A Conserved *cis*-Proline Precludes Metal Binding by the Active Site Thiolates in Members of the Thioredoxin Family of Proteins†

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ABSTRACT: Many thioredoxin-fold proteins possess a conserved *cis*-proline located in their C-terminal portions. This residue, as well as catalytic and resolving cysteines, is a key functional group in the active sites of these thiol−disulfide oxidoreductases. However, the specific function of the proline is poorly understood, and some thioredoxin-fold proteins lack this residue. Herein, we found that mutation of a *cis*-proline, Pro75, in human thioredoxin to serine, threonine, or alanine leads to the formation of an Fe_2-S_2 cluster in this protein. Further mutagenesis studies revealed that the first cysteine in the CxxC motif and a cysteine in the C-terminal region of the protein were responsible for metal binding. Replacement of Pro75 with arginine, a residue that occurs in place of Pro in peroxiredoxins, also led to the formation of the cluster in the thioredoxin. In addition, we found that mutation of the TxxC active site in a peroxiredoxin to the CxxC form could lead to coordination of an Fe_2-S_2 cluster in these proteins in vitro. Sco1, a distantly related thioredoxin-fold protein, has histidine in place of the *cis*-proline, and this residue binds copper. The Pro75His mutation led to increased copper binding by human thioredoxin when cells were grown in the presence of this trace element. Taken together, our data suggest that an important function of Pro75 in human thioredoxin, and likely other members of this superfamily, is to prevent metal binding by the reactive thiolate-based active site.

Thioredoxin is a ubiquitous redox protein that is present in all or almost all living organisms (*1*). It functions as protein disulfide reductase by transferring reducing equivalents to numerous proteins, such as ribonucleotide reductase (*1*), peroxiredoxin (*2*, *3*), methionine sulfoxide reductase (*4*), and transcription factors NF-κB (*5*) and AP-1 (*6*).

Multiple isoforms of thioredoxin exist in most organisms; for example, two thioredoxins are present in *Escherichia coli*, three in *Saccharomyces cerevisiae*, and two in mammals. In addition, numerous thioredoxin-like proteins are known in virtually every organism. Thioredoxins have been shown to be indispensable in many life forms. In mice, both cytosolic and mitochondrial thioredoxins are essential for development (*7*, *8*).

Thioredoxin and its structural homologues share the same structural fold, known as the thioredoxin fold (*9*, *10*). According to the SCOP (Structural Classification of Proteins) database (*11*, *12*), glutaredoxins, glutathione *S*-transferases (GSTs),[1] protein disulfide isomerases (PDI), DsbA, DsbC, glutathione peroxidases, and several other proteins are characterized by this fold. In addition, if circular permutations are considered, ribosomal protein L30e, tubulin C-terminal domain, cytidine deaminase, and phospholipase D could be viewed as thioredoxin-like fold structures (*13*).

Many proteins with the conventional thioredoxin fold (not considering circular permuted analogues or other variations) have two conserved features: a CxxC motif (two cysteines separated by two residues) located in the loop or at the beginning of an α1 helix and a *cis*-proline adjacent to the β3 strand of the fold. There may be further variations in the CxxC motif, namely, SxxC, TxxC, CxxS, or CxxT motifs, that are found in some members of the thioredoxin superfamily (*14*). The proline is conserved in the majority of thioredoxin-fold protein families, such as thioredoxins, glutaredoxins, GSTs, and DsbAs (*10*). However, in peroxiredoxins, arginine is present in place of the proline (*15*).

The CxxC motif or its variants are usually the active site residues in thioredoxin-fold proteins. While the importance of the proline in catalytic activities of thioredoxin-fold proteins has been recognized early on, its specific function is still not understood. It has been suggested that the *cis*-proline forms hydrogen bonds with the substrate (*10*). Mutation of this residue in DsbA (*16*) and GST (*17*) revealed that the *cis*-proline is important for the overall structure and structural stability of the active site, as well as for the catalytic activities of these proteins. In addition, mutation of the proline to serine or threonine led to stabilization of mixed disulfides between DsbA and its substrates or between DsbA and DsbB, a protein that oxidizes DsbA (*18*).

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[1] Abbreviations: GST, glutathione *S*-transferase; PDI, protein disulfide isomerase; Trx, thioredoxin; Grx, glutaredoxin, Prx, peroxiredoxin; CxxC motif, two cysteines separated by two residues; Fe_2-S_2 cluster, a cluster containing two iron and two sulfur atoms; Pro75, proline 75; ICP, inductively coupled plasma-emission spectrometry; DTT, dithiothreitol.

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Thioredoxin-fold proteins almost exclusively serve as thiol-based oxidoreductases and only rarely bind metal ions. However, a thioredoxin-fold ferredoxin is known that contains an Fe_2-S_2 cluster (*19*), and Sco1 proteins bind copper (*20*−*22*). Recently, it was reported that mammalian glutaredoxin Grx2 can bind an Fe_2-S_2 cluster (*23*). A bacterial thioredoxin was also found to be converted to an iron−sulfur-containing protein through either rational design (*24*, *25*) or mutant screening (*26*).

In the current study, we generated multiple mutants of human thioredoxin lacking the conserved proline, and many of them were found to bind an iron−sulfur cluster. Further mutagenesis experiments and comparison between metal-binding and oxidoreductase classes of thioredoxin-fold proteins revealed that the proline prevents metal binding by the active site thiolates.

MATERIALS AND METHODS

Sequence Analysis. Sequence alignments of thioredoxin-fold proteins were based on the secondary structure information obtained from the Protein Data Bank (www.pdb.org) and the alignment shown by Qi and Grishin (*13*). A phylogenetic tree was generated using PHYLIP (*27*). Protein structures were visualized and figures prepared with Chimera (*28*) (http://www.cgl.ucsf.edu/chimera).

Preparation of Recombinant Human Thioredoxin and Its Mutant Forms. Human thioredoxin cDNA was cloned into a pET20 vector (Novagen), and the indicated thioredoxin mutants were generated using the QuikChange site-directed mutagenesis kit from Stratagene.

Protein Expression and Purification. Recombinant wild-type and mutant human thioredoxins were expressed and isolated from *E. coli*. The plasmids were transformed into an *E. coli* strain BL21(DE3) (Novagen). Cells carrying the plasmids were induced with 0.1 mM IPTG and, after 3−4 h of protein synthesis at 37 °C, were collected. To supplement cells with copper or nickel, 100 μM cupric chloride or 100 μM nickel sulfate was added to the medium at the time of induction. Cells were centrifuged and proteins isolated using His-tag resins (QIAGEN) according to the pET System Manual (Novagen) and the QIAexpressionist (QIAGEN). Purity of proteins was verified by SDS−PAGE analysis. Recombinant rat thioredoxin reductase with a specific activity of 15−20 units/mg was expressed and purified as described in ref *29*.

Analysis of Metal Ion and Sulfur Content. Metal content of recombinant thioredoxins was determined by inductively coupled plasma-emission spectrometry (ICP) at the Chemical Analysis Facility, University of Georgia. Iron content was also analyzed by micro methods according to refs *30* and *31*. Labile sulfur content was measured as described in ref *32*.

In Vitro Reconstitution. The reconstitution assay was performed as described in ref *33* using 50 μM apoprotein, 5 mM DTT, 1 equiv of Fe(II), 1.5 equiv of cysteine, and either $^1/_{100}$ equiv of IscS and IscA or $^1/_{100}$ equiv of IscS and 1 mM GSH.

Activity Measurements. The activity of Trx was measured by following NADPH consumption during insulin reduction (*34*).

Substrate Binding of Thioredoxin Mutants. Recombinant human thioredoxin and its P75T and P75S mutants were immobilized on CNBr-activated Sepharose 4B (Amersham Biosciences) according to the manufacturer's protocol. Homogenized mouse liver or testis samples were applied onto the resins containing immobilized thioredoxin or its mutants. The columns were washed with PBS buffer, and the bound proteins were eluted with 10 mM DTT and analyzed by SDS−PAGE analysis.

RESULTS AND DISCUSSION

A Conserved cis-Proline in Thioredoxin Fold Proteins. A conserved *cis*-proline is located immediately upstream of β-strand 3 in many thioredoxin-fold proteins (Figure 1). This residue is even more conserved than the active site cysteines in these proteins. Conservation, proximity to the catalytic cysteines, and previous biochemical studies clearly demonstrate an important role of this proline in catalysis. However, the specific function that this residue plays in thioredoxins remains poorly understood. In a recent report, Qi and Grishin defined thioredoxin-fold proteins more broadly (*13*). In their structural alignment, the majority of thioredoxin-fold proteins possess the conserved proline, but circular permutated homologues lack this residue. It is also absent in some classical thioredoxin-fold proteins as well as in thioredoxin fold-like proteins, which lack one of the helixes present in thioredoxin. Some bacterial thioredoxins may replace one of the catalytic cysteines in the CxxC motif with selenocysteine, and many of these selenoproteins also lack the proline (*35*).

One of the previously proposed roles of the *cis*-proline is to maintain structural stability of thioredoxin-fold proteins. It was shown that mutation of the proline in TrxA destabilized protein structure (*36*), while the Pro-to-Gly mutation in GST A1-1 resulted in the loss of both catalytic activity and structural stability (*17*). Mutation of the proline in DsbA destabilized the structure and inhibited activity of this protein (*16*). This inactivation might be explained by the finding that proline mutants could form unproductive intermolecular disulfides with target proteins (*18*). This finding was employed to identify both reductants and oxidants of DsbA. Another possible role of the *cis*-proline is its involvement in redox reactions. In Trx (*37*) and Grx (*38*) structures, the proline residue plays a role in positioning of the substrate. In each case, the proline ring is positioned in parallel with the disulfide bond between the substrate and the N-terminal active site cysteine of thioredoxin. Such structural arrangement is believed to place the disulfide in position for attack by the C-terminal active site cysteine of thioredoxin.

Mutation of Proline 75 in Human Thioredoxin Results in the Formation of an Iron−Sulfur Cluster in the Protein. In human thioredoxin 1, the conserved proline is Pro75. We mutated this residue to serine or threonine, prepared recombinant proteins, and immobilized wild-type thioredoxin and the P75S and P75T mutants onto CNBr resins. Mouse liver or testis homogenates were incubated with the resins, and the proteins bound were eluted with DTT. However, in contrast to DsbA (*18*), we did not observe increased binding of target proteins to the two mutants compared to the wild-type thioredoxin (data not shown), suggesting that this approach is not universally applicable to thioredoxin-fold proteins.

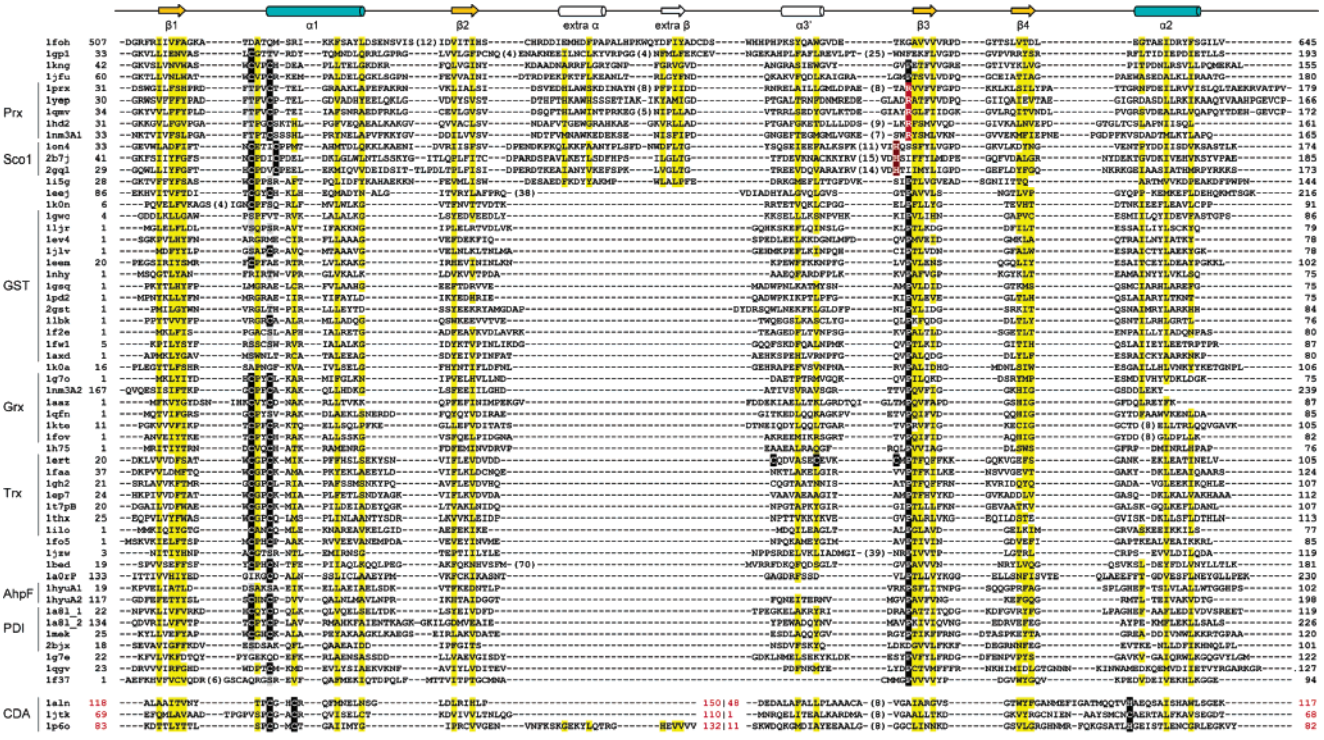

FIGURE 1: Alignment of thioredoxin-fold proteins. Information on the secondary structure was obtained from the PDB. Conserved active site cysteines and prolines are shown in black, conserved arginines in peroxiredoxins in red, and conserved histidines in Sco1 in maroon.

The *cis*-proline has been described as an important residue for enzyme activity. DsbA mutants lost around 50% of the wild-type activity (*16*, *39*), and a P56G mutant of human glutathione *S*-transferase A1-1 exhibited less than 2% activity compared with the wild-type protein (*17*). Similar to these findings, we found that the P75S mutant of human thioredoxin showed less than 1% activity of the wild-type protein.

Curiously, both human thioredoxin mutants were colored brown, whereas the wild-type protein was colorless. ICP metal analysis revealed the presence of significant amounts (0.41 equiv) of iron in the P75S mutant (Table 1). We also determined the iron content (0.42 equiv) in this protein using micro methods (*30*), which agreed well with the ICP data. To determine whether iron coordination was specifically the result of P75S and P75T mutations, we also changed the proline to alanine; the protein was still colored brown and contained iron. Therefore, it is the occurrence of proline that inhibited binding of iron to human thioredoxin rather than the presence of a certain residue that promoted iron binding.

Iron coordination in proteins could be mononuclear or in the form of iron−sulfur clusters. To distinguish between these two possibilities, we determined whether labile sulfur was present in the P75S mutant. The labile sulfur was indeed found at ∼0.32 mol/mol of protein, a value which was only slightly lower than the iron content (Table 1). Therefore, the major portion of the coordinated iron was likely in the form of an iron−sulfur cluster. Spectrophotometric analysis showed two additional absorption peaks around 320 and 420 nm, which is characteristic of iron−sulfur clusters, especially Fe_2-S_2 clusters. The spectrum was more distinct after subjecting the mutant to an in vitro reconstitution assay (*33*; Figure 2B). In contrast, the wild-type human thioredoxin was not able to coordinate an iron−sulfur cluster. There are several types of iron−sulfur clusters, the most common of which are Fe_2-S_2 and Fe_4-S_4 clusters. On the basis of the spectral analysis, and the determination of iron and acid-labile sulfur (Table 1), we suggest the coordination of an Fe_2-S_2 cluster between two monomers. This coordina-

Table 1: Spectroscopic Characteristics and Iron and Acid-Labile Sulfide Content of Proteins Used in This Work

protein	320, 420 nm bands after purification/ reconstitution	iron/protein after purification/ reconstitution	acid-labile sulfide/protein after purification/ reconstitution
Trx			
WT	−/−	0/0.028 ± 0.001	nd[a]/0.14 ± 0.03
C32S	−/nd	nd/nd	nd/nd
C35S	−/nd	nd/nd	nd/nd
C32S/C35S	−/nd	nd/nd	nd/nd
P75T	+/nd	nd/nd	nd/nd
P75S	+/+	0.41/0.62 ± 0.04	0.32/1.38 ± 0.35
P75SC32S	−/nd	0/nd	nd/nd
P75S/C35S	+/nd	nd/nd	nd/nd
P75S/C62S	+/nd	0.31/nd	nd/nd
P75S/C69S	+/nd	0.23/nd	nd/nd
P75S/C73S	−/nd	nd/nd	nd/nd
P75S/C32S/ C35S	−/nd	nd/nd	nd/nd
P75S/C62S/ C69S	+/nd	0.33/nd	nd/nd
P75A	+/nd	0.56/nd	nd/nd
P75R	+/+	0.2/0.57 ± 0.1	nd/nd
P75R/C32T	+/+	0.12 /0.53 ± 0.03	nd/nd
P75R/C73S	+/nd	nd/nd	nd/nd
P75R/C32T/ C73S	−/nd	nd/nd	nd/nd
P75R/C35S/ C73S	−/(−)	nd/0.37 ± 0.03	nd/nd
CSYC	+/+	nd/0.21 ± 0.02	nd/nd
Prx			
WT	−/−	nd/0.3 ± 0.12	nd/1.3 ± 0.33
CxxC	−/+	nd/0.51 ± 0.15	nd/1.39 ± 0.32

[a] nd = not determined.

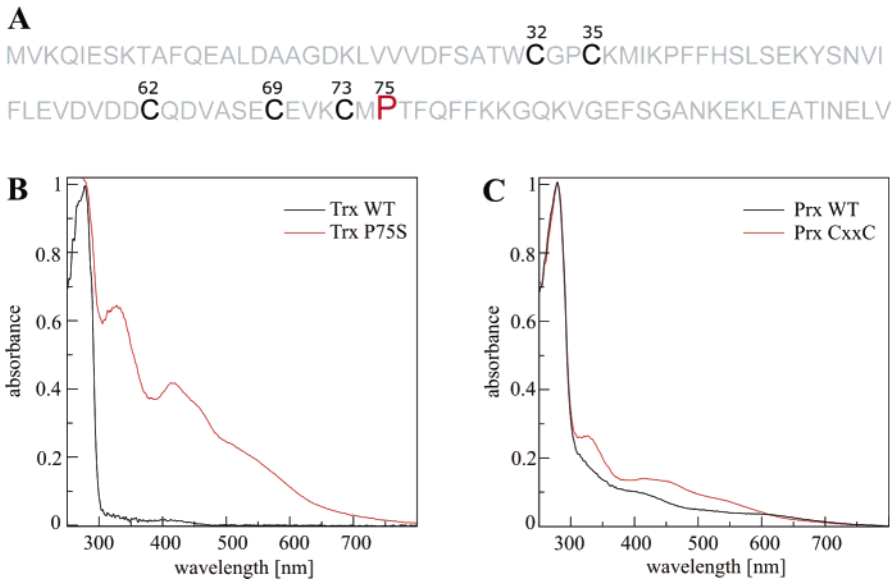

FIGURE 2: Mutation of the *cis*-Pro leads to Fe_2-S_2 cluster binding in thioredoxin-fold proteins containing the CxxC motif in the active site. Human thioredoxin sequence (A) in which the conserved *cis*-proline (shown in red) and five cysteines are numbered and enlarged. UV−vis spectra of human wild-type thioredoxin and its P75S mutant (B) and human wild-type peroxiredoxin and its CxxC mutant following a reconstitution assay (C).

tion was found in *E. coli* thioredoxin mutants containing CACC or CACA active site motifs instead of the CGPC sequence in the wild-type protein (*26*). The Fe_2-S_2-coordinating glutaredoxins were as well dimers bridged by this cofactor (*23*, *33*, *40*, *41*).

In glutaredoxins, as in the active site mutant forms of thioredoxin, the proline located in the active site seems to inhibit cluster formation. For example, human glutaredoxin 1 was found to bind an Fe_2-S_2 cluster when the protein was mutated from the CPYC active site form to the CSYC form that also occurs in human glutaredoxin 2 (*33*). We, therefore, constructed a human thioredoxin mutant containing the CSYC-type active site. The purified and reconstituted protein was an iron−sulfur protein, but at a reduced level compared with the P75 mutants (Table 1).

Iron−sulfur clusters are mostly bound in proteins through cysteines. In human thioredoxin, there are five cysteine residues: C32, C35, C62, C69, and C73 (Figure 2A). We separately mutated each cysteine in the P75S mutant and also prepared mutants that lacked various combinations of these residues (Table 1). By determining the iron content of selected mutant proteins, we found that the color of the purified proteins was indicative of iron content, and subsequently we simply used this property as a quick indicator of iron binding. We found that cysteines 32 and 73 were required for iron binding, whereas cysteines 62 and 69 were not (Table 1). Similar results were obtained for the P75T mutant. C73 is unique to mammalian thioredoxins; therefore, it would be interesting to determine if mutation of the proline in bacterial thioredoxins would also lead to metal binding.

We examined the structural basis for the formation of an iron−sulfur cluster in the human thioredoxin mutant. A structure of the Pro75Ser form was modeled and energy minimized and compared to the structure of the wild-type protein (Figure 3). We observed little difference between the two structures. The *cis*-proline is located in close vicinity to the pair of catalytic cysteines. It may provide rigidity to the active site, which precludes insertion of the cluster.

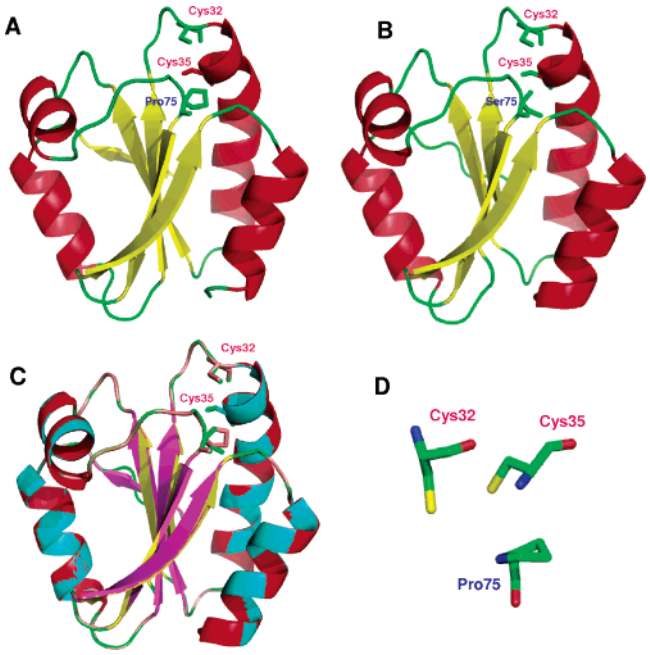

FIGURE 3: Structures of wild-type and mutant thioredoxins. (A) Structure of human thioredoxin (PDB accession number 1ERT). (B) Structural model of the Pro75Ser mutant of human thioredoxin. The structure was modeled with Modeler 8.0 and energy minimized using UCSF Chimera. Cys and Pro (or Ser that replaced the Pro) are labeled and shown in stick models. (C) Alignment of structures shown in (A) and (B). (D) Structural arrangement of active site cysteines 32 and 35 and proline 75. The distance between the sulfur atoms of Cys32 and Cys35 is 3.92 Å and between the sulfur atom of Cys35 and ring nitrogen of Pro75 is 3.59 Å.

Overall, the data suggested a new function of Pro75: preclusion of metal binding. Interestingly, to coordinate the cluster, thioredoxin-fold Fe_2-S_2-binding proteins require either additional cysteines (e.g., ferredoxins) (*19*), or non-protein thiols (e.g., glutaredoxins) (*33*, *40*, *41*). A thioredoxin mutant possessing additional cysteine residues was able to bind a mononuclear iron−sulfur cluster (*25*). Moreover, native thioredoxin-fold proteins lacking the *cis*-proline are

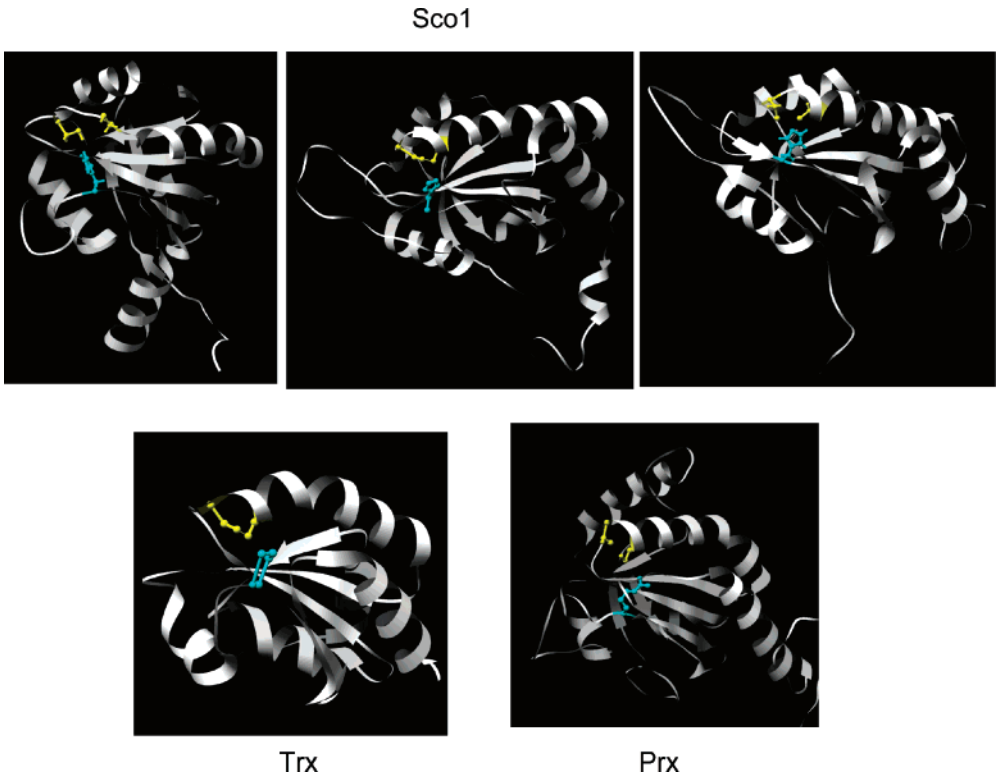

FIGURE 4: Structural comparison of Sco1 proteins with thioredoxin and peroxiredoxin. Three Sco1 structures (1on4, 2b7j, and 2gq1, from left to right), one thioredoxin structure (1ert), and one peroxiredoxin (1prx) structure are shown. The active site cysteines are shown in yellow and conserved proline, arginine, and histidine in blue.

often metal-binding proteins, e.g., Sco1 (*22*). In contrast, most thioredoxin-fold proteins lacking the CxxC motif also lack the *cis*-proline (e.g., peroxiredoxins). We, therefore, investigated the effect of the active site as well as various residues replacing the *cis*-proline on metal binding in other members of the thioredoxin-fold family of proteins.

An Arginine in Place of the cis-Proline in Peroxiredoxins. An alignment of thioredoxin-fold oxidoreductases and their homologues indicates that peroxiredoxins, a family of thioredoxin-fold peroxidases, have a conserved arginine in place of the proline (Figure 1) (*15*). 2-Cys peroxiredoxins utilize a distant resolving Cys instead of the second Cys in the CxxC motif in thioredoxins. Structural analyses of peroxiredoxins showed that the resolving Cys is normally shielded from the active site Cys until the latter is converted to Cys sulfenic acid during catalysis (*42*). To test a possible role of this residue, we mutated the *cis*-proline to arginine in human thioredoxin and found that the mutant protein also contained iron. We also mutated various cysteines in combination with the arginine mutation. Interestingly, the pattern of metal binding was slightly different in the case of these mutations. Each of the C32, C35, and C73 single mutants contained iron (Table 1), while mutation of any two of these residues resulted in the loss of metal binding, which was confirmed by reconstitution of both P75R/C32T and P75R/C35S/C73S mutants. It appears that either any two of these cysteines could coordinate the Fe_2-S_2 cluster or some cysteine mutations in the P75R background also affected protein structure, indirectly disrupting metal binding. We hypothesize that the use of arginine in peroxiredoxins is possible because of the lack of proximal resolving Cys. To further test the relationships between thioredoxin and peroxiredoxin, we mutated the CxxC motif in the P75R mutant of human thioredoxin to TxxC. The resulting protein was inactive as peroxiredoxin. We also mutated the TxxC motif of human peroxiredoxin 1 to CxxC and the arginine to proline, and the resulting protein was not active as thioredoxin (data not shown).

Table 2: Metal Content of the P75H Mutant of Human Thioredoxin Isolated from Cells Grown in Media Supplemented with Metals

	metal equivalents bound in protein	
metal ion	protein isolated from Cu-supplemented cells	protein isolated from Ni-supplemented cells
Co	0.07	0.03
Cu	0.11	0.0
Fe	0.08	0.06
Ni	0.0	0.0
Zn	0.65	0.72

The CxxC mutant of human peroxiredoxin was used to further examine the possibility that there is a correlation between the presence of the *cis*-proline and the CxxC active site motifs. For this, we subjected both wild-type and mutant proteins to iron−sulfur cluster reconstitution assays. In contrast to the wild-type protein, the peroxiredoxin mutant with the CxxC motif was able to coordinate an Fe_2-S_2 cluster, as shown by UV−vis spectral analysis (Figure 2C) as well as iron and acid-labile sulfur determination (Table 1).

The Proline to Histidine Mutation Leads to Copper Binding by Human Thioredoxin. A recently characterized thioredoxin-fold protein, Sco1, does not have the conserved *cis*-proline (Figure 1) (*20*−*22*). Instead, this protein utilizes a conserved histidine in the position structurally similar to that of proline (Figure 4). Sco1 is a copper-binding protein, and this histidine is one of the residues involved in copper coordination (*22*). We examined whether insertion of histi-

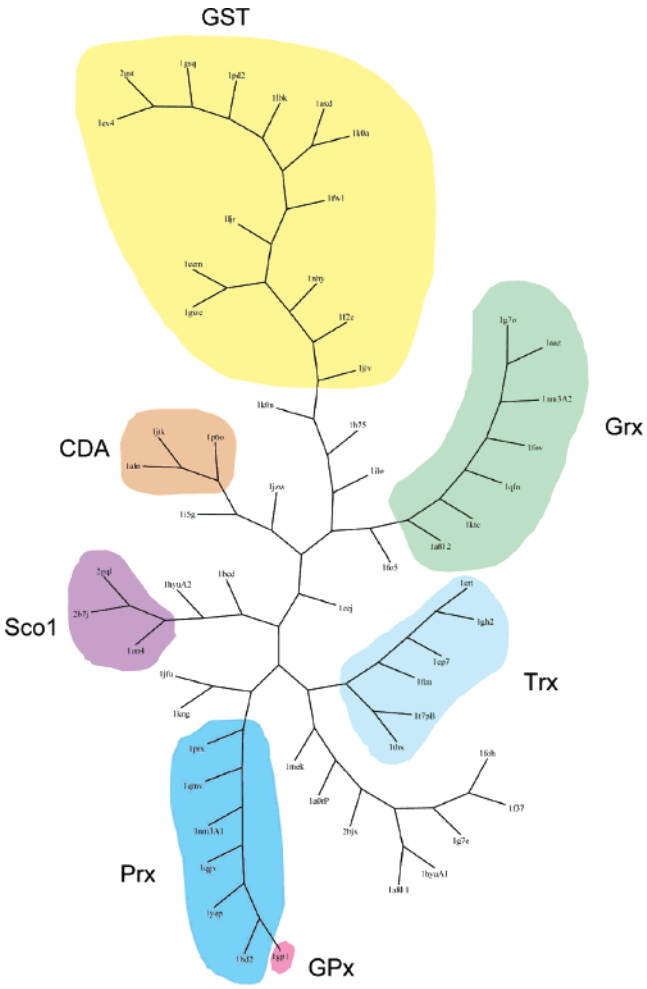

FIGURE 5: Phylogenetic analysis of thioredoxin-fold proteins. The tree was generated on the basis of sequence alignment in Figure 1. Various protein families within the thioredoxin superfamily are highlighted (*46*).

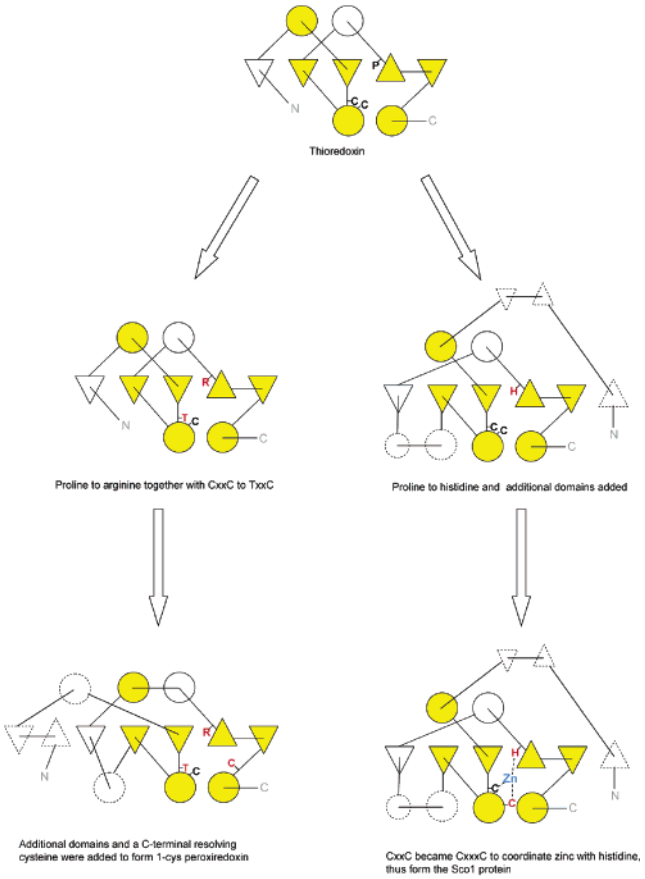

FIGURE 6: Evolution of the conserved proline in thioredoxin-fold proteins. We hypothesize that peroxiredoxins evolved from thioredoxins accompanied by changing the proline to arginine and the active site CxxC motif to TxxC, in part to avoid metal binding. Subsequently, a distant resolving cysteine evolved to form 1-Cys or 2-Cys peroxiredoxins. Sco1 proteins changed the proline with histidine. The active site cysteines of Sco1 proteins were capable of binding zinc, and subsequently the CxxC motif was replaced with CxxxC, which may be better adapted for metal binding. Topology diagrams (N and C for the N- and C-terminus, respectively) were generated using the TOPS program (*46*). Conserved secondary structures of thioredoxin-fold proteins are shown as yellow-filled circles and triangles for helices and strands, respectively. Dotted circles or triangles represent additional structural elements evolved in peroxiredoxins and Sco1. The conserved proline and the active site cysteines in thioredoxin are shown in black and the residues that changed during evolution in red.

dine in place of the proline could support metal binding in human thioredoxin and, if so, determined which metal is bound. Interestingly, the P75H mutation did not lead to the binding of iron; instead, metal ion analysis revealed the presence of zinc. We also grew bacterial cells expressing the P75H mutant in the presence of 100 μM copper or 100 μM nickel ions. When copper was present in the growth medium, more than 0.1 equiv of this metal was bound to the mutant protein, although zinc remained the major metal detected. The use of nickel, however, did not result in the nickel-containing thioredoxin (Table 2).

The CxxC motif is often found in zinc-finger proteins (*43*). Thus, a relevant question is how oxidoreductases containing catalytic CxxC sites preclude their highly reactive thiolates from binding metal ions. As discussed above, most CxxC motifs in thioredoxin-fold proteins are accompanied by the conserved proline with only few exceptions. Cytidine deaminases have a circular-permutated thioredoxin fold (Figure 1) (*13*), contain the CxxC motif, and lack the *cis*-proline. Interestingly, the two cysteines of the CxxC motif, together with a C-terminal Cys or His, coordinate zinc (PDB structures 1aln, 1jtk, and 1p6o). Thus, the studies on cytidine deaminases, Sco1, and ferredoxins clearly show that the CxxC motif in thioredoxin-fold proteins does have the tendency to bind various metal ions. The absence of the conserved proline in the metal-binding proteins mentioned above suggests that this residue restricts metal binding.

We further examined whether the *cis*-proline loss in thioredoxin-fold proteins can be traced back to a single evolutionary event. We carried out phylogenetic analysis of thioredoxin-fold proteins shown in Figure 1. This analysis (Figure 5) suggested that Sco1, cytidine deaminases, and peroxiredoxins lost the proline independently of each other. Indeed, each of these families had proline-containing proteins clustered with them in the same branch. Interestingly, glutathione peroxidases clustered with peroxiredoxins but had phenylalanine rather than arginine in place of the *cis*-proline.

Correlation between the Presence of a Proline and Two Cysteines in the Active Site. Following the experimental proof of the conversion of proteins lacking the cofactor to iron−sulfur proteins in both directions, and mutation analyses of *cis*-proline and CxxC sites in thioredoxin-fold proteins, we further examined a correlation between the conserved proline

and the active site cysteines by analyzing the general characteristics of the thioredoxin family as a COG0625 cluster. Among the 230 proteins in this group, the presence of the CxxC motif was almost always accompanied by the proline; however, many proline-containing proteins lacked the CxxC motif. This observation is consistent with the role of proline in inhibiting metal binding, but these data also suggest that this is not the only function of this residue. Indeed, it also has a strong effect on the activity as shown previously for DsbA and GST A1-1 (*16*, *17*) and for human thioredoxin in this work. Our view of the evolution and function of the conserved *cis*-proline is summarized in Figure 6.

Overall, our data suggest that the *cis*-proline in human thioredoxin is a guardian residue that protects the active site cysteines from binding iron in the form of iron−sulfur clusters and other metals. Consistent with this function, *cis*-proline is known to be a key residue in thioredoxin-fold proteins and could even be used for identification of these proteins in databases (*44*, *45*). It has been preserved during evolution in various subfamilies, such as thioredoxins, glutaredoxins, PDIs, AhpFs, and GSTs. The *cis*-proline in combination with the active site motif can now be used to distinguish between oxidoreductases and metal-binding proteins and to search for new metal-binding members of the thioredoxin-fold superfamily.

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