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Expression, purification, crystallization and preliminary X-ray diffraction analysis of mitochondrial thioredoxin Trx3 from *Saccharomyces cerevisiae*

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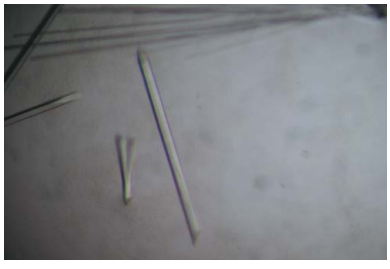


There are three thioredoxin isoforms in the yeast *Saccharomyces cerevisiae*: two cytosolic/nuclear thioredoxins, Trx1 and Trx2, and one mitochondrial thioredoxin, Trx3. In the present work, *S. cerevisiae* Trx3 overexpressed in *Escherichia coli* was purified and crystallized. The Trx3 crystals were obtained by the hanging-drop vapour-diffusion method. A data set diffracting to 2.0 Å resolution was collected from a single crystal. The crystal belongs to space group $P3_1$, with unit-cell parameters $a = b = 49.57$, $c = 94.55$ Å, $\alpha = \beta = 90$, $\gamma = 120^\circ$. The asymmetric unit is assumed to contain two subunits of Trx3, with a V_M value of 2.62 Å^3 Da^{-1} and a solvent content of 53%.

1. Introduction

Thioredoxins (Trxs) are a class of multifunctional redox-active proteins that are found ubiquitously from prokaryotes to man. They are small proteins (12 000 Da) with a conserved active-site motif (Trp-Cys-Gly-Pro-Cys). The reduced form of Trx reduces the oxidized thiols of target proteins, at the expense of being oxidized itself. Oxidized Trx is subsequently regenerated by the flavoenzyme thioredoxin reductase (TrxR). Thioredoxin was first described as a hydrogen donor for ribonucleotide reductase, assisting in the reduction of ribonucleotides to deoxyribonucleotides in *Escherichia coli* (Laurent *et al.*, 1964). More recently, Trx has been implicated in multiple biological functions, such as modulation of transcription-factor activity (Schenk *et al.*, 1994), disulfide reduction during protein synthesis (Hawkins *et al.*, 1991; Powis & Montfort, 2001), protection against oxidative damage by reducing antioxidant proteins, such as peroxiredoxin (Yoshida *et al.*, 2003), and protection against reductive stress (Trotter & Grant, 2002). Human Trx is also involved in the proliferation of some cells such as human tumour-cell lines (Powis *et al.*, 2000). A recent report shows that a C-terminally truncated human thioredoxin is secreted and acts as an eosinophilic cytotoxicity-enhancing factor, possessing cytokine-like activity (Pekkari & Holmgren, 2004).

As in mammals, there are two thioredoxin systems in yeast: a cytoplasmic system, including Trx1, Trx2 and a thioredoxin reductase TrxR1 (Gan, 1991), and a mitochondrial system consisting of Trx3 and TrxR2 (Pedrajas *et al.*, 1998). Trx3 is larger than its counterparts in the cytoplasm owing to a signal peptide extension at the N-terminus that directs its translocation into the mitochondria. It has been reported that Trx3 contributes to the defence against reactive oxygen species (ROS) as an electron donor to the thioredoxin peroxidase and regulates the activity of mitochondrial proteins by redox control (Pedrajas *et al.*, 1999). Recently, Trx3 has been found to have a functional relationship with GrxR1 (glutathione reductase) and an overlapping function with GSH (Trotter & Grant, 2005). A large variety of thioredoxin structures from various organisms have been determined (Rehse *et al.*, 2005; Smeets *et al.*, 2005; Friemann *et al.*, 2003; Capitani *et al.*, 2000; Weichsel *et al.*, 1996), but the structure of human thioredoxin 2 is that of a mitochondrial Trx. Recently, the crystal structure of Trx2 from the yeast *Saccharomyces cerevisiae* has also been solved by our group (Bao *et al.*, 2006). In the present work, we report the crystallization and preliminary X-ray diffraction analysis of Trx3. This is only the second mitochondrial thioredoxin

structure to be solved and Trx3 possesses two additional cysteines compared with its cytoplasmic homologues. Its structure will provide new insights into the specific functions of this mitochondrial enzyme.

2. Materials and methods

2.1. Cloning and expression

The coding sequence of Trx3/YCR083W was PCR-amplified from *S. cerevisiae* genomic DNA. The forward primer, which contains an *Nde*I site, 5′-CCTCCATATGTCCTCATACACCAGTATTAC-3′, was designed to construct the signal domain-truncated form and the reverse primer, which contains a *Not*I site, was 5′-CCGAGC-GGCCGCTTATAGATCTTTGATTCC-3′. The amplified fragments were cloned into a pET28a-derived expression vector (pET28ad) with a coding sequence for a hexahistidine (6×His) tag just after the start codon. Plasmid pET28ad-Trx3 was transformed into the host strain *E. coli* BL21(DE3) and the transformed cells were cultivated in 500 ml LB medium at 310 K until the OD_{600} reached about 0.6; induction then took place by adding IPTG to a final concentration of 0.8 m*M* and incubating for 20 h at 291 K. Cells were collected by centrifugation at 6000*g* for 15 min and were resuspended in 40 ml cold lysis buffer (20 m*M* Tris–HCl pH 8.5, 200 m*M* NaCl, 14 m*M* β-mercaptoethanol). After three cycles of freeze–thawing, 30 s of sonication and 30 min of centrifugation at 26 000*g*, the supernatant containing the soluble target proteins was collected for purification.

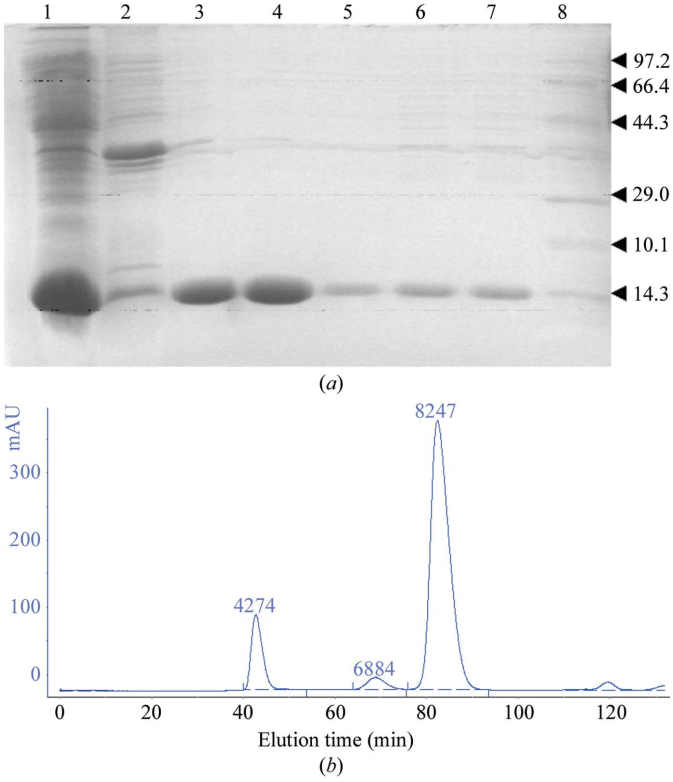


Figure 1
(*a*) 15% SDS–PAGE analysis of Trx3. Lane 1, supernatant from cell lysis; lanes 2, 3 and 4, elution from Ni–NTA affinity column at various concentrations of imidazole (10, 50 and 150 m*M*, respectively); lanes 5, 6 and 7, peaks I, II and III of Trx3 after gel filtration, respectively; lane 8, low-molecular-weight markers (kDa). (*b*) Chart of gel filtration of Trx3 using a HiLoad 16/60 Superdex 75 column. The flow rate was 1 ml min^{-1}. Three main peaks were collected: peak I (42.74 min), peak II (68.84 min) and peak III (82.47 min).

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	25.40–2.0 (2.11–2.0)
Space group	$P3_1$
Unit-cell parameters	
$a = b$ (Å)	49.57
c (Å)	94.55
$\alpha = \beta$ (°)	90.00
γ (°)	120.00
Wavelength (Å)	1.5418
Temperature (K)	100
Completeness (%)	99.7 (99.0)
$I/\sigma(I)$	9.6 (2.4)
R_{sym}†	0.074 (0.306)

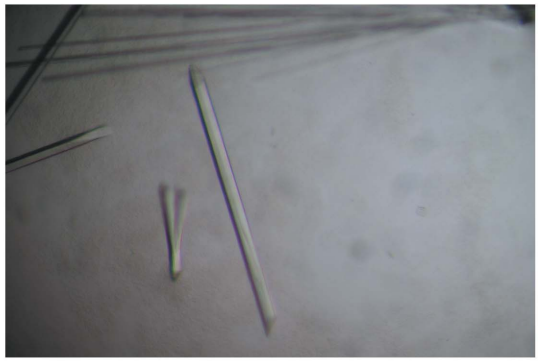
† $R_{sym} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the observed intensity of the ith measurement of reflection hkl and $\langle I(hkl)\rangle$ is the average intensity for multiple measurements.

2.2. Purification

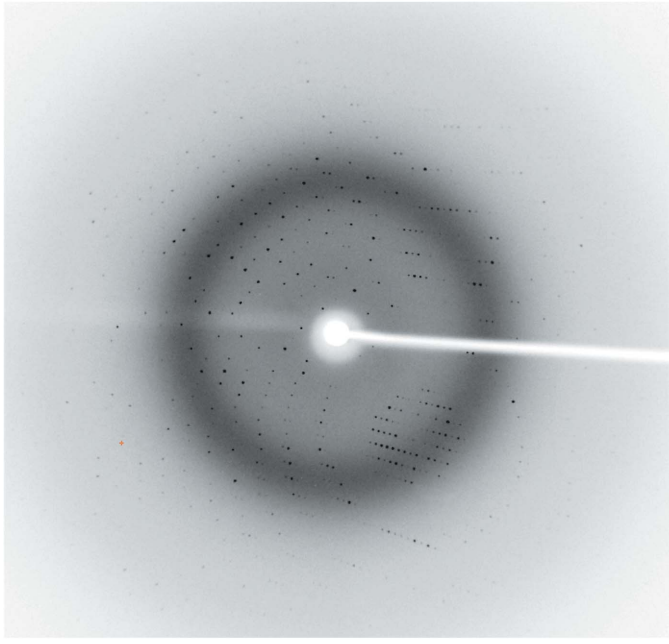
The supernatant was filtered and loaded onto a 2.5 ml Ni^{2+}-chelating Sepharose Fast Flow column (Amersham Biosciences) equilibrated with lysis buffer. The column was washed with the same buffer containing 10 m*M* imidazole and the recombinant Trx3 was then eluted with lysis buffer containing 150 m*M* imidazole. Eluted protein was further purified by gel filtration using a HiLoad 16/60 Superdex 75 column (Amersham Biosciences) equilibrated with buffer containing 20 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl and 14 m*M* β-mercaptoethanol. The purity of the protein was checked by SDS–PAGE and HPLC with a C_3 reverse-phase column (Agilent) and the molecular weight was determined by electrospray ion-trap mass spectrometry (QTrap, Applied Biosystems). To estimate the molecular weight for the peaks from the HiLoad 16/60 Superdex 75 column, the column was calibrated using MW-SDS-200 Kit molecular-weight markers (Sigma) and arrowhead proteinase inhibitor API-B (20.5 kDa) as molecular-weight standards. The purified protein was concentrated to 10 mg ml^{-1} using an Amicon Ultra 5000 MW concentrator (Millipore) and stored at 277 K for subsequent use.

2.3. Crystallization and X-ray data collection

Trx3 was crystallized using the hanging-drop vapour-diffusion method at 291 K. Initial screening was performed using Crystal Screens I and II (Hampton Research Inc.). Each drop contained 2 µl reservoir solution and 2 µl protein sample (10 mg ml^{-1} protein in buffer consisting of 100 m*M* Tris–HCl pH 7.5, 200 m*M* NaCl) and 0.5 ml of precipitant solution was added to the reservoirs. After optimization, single crystals suitable for X-ray diffraction measurement grew within a week in a condition consisting of 0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate buffer pH 4.6, 8%(*w*/*v*) PEG 4000 and 15% glycerol. Crystals were transferred to a cryoprotectant buffer consisting of the reservoir solution plus 15% glycerol and flash-frozen in liquid nitrogen. Diffraction data were collected at 100 K on an in-house R-AXIS IV^{++} image-plate detector using Cu $K\alpha$ radiation (wavelength 1.5418 Å) generated with a Rigaku rotating-anode generator (operated at 50 kV and 100 mA) and focused with a confocal mirror. The data were indexed and integrated with *MOSFLM* (Leslie, 1999) and scaled using *SCALA* (Evans, 1997) from the *CCP*4 suite (Collaborative Computational Project, Number 4, 1994). A summary of the data-collection statistics used for structure determination is given in Table 1. The crystals belong to space group $P3_1$, with unit-cell parameters $a = b = 49.57$, $c = 94.55$ Å, $\alpha = \beta = 90$, $\gamma = 120°$.



(*a*)



(*b*)

Figure 2
(*a*) Crystals of *S. cerevisiae* Trx3 obtained by the hanging-drop vapour-diffusion method. The average dimensions of these crystals were 0.3 × 0.05 × 0.06 mm. (*b*) X-ray diffraction pattern at a resolution of 2.0 Å.

3. Results and discussion

The open reading frame of TRX3/YCR083W is 381 bp in length and codes for 127 amino-acid residues. The first 21 residues are predicted to form a signal peptide that is necessary for the protein to be located at the mitochondria (Pedrajas *et al.*, 1999). We designed the PCR primers to amplify the sequence coding for the mature form of Trx3 without the signal sequence. The expressed protein encodes residues 22–127 of the open reading frame preceded by the sequence MGHHHHHHM as an affinity tag. The molecular weight of Trx3 is 12 849 Da as determined by mass spectrometry and SDS–PAGE (Fig. 1*a*), which matches the expected theoretical molecular weight of 12 848.96 Da. During gel-filtration chromatography, three peaks were observed at elution times of 82.47, 68.84 and 42.74 min. Calibration of the column with molecular-weight standards showed that these peaks correspond to molecular weights of 12.8, 26 and ≥70 kDa, respectively. This implied that Trx3 also forms dimers and multimers in solution, as reported for thioredoxins from other species (Rehse *et al.*, 2005; Smeets *et al.*, 2005; Friemann *et al.*, 2003; Weichsel *et al.*, 1996). The two additional cysteines of Trx3 may contribute to dimerization or the further formation of multimers.

Hampton Research Crystal Screens I and II gave crystals from several conditions. We chose the buffer consisting of 25% PEG 4000, 0.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.6 as the initial condition for optimization. The initial crystals were single needles, so we decreased the concentration of PEG 4000 and added glycerol in order to reduce the nucleation rate. Finally, we obtained a single crystal with dimensions of 0.3 × 0.05 × 0.06 mm that was suitable for diffraction experiment (Fig. 2*a*). The data revealed significant diffraction to 2.0 Å resolution (Fig. 2*b*). Data processing showed that the crystal belongs to space group $P3_1$ and data statistics are presented in Table 1. The Matthews coefficient suggests that there are two subunits in the asymmetric unit, which gives a V_M value of 2.62 Å^3 Da^{-1} and a solvent content of 53%. Molecular replacement was performed with *CNS* using the human thioredoxin (PDB code 1erv; 44% identity) as a search model and two solutions, one for each monomer in the asymmetric unit, were indeed found; the results also confirmed that the space group was $P3_1$. Structure refinement and analysis are in progress.

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