PROTEIN STRUCTURE REPORT

Crystal structure of human thioredoxin revealing an unraveled helix and exposed S-nitrosation site

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Abstract: Thioredoxins reduce disulfide bonds and other thiol modifications in all cells using a CXXC motif. Human thioredoxin 1 is unusual in that it codes for an additional three cysteines in its 105 amino acid sequence, each of which have been implicated in other reductive activities. Cys 62 and Cys 69 are buried in the protein interior and lie at either end of a short helix (helix 3), and yet can disulfide link under oxidizing conditions. Cys 62 is readily S-nitrosated, giving rise to a SNO modification, which is also buried. Here, we present two crystal structures of the C69S/C73S mutant protein under oxidizing (1.5 Å) and reducing (1.1 Å) conditions. In the oxidized structure, helix 3 is unraveled and displays a new conformation that is stabilized by a series of new hydrogen bonds and a disulfide link with Cys 62 in a neighboring molecule. The new conformation provides an explanation for how a completely buried residue can participate in SNO exchange reactions.

Keywords: nitrosylation; unfolding; protein dynamics; oxidation; reduction; signaling pathways

Introduction

Thioredoxins reduce protein disulfide bonds and other cysteine oxidations in the reducing cellular compartments in all kingdoms of life.^{1,2} Thioredoxin exchanges the disulfide bond or other thiol modification in a target protein for a disulfide bond in a Cys-Gly-Pro-Cys motif, which is subsequently reduced to the dithiol by thioredoxin reductase. In humans, there are two thioredoxins, thioredoxin 1 (hTrx1), which is found in the cytosol and the nucleus, and thioredoxin 2 (hTrx2), which is targeted to the mitochondria. hTrx1 is increasingly implicated in the

regulation of many complex diseases and physiological processes, including cancer,^{3,4} cardiovascular disease,⁵ and aging.⁶

hTrx1 has three cysteines, Cys 62, Cys 69, and Cys 73, in addition to the active site dithiol pair (Cys 32 and Cys 35). Each of these has been implicated in S-nitrosation activities (also called S-nitrosylation), particularly with respect to regulation of apoptosis.^{7–14} S-nitrosation/nitrosylation is the addition of NO to a cysteine residue, a redox reaction requiring one electron oxidation, and numerous proteins have been suggested to be regulated in this manner.¹⁵ Removal of the nitroso group in target proteins, or transfer of the group to other proteins, is likely to involve hTrx1 in human cells. To investigate which of the five cysteine residues might be involved, we previously determined the hTrx1 crystal structure after transnitrosation with S-nitroso-glutathione (GSNO) and, unexpectedly, found the

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Table I. Crystallographic Data for C69S/C73S Thioredoxin

Structure	Reduced	Oxidized
PDB entry	3M9J	3M9K
Crystal		
Space group	$P2_1$	$P6$
Cell dimensions	$a = 37.23 \text{ \AA}$ $b = 46.21 \text{ \AA}$ $c = 52.33 \text{ \AA}$ $\beta = 93.25^\circ$	$125.62 \text{ \AA}$ $125.62 \text{ \AA}$ $33.55 \text{ \AA}$
Z	4	12
$V_M (\text{\AA}^3 \text{ Da}^{-1})$	1.64	2.62
Data collection		
X-ray source	SSRL 9-2	SSRL 9-2
Wavelength ($\text{\AA}$)	0.97946	0.97946
Resolution ($\text{\AA}$)	1.10	1.50
Unique reflections	69636	48316
Multiplicity ^a	4.9 (4.1)	9.9 (9.2)
Completeness (%) ^a	96.9 (91.3)	98.6 (97.8)
$I/\sigma(I)$ ^a	40.0 (3.2)	15.2 (2.8)
R_{merge} ^a	0.047 (0.398)	0.053 (0.631)
Refinement		
R_{cryst} ^a	0.144 (0.306)	0.184 (0.303)
R_{free} ^{a,b}	0.172 (0.321)	0.211 (0.347)
RMSD bonds ($\text{\AA}$)	0.017	0.020
RMSD angles (deg)	1.70	1.73
Region of Ramachandran plot		
Most favorable (%)	92.6	91.5
Additionally allowed (%)	7.4	8.5

V_M , Matthews coefficient.

^a Overall (outer shell).

^b Five percent of data not used in refinement.

most buried cysteine, Cys 62, was readily and stably modified at pH 7.0, while the more exposed Cys 69 required a higher pH for nitrosation. Cys 32 and Cys 73 presumably also reacted with GSNO at pH 7.0, producing crystals with an intramolecular disulfide bond between Cys 32–Cys 35, and an intermolecular disulfide bond found between Cys 73–Cys 73' from a second thioredoxin molecule. Additional indications of Cys 62 activity have also been reported, including the finding of an intermolecular disulfide linkage between Cys 62 and actin,¹⁶ and the finding of an intramolecular disulfide linkage between Cys 62 and Cys 69, despite their locations on either end of helix 3.¹⁷

Results

S-Nitrosation of the C69S/C73S thioredoxin double mutant

We sought to simplify characterization of Cys 62 S-nitrosation by mutating cysteines 69 and 73 to serine. The resulting protein behaved much like wild type and was readily nitrosated with GSNO, yielding 0.61 ± 0.06 SNO moieties per thioredoxin molecule as estimated using the Griess/Saville method and analytical gel filtration column chromatography ($n = 3$). The protein eluted from the column as a monomer.

Crystal structure of the reduced protein

The reduced mutant protein crystallized in a new crystal form, and yielded the highest resolution structure yet for a human thioredoxin (1.1 $\text{\AA}$, Table I). The asymmetric unit contained two very similar copies of the protein (RMSD = 0.54 $\text{\AA}$ for carbon alpha atoms), each of which was nearly identical to the wild type protein (RMSD = 0.55 and 0.44 $\text{\AA}$ for molecules A and B, respectively). A total of 16% of the protein was modeled with more than one conformation, including residues 42–50 of molecule A, which shift as a helical unit and occupy two distinct positions approximately 1.5 $\text{\AA}$ apart, possibly in response to the presence or absence of an N-terminal methionine. The N-terminal methionine is incompletely removed during bacterial expression¹² and modeled here as 50% occupied. Cys 32 and Cys 35 are completely reduced. The two thioredoxin molecules display the same homodimeric interface previously observed in the wild type protein, but with an approximately 5 degree rotation (see below).

Crystal structure of the oxidized protein

Crystallization drops that were allowed to equilibrate over several weeks yielded a second crystal form, also with two molecules in the asymmetric unit, but in this case disulfide linked between Cys 62 of each molecule. The active site dithiol pair, Cys 32 and Cys 35, was also disulfide linked in each molecule. Presumably, this crystal form could only occur once all reductant was oxidized, allowing for disulfide bond formation. The interactions between monomers in the disulfide-linked interface were minimal; however, the typical homodimeric interface found in all of our hTrx1 structures was again present in this crystal, but in this case generated through crystallographic symmetry (see below). As a result of this arrangement, two covalently linked dimers form a tetrameric unit around a crystallographic two-fold rotation axis [Fig. 1(A)].

The most striking finding in the oxidized structure is that the helix containing Cys 62 is unraveled in one of the molecules (molecule B) and rotated slightly outward in the other, exposing both sulfhydryls and allowing for disulfide bond formation. The unraveled conformation is well-ordered in the crystal and fully described [Fig. 1(B)]. The new conformation, affecting residues 60–72, forms completely new interactions [Fig. 1(C)]. The largest changes are in residues 61–63 and 68–69, with shifts of up to 10 $\text{\AA}$ with respect to the position of these residues in the reduced structure [Fig. 1(D)]. Both Cys 62 and Ser 69 (corresponding to Cys 69 in the wild type protein) are completely exposed in molecule B, but largely buried in molecule A, which has a conformation similar to that observed in the structure of the wild type protein (PDB entry 1ERT). The

Figure 1. Crystal structure of oxidized C69S/C73S hTrx1. (A) Ribbon drawing highlighting the tetrameric packing in the crystal, with Cys 62 side chains illustrated with solid bonds. A disulfide-linked dimer is found in the asymmetric unit (Chain A in blue and Chain B in red). The additional two chains are generated through a crystallographic two-fold rotation. (B) Electron density for residues 59–63 in chain B, which adopt the new orientation (final $2F_o - F_c$ map, 1.5σ). (C) Hydrogen bonds stabilize the new arrangement in chain B and the position for oxidized dithiothreitol (DTT). This region is helical in chain A. (D) Superposition of chains A and B (stereoview). Note the shift of residues Cys 62 and Ser 69 from buried to exposed positions.

backbone hydrogen bonds normally formed by residues 65 and 66 as part of helix 3 are replaced in the unraveled structure with three hydrogen bonds to the side chain of Gln 63, which changes ϕ/ψ angles from those of a helix to those of a beta sheet. The Glu 68 side chain forms a new hydrogen bond, through water, with Trp 31. A nonpolar pocket, lined by residues Phe 27, Ala 66, Thr 76, Glu 70, Val 71, and Met 74, opens up near Ala 66 and is filled by an oxidized molecule of dithiothreitol (DTT). Possibly, DTT insertion into the pocket stabilizes the new conformation [Fig. 1(C)].

Homodimer interface

Wild type human thioredoxin 1 was found to be a disulfide-linked homodimer in the original crystal struc-

ture, with an intermolecular disulfide link between Cys 73 of each monomer;¹⁸ a similar dimer is formed on treatment of the protein with GSNO.¹² C73S mutant thioredoxin yields the same dimer interface as the wild type protein but without disulfide linkage.^{12,18} The C69S/C73S double mutant described herein displays this same dimer interface despite crystallizing in two new crystal forms and, for the oxidized protein, being disulfide linked through Cys 62. As noted above, the interface in the oxidized crystals is through crystallographic symmetry, giving rise to a repeating unit that contains four thioredoxin chains [Fig. 1(A)].

Discussion

The means by which Cys 62 can participate in inter- and intramolecular functions have been unclear due to its buried state in all structures previously reported. We have previously postulated that helix 3 must be particularly dynamic to account for these findings,^{12,17} but the helix has in general been quite well-resolved in the crystal. Here, we show helix 3 can completely unravel, giving rise to new protein surfaces and complete exposure of residues 62 and 69. The mechanism for this appears to be at least twofold. First, helix 3 is linked to the rest of the protein through two flexible loops at either end of the helix, and through relatively nonspecific, mostly hydrophobic, contacts. Second, conserved residue Gln 63 is able to move from helical ϕ/ψ angles to those for a beta sheet, allowing for a side-chain shift of 6 Å and the formation of hydrogen bonds to the backbone atoms of residues 65 and 66 [Fig. 1(C)]. The dynamics in helix 3 likely accounts for the unexpected involvement of Cys 62 in disulfide bond formation or *S*-nitrosation. The stability of the Cys 62-SNO modification may also be enhanced by reformation of helix 3 and burial of the hydrophobic SNO group in the hydrophobic interior of the protein. One can further envision protein-dependent unraveling of helix 3 for SNO exchange or other activities through binding in the crevice created near residues Ala 66, Val 71, and Met 74, which is occupied by DTT in the present structure. Interestingly, both Val 71 and Met 74 line both the crevice and the homodimeric surface, suggesting target protein binding and helix 3 dynamics could be coupled together (see below).

Additional support for our model comes from the recent structure of the hTrx1 double mutant C35S/C73R, which also has Cys 62 in an intermolecular disulfide bond, in this case with Cys 32 (PDB entry 3E3E)¹⁹. In this structure, helix 3 is also unraveled and the new conformation stabilized through hydrogen bonding by Gln 63, much as in the structure reported herein [Fig. 1(C)]. Additionally, a molecule of hexaethylene glycol occupies the same pocket in the C35S/C73R mutant as does DTT in the present structure.

Functional protein–protein interface

The consistent appearance of the same interface in four crystal forms suggests hTrx1 homodimerization may have a physiological role. We previously characterized a weak pH-dependent noncovalent dimer in solution by size exclusion chromatography with an apparent dissociation constant ranging between 6 and 164 μ M for pH values between 3.8 and 8.0²⁰; the pH dependence is due to Asp 60, which is buried in the dimer interface and has an apparent pK_a of 6.5. However, the C62A/C69A/C73A mutant does not appear to be dimeric in studies using ultracentrifugation and NMR,²¹ nor is the interface predicted to be a dimer in solution by the Protein Interfaces, Surfaces and Assemblies (PISA) algorithm.²² We were also unable to detect disulfide-linked thioredoxin in cell extracts treated with GSNO.¹² Thus, although the homodimer persists in the crystal, its role *in vivo* remains doubtful. Nevertheless, the dimer interface involves the same interface reported in two crystal structures of thioredoxin in complexes with protein targets,^{23,24} and two NMR structures bound to peptides from target proteins Ref1 and NF κ B.^{25,26} Most likely, this interface is generally used for specificity in interaction, and possibly also to generate correct alignment of target disulfide bonds with Cys 32, which single-molecule studies suggest requires rearrangement at the interface for formation of the intermolecular disulfide bond intermediate.^{27,28}

S-Nitrosation of Cys 62

Our original motivation for this study was to further investigate S-nitrosation of Cys 62, for which differing results have been published. In our hands, Cys 62 forms a stable nitrosation product at neutral pH, while nitrosation of Cys 32 leads to a Cys 32–Cys 35 disulfide bond, nitrosation of Cys 73 leads to disulfide bond formation with Cys 73 of another molecule, and nitrosation of Cys 69 occurs only at higher pH.¹² In contrast, two studies using a His-tagged version of hTrx1 detected Cys 73 SNO but not Cys 62 SNO.^{9,29} The reason for this inconsistency is not yet clear but may be due to the His-tag itself, which might interfere with homodimerization through increased charge repulsion, or alter the dynamics of helix 3, leading to a change in the Cys 73 SNO and Cys 62 SNO stabilities. Both tagged and untagged proteins are readily S-nitrosated at Cys 73 and this modification appears to be crucial for SNO exchange with caspase-3.^{8,9} However, the SNO modification is readily lost in the untagged protein through Cys 73–Cys 73' disulfide bond formation. Cys 62 SNO has yet to be detected in the tagged protein, perhaps due to a change in Cys 62 reactivity, a change in Cys 62–Cys 69 disulfide bond propensity, or to the difficulty in detecting buried SNO moieties by mass

spectrometry or chemical approaches such as the biotin switch. Nonetheless, that Cys 62 SNO is readily formed in the untagged protein is clear from the present studies and by crystal structure determination,¹² and thus may also be of importance in the biology of SNO signaling.

Materials and Methods

Materials

GSNO was prepared from reduced L-glutathione and sodium nitrite (Sigma) as described³⁰ and recrystallized from a water/acetone mixture. Other reagents were purchased from Sigma unless otherwise noted.

Expression and purification of C73S/C69S thioredoxin

The C69S/C73S mutation was introduced into the human thioredoxin-1 gene in the pET3a plasmid using the QuikChange Lightning Site-Directed Mutagenesis Kit (Stratagene) and the following forward and reverse primers:

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GTTCCTTCAGAGAGTGAAGTCAAAAGCATGCC
AACATTCCAG
CTGGAATGTTGGCATGCTTTTGACTTCACTCTC
TGAAGCAAC
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The plasmid containing the mutant variants was transformed into *E. coli* strain BL21(DE3)pLysS and grown at 37°C in 3 L Issa's minimal media supplemented with ampicillin. Protein expression was induced with 0.4 mM IPTG at OD₆₀₀ = 0.6 and cell growth was continued over night. All subsequent steps were carried out at 4°C. The bacterial pellet (19.3 g) was resuspended in a buffer containing 100 mM Tris.HCl (pH 8), 1 mM EDTA, and 1 mM PMSF, followed by cell lysis using sonication and centrifugation in a 45 Ti rotor at 40,000 rpm. The supernatant was supplemented with 5 mM DTT, loaded onto a Q Sepharose FF column and eluted with 200 mL of a NaCl gradient (0–200 mM). Fractions containing the protein, as judged by SDS-PAGE, were pooled, precipitated with 90% ammonium sulfate, and resuspended in 10 mM Tris.HCl (pH 8), 1 mM EDTA, 2 mM DTT. The material was passed through a Sephacryl S-100 26/60 gel filtration column (GE Healthcare) previously equilibrated with 10 mM Tris.HCl (pH 8), 1 mM EDTA, 200 mM NaCl. The protein was concentrated in a 5 K Vivaspin column and equilibrated with 20 mM MES and 2 mM DTT, resulting in 185 mg of more than 95% pure protein.

Quantifying hTrx-SNO formation

S-nitrosation of C69S/C73S thioredoxin was examined as previously described¹² with slight modification. Briefly, 5 μ M protein was incubated on ice in the dark with fourfold excess GSNO for 2 h in a 50 mM Tris.HCl buffer (pH 7.4), containing 300 mM

NaCl and metal chelators diethylenetriaminepentaacetic acid and neocuprione, 50 μM each. After equilibrating, excess GSNO was removed by passing the sample through a Superdex 75 10/30 analytical gel filtration column (Pharmacia). The SNO content in the protein-containing fractions was determined using the Griess/Saville method;^{31,32} protein concentration was estimated based on absorption at 280 nm ($\epsilon = 0.626 \text{ mL mg}^{-1} \text{ cm}^{-1}$).

Crystal structure determinations

Crystals were obtained using the vapor diffusion hanging drop method by combining 2 μL of the protein solution with 2 μL of the precipitant solution and equilibrating the drop with 1 mL precipitant solution at room temperature. The monoclinic crystals of the reduced form grew from 30% PEG 4000, 200 mM ammonium acetate, 100 mM sodium citrate pH 5.6, 5 mM DTT. The elongated hexagonal bipyramidal crystals of the oxidized form appear after 4 weeks equilibration against 1.8 M ammonium sulfate, 100 mM MES pH 5.6, 10 mM cobalt (II) chloride, 2 mM DTT. Data were measured remotely at SSRL beamline 9-2 (100 K) using the beamline robotics. Both crystal structures were determined by molecular replacement using the program MOLREP in the CCP4 program suite,³³ and wild type thioredoxin as a starting model (PDB entry 1ERT).¹⁸ Model adjustments were made with COOT³⁴ and refined with REFMAC5.³³ Anisotropic temperature factors and hydrogen atoms in their riding positions were used in the refinement of both structures. Structural figures were prepared using PyMOL (Delano Scientific, San Carlos, CA, <http://www.pymol.org/>).

Atomic Coordinates

The atomic coordinates and structure factors have been deposited with the Protein Data Bank (PDB entries 3M9J and 3M9K for the reduced and oxidized forms, respectively).

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