Crystal Structure of an Unusual Thioredoxin Protein with a Zinc Finger Domain*

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Many Gram-negative bacteria have two cytoplasmic thioredoxins, thioredoxin-1 and -2, encoded by the trxA and trxC genes, respectively. Both thioredoxins have the highly conserved WGCPC motif and function as disulfide-bond reductases. However, thioredoxin-2 has unique features: it has an N-terminal motif that binds a zinc ion, and its transcription is under the control of OxyR, which allows it to be up-regulated under oxidative stress. Here, we report the crystal structure of thioredoxin-2 from Rhodobacter capsulatus. The C-terminal region of thioredoxin-2 forms a canonical thioredoxin fold with a central β-sheet consisting of five strands and four flanking α-helices on either side. The N-terminal zinc finger is composed of four short β-strands (S1–S4) connected by three short loops (L1–L3). The four cysteines are at loops L1 and L3 and form a tetragonal binding site for a zinc ion. The zinc finger is close to the first β-strand and first α-helix of the thioredoxin fold. Nevertheless, the zinc finger may not directly affect the oxidoreductase activity of thioredoxin-2 because the zinc finger is not near the active site of a protomer and because thioredoxin-2 is a monomer in solution. On the basis of structural similarity to the zinc fingers in Npl4 and Vps36, we propose that the N-terminal zinc finger of thioredoxin-2 mediates protein-protein interactions, possibly with its substrates or chaperones.

Oxidative stress causes the damage of DNA, protein, and lipid molecules and also affects their biosynthesis. All cells possess systems that serve as a defense against oxidative stress. These include reducing systems provided by GSH, glutaredoxins, and thioredoxins (1, 2). The thioredoxins are small cytoplasmic proteins present in all cells. They serve as reductases that reduce disulfide bonds in a wide range of proteins, including 3’-phosphoadenylylsulfate reductase, ribonucleotide reductase, methionine sulfoxide reductase, and the membrane protein DsbD. Thioredoxins have a highly conserved WGCPC motif; during the catalytic cycle, the two cysteines alternate between reduced and oxidized forms. When thioredoxin reduces a disulfide bridge, it itself becomes oxidized. The disulfide in thioredoxin must therefore be regenerated by a thioredoxin reductase, which in turn uses the reducing equivalents derived from NADPH (2, 3). In addition to functioning as an electron donor, thioredoxin is an essential component of the T7 DNA polymerase and is involved in the assembly of a number of filamentous viruses (4).

Bacterial thioredoxin-2 is a recently described cytoplasmic thioredoxin homolog. It shares ~30% overall sequence identity with thioredoxin-1 and contains an N-terminal extension of about ~40 residues (5–8). Thioredoxin-2 seems to play a role in the protection against oxidative stress, as the deletion of its gene in Rhodobacter capsulatus results in increased sensitivity to oxidative agents (8, 9). Furthermore, under oxidative stress conditions, the expression of thioredoxin-2 is up-regulated by the transcription factor OxyR (6, 9), whereas the expression of thioredoxin-1 does not change. The N-terminal extension contains two additional CXXC motifs and binds a zinc ion with high affinity. Based on in vitro experiments with the oxidative agent hydrogen peroxide, it has been proposed that the zinc ion is released from thioredoxin-2 under oxidative stress (7). However, it is not clear whether the zinc ion is released in intact cells particularly because thioredoxin-2 remains in its reduced state even under oxidative stress (6). Mutation of the zinc-binding domain does not affect the oxidoreductase activity of thioredoxin-2 in vivo, at least not for the substrates analyzed (6). However, it has been reported that the deletion of the zinc finger domain decreases the reductase activity in vitro (5). Thioredoxin-2 has been found in ~20 different bacterial species. Proteins with the same name in eukaryotes lack a zinc finger domain.

As the zinc finger of bacterial thioredoxin-2 is found in different organisms, it is unlikely to be without function. Similar domains are found in many proteins that are involved in DNA, RNA, or lipid binding; in protein-protein interactions; in protein stability; or in the sensing of oxidative stress (12). The zinc finger motif usually contains ~20–60 residues with cysteines and/or histidines folded into a tetragonal binding site for a zinc ion. The zinc ion is important in stabilizing the structural fold and binds with high affinity. Although all zinc finger domains share the tetragonal zinc coordination, their structures are as diverse as their functions. It is therefore not easy to predict the structure of a zinc finger domain on the basis of the amino acid
Crystal Structure of an Unusual Thioredoxin Protein

sequence. Specifically, a BLAST search using thioredoxin-2 does not reveal any zinc fingers with known structures. The thioredoxin-2 zinc finger contains two CXXC motifs separated by 15–16 residues. A similar pattern is found in DksA (CXXCX_{17}CXXC), transcription factor IIB (CXXCX_{15}CXXC), and ClpX (CXXCX_{16}CXXC) (13–16), but the structures of these zinc fingers are quite different. The DksA zinc finger has the typical βαβ-fold found in many transcription factors that bind directly to DNA; the transcription factor IIB zinc finger has four short strands that are involved in the interaction with RNA polymerase II; and the zinc finger in ClpX has two β-hairpins followed by an α-helix and is involved in homodimerization and substrate interaction (14, 15). It has been proposed that the zinc finger of thioredoxin-2 is similar to that in the redox sensor protein Hsp33 (7), but there is essentially no sequence similarity. The primary sequence of thioredoxin-2 does not reveal much of its function or structure.

Here, we report the crystal structure of R. capsulatus thioredoxin-2 at 1.92-Å resolution. Thioredoxin-2 has an N-terminal zinc finger and a C-terminal canonical thioredoxin fold. Our results show that the structure of the N-terminal zinc finger is similar to those of Npl4, Vps36, and transcription factor IIB (16–18). We propose that the zinc finger of thioredoxin-2 mediates protein-protein interactions with its substrates or chaperones during the defense against oxidative stress.

EXPERIMENTAL PROCEDURES

Cloning and Protein Expression—The R. capsulatus thioredoxin-2 gene was amplified from genomic DNA by PCR and cloned into pET28a between the NdeI and HindIII sites. The recombinant protein has an N-terminal histidine tag and a thrombin site between the tag and thioredoxin-2. The expression vector was transformed into C43(DE3) cells. The transformed cells were grown to A_{600} = 0.7 and induced by the addition of isopropyl β-D-thiogalactopyranoside to a final concentration of 0.5 mM. Cells were harvested after culturing at 37 °C for an additional 4 h. The cell pellet was resuspended in Tris-buffered saline (20 mM Tris, 0.3 mM NaCl, and 10% glycerol, pH 8.0), and cells were broken with a Microfluidizer. The recombinant thioredoxin-2 protein was in the supernatant and purified by Ni-NTA chromatography under native conditions. Thrombin was added to the purified sample at a ratio of 10 units of thrombin to 1 mg of recombinant thioredoxin-2. The cleaved sample was concentrated and further purified by size-exclusion fast protein liquid chromatography in a running buffer of 20 mM HEPES and 0.1 mM NaCl, pH 7.4. Fractions containing thioredoxin-2 were pooled and concentrated to a final concentration ~10 mg/ml.

Crystallization, Data Collection, and Structure Determination—Before trays were set up, dithiothreitol was added to a final concentration of 5 mM, and the protein solution was incubated at room temperature for 1 h. Crystals were grown in 28–36% polyethylene glycol 400, 10 mM ZnSO_{4}, and 50 mM N-(2-acetamido)imidodiacetic acid, pH 6.5, by the hanging drop method at 22 °C. Crystals usually grew to 200 × 200 × 50 μm³ in 1–2 weeks. Crystals were looped directly from the drop and flash-frozen in liquid nitrogen. Data were collected using synchrotron radiation at a zinc peak wavelength of 1.2826 Å at beam line ID19 of the Advanced Photon Source. A data set for single-wavelength anomalous diffraction (SAD) phasing was collected. The images were indexed and scaled by HKL3000 (19). Phasing, map calculation and improvement, and automated model building were done in HKL3000. Briefly, using the zinc SAD data set, the SHELEX module in HKL3000 found three zinc sites (20). The zinc sites were refined and phases were calculated using mthlphare (21). Automated model building was carried out using the ARP/wARP program (22). The electron density map was further improved by NCS averaging using the NCS operator derived from the partially built model (21). The map was used for a second round of ARP/wARP automated model building. Most of the model (~90%) was built. The rest was manually built using Coot (23). The refinement was carried out in CNS (24) against a second data set at 1.92-Å resolution.

Analytical Ultracentrifugation—Analytical ultracentrifugation was carried out on reduced thioredoxin-2 in a Beckman Coulter Optima XL-A ultracentrifuge. Three samples with initial concentrations of ~15, ~40, and ~80 μM thioredoxin-2 were loaded on a 6-well cell and centrifuged at three speeds of 24,000, 28,000, and 36,000 rpm. Data were collected after equilibrium was achieved as indicated by no changes in two successive scans separated by 4 h. Data were analyzed by Beckman XL-A data analysis software using MULTIFIT to an ideal self-association model (25).

Ni-NTA Pulldown of His-tagged Thioredoxin-2 and Identification of Its Binding Partner DnaK—Escherichia coli strain DHB4 (Δ[ara-leu])7697 araD139 ΔlacX74 galE galK rpsL phoR Δ(phoA)PuvII ΔmalF3 thi (26) was transformed with plasmid pDR1005 (pBAD33-His_{6}-trxC) or pDR1006 (pBAD33-His_{6}-Δ2–29trxC) or with the pBAD33 vector (6). Protein expression was induced by 0.2% L-arabinose at A_{600} = 0.1. Cells were grown at 37 or 42 °C after induction and harvested when the cell density reached A_{600} = 0.5. Cells from 10 ml of culture were lysed in 1 ml of buffer containing 20 mM Tris and 0.3 mM NaCl, pH 8. The supernatant was mixed with 100 μl of Ni-NTA beads.

| TABLE 1 |
| Data collection and refinement statistic |
| Data set | For SAD phasing | For structure refinement |
| --- | --- | --- |
| Resolution (Å) | 50.0 to 2.0 | 50.0 to 1.92 |
| Unique reflections | 22,441 | 24,706 |
| Redundancy (×) | 6.7 (4.1)* | 2.9 (1.9) |
| Completeness (%) | 99.5 (95.3) | 96.6 (75.1) |
| Rmerge | 0.089 (0.450) | 0.066 (0.252) |
| Reflections for Rwork/Rfree | 23,512/1194 | 20.5/21.9 |
| r.m.s.d. bond length (Å) | 0.015 | |
| Mean B-factor | 25.4 | |
| Ramachandran plot (most favored/additionally allowed; %) | 93.2/6.3 | |

* Values in parentheses refer to data in the highest resolution shell (2.07 to 2.00 Å in the first data set and 1.99 to 1.92 Å in the second).

r.m.s.d. = root mean square deviation.

The abbreviations used are: Ni-NTA, nickel-nitrilotriacetic acid; SAD, single-wavelength anomalous diffraction.
Crystall Structure of an Unusual Thioredoxin Protein

The Ni-NTA beads were washed three times with 1 ml of the same buffer and then washed three times with 1 ml of the same buffer containing 30 mM imidazole. The proteins were eluted with 300 mM imidazole. Samples eluted from the Ni-NTA columns were run on SDS-polyacrylamide gel under reducing conditions. The bands in the SDS gel were cut out and analyzed by liquid chromatography/tandem mass spectrometry (Taplin Mass Spectrometry Facility, Harvard Medical School).

RESULTS AND DISCUSSION

Overall Structure of Thioredoxin-2—R. capsulatus thioredoxin-2 was crystallized, and its structure was determined using SAD originating from the bound zinc ion (Table 1). Thioredoxin-2 has an N-terminal zinc finger motif and a C-terminal canonical thioredoxin fold (27). The thioredoxin fold consists of a five-stranded β-sheet with flanking α-helices (Fig. 1A). The secondary structural elements are βαβεβαβα, as shown in Fig. 1B. The distance between the sulfur atoms of Cys73 and Cys76 at the active site is 3.7 Å, indicating that, in the crystal, thioredoxin-2 is in its reduced state. This is expected because 5 mM dithiothreitol was added to the protein solution during crystallization. Nevertheless, the structure can be superimposed on the oxidized form of E. coli thioredoxin-1 with a backbone root mean square deviation of 0.93 Å (Fig. 1C), in agreement with the previous observation that the reduced and oxidized forms of thioredoxin-1 are almost identical (28). The active sites of thioredoxin-1 and -2 are also very similar (Fig. 1D). The highly conserved WCGPC motif is located at the loop following the central β-strand (β2) and the beginning of the second α-helix. The active-site cysteine (Cys73 in thioredoxin-2 and Cys82 in thioredoxin-1) is at the beginning of helix α2 and is solvent-exposed. This cysteine has a relatively low pK_a (29). The deprotonated thiolate is stabilized by the helical dipole moment of helix α2 (30). The second conserved cysteine (Cys76 in thioredoxin-2 and Cys81 in thioredoxin-1) has a higher pK_a. It is likely to be deprotonated through a general base catalysis by an aspartic residue in the β2-strand (Asp67 in thioredoxin-2 and Asp68 in thioredoxin-1). Because the carboxyl group of this aspartic residue is ~5 Å away from the second sulfur atom, the deprotonation is probably mediated by the polarization of a water molecule. Another common feature among the thioredoxins is a positively charged residue (Arg77 in thioredoxin-2 and Lys36 in thioredoxin-1) that interacts with the backbone carbonyl group of the residue preceding the conserved tryptophan (Trp72 in thioredoxin-2 and Trp74, respectively). This interaction might be important to maintain the active-site conformation. Overall, thioredoxin-1 and -2 likely use the same mechanism to catalyze disulfide reduction of their substrates.

The N-terminal Zinc Finger—The N-terminal extension of thioredoxin-2 is folded into a zinc-binding finger, consisting of four short strands (S1–S4) connected by three short loops.
The S1/S2 strands are hydrogen-bonded with each other and connected by loop L1. The S3/S4 strands are shorter than the S1/S2 strands with fewer hydrogen bonds formed between them; they are connected by the short loop L3. The two CXXC motifs are located in the loops (L1 and L3) at the end of the four strands. The four cysteines form a tetragonal zinc-binding site. The zinc ion is clearly visible in the anomalous difference map (Fig. 2B). The S1/S2 strands are associated with the S3/S4 strands through the zinc ion and through hydrophobic interactions. A number of hydrophobic residues (Leu12 and Val23 in the S1/S2 strands and Pro32, Ile42, and Leu43 in the S3/S4 strands) are involved in packing these two structural elements together (Fig. 2A). These residues are relatively well conserved. Although the hydrophobic interactions are likely to be important, the zinc ion probably plays a dominant role in keeping the S1/S2 and S3/S4 structural elements together. The distance between the zinc ion and the sulfur atoms in the cysteines is \( <2.3 \, \text{Å} \), indicating a strong interaction.

In addition to the zinc ion in the zinc finger domain, a second zinc ion, coordinated by His \(^{111} \) and Asp \(^{58} \), is observed in one protomer in the crystal (Fig. 2B). The zinc ion at this site has a relatively low occupancy and is probably bound with low affinity.

The Zinc Finger Is Not Near the Active Site of Thioredoxin-2—In our structure, the zinc finger is not close to the WCGPC active site. Rather, it is located at one side of the thioredoxin fold defined by the first strand (\( \beta_1 \)) and the first \( \alpha \)-helix (\( \alpha_1 \)) (Fig. 1, A and B). The zinc ion is almost at the opposite end of the active site, with a distance of \( \sim 30 \, \text{Å} \) between them. It is thus quite unlikely that the zinc finger can directly affect the reactivity of the active site within the same molecule. It is also unlikely that the zinc finger would directly compete for substrate binding within a protomer. The known structures of complexes between a thioredoxin fold and a substrate (DsbD-DsbD (31), DsbA-DsbB (32), and thioredoxin-NF-κB (33)) indicate that the substrate-binding region is defined by the WCXAC active site at the \( \beta_2-\alpha_2 \) region and the beginning of the \( \beta_4 \)-strand where the Gly-Ile-Pro conserved motif is located (Fig. 1D). These regions are far away from the zinc finger domain.

Dimer in Crystal and Monomer in Solution—Although the zinc finger is not close to the WCGPC active site or the substrate-binding groove within a protomer, it is conceivable that it could be close to one of these in an oligomer. In our crystals, the two protomers in the asymmetric unit are arranged such that the zinc finger of one protomer is close to the active site of
the second protomer (Fig. 3A). Notably, the side chain of the con-
served residue Arg77 forms a hydrogen bond with the backbone car-
bonyl of Cys37 in loop L3 of the zinc finger. In addition, the side chain of
Gln102 is hydrogen-bonded with the carbonyl of Ala16 in loop L1. Finally,
residues in loops L1 (Leu15, Ala16, and Cys17) and L3 (Cys37, Gly38, and
Ala39) are in van der Waals contact with residues around the active site
(Pro71, Trp72, Cys73, Pro75, and Arg77) (Fig. 3A). These interactions
suggest that the zinc finger might
affect the oxidoreductase activity of
the thioredoxin fold in a dimer or
higher oligomer. However, several
lines of evidence suggest that thio-
reductin-2 is monomeric in solu-
tion. First, the buried surface
between the protomer is relatively
small, ~1016 Å² (7.5% of the total
surface). Second, the reduced form
of R. capsulatus
thioredoxin-2 runs
at the position of a monomer in gel
filtration experiments (data not
shown). Similar results have also
been reported for both the reduced
and oxidized forms of E. coli
thioredoxin-2 (7). To exclude that thi-
oredoxin-2 dimerizes with low affinity,
we performed analytical ultracen-
trifugation experiments, which can
measure $K_d$ values as low as 1 mM.
Thioredoxin-2 at three different
concentrations (15, 40, and 80
M) was used and centrifuged to
equilibrium at three different
speeds. The data were fit to a self-
association model. The best fit was
obtained with a monomer species of
14.3 kDa (theoretical molecu-
lar mass of 15.5 kDa) (Fig. 3B). The
deviations from the theoretical val-
ues (Fig. 3B, upper panels)
were close to zero and randomly distrib-
uted. The results indicate that thi-
oredoxin-2 is a monomer in solu-
tion even at concentrations as high
as 80 μM. The dimer observed in the
crystal is thus likely to be a non-
physiological artifact. It should be
noted that in other thioredoxin
crystals, dimers or oligomers were
also observed in the asymmetric
unit (34–36). Given that the inter-
faces between the monomers are

FIGURE 4. A, overlay of the zinc fingers of thioredoxin-2, Vps36, and Npl4. The zinc fingers of Vps36 (blue) and Npl4
(yellow) are superimposed onto that of thioredoxin-2 (pink). The zinc ions are also colored the same way as their
respective ribbon model. The side chains of thioredoxin-2 cysteines are shown in green. B, binding determinants in
thioredoxin-2 (left), Vps36 (middle), and Npl4 (right). The zinc fingers are oriented as described for A. The side chains
of residues involved in protein-protein interactions and the cysteines in zinc ion coordination are shown in green.
The zinc ions are shown in silver. C, the second protein-protein interaction region of the thioredoxin-2 zinc finger in
the crystal. The extended N terminus of one protomer (shown in green) binds to the side of the zinc finger of a
symmetry-related protomer (shown in pink). Met² and Lys² of the N terminus form hydrogen bonds (shown as blue
dashed lines) with Ala²⁰, Lys²¹, and Lys²⁵ (shown in gray).
Crystal Structure of an Unusual Thioredoxin Protein

different in these structures and that the existence of dimers or oligomers in solution has not been demonstrated for any of the wild-type thioredoxins, it appears that all members of the family function as monomers.

The Zinc Finger of Thioredoxin-2 Is Homologous to Those That Mediate Protein-Protein Interactions—We performed a DALI search using the zinc finger of thioredoxin-2 (residues 7–44) to find homologous structures. Four structures (Vps36 (Protein Data Bank code 2J9U), Npl4 (code 1NJ3), 30 S ribosomal protein S27e (code 1NVH), and YAF2 (code 2D9G)) were found with Z scores >2 (37). The structures are considered to be similar if the DALI Z score is >2. All of them contain two CXXC motifs that form a tetragonal zinc-binding site. The length between the C motif is also important for ubiquitin binding. The C motif has been implicated in ubiquitin binding as shown by chemical shift perturbation and mutagenesis studies. In addition, Met<sup>25</sup> near the second CXXC motif is also important for ubiquitin binding. The TF binding surface is not exactly the same as that of Vps36 (Fig. 4B) (16). Nevertheless, both are located at loops L1 and L3.

We noticed two surfaces of the thioredoxin-2 zinc finger that are involved in protein-protein contacts in the crystal. The first binding surface is the one in the dimerization interface (Figs. 3A and 4B). This binding surface resembles that of Vps36 and, to a lesser extent, that of Npl4. The second binding surface is shown in Fig. 4C. It involves the unstructured N terminus (residue 1–7) of one protomer interacting with one side of the zinc finger domain of a symmetry-related molecule. Met<sup>2</sup> and Lys<sup>5</sup> in the N-terminal segment form four hydrogen bonds with Ala<sup>20</sup>, Lys<sup>22</sup>, and Lys<sup>33</sup> in the zinc finger (Fig. 4C). On the basis of these interactions and the similarity to other zinc finger domains, we propose that this type of zinc finger mediates protein-protein interactions. Specifically, the zinc finger of thioredoxin-2 may be involved in the binding of substrates and/or chaperones. Thioredoxin-2 has been found to interact with a number of proteins, including the DnaJ and DnaK chaperones, in a large-scale search for interacting proteins in E. coli (38). Our attempts to confirm the interaction with DnaJ by direct pulldown experiments have been unsuccessful (data not shown). However, the interaction with DnaK could be confirmed by us in experiments in which we pulled down His-tagged thioredoxin-2 with Ni-NTA beads and identified bound proteins by mass spectrometry (Fig. 5). A thioredoxin-2 mutant lacking the zinc domain appeared to bind less DnaK (Fig. 5, lane 3 versus lane 4). These data suggest that the zinc domain may interact with the DnaK chaperone, but they do not exclude the possibility that a fraction of this domain may be unfolded and thus be bound by DnaK.

The zinc finger of thioredoxin-2 is different from that of Hsp33, a redox sensor, and from those of DNA-binding proteins. These results indicate that thioredoxin-2 does not act as a redox sensor, is not involved in protecting DNA against oxidative damage, and is not involved in transcription, yet conservation of this domain suggests that it has an important function. On the basis of the structural homology to the zinc fingers of Npl4 and Vps36, we propose that the zinc finger of thioredoxin-2 mediates protein-protein interactions.

Thioredoxin-2 Is Conserved among Proteobacteria, and Its Zinc Finger Is Seen in Other Proteins—Although thioredoxin-2 was initially found only in E. coli and Corynebacterium nephridii (5), we found 85 homologs using the zinc finger domain (residues 1–40) of the protein from R. capsulatus in a BLAST search. All of these proteins are from Gram-negative pro-
teobacteria, representing all five (α–ε) classes. The only exception is the protein from *C. nephridii*, a Gram-positive actinobacterium. We then performed a pattern search using CXXCX_{10–20}CXXCX_{30–40}WCXXC in which we allowed 10–20 residues between the two CXXC motifs in the zinc finger and 30–40 residues between the second CXXC motif and the WCXXC active site of thioredoxin. We identified 12 additional homologs from Gram-negative proteobacteria, four from Gram-positive actinobacteria (*Streptomyces avermitilis, Arthrobacter aurescens, Mycobacterium vanbaalenii,* and *Mycobacterium flavescentis*), and one from a *Deinococcus-Thermus* species (*Thermus thermophilus*). Thus, thioredoxin-2 is conserved in >100 different bacteria. It is interesting that a zinc finger motif homologous to that of thioredoxin-2 is also present in the Paraquat-inducible membrane protein PqiVT8. The zinc finger may thus have evolved from a common ancestor and be used in various proteins as a functional module. This is consistent with our proposal that the N-terminal zinc finger is involved in protein-protein interactions.

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Crystal Structure of an Unusual Thioredoxin Protein

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