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Expression, purification and X-ray crystallographic analysis of thioredoxin from *Streptomyces coelicolor*

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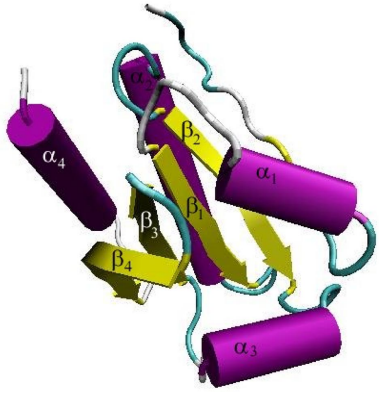

Thioredoxins are ubiquitous proteins that serve as reducing agents and general protein disulfide reductases. In turn, they are reduced by electrons obtained from the NADPH-containing thioredoxin reductase. Thioredoxins have been isolated and characterized from a large number of organisms. The Gram-positive bacterium *Streptomyces coelicolor* contains three thioredoxins that are involved in unknown biological processes. *trxA* from *S. coelicolor* was cloned and expressed in *Escherichia coli* and the protein purified and crystallized using the hanging-drop method of vapour diffusion. The crystal structure of thioredoxin A has been determined at 1.5 Å resolution using a synchrotron-radiation source. The protein reveals a thioredoxin-like fold with a typical C*XX*C active site. The crystal exhibits the symmetry of space group $P2_12_12$, with unit-cell parameters a = 43.6, b = 71.8, c = 33.2 Å.

1. Introduction

Streptomyces, the Gram-positive spore-forming soil bacterium, belongs to the sporoactinomycetes group. Unusually for bacteria, *Streptomyces* exhibits complex multicellular development, with differentiation of the organism into distinct 'tissues': a branching filamentous vegetative growth gives rise to aerial hyphae bearing long chains of reproductive spores. In addition, *S. coelicolor* is a filamentous bacterium responsible for producing most natural antibiotics used in human and veterinary medicine. Its linear chromosome, almost 9 million base pairs long, contains the largest number of predicted genes so far discovered in a bacterium (Bentley *et al.*, 2002).

Streptomyces may also serve as a suitable model organism for the study of the thioredoxin system. Generally, proteins inside cells are kept reduced and usually contain many free sulfhydryl groups, with disulfide bonds being rare (Gilbert, 1990). The major ubiquitous disulfide reductase responsible for maintaining proteins in their reduced state is thioredoxin, which is reduced by electrons from NADPH *via* thioredoxin reductase. However, the cysteine-containing tripeptide glutathione can also act to preserve the thiol–disulfide status of proteins in the cell in a manner similar to thioredoxin. Usually, in a bacterial cytoplasm that is maintaining a reducing environment both redox systems are present. In contrast, actinomycetes, including streptomycetes and mycobacteria, which do not synthesize glutathione, maintain the thiol–disulfide status of proteins mainly using thioredoxin. A number of actinomycetes also produce the unusual thiol mycothiol, which can play a role analogous to glutathione and protects actinomycetes against oxygen toxicity (Newton *et al.*, 1996). The complete genome sequence of *S. coelicolor* revealed several possible thioredoxin genes and thus *Streptomyces* seems to have a more complex redox system in comparison with other bacterial species.

Thioredoxins are small and very stable redox-active proteins that are present in all living organisms. Although thioredoxins have been isolated and characterized from numerous species, they are still very attractive for further investigation owing to their wide involvement in diverse cell physiology processes such as blood clotting, division and proliferation of animal cells, germination of seeds and regulation of photosynthesis (for a review, see Buchanan *et al.*, 1994). At the molecular level, thioredoxins serve as hydrogen donors in a wide

variety of reactions, such as ribonucleotide reduction (Laurent *et al.*, 1964) and methionine sulfoxide reduction (Gonzales-Porque *et al.*, 1970). They also modulate the activity of the transcription factors NFkB and AP-1 in human cells (Schenk *et al.*, 1994). In addition to their redox function, thioredoxins have been shown to play a structural role in several systems. They are important for the life cycle of bacteriophage T7 (Tabor *et al.*, 1987) and essential for the assembly of the filamentous phages M13 and f1 (Russel & Model, 1985).

Thioredoxins possess an active site made up of two neighbouring cysteines in a conserved motif, WCGPC, also referred to as the 'thioredoxin-motif'. Similar sequences with two cysteines in a conserved motif C*XX*C have been found in thioredoxin-like domains in several other proteins. These proteins, which are not exclusively thioredoxins, represent a very broad family of multifunctional proteins with varying roles in living cells.

The primary structures of many thioredoxins are known. Their sequences vary in length from 105 to 110 amino acids and show from 27 to 69% sequence identity. Interestingly, all thioredoxins have similar three-dimensional structures despite large variation in amino-acid sequences (Eklund *et al.*, 1991; Martin, 1995). This common structure is often called the thioredoxin fold. In addition to thioredoxins, this structure has been found in another nine protein classes, including the redox proteins glutaredoxin, Dsb protein from *Escherichia coli*, glutathione peroxidase, glutathione *S*-transferase, protein disulfide isomerase, cytochrome *c* oxidase (COX protein, conserved among prokaryotes and eukaryotes), peroxiredoxins, evolutionary conserved Dim 1 protein and iodothyronine selenodeiodinases, together forming the thioredoxin-like-fold superfamily. Interestingly, the three-dimensional structures of proteins with thioredoxin folds are very similar despite their functional differences and low sequence identity, suggesting that all these proteins may possibly stem from a common ancestor (Martin, 1995; Kemmink *et al.*, 1996).

Here, we report the cloning, expression, purification and crystallization of TrxA protein. This study describes the first three-dimensional structure of an *S. coelicolor* thioredoxin.

2. Materials and methods

2.1. Cloning, expression and purification

The *trxA* gene from *S. coelicolor* was amplified by the polymerase chain reaction (PCR) using genomic DNA of *S. coelicolor* A3(2) as a template, which was isolated according to the protocol previously reported by Hopwood *et al.* (1985). The oligonucleodide primers 5′-GGA ATT CCA TAT GGC CGG CAC CCT GAA CCA-3′ and 5′-CGC GGA TCC TCA GTC GGC GAT GAA GTC CTC-3′ were used for the PCR. The amplified fragment was digested with the restriction enzymes *Nde*I and *Bam*HI and cloned into the expression vector pET-15b (Novagen, USA). For cloning we used the bacterial strain *E. coli* MM 294 [F^+, endAJ, hsd17(rk^-, mk), supE44, thi-1], which was grown in LB medium as reported by Ausubel *et al.* (1987). The recombinant pET-15b-trxA plasmid was transformed into *E. coli* λDE3 lysogen strain BL21 [F^-, omp T, hsdsB ($r^-{}_B{}^-$, $m^-{}_B{}^-$), gal, dcm (DE3)] (Novagen, USA), which yielded the overproducing strain *E. coli* Bl21trxA. The cells of overproducing *E. coli* strain were grown at 310 K on LB medium, which was supplemented with 100 µg ml^{-1} ampicillin. The culture was grown to an absorbance of 0.6 at OD_{600} and IPTG (isopropyl-β-thiogalactoside) was then added to the medium to a final concentration of 1 *M*. Growth of the culture continued for an additional 3 h. The cells were then harvested by centrifugation, resuspended in 100 m*M* Tris–HCl pH 8.0 and 2 m*M* EDTA and disrupted by sonication using a Soniprep 150 (MSE, Crawley, UK). The recombinant protein was obtained from the cytoplasmatic fraction after ultracentrifugation at 39 000*g* using a Beckman L8-50M/E ultracentrifuge (Beckman PA, CA, USA). The clarified supernatant was applied to specific chelating columns (Novagen, USA). After elution of proteins with non-specific interactions, increasing the imidazole concentration from 100 to 400 m*M* in a stepwise manner eluted the recombinant protein. All the fractions were analysed by 15% SDS–PAGE and Coomassie blue staining (Laemmli, 1970).

2.2. Protein crystallization

Preliminary crystallization conditions for thioredoxin were obtained by screening with Crystal Screen. TrxA was crystallized by the hanging-drop vapour-diffusion method. Each drop consisted of 2 µl protein solution and 2 µl reservoir solution. All experiments were performed at 293 K. Crystals of X-ray quality were obtained as described in §3. They were then flash-frozen for data collection using a cryoprotectant consisting of 0.1 *M* sodium citrate pH 5.6, 0.16 *M* ammonium acetate, 28% PEG 4000 and 20% ethyleneglycol.

2.3. Data collection

Diffraction data were collected on beamline 19BM of the Structural Biology Center at the Advanced Photon Source, Argonne National Laboratory. The crystal was mounted in a nylon loop. The data were collected at 100 K at a wavelength of 0.97918 Å using an SBC3 CCD detector. Diffraction data to 1.5 Å resolution were processed using the *HKL*2000 package suite (Otwinowski & Minor, 1997).

2.4. Molecular replacement and refinement

The crystal structure of the protein was solved by the molecular-replacement method (MR). Initial phases were obtained using the full-length thioredoxin from *Alicyclobacillus acidocaldarius* (PDB code 1nw2; Bartolucci *et al.*, 2003) as the search model. The sequence identity between the *A. acidocaldarius* and *S. coelicolor* thioredoxins is 47% (Fig. 1). A cross-rotational search followed by a translational search was performed using the program *MOLREP* (Vagin & Teplyakov, 1997). The initial model was rebuilt using the program *O* (Jones & Kjeldgaard, 1997) based on manual inspection of the $2F_o - F_c$ map calculated after rigid-body refinement. All further refinement was performed using the program *REFMAC* (Murshudov *et al.*, 1997). Subsequent rounds of model building and refinement were carried out using the maximum-likelihood-based approach implemented in *REFMAC* using all data to the highest resolution. 5%

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Aa  ----ATMTLTDANFQQAIQGDKPVLVDFWAAWCGPCRMMAPVLEEFAEAHADKVTVAKLNVDENPETTSQFGIMSIPTLILFKGGRPVKQLIGYQPKEQLEAQLADVLQ-
Sc  MAGTLKHVTDDSFEQDVLKNDKPVLVDFWAAWCGPCRQIAPSLEAIAAEYGDKIEIVKLNIDENPGTAAKYGVMSIPTLNVYQGGEVAKTIVGAKPKAAIVRDLEDFIAD
Ec  MSDKIIHLTDDSFDTDVLKADGAILVDFWAEWCGPCKMIAPILDEIADEYQGKLTVAKLNIDQNPGTAPKYGIRGIPTLLLFKNGEVAATKVGALSKGQLKEFLDANLA-
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Figure 1
The TrxA sequence from *S. coelicolor* (Bentley *et al.*, 2002) was aligned with thioredoxins from *A. acidocaldarius* (Bartolucci *et al.*, 2003) and *E. coli* (Holmgren, 1995), with sequence identity above 47%. The sequences were aligned using the program *Vector NTI* (InforMax Incorporated, USA). Conserved residues are highlighted by a yellow background, identical residues are highlighted by a blue background and active-site residues are shown in red and highlighted by a yellow background.

of reflections were randomly selected for the calculation of R_{free} and were not used for the refinement. The final electron density is well defined for the whole main chain and most of the side chains, except for the last residue Asp110. Water molecules were added automatically using the program *ARP/wARP* (Perrakis *et al.*, 1997). During the final stages of refinement, 152 water molecules were added. The final structure of TrxA has an R_{free} value of 21.5%, an R_{work} value of 17.4% and an average temperature factor of 17 Å^2. The model displays root-mean-square deviations from ideal values of 0.014 Å for bond lengths and of 1.469° for bond angles. The validity of the model was assessed using the *PROCHECK* program (Laskowski *et al.*, 1996) and there were no outliers in the Ramachandran plot. Figures were prepared using the program *VMD* (Humphrey *et al.*, 1996).

3. Results and discussion

3.1. Purification and crystallization

SDS–PAGE analysis of the soluble fraction of the BL21(DE3) cells containing the overexpressed TrxA showed a large band corresponding to a protein of approximately 12 kDa. This high level of expression facilitated purification by the simple three-step protocol described in §2. TrxA was eluted from the nickel column using an elution buffer containing 0.2–0.3 *M* imidazole. The purity of the preparation was checked by SDS–PAGE (Fig. 2). The typical yield was about 4–5 mg protein from 100 ml induced cell culture as estimated by the method of Bradford (1976).

The optimal concentration for the crystal growth of thioredoxin was determined by examination of the results from a PCTTM Pre-Crystallization Screen. We used different concentrations of the protein ranging from 7 to 11 mg ml^{-1}. For crystallization trials, purified protein with a concentration of 10 mg ml^{-1} in 100 m*M* Tris–HCl pH 7.5 and 1 m*M* dithiothreitol was used. The preliminary crystallization conditions were taken from the Crystal Screen crystallization screen and crystals were observed in five conditions: (i) 0.1 *M* sodium citrate pH 5.6, 0.2 *M* ammonium acetate, 30% PEG 4000, (ii) 0.1 *M* sodium acetate pH 4.6, 0.2 *M* ammonium acetate, 30% PEG 4000, (iii) 0.1 *M* sodium cacodylate pH 6.5, 0.2 *M* magnesium acetate, 20% PEG 8000, (iv) 0.2 *M* ammonium sulfate, 30% PEG 8000 and (v) 0.1 *M* Na HEPES pH 7.5, 2.0 *M* ammonium sulfate, 2% PEG 400. After optimization of the crystallization conditions obtained from the screen, crystals suitable for diffraction analysis were obtained from a 4 µl drop containing 0.1 *M* sodium citrate pH 5.6, 0.1 *M* ammonium acetate and 25% PEG 4000 using the hanging-drop vapour-diffusion method at 293 K as described in §2.

Table 1
Crystallographic data statistics of thioredoxin.

Values in parentheses are for the last shell.

Data collection	
Wavelength (Å)	0.97918
Resolution (Å)	50.0–1.5
Space group	$P2_12_12$
Unit-cell parameters	
a (Å)	43.579
b (Å)	71.756
c (Å)	33.187
$\alpha = \beta = \gamma$ (°)	90.0
Total No. of reflections	94419
No. of unique reflections	16654
Completeness (%)	96.5 (85.3)
Redundancy	5.7
R_{merge}	0.086 (0.313)
$I/\sigma(I)$	21.8 (2.08)
Structural refinement and geometry	
Resolution range (Å)	25.0–1.5
No. reflections	15784
R factor/R_{free} (%)	17.4/21.5
Average B factor (Å^2)	16.74
Total No. of atoms	1022
Total No. of waters	152
R.m.s.d. bonds (Å)	0.014
R.m.s.d. angles (°)	1.469

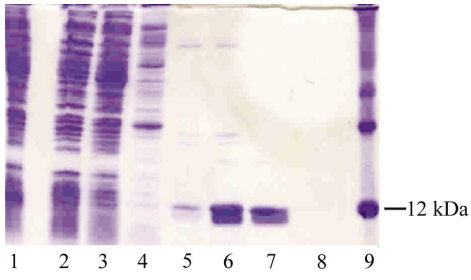

Figure 2
SDS–PAGE [15%(*w*/*v*)] of the isolation of *S. coelicolor* A3(2) Trx from the overexpression *E. coli* BL21trxA strain. Lane 1, cell extract after sonication and ultracentrifugation (sample). Lane 2, fraction after loading sample. Lane 3, fraction after elution with binding buffer (5 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). Lane 4, fraction after elution with wash buffer (60 m*M* imidazole, 0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). Lane 5, Fraction after elution by elution buffer with 0.1 *M* imidazole (0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). Lane 6, enzyme preparation of Trx obtained using elution buffer with 0.2 *M* imidazole (0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). Lane 7, enzyme preparation of Trx obtained using elution buffer with 0.3 *M* imidazole (0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). Lane 8, fraction after elution with elution buffer with 1 *M* imidazole (0.5 *M* NaCl, 20 m*M* Tris–HCl pH 7.9). Lane 9, protein molecular-weight standard (cytochrome *c*, 12 kDa).

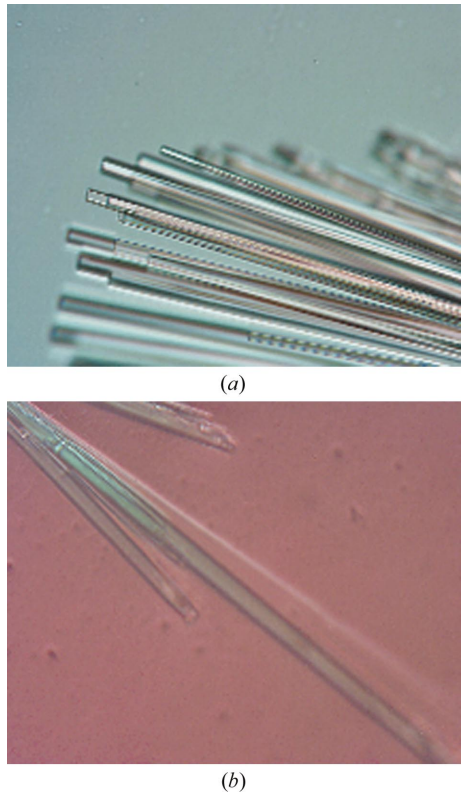

Figure 3
Crystals of TrxA from *S. coelicolor*. (*a*) Cluster of thioredoxin crystals. (*b*) Crystal used for data collection. Crystals were grown by the hanging-drop vapour-diffusion method at room temperature in clusters and attained dimensions of 350 × 80 × 20 µm. Crystallization conditions: 0.1 *M* sodium citrate pH 5.6, 0.16 *M* ammonium acetate, 25% PEG 4000. Protein concentration: 10 mg ml^{-1} in 100 m*M* Tris–HCl pH 7.5 and 1 m*M* DTT. The cryoprotectant consisted of 0.1 *M* sodium citrate pH 5.6, 0.16 *M* ammonium acetate, 28% PEG 4000, 20% ethylene glycol.

The prism-shaped crystals grew in clusters and attained dimensions of 350 × 80 × 20 µm in 10 d (Fig. 3*a*). Separation of one crystal (Fig. 3*b*) from the cluster was performed with needles and tools intended for crystal mounting. The crystal parameters and data-collection statistics were as reported in Table 1.

3.2. Overall structure

The three-dimensional structure of TrxA is very similar to those of thioredoxins from other sources (Holmgren, 1995; Katti *et al.*, 1990). This structure is a monomer comprised of 110 amino acids. All other thioredoxins crystallized in a monomeric form, except for human thioredoxin, which crystallized in an inactive dimer where the active site is blocked by the other monomer.

The thioredoxin from *S. coelicolor* consists of a central core made up of four β-strands surrounded by four α-helices to form two conformational domains with topologies $\alpha\beta\alpha\beta\alpha$ and $\beta\beta\alpha$ (Fig. 4). Thus, the typical thioredoxin fold that consists of four β-strands and three flanking α-helices is present in TrxA from *S. coelicolor* (Martin, 1995). In addition to its presence in all thioredoxins, the thioredoxin fold can also be found in other protein families.

TrxA from *S. coelicolor* crystallized in the reduced form because the crystallization drop contained DTT [5,5′-dithio-bis(2-nitro-benzoic acid)].

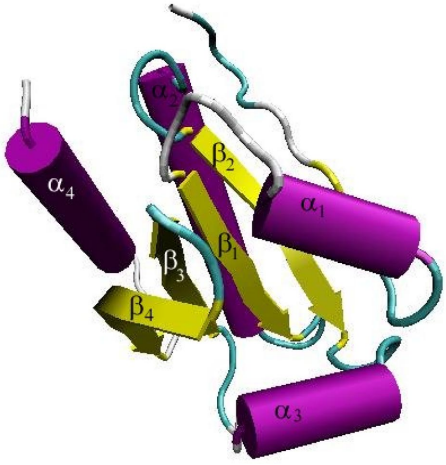

Figure 4
Overall structure of the TrxA molecule from *S. coelicolor*. α-Helices are shown in purple and β-strands in yellow.

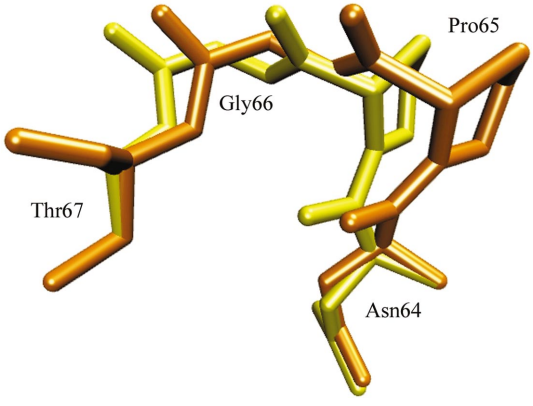

Figure 5
Alternate conformations of main-chain residues 64–67. The *A* conformation of the residues is shown in orange and the *B* conformation of the residues is shown in yellow.

3.3. Alternate conformations

In the structure of thioredoxin from *S. coelicolor*, residue Glu55 was refined in two alternate conformations. Similar alternate conformations of single amino-acid residues (Ile5, Leu41, Ser89) were observed in the structure of Trx-2 from *Anabaena* (Saarinen *et al.*, 1995). However, in the TrxA structure of *S. coelicolor* we observed conformational variability of the main chain for residues Asn64, Pro65, Gly66 and Thr67, where this region can adopt two different conformations (Fig. 5). Although this region is not important for protein redox function, subtle changes in residues and/or conformation may play a role in species-specific protein–thioredoxin interactions.

3.4. Active site

The active-site sequence is Trp-Cys-Gly-Pro-Cys in most thioredoxins. In TrxA the distance between the two S atoms of the active-site cysteines (33 and 36) is 3.15 Å(Fig. 6) and is comparable with the structure of reduced human thioredoxin, in which the distance between two S atoms of the active-site cysteines (32 and 35) is 3.9 Å (Weichsel *et al.*, 1996). The distances differ from those in the active sites of oxidized human thioredoxin and thioredoxin from *E. coli*, where the active-site cysteines (32 and 35) form a disulfide bond with a bond length of about 2 Å (Weichsel *et al.*, 1996; Katti *et al.*, 1990).

In all thioredoxins, the active-site helix α2 (32–50) is divided into two parts by the presence of an invariant proline residue at position 41. The first part of this helix (residues 32–41) displays regular helical bonding and the central residues in this turn are Asn-Gly as in *E. coli* thioredoxin. Most of the residues conserved in the 39 known thioredoxin sequences are located around the active site (Saarinen *et al.*, 1995). The residues in the central part constitute the suggested thioredoxin protein-interaction area. Clusters of hydrophobic residues are found in the region around the redox-active disulfide in *S. coelicolor*.

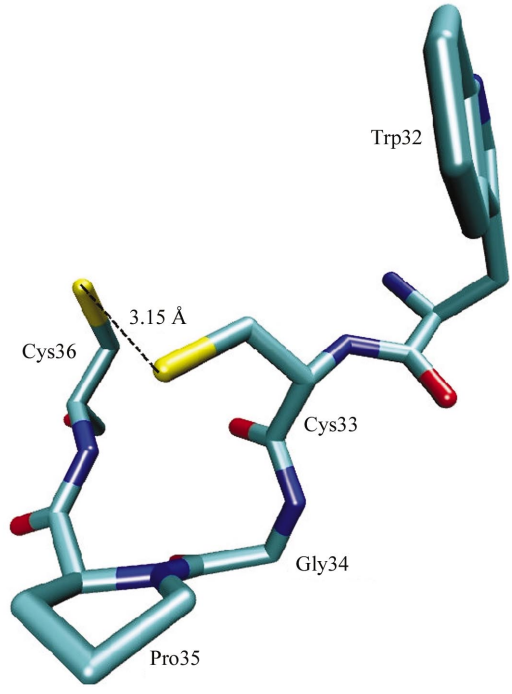

Figure 6
Active-site residues 33–36 of TrxA.

In the vicinity of the active-site residues is Asp27 (Asp26 in *E. coli*), which is important for enzymatic activity (Saarinen *et al.*, 1995). This residue is highly conserved in all thioredoxins and is the subject of numerous studies aimed at defining its role in thioredoxin stability and function. A water molecule hydrogen bonds to the side-chain carboxylate of Asp27 and the main-chain carbonyl of the active site Cys36 (Katti *et al.*, 1990; Saarinen *et al.*, 1995). In the structure of TrxA from *S. coelicolor*, water molecule 128 makes one hydrogen bond (2.87 Å) with the active-site oxygen of Cys36 and another hydrogen bond (2.65 Å) with the oxygen of Asp27. The conserved Asp residue serves as an acid/base in the oxidation/reduction reactions catalysed by thioredoxins, by protonating (during substrate oxidation) or deprotonating (during substrate reduction) the thiol of the most buried substrate of cysteine residue *via* this bridging water molecule (Menchise *et al.*, 2001).

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