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Received 14 March 2013

Accepted 18 April 2013

PDB References: reduced LvTrx, 4aj6; partially reduced LvTrx, 4aj8; thioredoxin, 3zxx; oxidized LvTrx, 4aj7

Expression, purification, crystallization and X-ray crystallographic studies of different redox states of the active site of thioredoxin 1 from the whiteleg shrimp *Litopenaeus vannamei*

Thioredoxin (Trx) is a 12 kDa cellular redox protein that belongs to a family of small redox proteins which undergo reversible oxidation to produce a cystine disulfide bond through the transfer of reducing equivalents from the catalytic site cysteine residues (Cys32 and Cys35) to a disulfide substrate. In this study, crystals of thioredoxin 1 from the Pacific whiteleg shrimp *Litopenaeus vannamei* (LvTrx) were successfully obtained. One data set was collected from each of four crystals at 100 K and the three-dimensional structures of the catalytic cysteines in different redox states were determined: reduced and oxidized forms at 2.00 Å resolution using data collected at a synchrotron-radiation source and two partially reduced structures at 1.54 and 1.88 Å resolution using data collected using an in-house source. All of the crystals belonged to space group *P*3₂12, with unit-cell parameters *a* = 57.5 (4), *b* = 57.5 (4), *c* = 118.1 (8) Å. The asymmetric unit contains two subunits of LvTrx, with a Matthews coefficient (*V*_M) of 2.31 Å³ Da^{−1} and a solvent content of 46%. Initial phases were determined by molecular replacement using the crystallographic model of Trx from *Drosophila melanogaster* as a template. In the present work, LvTrx was overexpressed in *Escherichia coli*, purified and crystallized. Structural analysis of the different redox states at the Trx active site highlights its reactivity and corroborates the existence of a dimer in the crystal. In the crystallographic structures the dimer is stabilized by several interactions, including a disulfide bridge between Cys73 of each LvTrx monomer, a hydrogen bond between the side chain of Asp60 of each monomer and several hydrophobic interactions, with a noncrystallographic twofold axis.

1. Introduction

Thioredoxin (Trx) is a protein that reduces disulfide bonds in many proteins, the best studied of which is the reduction of ribonucleotide reductase (Thelander & Reichard, 1979). Trx plays a structural role in coliphages such as coliphage f1 from phage M13, participates in the regulation of photosynthetic active centres and acts as a transcription factor in some eukaryotes (Holmgren *et al.*, 1975; Holmgren, 1985; Jacquot *et al.*, 1994). Trx has also been shown to stimulate the growth of human T cells (Tagaya *et al.*, 1989) and to protect against oxidative damage by reducing antioxidant proteins such as peroxiredoxin (Yoshida *et al.*, 2003).

Trx is a member of a family of redox proteins with a highly conserved primary structure and 27–69% sequence identity (Eklund *et al.*, 1991). All Trxs have almost identical three-dimensional structures despite their large variation in amino-acid sequence and consist of a central five-stranded β-sheet flanked by three or four α-helices (Holmgren, 1985; Martin, 1995). The active site of Trx is highly conserved and has two redox-active cysteine residues in the sequence -Trp-Cys-Gly-Pro-Cys-, which is localized on the surface of the protein. Oxidized Trx is reduced by the flavoenzyme thioredoxin reductase, and together they form the 'thioredoxin system' (Raddatz *et al.*, 1997). Several factors affect thiol–disulfide exchange in the Trx

active site: for example, the pK_a of the nucleophilic Cys32, the electrostatic environment of the neighbouring residues and the pH of the medium (Collet & Messens, 2010).

The determination of the three-dimensional structures of both oxidized and reduced forms of human Trx (PDB entries 1eru and 1ert), *Drosophila melanogaster* Trx (PDB entries 1xw9 and 1xwc) and *Escherichia coli* Trx (PDB entries 1xoa and 1xob) has presented new opportunities for understanding the catalytic activity of Trx in disulfide exchange, and these enzymes have been the subject of many investigations, including studies of their structures, thiol redox regulation and reaction mechanisms (Weichsel *et al.*, 1996; Wahl *et al.*, 2005; Jeng *et al.*, 1994).

Information about Trx in invertebrates is scarce. However, there are a few reports of crustacean Trxs, including studies of the antioxidant capacity of Trx from the Pacific whiteleg shrimp *Litopenaeus*

vannamei (LvTrx; Aispuro-Hernandez *et al.*, 2008) and investigations of the behaviour of Trx towards viral infections in the Chinese shrimp *Fenneropenaeus chinensis* and *L. vannamei* (Ren *et al.*, 2009; Garcia-Orozco *et al.*, 2012).

In the present work, the crystal structure determination of LvTrx revealed a dimeric form of the protein covalently linked through a disulfide bond involving Cys73 from each monomer. Dimer formation under reducing acidic conditions *via* noncovalent and covalent binding has been reported for *E. coli* and human Trxs (Holmgren *et al.*, 1975; Andersen *et al.*, 1997). In human Trx, a dimer was shown to be formed by an intermolecular disulfide involving Cys73, a residue that is not involved in catalytic activity (Weichsel *et al.*, 1996). Whether or not the dimer has a physiological role remains unclear. Our ultimate goal is to provide information to assist in understanding the physiological function of the antioxidant enzyme system in LvTrx. These crystallographic structures will enable us to gain a comprehensive understanding of the catalytic reduction of LvTrx at high

Figure 1

Micrograph of crystals of recombinant LvTrx obtained using the hanging-drop vapour-diffusion method. 10 mM DTT was added to every buffer used during all of the purification steps. 5 mM DTT was added to the crystallization droplet.

Figure 3

Crystal of recombinant LvTrx obtained using the hanging-drop vapour-diffusion method with exposure to 10 mM DTT in the first purification step only.

Figure 2

Crystals of recombinant LvTrx obtained using the hanging-drop vapour-diffusion method with 10 mM DTT added during all purification steps. The blue colour observed arises from the addition of Izit crystal dye (Hampton Research, USA) to the droplet.

Figure 4

Crystals of recombinant LvTrx obtained using the hanging-drop vapour-diffusion method without the addition of DTT to any of the purification or crystallization processes.

resolution, thus allowing us to propose experiments to determine whether or not dimer formation in solution is possible.

2. Materials and methods

2.1. Expression and purification of LvTrx

The recombinant LvTrx was obtained in a similar manner as in previously published studies (Aispuro-Hernandez *et al.*, 2008). The recombinant clone was sequenced on both strands and was used to transform *E. coli* BL21 competent cells (Novagen). The plasmid pET11a was transformed into the host strain *E. coli* BL21 and the transformed cells were cultivated and grown on Luria–Bertani [LB; 1% (w/w) tryptone, 0.5% (w/v) yeast extract, 1% (w/v) NaCl] agar plates containing 200 $\mu\text{g ml}^{-1}$ ampicillin at 310 K. A single colony was picked and grown for plasmid isolation and was verified by sequencing. A colony was inoculated into 50 ml LB medium containing 200 $\mu\text{g ml}^{-1}$ ampicillin and incubated for 12 h at 310 K; part of the culture (25 ml) was used to inoculate 1 l LB medium with 200 $\mu\text{g ml}^{-1}$ ampicillin. The culture was grown to an OD_{600} of 0.6. Isopropyl β -D-1-thiogalactopyranoside (IPTG) was then added to the

medium to a final concentration of 0.4 mM and the culture was grown for an additional 5 h. The cells were then harvested by centrifugation (24 000g, 30 min, 277 K) using a Beckman JA-14 rotor centrifuge (Beckman, California, USA) and washed in 0.9% (w/v) NaCl. The cell suspension was resuspended in cold lysis buffer consisting of 100 mM Tris–HCl pH 8.0, 10 mM DTT [dithiothreitol; (2S,3S)-1,4-bis-(sulfanyl)butane-2,3-diol] with Complete EDTA-free protease-inhibitor cocktail (Roche Molecular Biochemical, USA) and sonicated on ice (three pulses of 1 min with a rest interval of 5 min at 277 K). Cell debris was removed by centrifugation (24 000g, 20 min, 277 K) and the supernatant containing the soluble target protein was collected for purification. All fractions were analyzed using 15% SDS–PAGE with Coomassie Blue staining (Laemmli, 1970).

The supernatant containing recombinant LvTrx was fractionated by two consecutive precipitation steps at 50 and 85% ammonium sulfate saturation. The precipitate was resuspended in 10 mM Tris–HCl pH 7.5, 10 mM DTT and heated to 343 K for 20 min. Cell debris was then removed by centrifugation (24 000g, 20 min, 277 K). The supernatant was dialyzed two times at 277 K against ten times its volume of 10 mM Tris–HCl pH 7.5. The supernatant was loaded onto a 15 ml Q Sepharose ion-exchange column (GE Healthcare, Sweden)

Figure 5
2F_o - F_c electron-density maps for different LvTrx redox states in the catalytic site from the reduced to the oxidized state (the process is highlighted by an arrow), showing the catalytic site of each chain and the inter-monomeric disulfide bridge that is present in all of the redox states determined here (the maps were contoured at 1.0 σ above the mean value and the figures were generated with CCP4mg).

Table 1

Summary of crystallographic data-collection and refinement statistics.

Values in parentheses are for the highest resolution shell.

	<i>Lv</i> Trx, reduced (PDB entry 4aj6)	<i>Lv</i> Trx, partially reduced (PDB entry 4aj8)	<i>Lv</i> Trx, oxidized (PDB entry 4aj7)	<i>Lv</i> Trx, partially reduced (PDB entry 3zzx)
Data-collection statistics				
X-ray source	Beamline X6A, NSLS	Rigaku MicroMax-007 HF rotating anode	Beamline X6A, NSLS	Rigaku MicroMax-007 HF rotating anode
Crystal dimensions (μm)	33 × 33 × 33	139 × 30 × 30	61 × 80 × 30	100 × 30 × 30
Absorbed dose (MGy)	0.510	0.019	0.670	0.013
Detector	ADSC Q270	R-Axis IV ⁺⁺	ADSC Q270	R-Axis IV ⁺⁺
Wavelength (Å)	0.9795	1.5418	0.9795	1.5418
Space group	<i>P</i> ₃ 12	<i>P</i> ₃ 12	<i>P</i> ₃ 12	<i>P</i> ₃ 12
Unit-cell parameters (Å, °)	<i>a</i> = <i>b</i> = 57.99, <i>c</i> = 118.61, α = β = 90.0, γ = 120.0	<i>a</i> = <i>b</i> = 57.52, <i>c</i> = 118.02, α = β = 90.0, γ = 120.0	<i>a</i> = <i>b</i> = 57.90, <i>c</i> = 117.87, α = β = 90.0, γ = 120.0	<i>a</i> = <i>b</i> = 57.83, <i>c</i> = 118.18, α = β = 90.0, γ = 120.0
Δφ (°)	1.0	0.5	0.5	1.0
Resolution range (Å)	19.16–2.00 (2.10–2.00)	24.37–1.54 (1.64–1.54)	19.64–2.00 (2.10–2.00)	23.29–1.88 (1.98–1.88)
No. of reflections	87019	212026	94693	56768
No. of unique reflections	15712	33397	14756	17740
Completeness (%)	99.9 (96.3)	99.7 (98.6)	99.4 (96.6)	94.5 (91.6)
<i>R</i> _{merge} [†] (%)	6.7 (35.4)	5.7 (40.0)	5.8 (24.2)	6.0 (44.0)
<i>R</i> _{meas} [‡] (%)	7.4 (39.0)	6.2 (27.6)	6.3 (27.4)	7.4 (57.2)
⟨ <i>I</i> /σ(<i>I</i>)⟩	18.8 (4.8)	17.0 (4.9)	20.3 (5.2)	13.3 (3.1)
Multiplicity	5.5 (5.6)	6.3 (5.5)	6.4 (4.3)	3.2 (3.1)
Asymmetric unit content	Dimer	Dimer	Dimer	Dimer
Refinement statistics				
<i>R</i> _{work} / <i>R</i> _{free} (%)	19.37/24.54	17.76/21.41	17.35/22.59	18.47/23.12
<i>B</i> values (Å ²)				
Protein	18.99	17.20	21.74	24.20
Ion/ligand	45.42	39.79	46.64	45.82
Water	26.00	24.52	27.37	28.56
All atoms	22.86	21.21	24.73	25.08
Wilson plot <i>B</i> value (Å ²)	22.29	19.27	24.49	23.81
R.m.s.d. from ideal stereochemistry				
Bond lengths (Å)	0.018	0.007	0.008	0.007
Bond angles (°)	1.170	1.053	0.976	1.026
Coordinate error (maximum-likelihood basis) (Å)	0.12	0.34	0.27	0.51
Ramachandran plot, residues in (%)				
Most favoured regions	98.18	94.59	97.74	98.00
Additional allowed regions	1.82	5.41	2.26	2.00
Disallowed regions	0	0	0	0

[†] $R_{\text{merge}} = \sum_{hkl} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$, where $I_i(hkl)$ and $\langle I(hkl) \rangle$ represent the diffraction-intensity values of the individual measurements and the corresponding mean values. The summation is over all unique measurements. [‡] R_{meas} is a redundancy-independent version of R_{merge} : $R_{\text{meas}} = \sum_{hkl} [N(hkl)/[N(hkl) - 1]]^{1/2} \sum_i |I_i(hkl) - \langle I(hkl) \rangle| / \sum_{hkl} \sum_i I_i(hkl)$.

pre-equilibrated and washed with three column volumes of 10 mM Tris pH 7.5, 10 mM DTT. The sample was eluted with a linear gradient of NaCl (0–1 M) or a pulse of 300 mM NaCl was applied in the equilibration buffer at a flow rate of 1 ml min^{−1}. The collected fractions were analyzed at 280 nm and the fractions corresponding to *Lv*Trx were pooled and concentrated by ultrafiltration (Amicon Ultra-3 cellulose filter, Millipore; 2 kDa molecular-weight cutoff). After each chromatography step had been performed, denaturing electrophoresis (15% SDS–PAGE) was used to detect the peak corresponding to a molecular mass of 12 kDa. In all cases the gels were stained with Coomassie Blue (Sigma, USA) and the protein was quantified using the Bradford assay method (Bradford, 1976) with bovine serum albumin as a standard. The purification yield was approximately 5 mg purified protein from 1 l culture. To carry out the purification of *Lv*Trx without DTT the same steps were used as described above, with the exceptions that DTT was not added during the purification and that as the last step the sample was dialyzed in 10 mM sodium acetate buffer pH 3.8 and again passed through anion-exchange resin (Q Sepharose, GE Healthcare, Sweden), thereby obtaining pure protein in the absence of reducing agent.

2.2. Protein crystallization

Initial screening was performed using Crystal Screen and Crystal Screen 2 from Hampton Research. As a first approach to the crystallization of *Lv*Trx, these kits were used with the microbatch

technique in Greiner plates and crystals of *Lv*Trx were obtained after one month using condition No. 47 of Crystal Screen (0.1 M sodium acetate trihydrate pH 4.6, 2.0 M ammonium sulfate). Since the initial crystals were too small to test for diffraction, the hanging-drop vapour-diffusion method was used to improve the crystal size. Each drop consisted of 2 μl reservoir solution and 2 μl protein sample (at 6 mg ml^{−1}), with 1 ml precipitant solution in the reservoir. After optimization, crystals suitable for X-ray diffraction measurements grew within two weeks. All crystals were obtained in this manner using one of four conditions: (i) DTT was placed directly in the crystallization drop (Fig. 1), (ii) the protein was exposed to DTT throughout the entire purification (Fig. 2), (iii) the protein was exposed to DTT only in the first purification step (Fig. 3) or (iv) the protein was not exposed to DTT (Fig. 4). The crystals were then flash-cooled in liquid nitrogen using a cryoprotectant buffer comprised of crystallization buffer containing 30% (v/v) glycerol prior to data collection.

2.3. Data collection

Diffraction data were collected on an in-house R-Axis IV⁺⁺ image-plate detector using Cu Kα radiation (wavelength of 1.5418 Å) generated by a Rigaku MicroMax-007 HF rotating-anode generator at the Laboratorio Nacional de Estructuras de Macromoléculas (LANEM), Instituto de Química, Universidad Nacional Autónoma de México and on beamline X6A of the National Synchrotron Light

Source (NSLS), Brookhaven National Laboratory (BNL), USA (wavelength of 0.9795 Å) using an ADSC Quantum 270 detector. X-ray diffraction data for the different *Lv*Trx crystals were collected

from a single crystal in each case. The crystals were flash-cooled as mentioned above and maintained at 100 K in a stream of nitrogen during data collection.

2.4. Molecular replacement and refinement

The first crystal structure of recombinant *Lv*Trx was determined by the molecular-replacement method. Initial phases were obtained using the coordinates of *D. melanogaster* Trx (PDB entry 1xwa; Wahl *et al.*, 2005) as the search model. The sequence identity between *D. melanogaster* Trx and *Lv*Trx is 60%. We used this first structure as a model for solution of the three remaining data sets. The diffraction images in each data set were integrated using *XDS* (Kabsch, 2010) and scaling was performed with *SCALA* from the *CCP4* suite (Winn *et al.*, 2011). The crystals belonged to space group *P3₂12*, with unit-cell parameters $a = 57.5$ (4), $b = 57.5$ (4), $c = 118.1$ (8) Å, $\alpha = 90$, $\beta = 90$, $\gamma = 120^\circ$. Additionally, *POINTLESS* (Evans, 2006) clearly supported the space-group selection. A cross-rotational search followed by a translational search was performed using *Phaser* (McCoy *et al.*, 2007) to obtain initial models and phases (LLG = 3810, RFZ = 30.0, TFZ = 56.7, with a dimer found in the asymmetric unit). The models were improved based on manual inspection of the $2F_o - F_c$ map after rigid-body refinement with geometric constraints in *REFMAC* (Murshudov *et al.*, 2011). All further refinements were performed using *PHENIX* (Adams *et al.*, 2010). The final models were completed and refined using *PHENIX* and *Coot* (Emsley *et al.*, 2010). Crystal structures of *Lv*Trx were determined for different redox forms of the active-site cysteines: reduced, two partially reduced forms and oxidized at resolutions of 2.00 Å (PDB entry 4aj6), 1.54 Å (PDB entry 4aj8), 1.88 Å (PDB entry 3zzx) and 2.00 Å (PDB entry 4aj7), respectively (Fig. 5). The data-collection and refinement statistics are summarized in Table 1. The absorbed dose during data collection is also given in Table 1 and it can be seen that the dose for the two data sets collected using an in-house generator (PDB entries 4aj8 and 3zzx) is an order of magnitude lower than that for the two structures determined using data collected at the NSLS (PDB entries 4aj6 and 4aj7). As expected, the chemical reduction produced by radiation damage at an absorbed dose of 0.67 MGy (PDB entry 4aj7) needs to be supplemented by the presence of DTT (PDB entry 4aj6, with an absorbed dose of 0.51 MGy) in order to reduce the two disulfide bridges formed between the two pairs of catalytic cysteines found in the asymmetric unit without affecting the intermolecular disulfide of Cys73.

In this contribution, crystallographic structures of *Lv*Trx have been identified and analyzed with their catalytic cysteines Cys32 and Cys35 (Powis & Montfort, 2001) in different redox states. However, in the four structures presented here a dimer mediated by a disulfide bridge between Cys73 of the two monomers was always present (Fig. 6a). Formation of the *Lv*Trx dimer involved relatively weak interactions which are mainly hydrophobic and are mediated by 12 amino-acid residues, notably a disulfide bond between the Cys73 residues of the monomers. Moreover, the area buried at the interface as determined by the *PISA* (*Protein Interfaces, Surfaces and Assemblies*) server at the European Bioinformatics Institute (Krissinel & Henrick, 2007) supports the structural stability of this interaction. The interface area is 609.75 Å² and the solvation free-energy gain upon formation of the interface, $\Delta_i G$, is -31.4 kJ mol⁻¹ on average (Figs. 6b and 6c). These structures also demonstrated movement of the active-site cysteine residues Cys32 and Cys35 owing to chemical reduction and of the side chain of Asp60, which is also present in the interface between the monomers and forms a hydrogen bond to the Asp60 side chain of the other monomer. In the oxidized active-site structure (PDB entry

Figure 6
*Lv*Trx crystallographic structures. (a) General organization of *Lv*Trx in the asymmetric unit, showing the disulfide bridge between the Cys73 residues and a hydrogen bond between the Asp60 residues of each monomer; both residues are involved in dimer stabilization. (b) The contact interface between monomers in the *Lv*Trx asymmetric unit is represented by yellow and blue complementary meshes. (c) Front view of one monomer making up the dimeric interface, showing the 12 residues involved in the inter-monomer interaction; the $2F_o - F_c$ map contoured at 1σ is also displayed as grey dots and water molecules in the vicinity are represented by red spheres. It is notable that no water molecules are localized at the dimeric interface (PDB entry 4aj6 was used to generate these images with *CCP4mg*).

4aj7) the latter interaction is 3.0 Å in length, while in the reduced active-site structure (PDB entry 4aj6) this hydrogen bond is 2.5 Å in length. If we consider the coordinate error (maximum-likelihood basis) for PDB entries 4aj6 and 4aj7, such a difference in distance seem negligible (Table 1). However, it is important to note that even when the active cysteines are fully oxidized the hydrogen bond between the Asp60 side chains is still present. As previously reported, the majority of Trxs have been found in a monomeric form in solution. However, like human Trx, LvTrx exhibits a dimeric crystallographic behaviour that has been described here at the molecular level. Currently, further biochemical assays are being performed in order to describe the nature and the potential biological implications of the dimeric form found by crystallographic studies. Although the biological function of Trx dimers has not yet been fully determined, the monomer–monomer interaction described here would suggest that LvTrx dimerization seems to be possible both in solution as well as in crystals. However, it is pivotal to demonstrate whether or not this dimer is involved in the regulatory functions of LvTrx in living cells.

AAC-A was supported by an MSc fellowship from CONACyT. ER-P acknowledges financial support from CONACyT project No. 102370 and PAPIIT IN204611. The authors thank LANEM and its staff, specifically Adela Rodríguez-Romero and Georgina E. Espinosa-Perez, at Instituto de Química, UNAM for the allocation of data-collection time and for assistance during data collection, respectively. We thank the staff of NSLS beamline X6A for data-collection facilities, in particular Vivian Stojanoff. Beamline X6A is funded by NIGMS (GM-0080) and the US Department of Energy (contract No. DE-AC02-98CH10886). The authors thank Biol. Sonia P. Rojas-Trejo for very committed technical assistance.

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