domain but are not well conserved. To determine if and how many cysteine residues are involved in zinc binding, we used the PAR/PMPS assay (17). PAR is a dye that chelates zinc to form a red Zn(PAR)₂ complex. This complex strongly absorbs at 500 nm and its formation can therefore be easily monitored. However, the affinity of PAR for zinc is lower than the affinity of many cysteine coordinating zinc centers ($K_a = 2 \times 10^{12} \text{ M}^{-1}$). Therefore, tightly bound zinc cannot be extracted by PAR. To be able to detect zinc bound to high affinity thiol centers, thiol-modifying reagents such as PMPS are used. PMPS reacts stoichiometrically with reduced cysteines to form mercaptide-thiol bonds using the following reaction mechanism $\text{R-HgOH} + \text{P-SH} \rightarrow \text{R-Hg-S-p} + \text{H}_2\text{O}$ where the R stands for the phenyl-sulfonic acid group of PMPS and the P stands for a protein. If the thiols on the protein are involved in coordinating a metal such as a zinc, this covalent modification leads to the release of the zinc. The modification of the cysteine causes zinc release which immediately leads to the formation of the red Zn(PAR)₂ complex.

When freshly purified Trx2 was incubated with PAR, we did not observe a significant increase in A_{500} , indicating that PAR was unable to extract the zinc from the protein. Stepwise additions of a PMPS stock solution led to a progressive increase in A_{500} . When all 6 cysteines of Trx2 were titrated with PMPS, no further increase was observed (Fig. 2A). This indicated that all 6 reduced cysteines had to be modified by PMPS to completely release zinc. Noteworthy, a short lag phase was observed at the beginning of the titration, suggesting that at least one, possibly two, cysteine residues are more accessible to the PMPS titration solution than the zinc coordinating cysteines.

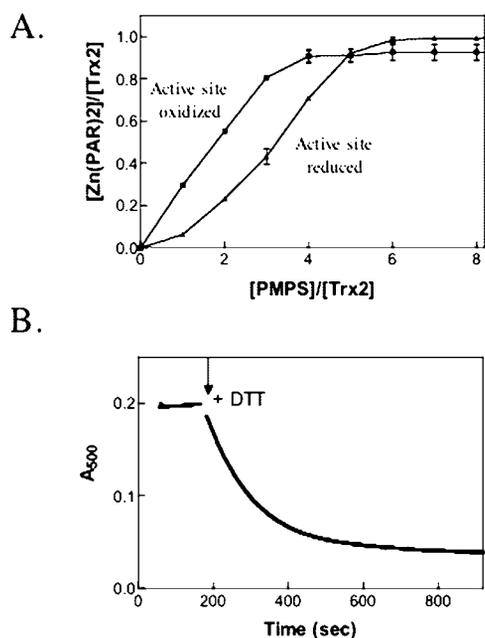


FIG. 2. **Titration of Trx2.** A, reduced (\blacktriangle) Trx2 ($2 \mu\text{M}$) was incubated in 40 mM Hepes, pH 7.0 with $100 \mu\text{M}$ PAR. A_{500} was monitored. Stepwise additions of $2 \mu\text{M}$ PMPS induced zinc release. The ratio PMPS/Trx2 corresponds to the number of cysteine residues that have to be titrated to allow zinc release. $[\text{Zn}(\text{PAR})_2]$ is the concentration of zinc released from Trx2. The same experiment was performed using Trx2 with the two catalytic cysteines oxidized (\blacksquare). B, addition of $40 \mu\text{M}$ DTT reduced the mercaptide-thiol bonds and allowed the reassociation of the zinc center.

These cysteines appear to be modified first by PMPS before the zinc coordinating cysteines were modified.

To evaluate whether zinc is coordinated by the 4 N-terminal cysteines, Trx2 whose active site cysteines were oxidized ($\text{Trx2}_{2\text{catcysox}}$) was prepared by a short incubation with oxidized glutathione, allowing the transfer of one disulfide bond from oxidized glutathione to Trx2 via the following mechanism: $\text{Trx2}(\text{SH})_2 + \text{GSSG} \rightarrow \text{Trx2S}_2 + 2\text{GSH}$. When the four cysteines in the N-terminal domain were kept in the reduced state while both active site cysteines were oxidized, the titration of 4 thiol groups was sufficient to allow the complete zinc release (Fig. 2A). Importantly, disulfide bond formation in the active site did not change the total amount of zinc bound to the protein. These results suggested that zinc is coordinated by presumably all 4 cysteines in the N-terminal domain of Trx2 and that the two active site cysteines are not involved in zinc coordination.

As shown in Fig. 2B, addition of DTT, which reduces the mercaptide-thiol bonds between PMPS and the cysteines in Trx2, allows Trx2 to quickly re-extract the zinc from the $\text{Zn}(\text{PAR})_2$ complex, indicating that the zinc center can be reformed readily *in vitro*. Zinc is usually coordinated by four ligands. We therefore suggest that all four cysteines, which are highly conserved in the N-terminal domain, are involved in zinc coordination.

Affinity Constant of Trx2 for Zn—A straightforward approach to determine the binding constant (K_a) of a protein for metals is to incubate the metal-containing protein with specific chelators whose binding constants are known. At equilibrium, the following Equation 1 applies (12),

$$K_a(\text{Trx2}) = \frac{K_a(\text{chelator}) \times [\text{Trx2:Zn}] \times [\text{chelator}_{\text{free}}]}{[\text{chelator:Zn}] \times [\text{Trx2}_{\text{free}}]} \quad (\text{Eq. 1})$$

where $[\text{Trx2:Zn}]$ and $[\text{chelator:Zn}]$ are the concentrations of zinc-containing Trx2 and zinc-bound chelator, respectively.

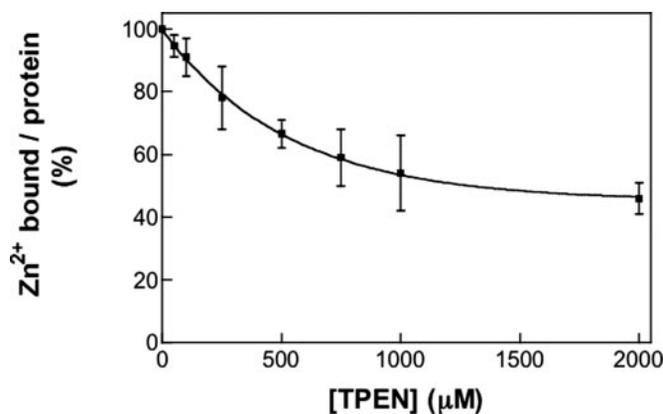


FIG. 3. **Zinc binding competition experiments.** Zinc containing Trx2 ($40 \mu\text{M}$) was incubated with increasing concentrations of TPEN in 40 mM Hepes, pH 7.5 and 23°C . After 18 h, the protein was purified from free TPEN and zinc bound TPEN by gel filtration and the amount of zinc bound to the protein was determined using the PAR/PMPS assay.

We explored the use of two different zinc chelators, EDTA and N,N,N',N' -tetrakis(2-pyridyl-methyl)ethylenediamine (TPEN). EDTA was not useful as a chelator because we found that EDTA binds to the Trx2-holoprotein to form a $[\text{Trx2-Zn-EDTA}]$ complex (data not shown). We, therefore, used the metal chelator TPEN, which has a very high binding constant for zinc ($K_a = 10^{16} \text{ M}^{-1}$). Zinc-saturated Trx2 ($40 \mu\text{mol}$; 1 Zn/molecule) was incubated with increasing concentrations of TPEN and allowed to equilibrate for 18 h. The samples were then gel filtrated against metal-free buffer to separate the protein from zinc-free and zinc-complexed TPEN. After this step, the protein concentration in the different samples was $\sim 10 \mu\text{M}$. The amount of zinc, which remained bound to the protein via its 4 conserved cysteines after the incubation, was determined using the PAR/PMPS assay. As shown in Fig. 3, not even millimolar concentrations of TPEN could dissociate more than 60% of the zinc bound to $40 \mu\text{M}$ Trx2, showing that the zinc binding constant of Trx2 is even higher than that of TPEN. Using Equation 1, we determined the binding constant of Trx2 for zinc to be higher than 10^{18} at 23°C and pH 7.0.

Zinc Is Released upon Oxidation—Several zinc-binding proteins have been shown to release zinc upon oxidation. A recently reported example is the chaperone Hsp33 of *E. coli*. Under physiological conditions, Hsp33 is reduced and all 4 conserved cysteines are involved in the high affinity binding ($K_a = 2.5 \times 10^{17} \text{ M}^{-1}$) of zinc. Upon exposure of Hsp33 to oxidative stress (e.g. H_2O_2), however, 2 intramolecular disulfide bonds are formed and the zinc is released; this turns on the chaperone activity of the protein (13). To test whether the zinc center of Trx2 is also oxidation sensitive, we incubated Trx2 with hydrogen peroxide. At different time points, aliquots were taken and the amount of zinc present in solution was determined using the PAR assay. To monitor the thiol/disulfide status of Trx2, AMS trapping experiments were performed in parallel. AMS is a 490 Da molecule that alkylates free thiols causing a mobility shift in SDS-PAGE gels. As shown in Fig. 4A, incubation of Trx2 with hydrogen peroxide releases the zinc from the protein. The half-time of this reaction is ~ 38 min at 43°C . Importantly, this zinc release is paralleled by the oxidation of the zinc-coordinating cysteines (Fig. 4B). Complete oxidation of the protein is reached when complete release of zinc has been achieved. Interestingly, within the first 5 min of exposure of Trx2 to H_2O_2 , 2 cysteines are completely oxidized. These two residues are likely to be the two cysteines of the thioredoxin domain of Trx2.

We also show that the zinc release is temperature sensitive

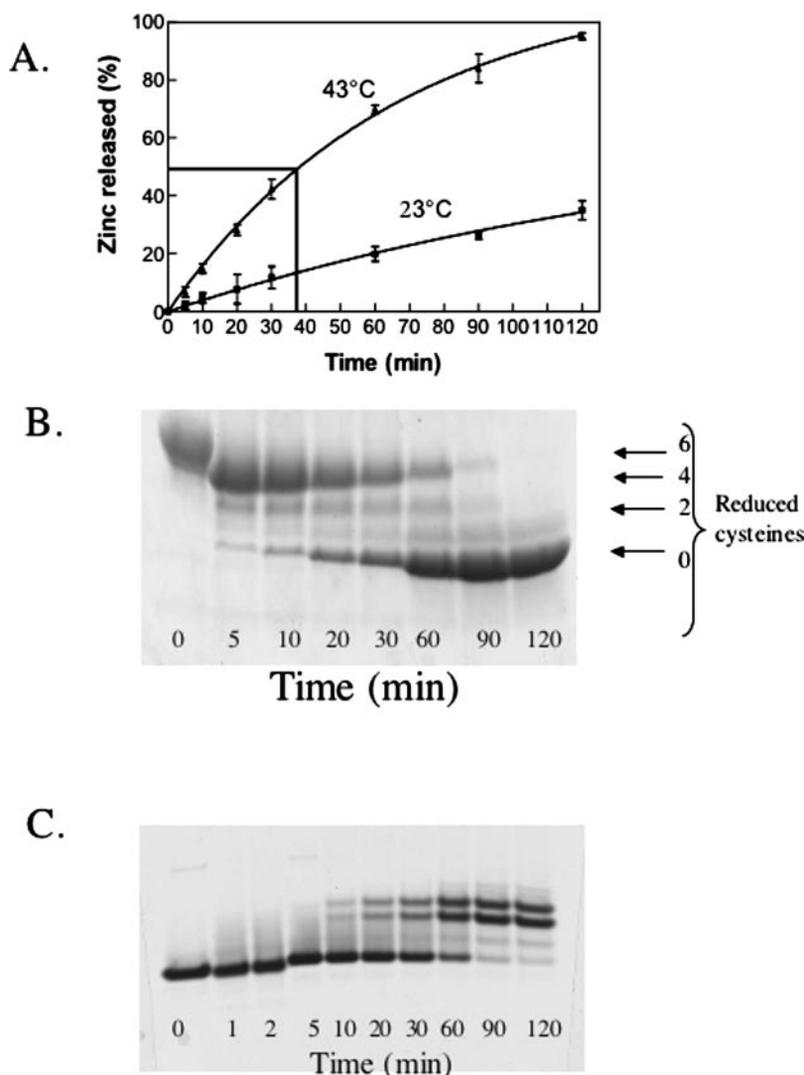


FIG. 4. Oxidation of Trx2 by hydrogen peroxide. *A*, zinc release upon oxidation. Trx2 (50 μ M) was incubated with H_2O_2 (2 mM) at 23 °C and 43 °C. At different time points, the zinc concentration was determined using PAR. *B*, aliquots from the reaction at 43 °C were precipitated with 5% trichloroacetic acid, free thiols were trapped with AMS and protein samples were analyzed by SDS-PAGE. *C*, in the course of the oxidation reaction, aliquots were loaded on native gels.

and occurs much faster at 43 °C than 23 °C (Fig. 4A). Such phenomenon has already been reported for other proteins such as Hsp33 (18). We have also found that addition of substoichiometric amounts of a redox active metal like copper, which catalyzes the production of hydroxyl radicals from hydrogen peroxide, increases the rate of oxidation of Trx2 by more than 10-fold (data not shown).

The Oxidation Is Reversible—The CXXC active cysteines of TrxA and Trx2 are both kept reduced by thioredoxin reductase (TrxB) in a NADPH-dependent manner (3). To determine if TrxB is also capable of directly or indirectly reducing the CXXC_{n1} and CXXC_{n2} cysteines, we incubated oxidized Trx2 with NADPH and catalytic amounts of TrxB. Because NADPH absorbs at 340 nm, it is possible to follow Trx2 reduction by monitoring the change in absorbance at 340 nm. At different time points during the reduction reaction, aliquots were taken and analyzed by AMS trapping and SDS-PAGE electrophoresis. As shown in Fig. 5A, TrxB is able to efficiently reduce completely oxidized Trx2. The amount of NADPH consumed in the reaction corresponded to the reduction of 3 disulfide bonds in Trx2. This is in agreement with the results of the AMS-trapping experiment (Fig. 5B), which shows that the protein is completely reduced at the end of the reaction. Interestingly, the TrxB-catalyzed reduction reaction occurs in two phases. Analysis of the thiol status reveals that the first phase corresponds to the rather slow reduction of two cysteines residues. These could be the active site cysteines. As soon as these 2 cysteines

are reduced, the reduction of the 4 other occurs very quickly.

We then performed a PAR/PMPS assay to determine whether zinc was re-coordinated by TrxB-reduced Trx2 and found that about 25% of Trx2 was zinc-coordinated. This is presumably endogenous zinc in buffers and protein solution because no exogenous zinc was added. Moreover, completely oxidized Trx2 had been separated from hydrogen peroxide by gel filtration. This process is likely to remove most of the unbound zinc. When zinc was added after the TrxB-catalyzed Trx2 reduction, about 85% of the protein was zinc-coordinated. Our results show that TrxB is able to recycle Trx2 after its complete oxidation, allowing the effective re-formation of its high-affinity zinc center.

Zinc Release Induces Major Conformational Changes in Trx2—In Hsp33, the oxidation of the zinc center induces a conformational change, which results in the dimerization of the protein and the activation of the chaperone activity (13). To test whether Trx2 conformation was also reversibly modified upon oxidation of its zinc center, reduced, zinc-saturated Trx2 was incubated with hydrogen peroxide. At different time points, aliquots were analyzed by electrophoresis using non-denaturing gels (Fig. 4C) and the free zinc concentration was determined using the PAR assay (see Fig. 4A). As shown in Fig. 4C, the release of zinc is exactly paralleled by the appearance of two bands migrating significantly more slowly than reduced Trx2. This suggests that the oxidation of the zinc center modifies the conformation of the protein.

A.

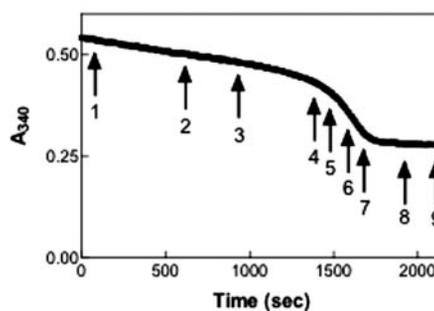
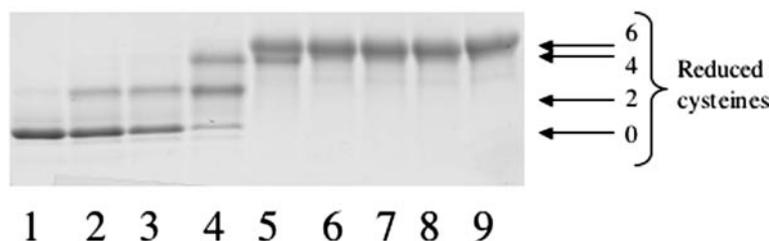


FIG. 5. **Reduction of completely oxidized Trx2 by TrxB.** Completely oxidized Trx2 (20 μ M) was incubated with a catalytic amount of TrxB (10 nM) in presence of NADPH (50 μ M). *A*, the decrease in A_{340} corresponding to NADPH consumption was monitored over time. *B*, at different time points, labeled from 1 to 9, aliquots were taken. Trx2 was precipitated with trichloroacetic acid, free thiols were trapped with AMS, and the samples were analyzed by SDS-PAGE.

B.



To determine whether this conformational change was the result of the formation of internal disulfides or intermolecular disulfide bonds between two or more Trx2 polypeptides, the same samples were also loaded on a denaturing SDS-PAGE under non-reducing and reducing conditions. If the shift observed on the native gels resulted from the formation of intermolecular disulfide bonds, Trx2 oligomers should be observed under non-reducing conditions and should dissociate under reducing conditions. However, more than 80% of Trx2 migrated as a monomer on denaturing SDS-PAGE and only a small percentage was present as disulfide cross-linked dimers (data not shown). This indicated that the upper bands observed on native gels are not the result of intermolecular disulfides, but rather due to other significant conformational changes in Trx2 that occur upon oxidation.

To determine whether Trx2 forms oligomers upon oxidation, we analyzed the oligomerization state of oxidized and reduced Trx2 using size exclusion chromatography. The apparent molecular mass of both proteins was calculated according to a standard curve established under the same buffer conditions. The reduced protein eluted as a 13.5 kDa protein and the oxidized protein eluted as a 18 kDa protein (data not shown). The fact that oxidized Trx2 eluted as a 18 kDa polypeptide was consistent with the conformational changes that were observed in the native gels. To clearly define the oligomeric state of Trx2, ultracentrifugation experiments of reduced and oxidized Trx2 were performed. These experiments were performed using 10 μ M Trx2. This concentration was sufficient to monitor conformational changes of Trx2 in native PAGE. Both fully oxidized and reduced Trx2 were found to have a calculated molecular mass of $16,470 \pm 710$ Da. This indicated that Trx2 is a monomer in both redox states and that the conformational changes that occur upon oxidation do not induce the oligomerization of the protein.

The fact that oxidation of the zinc center affects the conformation of the protein was further confirmed by circular dichroism spectroscopy and proteolytic digestion (Fig. 6). The CD spectrum of the oxidized sample (Trx2_{6cysox}) was indeed markedly different to this of the reduced sample (Trx2_{6cysred}). The

more negative CD values at 220 nm suggest that the oxidized form could be more α -helical and the tryptic digestion experiments showed that reduced Trx2_{6cysred} is much more stable than the Trx2_{6cysox} (Fig. 6).

Zinc-free Trx2 Conserves Its Reductase Activity—Both Trx2 and TrxA have a dithiol-disulfide-oxidoreductase activity and are able to catalyze the reduction of insulin by DTT (6, 19). Reduction of the disulfide bonds present in insulin causes the precipitation of the B chain, which can be monitored by following the increase of turbidity at 650 nm (19). To determine whether zinc is required for the oxidoreductase activity of Trx2, we compared the ability of both the zinc-free and the zinc-containing enzyme to reduce insulin. As shown in Fig. 7, the absence of zinc does not affect the rate of insulin reduction. This suggests that the zinc center of Trx2 is not directly involved in the reductase activity of the protein. We also show that Trx2, with or without zinc, catalyzes the reduction of insulin by DTT more efficiently than TrxA.

It would have been interesting to study the effect of the oxidation of the zinc center on the oxidoreductase activity of Trx2. However, we could not perform this experiment as incubation of oxidized Trx2 (Trx2_{6cysox}) with either DTT or TrxB/NADPH led to the reduction of all 6 cysteine residues.

DISCUSSION

Thioredoxins are present in almost all sequenced genomes and many of them have been extensively studied for more than 40 years. Thioredoxins have been shown to be responsible for thiol-disulfide exchange reactions and serve as electron donors for PAPS reductase, ribonucleotide reductase, and methionine sulfoxide reductase (1–3). They also play a role in the reduction of deleterious disulfides in cytosolic proteins that may have been formed as a result of oxidative stress. Crucial for these reactions is a CGPC active site sequence that undergoes cycles of oxidation and reduction as part of its catalytic mechanism (1–3).

We have identified a subfamily of thioredoxins present in at least 20 different organisms that is characterized by the additional presence of an N-terminal domain that contains two

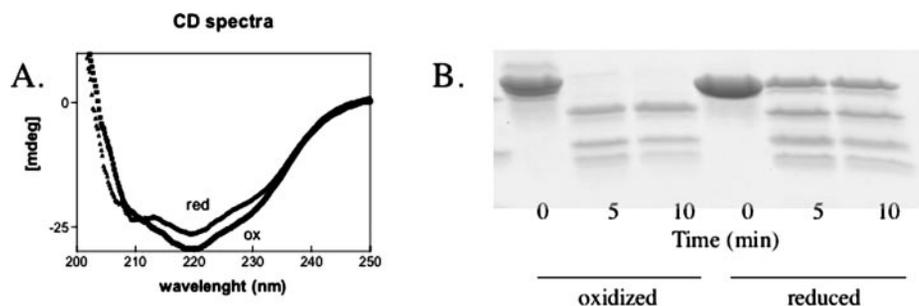


FIG. 6. **Oxidation of the zinc center induces a conformational change.** Reduced zinc-saturated Trx2 (50 μM) was incubated with H_2O_2 (2 mM) at 43 $^\circ\text{C}$. At the end of the reaction, the CD spectrum of the oxidized protein ($\text{Trx2}_{6\text{cysox}}$) was recorded and compared with the spectrum of the reduced protein ($\text{Trx2}_{6\text{cysred}}$) (A). The protease sensitivity of both $\text{Trx2}_{6\text{cysred}}$ and $\text{Trx2}_{6\text{cysox}}$ was also compared at different time points (B). CD spectroscopy and tryptic digestion were performed as described under “Experimental Procedures.”

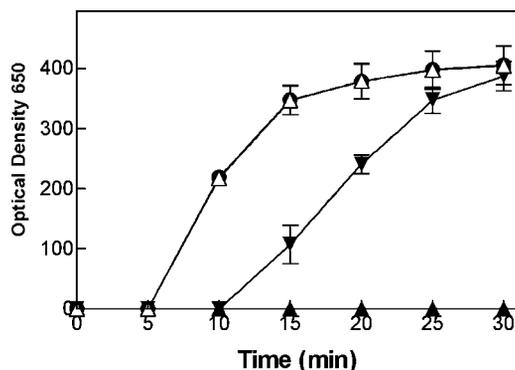


FIG. 7. **Zinc-free Trx2 is able to catalyze the reduction of insulin by DTT.** Insulin (130 μM) was incubated with DTT (0.6 mM) and 1 μM of either zinc-containing Trx2 (●), zinc-free Trx2 (△), or TrxA (▼). The non-catalyzed reaction (DTT alone) was also monitored (▲). Zinc-free Trx2 was prepared as described under “Experimental Procedures.” The reactions were followed by monitoring the optical density at 650 nm.

highly conserved CXXC motifs separated by 15–16 residues. We have found that the two CXXC motifs tightly coordinate one zinc atom. *In vitro* oxidation of these CXXC motifs releases the zinc and causes significant conformational changes in the protein. So far, there have only been two other reports of thioredoxin-like molecules that bind zinc. Zinc is present in the crystal structure of a protein disulfide oxidoreductase from the archaeon *Pyrococcus furiosus*, a protein that contains two thioredoxin folds (20). Here, however, the zinc is only loosely coordinated by two poorly conserved residues Glu-15 and His-102 and two water molecules. Moreover, 0.2 M zinc was present during crystal growth, raising the possibility that the presence of zinc in this protein might be a crystallization artifact. The other reported zinc binding in thioredoxin is based on biochemical engineering, where two cysteine residues and two histidine residues were introduced into *E. coli* TrxA at appropriate locations (21). Therefore, to our knowledge, the Trx2 subfamily appears to be the first report of a naturally occurring zinc site present in members of the thioredoxin family. The conservation of the two CXXC motifs CXXC_{n1} and CXXC_{n2} in Trx2 homologues and in paraquat-inducible membrane proteins strongly suggests that the latter also contain bound zinc.

Several hundred different enzymes have been reported to require zinc. Unlike other first-row transition metals, the zinc atom contains a filled d-orbital and does not participate in redox reactions (22). This lack of redox activity makes Zn^{2+} an ideal metal cofactor for enzyme catalysis, where it functions as a Lewis acid and accepts a pair of electrons. Zinc can also play a structural role and enhances protein stability. This is, for instance, the case in zinc finger proteins. Many different zinc binding motifs have been described by the Prosite (release

17.54) and Pfam databases (version 9.0) (23, 24). None of these, however, corresponds to the zinc binding motif present in Trx2 [CXXC(15–16)CXXC]. Thus, we concluded that we have identified a new specific zinc binding sequence that is present in two classes of proteins, thioredoxins and paraquat-inducible proteins.

One of the unique characteristics of the zinc binding motif in Trx2 is its extreme affinity for zinc ($K_a > 10^{18} \text{ M}^{-1}$), which is much higher than the affinity constant that has been reported for carbonic anhydrase ($0.8 \times 10^{12} \text{ M}^{-1}$) (25), metallothionein ($1.4 \times 10^{13} \text{ M}^{-1}$) (26), or the chaperone Hsp33 ($2.5 \times 10^{17} \text{ M}^{-1}$) (12). We are not aware of any zinc-binding protein with comparable or greater affinity to zinc than Trx2. There is a thermodynamic relationship between the strength of cofactor binding and the increase in protein stability that accompanies the binding of the cofactor. It is, therefore, not surprising that the zinc-bound protein is significantly more stable toward proteolytic digestion (not shown).

Interestingly, the zinc binding center of Trx2 was found to be redox sensitive. Incubation of Trx2 in H_2O_2 quickly induces the formation of 2 intramolecular disulfide bonds and the release of zinc. Redox-sensitive cysteines have been described in several transcription factors (*e.g.* OxyR and HoxB) and in the chaperone Hsp33. In these proteins, oxidation of cysteine residues induces a conformational change. This causes an alteration in the activity of OxyR (27) and Hsp33 (13) and affects the binding characteristics of HoxB5 (28). This is also the case with Trx2 where major conformational changes accompany the oxidation process. This is likely to affect strongly the properties of the protein and could trigger another activity. There are several parallels between Trx2 and Hsp33. Both proteins coordinate a zinc, that is released upon oxidation, and in both cases a major conformational change is triggered by zinc release. Hsp33 is activated directly by oxidative stress and Trx2 is under the control of the oxidative stress sigma factor OxyR. These striking parallels suggest that Trx2 like Hsp33 has a specific function in response to oxidative stress.

Another important point will be to evaluate to what extent the zinc center of Trx2 is oxidized upon oxidative stress *in vivo*. Ritz *et al.* (9) reported that all 6 cysteines of Trx2 stay in the reduced form even in strains with a more oxidizing cytoplasm. These conditions do not reflect the presence of reactive oxygen species and we are currently investigating the effect of addition of hydrogen peroxide on the oxidation state of Trx2 *in vivo*. It has been reported that Trx2 could be equivalent to TrxA and could work as a backup system expressed under oxidative stress conditions. If this is indeed the case, why is zinc bound to Trx2 and why would the N-terminal domain be conserved in more than 20 other bacteria? We show that the oxidoreductase activity of the protein on insulin is independent of the presence of zinc. It is therefore likely that the highly conserved zinc-

center of Trx2 plays another role, yet to be discovered. Interestingly, the zinc binding domain of Trx2 is conserved in paraquat-inducible proteins, another family of oxidative stress induced proteins. It is intriguing that the zinc center we describe here is found in two proteins families, both of which are induced by oxidative stress.

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