PROTEIN STRUCTURE REPORT

Structure of human thioredoxin exhibits a large conformational change

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Abstract: Thioredoxin is an oxidoreductase, which is ubiquitously present across phyla from humans to plants and bacteria. Thioredoxin reduces a variety of substrates through active site Cys 32, which is subsequently oxidized to form the intramolecular disulphide with Cys 35. The thioredoxin fold is known to be highly stable and conformational changes in the active site loops and residues Cys 32, Cys 35 have been characterized between ligand bound and free structures. We have determined a novel 2.0 Å resolution crystal structure for a human thioredoxin, which reveals a much larger conformational change than previously characterized. The principal change involves unraveling of a helix to form an extended loop that is linked to secondary changes in further loop regions and the wider area of the active site Cys 32. This gives rise to a more open conformation and an elongated hydrophobic pocket results in place of the helix. Buried residue Cys 62 from this helix becomes exposed in the open conformation. This provides a structural basis for observations that the Cys 62 sidechain can form mixed disulphides and be modified by thiol reactive small molecules.

Keywords: thioredoxin; unfolding; conformational change; redox

Introduction

The thioredoxin redox system, consisting of thioredoxin (Trx) and thioredoxin reductase (trxR), is a ubiquitous system found across all phyla.^{1,2} All thioredoxin proteins share a common structure, consisting of four α -helices and five β -sheets, and have the highly conserved active site sequence Cys-Gly-Pro-Cys.³ This system is known to have a wide variety

of biological functions including maintaining an intracellular reduced state,⁴ as a regulator of the transcription factors NF- κ B and AP-1,⁵ donating reducing equivalents for ribonucleotide reductase,⁶ and inhibiting the apoptosis signalling kinase, ASK1.⁷ Because of the importance of redox processes in the pathophysiology of cancer and survival of pathogens, chemotherapeutic targeting of thioredoxin has been widely investigated and discussed.^{8–10}

Extensive structural data exists for thioredoxin, with examples of crystal structures available for *E.coli*,^{11,12} *Anabaena*,¹³ *T.vaginalis*,¹⁴ and *M.tuberculosis*.¹⁵ Unlike many other thioredoxins, the human cytoplasmic thioredoxin has three cysteine residues (Cys 62, Cys 69, Cys 73) additional to the active site Cys 32 and Cys 35. The human cytoplasmic thioredoxin crystal structure reveals a homodimer with Cys 73 forming an intermolecular disulphide bridge.¹⁶

Abbreviations: Trx, thioredoxin; trxR, thioredoxin reductase.

Additional Supporting Information may be found in the online version of this article.

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It is not known whether this dimer exists *in vivo*, or is an artefact of the crystallisation process, but it does obscure the active site groove and Cys 32. This dimer has also been shown to form in the crystal structure of the human thioredoxin mutant C73S, demonstrating that noncovalent interactions, other than the disulphide bridge, mediate the dimer.

Although Cys 73 is available on the surface of the thioredoxin structure, Cys 62 and Cys 69 are both partially buried beneath helix $\alpha 3$. Thus, findings that Cys 62 and Cys 69 are able to form covalent adducts with small molecules and form disulphide bridges came as a surprise and has led to speculation that these residues become exposed under certain conditions.^{17–19} We report a novel human thioredoxin crystal structure that when compared to wild type has undergone a conformational change involving unraveling of a helix $\alpha 3$ and exposure of residues Cys 62 and Cys 69 resulting in a more open conformation.

Results

Structure of human thioredoxin in an open conformation

As crystallization of wild-type human thioredoxin normally results in a dimer obscuring the active site, we prepared a double mutant of C35S and C73R (hTrxC35S/C73R) with the aim of trying to discover novel crystal forms where the active site is exposed. Engineered introduction of the bulkier, charged arginine sidechain in place of Cys 73 should disrupt the dimer interface through steric and charge effects. The C35S mutation was engineered to maintain a reduced state. Crystals of hTrxC35S/C73R were grown from 0.1M sodium acetate, pH 4.6, and 30% (v/v) PEG 300 in the trigonal space group P3₁21. The structure has two molecules in the asymmetric unit (chains are termed TrxA and TrxB and both span residues 1–105). TrxA is structurally indistinguishable to wild-type human thioredoxin with equivalent residues having an average r.m.s.d. of 0.474 Å (PDB: 1ERT¹⁶). TrxB contains prominent structural differences to the wild type (average r.m.s.d. 0.986 Å) particularly in the region of helix $\alpha 3$ and loops which surround the active site and the overall [Fig. 1(A,B)].

Rearrangement of helix $\alpha 3$ and exposure of Cys 62, Cys 69

The region of greatest conformational change spans residues 60 to 72 encompassing helix $\alpha 3$ and loops connecting to $\beta 3$ and $\beta 4$ with movements in the range of 8–10 Å from positions in the wild type (Supporting Information Fig. 1, Supporting Information Movie 1). In the wild-type structure, Cys 69 occupies a position on the underside of $\alpha 3$ where the sidechain packs against the Phe 11 sidechain in the hydrophobic core, and the main chain carbonyl forms a buried hydrogen bond with the side chain of

Gln 78. Cys 62 is also partially buried with the sidechain thiol packing against the main chain carbonyl group from Ile 5 and the peptide bond of Ser 7. In the TrxB structure, Cys 69 and Cys 62 become exposed in a more extended conformation at the top of the fold [Fig. 1(B)]. The unraveling of helix $\alpha 3$ results into a more open conformation with an elongated pocket formed exposing residues Phe 11, Phe 27, Gln 78 from the hydrophobic core. In the TrxB, but not TrxA, structure a single polyethylene glycol 300 (PEG) molecule is bound here occupying the pocket and effectively replacing the space occupied by residues Cys 69 to Lys 72 in the wild-type structure [Fig. 1(C)]. One side the PEG molecule interacts with hydrophobic core sidechains from strands $\beta 2$, $\beta 4$, $\beta 5$, and the other is partially buried by sidechain and mainchain interactions from residues Val 65 to Glu 68. The presence of PEG is not the only factor contributing stabilizing the more open conformation as both Cys 62 and Cys 69 form disulphides to residue Cys 32 from two separate symmetry related molecules. In addition, the introduced residue Arg 73B side chain forms a salt bridge to the carboxyl of Asp 61B from a symmetry related molecule, also stabilizing the extended form of this region.

Changes in the active site Cys 32 loop

A second region of conformational change is the active site Cys 32, Cys 35 loop region between strand $\beta 2$ and helix $\alpha 2$. [Fig. 1(A)]. This conformational change includes a 170° rotation about the ψ -angle of the Trp 31 residue and a subsequent 190° twist about the ϕ -angle of the Gly 33 C α , causing a shift of 4.2 Å in the C α of Cys 32 from the TrxA (wild type) conformation. This rearrangement results in both the indole ring of the tryptophan residue and the thiol of the nucleophilic cysteine pointing up and away from the active site loop. As a consequence of the conformation, Cys 32 is brought into a position to form an intermolecular disulphide bond with Cys 62B, from a symmetry related TrxB molecule. This conformational change observed for the Cys 32 loop is not found in other thioredoxin structures available in the database for either ligand bound structures such as *E. coli* thioredoxin bound to thioredoxin reductase (TrxR) (PDB: 1F6M²⁰) or NMR structures such as the human thioredoxin NF- κ B complex (PDB: 1MDI²¹). The disulphide observed between Cys 32A and Cys 69B results in a packing of the two molecules which is heterodimeric [Fig 1(D)]. In this dimeric structure, residues Ala 66 to Arg 73 are observed to occupy the peptide binding groove in a fashion almost identical to the structure observed in the NMR structure of the thioredoxin NF- κ B complex [Fig. 1(E,F)].

Discussion

Our structural data describes a more open conformation of the thioredoxin structure than has been

Figure 1. hTrxC35S/C73R crystal structure. (A) Stereo cartoon topology diagram of hTrxC35S/C73R A chain (white) and hTrxC35S/C73R B chain (orange) superposed. The same coloring is used through all panels. (B) Unraveling of helix $\alpha 3$ is shown with changes in the positions of Cys 62 and Cys 69, whereas Val 65 retains its position. (C) Electron density for bound PEG molecule calculated using 2Fo-Fc coefficients contoured at 1.0 r.m.s. and shown in blue. Stick representation of side chains surrounding the PEG are shown in orange and green for modelled PEG. (D) Cartoon topology diagram of the dimer formed by hTrxC35S/C73R A and B chains. (E) The loop region of chain B packs along the hydrophobic groove of chain A making hydrogen bond contacts and a Cys 69B-Cys32A disulphide. (F) Human thioredoxin-NF κ B complex from the NMR structure (1MDI) shows an equivalent disulphide to Cys32 and similar hydrogen bonding interactions to (E) (red dotted lines).

previously observed with helix $\alpha 3$ containing Cys 62 and Cys 69 at either end unraveling to form an extended loop. This open conformation is likely to be transient and unstable and in the crystal it is stabilized by several factors including lattice contacts and a bound PEG molecule. A more open conformation is consistent with data showing that partially buried

Cys 62 and Cys 69 are capable of forming covalent complexes with small molecules.^{17–19} Supportive of these observations is a recent structure of the thioredoxin double mutant C69S/C73S (PDB: 3M9K; (A. Weichsel, M. Kem and W.R. Montfort accepted for publication Protein Science)) which shows a very similar conformational change with helix $\alpha 3$ also

Table I. Crystallographic Data for Thioredoxin C35S/C73R

<i>Data collection</i>	
Space group	P3(1)21
Cell dimensions	
<i>a</i> , <i>b</i> , <i>c</i> (Å)	62.05, 62.05, 90.61
α , β , γ (°)	90, 90, 120
Resolution (Å)	2.01
R_{merge} ^a	0.04 (0.19)
$I/\sigma I$	14.5 (5.1)
Completeness (%)	99.8 (100.0)
Redundancy ^b	3.70 (3.58)
Mathews Coefficient	1.9
Solvent Content (%)	35
<i>Refinement</i>	
No. reflections	13878
$R_{\text{work}}/R_{\text{free}}$ ^c	0.218/0.257
No. atoms	
Protein	1658
Solvent	362
<i>B</i> -factors (Å ²)	
Protein	19.2
Solvent	28.7
R.m.s. deviations	
Bond lengths (Å)	0.012
Bond angles (°)	1.915
Ramachandran plot	
Most favored (%)	91.1
Allowed (%)	8.9

^a R_{merge} calculated with SCALA.²²^b Values in parentheses are for highest-resolution shell.^c R_{work} and R_{free} calculated using REFMAC.²⁴

unraveling to form an extended pocket. Here, the pocket is occupied by a DTT molecule from the crystallization medium in the same position as PEG. Remarkably, the C69S/C73S structure also has two thioredoxin molecules in the asymmetric unit showing the closed and open forms. Unlike the C35S/C73R structure, the two C69S/C73S monomers are arranged into a dimer identical to the wild-type homodimer, and the active site Cys 32 is oxidized with Cys 35. The structural data described here revealing a more open conformation of human thioredoxin may provide a useful scaffold for design of agents in the chemotherapeutic targeting of thioredoxin in the pathophysiology of cancer.⁸

Materials and Methods

Cloning, expression, and purification

Mutations of C35S and C73R were introduced to the wild-type thioredoxin cDNA using the QuikChangeTM kit (Stratogene). The open reading frame for thioredoxin C35S/C73R was cloned into the expression vector pETBlue-1 (Novagen) and transformed into *E. coli* Tuner (DE3) cells. Cultures were grown at 37°C until OD₅₉₅ 0.6 was reached, and the cells induced with 2.0 mM isopropyl β-D-thiogalactopyranoside (IPTG) and left to incubate overnight at 37°C. The cells were harvested by centrifugation, and resuspended in 50 mM

Tris-HCl at pH 7.4, 1 mM EDTA and lysed by sonication (Branson). Human thioredoxin was purified from the supernatant by anion exchange chromatography using a HiPrep 16/10 DEAE column (GE Healthcare), and a 0–500 mM NaCl gradient. The protein was further purified by gel filtration using a Superdex 75 26/60 column (GE Healthcare) equilibrated with 50 mM Tris-HCl at pH 7.4 and 200 mM NaCl, after which the protein was concentrated to 60mg/mL.

Crystal structure determination

Crystals were grown by sitting drop vapour diffusion using 0.1M Na acetate, pH 4.6, and 30% (v/v) PEG 300. The crystals formed the trigonal space group P3₁21, with unit cell parameters $a = 62.05$ Å, $b = 62.05$ Å, and $c = 90.61$ Å; $\alpha = 90.0^\circ$, $\beta = 90.0^\circ$, and $\gamma = 120.0^\circ$. Data were collected to 2.0 Å at the University of Nottingham home source Rigaku Micromax-007 X-ray generator ($\lambda = 1.5418$ Å). Data were reduced, scaled, and refined to 2.0 Å resolution using MOSFLM²² with a final R_{merge} of 0.04 (Table 1). The hTrxC35S/C73R structure was solved by molecular replacement using PHASER²³ (CCP4 suite) and utilizing the existing human thioredoxin wild-type crystal structure (PDB: 1ERT¹⁶). Molecular replacement identified two clear solutions, corresponding to two molecules in the asymmetric unit. The highest peak in the cross-rotation function gave the correct orientation for the monomers and an R_{factor} of 0.36. The resulting $2F_o - F_c$ map gave clear electron density, allowing model building of the 210 residues. Extensive model rebuilding was required for significant portions of the TrxB chain, due to conformational changes. Refinement was performed using REFMAC5,²⁴ with model building carried out using Coot.²⁵ The final model has an R_{factor} of 0.22 and R_{free} 0.26 with 91.1% of residues in the most favored region and the remainder in the additional allowed area of the Ramachandran plot. Structural figures were prepared using PyMol (Delano, W.L. (2002) The PyMOL molecular graphics system. Delano Scientific San Carlos, CA, USA).

Atomic coordinates

The atomic coordinates and structure factors have been deposited with the Protein Data Bank (PDB entry 3E3E).

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