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Purification, crystallization and preliminary X-ray diffraction analysis of glutathionylated Trx1 C33S mutant from yeast

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Thioredoxins (Trxs) are a family of small redox-active proteins that are found in all living organisms. In *Saccharomyces cerevisiae*, two cytosolic Trxs (Trx1 and Trx2) and one mitochondrial Trx (Trx3) have previously been identified. In this work, cytosolic Trx1 containing a C33S mutant was overexpressed, purified, glutathionylated and crystallized using the hanging-drop vapour-diffusion method. A set of X-ray diffraction data was collected to 1.80 Å resolution. The crystal belonged to space group $P1$, with unit-cell parameters a = 38.53, b = 38.81, c = 41.70 Å, α = 72.91, β = 87.51, γ = 60.58°.

1. Introduction

Thioredoxin (Trx) is a small redox-active protein with a conserved catalytic active site (-Trp-Cys-Gly-Pro-Cys-Lys-) that undergoes reversible oxidation–reduction of two thiol groups. Trx was originally identified as a hydrogen donor for ribonucleotide reductase from *Escherichia coli* (Laurent *et al.*, 1964). Since then, Trx has been implicated in multiple biological functions, especially in protection against oxidative damage by reducing antioxidant proteins such as thioredoxin peroxidase (Berggren *et al.*, 2001) and glutathione peroxidase (Björnstedt *et al.*, 1994).

Trx, NADPH and thioredoxin reductase (TrxR) comprise the Trx system; this is one of the two main cellular antioxidant defence systems, with the other being the glutathione (GSH) system. The GSH system, which was first discovered as a hydrogen donor for ribonucleotide reductase in an *E. coli* mutant lacking Trx1 (Holmgren, 1976), consists of GSH, glutaredoxin (Grx), glutathione reductase (GR) and NADPH. Although these two systems were not initially believed to be coupled in cells, recent work has suggested that there may be cross-talk between them. For example, glutathionylation of Cys73 of human Trx has been identified and has been demonstrated to regulate the enzymatic activity and function of Trx (Casagrande *et al.*, 2002). Furthermore, the Trx system can substitute for GR as a reductant for oxidized GSH (GSSG) in the absence of GR in *Drosophila melanogaster* (Kanzok *et al.*, 2001). GSSG reduction by the Trx system has also been observed in other organisms such as humans, *E. coli* and *Plasmodium falciparum* (Kanzok *et al.*, 2000). Although TrxR is closely related to GR both structurally and mechanistically, it cannot reduce GSSG (Holmgren & Björnstedt, 1995; Arner *et al.*, 1999). Studies indicate that Trx acts as an electron shuttle between reduced TrxR and GSSG and the following reaction sequence summarizes the reduction of GSSG by the Trx system:

$$\mathrm{NADPH} + \mathrm{TrxS_2} + \mathrm{H^+} \rightarrow \mathrm{NADP^+} + \mathrm{Trx(SH)_2} \quad (1)$$

$$\mathrm{Trx(SH)_2} + \mathrm{GSSG} \rightarrow \mathrm{TrxS_2} + 2\mathrm{GSH} \quad (2)$$

(Kanzok *et al.*, 2000, 2001; Holmgren, 1985). Trx may catalyze GSSG reduction *via* a ping-pong mechanism. After reduced Trx binds to GSSG, the N-terminal active-site Cys30, acting as a nucleophile, attacks the GSSG disulfide to form a covalently linked mixed-disulfide transition state and release a GSH; nucleophilic attack of the C-terminal active-site Cys33 then generates oxidated Trx and the other GSH (Holmgren, 1985, 1995).

Recently, the crystal structures of three isoforms of Trx from *Saccharomyces cerevisiae* have been solved by our group (Bao *et al.*, 2006, 2007; Zhang *et al.*, 2008). Here, we report the crystallization and preliminary X-ray diffraction analysis of glutathionylated Trx1 C33S mutant (Trx1C33S-SG), which should present the transient mixed-disulfide state of Trx1 Cys30 with GSH as described above. This structure will enable us to understand the details of Trx-based GSSG-reduction mechanisms.

2. Materials and methods

2.1. Cloning and protein expression

The *TRX1*/*YLR043C* open reading frame was amplified by PCR from *S. cerevisiae* genomic DNA. *Nde*I and *Not*I restriction sites (bold) were incorporated into the sequences of the sense and anti-sense primers 5′-GGGG**CATATG**GTTACTCAAT-3′ and 5′-CCCC-**GCGGCCGC**TTAAGCATTAGC-3′, respectively. The PCR product was cloned into a pET28a-derived expression vector, which gave a protein with a hexahistidine (6×His) tag just after the start codon. The resulting construct was sequenced to ensure that no mutations had occurred during amplification. The C33S mutant was generated using the PCR-based site-directed mutagenesis method. The primers used to generate the nucleic acid mutant were 5′-TGGTGCGGT-CCAT**C**TAAAATGATTG-3′ and 5′-CAATCATTTTA**G**ATGGAC-CGCACCA-3′ (the nucleotide substitutions are shown in bold). DNA sequencing confirmed that only the appropriate mutation was incorporated into the construct. *E. coli* BL21 (DE3) strain cells were transformed with the construct and grown on LB–agar plates containing 10 µg ml^{-1} kanamycin. A single colony was verified and cultured in 600 ml LB medium containing 10 µg ml^{-1} kanamycin at 310 K until the OD_{600} reached 0.6. Target-protein expression was then induced for 20 h at 291 K by adding isopropyl β-D-1-thio-galactopyranoside (IPTG) to a final concentration of 0.2 m*M* in the medium. Cells were harvested by centrifugation at 7330*g* for 10 min and resuspended in cold lysis buffer (20 m*M* Tris–HCl pH 7.5, 100 m*M* NaCl). The suspension was sonicated and clarified by centrifugation at 29 000*g* for 25 min at 277 K.

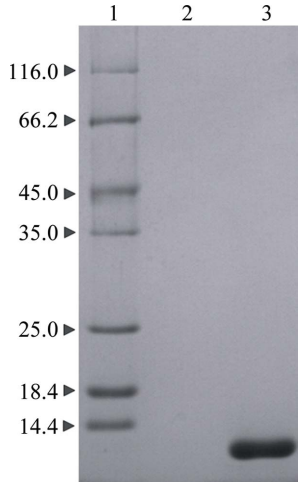


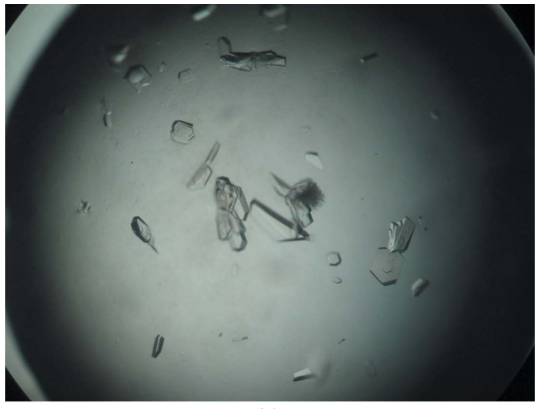
Figure 1
15% SDS–PAGE analysis of Trx1 C33S mutant. Lane 1, low-molecular-weight markers (kDa); lane 2, blank; lane 3, purified protein after gel filtration.

2.2. Protein purification and glutathionylation

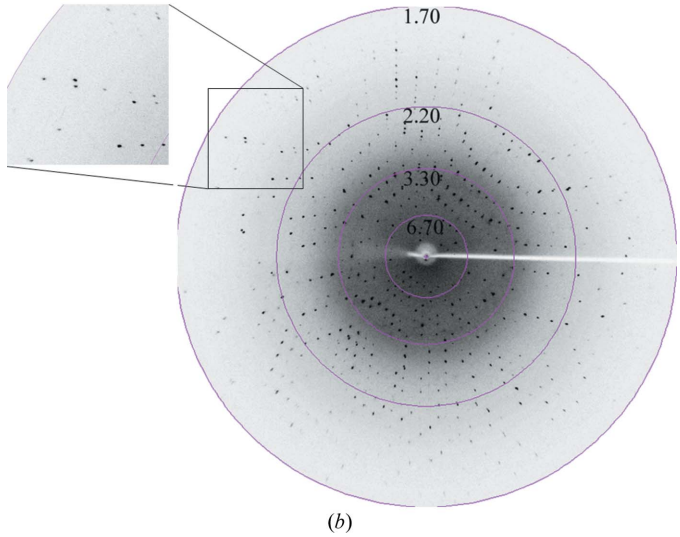
The supernatant was loaded onto 2 ml nickel–agarose affinity resin (Qiagen) pre-equilibrated with lysis buffer. After washing with ten volumes of lysis buffer plus 10 m*M* imidazole, the target protein was eluted with lysis buffer containing 250 m*M* imidazole, treated with 10 m*M* dithiothreitol (DTT) for 1 h and then further purified on a HiLoad 16/60 Superdex 75 column (Amersham Biosciences) equilibrated with lysis buffer. Purified reduced Trx1 C33S mutant protein (at a concentration of about 0.1 m*M*) was immediately mixed with GSSG (at a final concentration of 10 m*M*) for incubation at 277 K overnight. The excess GSSG and the GSH generated were also removed using a HiLoad 16/60 Superdex 75 column (Amersham Biosciences) equilibrated with lysis buffer. The fractions containing the target protein were verified using SDS–PAGE.

2.3. Crystallization and X-ray data collection

The target protein was concentrated to 10 mg ml^{-1} by ultrafiltration (Millipore Amicon). Crystallization conditions were screened using the hanging-drop vapour-diffusion method at 291 K using Crystal Screens I and II (Hampton Research). Each drop consisted of 1 µl protein solution and 2 µl reservoir solution and was equilibrated against 500 µl reservoir solution. After optimization, crystals suitable for X-ray diffraction grew within one week in a condition consisting of 2.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.4. Crystals were flash-frozen in liquid nitrogen using a cryoprotectant consisting



(*a*)



(*b*)

Figure 2
(*a*) Crystals of Trx1C33S-SG. (*b*) X-ray diffraction pattern of the crystal.

Table 1
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Nominal resolution range (Å)	33.81–1.80 (1.90–1.80)
Space group	*P*1
Temperature (K)	100
Wavelength (Å)	1.54180
Unit-cell parameters (Å, °)	a = 38.53, b = 38.81, c = 41.70, α = 72.91, β = 87.51, γ = 60.58
Unique reflections	17174 (2416)
Completeness (%)	92.73 (89.30)
Redundancy	3.9 (3.8)
Mean $I/\sigma(I)$	31.9 (22.5)
$R_{\rm merge}$†	0.033 (0.057)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $\langle I(hkl)\rangle$ is the mean intensity of the set of equivalent reflections defined by space group *P*1 for the reflections denoted $I_i(hkl)$.

of the reservoir solution plus 25%(*v*/*v*) glycerol. A complete diffraction data set consisting of 360 images was collected with an oscillation angle of 1° per image at 100 K using an in-house Rigaku MM007 X-ray generator (λ = 1.54180 Å) with a MAR Research 345 detector at the School of Life Sciences, University of Science and Technology of China (USTC, Hefei, People's Republic of China).

3. Result and discussion

The Trx1 C33S mutant was expressed in *E. coli* BL21 (DE3) in a soluble form; it was purified to homogeneity and the purity of the protein was checked using SDS–PAGE (Fig. 1). After crystallization trials and optimization, diffraction-quality crystals grew within one week using the hanging-drop vapour-diffusion method and a reservoir consisting of 2.2 *M* ammonium sulfate, 0.1 *M* sodium acetate pH 4.4. The crystallization conditions and shape of the Trx1C33S-SG crystals were found to differ greatly from those of native Trx1 (Zhang *et al.*, 2008; Fig. 2*a*). A set of diffraction data was collected to 1.80 Å resolution (Fig. 2*b*). The data were processed using the program *iMOSFLM* (Leslie, 1994). The crystal belonged to space group *P*1, with unit-cell parameters a = 38.53, b = 38.81, c = 41.70 Å, α = 72.91, β = 87.51, γ = 60.58°. The data-collection statistics are listed in Table 1. The Matthews calculation assumed that there were two subunits in the asymmetric unit of the crystal, with a $V_{\rm M}$ value of 2.15 Å^3 Da^{-1} and a solvent content of 42.75% (Matthews, 1968). Phase determination using the molecular-replacement method is ongoing and structure refinement and functional interpretation are in progress. The mixed disulfide with GSH has already been observed.

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