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Purification, crystallization and preliminary crystallographic data for rat cytosolic selenocysteine 498 to cysteine mutant thioredoxin reductase

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Mammalian cytosolic thioredoxin reductase is a homodimer of 55 kDa subunit containing an essential penultimate selenocysteine residue. An active analogue of the rat enzyme in which cysteine replaces selenocysteine has been expressed in *Escherichia coli* cells at high levels and purified to homogeneity. The pure enzyme contains one FAD per subunit and shows spectral properties identical to that of the wild-type thioredoxin reductase. The isolated enzyme in its oxidized and reduced forms or the enzyme complexed with $NADP^+$ was crystallized by the hanging-drop vapour-diffusion method. The diffraction pattern extends to 3 Å resolution. The crystals are monoclinic, space group $P2_1$, with unit-cell parameters a = 78.9, b = 140.5, c = 170.8 Å, α = 94.6°. There are three dimeric molecules in the asymmetric unit.

1. Introduction

Thioredoxin reductase (TrxR) belongs to the pyridine nucleotide–disulfide oxidoreductase family (Williams, 1992) and is present in all prokaryotes and eukaryotes. Mammalian TrxR, which catalyzes reduction of the active-site disulfide in thioredoxin by NADPH, has different characteristics from the enzyme of bacteria, yeast or plants, exhibiting a broader substrate specificity, reducing thioredoxins from different species and also selenite, vitamin K, lipoic acid, alloxan or 5,5′-dithio(bis 2-nitrobenzoic acid) (DTNB) as well as hydroperoxides but not GSSG (Holmgren, 1989; Arnér *et al.*, 1999). The enzyme itself is one of the target molecules of the cytostatic drugs of the nitrosourea type (Gromer *et al.*, 1997) and is irreversibly inhibited by the immunostimulatory agent 1-chloro-2,4-dinitrobenzene (DNCB; Arnér *et al.*, 1995; Nordberg *et al.*, 1998). Mammalian cytosolic TrxR has a subunit size of ∼55 kDa, in contrast to 35 kDa for *E. coli* TrxR. The enzyme is homologous to glutathione reductase but is elongated by 16 residues and has an essential selenocysteine (SeCys) residue within the conserved C-terminal -Gly-Cys-SeCys-Gly sequence motif (Tamura & Stadtman, 1996; Zhong *et al.*, 1998; Zhong & Holmgren, 2000). The redox-active disulfide is identical to that of glutathione reductase (Zhong *et al.*, 1998). In *E. coli* TrxR, the redox-active disulfide is instead within the sequence -Cys-Ala-Thr-Cys- and is located in the NADPH domain rather than in the FAD domain (Kuriyan *et al.*, 1991). Recently, a mitochondrial TrxR has been identified (Rigobello *et al.*, 1998; Lee *et al.*, 1999; Watable *et al.*, 1999) showing the same glutathione reductase-like active-site sequence and also a selenocysteine residue located in an identical sequence motif to that of cytosolic TrxR.

Since sulfur and selenium are similar in their chemical properties but differ in ionic radius, electronegativity and covalent radius, substitution of selenocysteine by cysteine will therefore result in altered biological activities. The active sulfur analogue of rat TrxR (SeCys498Cys TrxR) has been successfully expressed in *E. coli* cells in amounts sufficient for detailed kinetic studies and further characterization (Zhong & Holmgren, 2000). In this paper, we report a simple procedure for large-scale purification, determination of crystallization conditions and initial X-ray characterization of these crystals. Analysis of the three-dimensional structure will be required in order to understand the catalytic mechanisms of mammalian TrxR, with its unique requirement for selenium.

2. Materials and methods

2.1. Purification of rat SeCys498Cys thioredoxin reductase

Construction of the SeCys498Cys thioredoxin reductase has been described elsewhere (Zhong & Holmgren, 2000). The mutant protein was overexpressed in *E. coli* BL21(DE3)pLysS cells. The cells harboring the recombinant TrxR plasmid (pET-3d TrxRU498C) were grown at 310 K in LB medium containing 50 μg ml^{-1} carbenicillin, 34 μg ml^{-1} chloramphenicol and 5 m*M* EDTA. Gene expression was induced with 0.5 m*M* IPTG for 4 h at $A_{600nm} \simeq 0.6$. Cells were

Table 1
Purification of recombinant rat SeCys498Cys TrxR from *E. coli* BL21/pET-3d TrxRU498C cells (1 l culture).

Step	Volume (ml)	Protein (mg)	Activity (A_{412} min^{-1} ml^{-1})	Specific activity (A_{412} min^{-1} mg^{-1})	Yield (%)
Crude extracts	100	1090	41	3.8	100
Pool from 2′,5′-ADP Sepharose	10	25	176	70	43

harvested by centrifugation at 6000 rev min^{-1} for 10 min at 277 K and the cell pellets were stored at 193 K until use. After two rounds of freezing and thawing, the cells were resuspended in cold 50 m*M* potassium phosphate pH 7.5, 2 m*M* EDTA, 1 m*M* PMSF and 0.1% Triton X-100 and the suspension was then incubated at 303 K for 15 min prior to sonication. Following centrifugation for 30 min at 12000 rev min^{-1} and 277 K, the supernatant was applied to a DEAE-Sephacel column (2.5 × 15 cm). (This step is only required for the supernatant produced from more than 1 l of cell cultures. Smaller volumes could be loaded directly onto a 2′,5′-ADP Sepharose column.) Bound protein was eluted with a linear gradient of 0–0.5 *M* NaCl in 50 m*M* potassium phosphate, 1 m*M* EDTA pH 7.5 (1000 ml each). Fractions containing enzyme activity with DTNB were pooled and dialyzed against 50 m*M* potassium phosphate, 1 m*M* EDTA pH 7.5. The dialysate was loaded onto a 2′,5′-ADP Sepharose 4B column (1.5 × 7 cm) equilibrated with the same buffer. The enzyme was eluted with a linear gradient of 50–300 m*M* potassium phosphate pH 7.5, 1 m*M* EDTA (400 ml each). The fractions containing DTNB reductase activity were pooled and concentrated in an Ultrafiltration system (Diaflo) using a YM30 membrane, then washed three times with 50 m*M* potassium phosphate buffer, 1 m*M* EDTA pH 7.5 and 20% glycerol to completely remove salt. The purified protein was stored in liquid nitrogen or at 193 K. It exhibits a single band on SDS–PAGE stained with Coomassie brilliant blue.

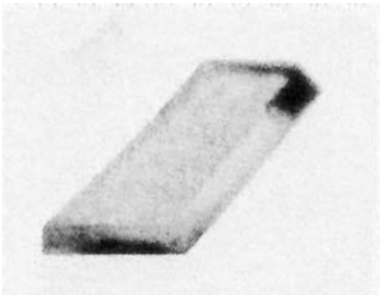

Figure 1
Crystal of recombinant rat SeCys498Cys TrxR. The crystals were grown using the hanging-drop method and were obtained from enzyme–NADP$^+$ mixtures at pH 4.2.

2.2. Protein determination

Two methods were used. At a high concentration, the enzyme was quantified by measuring absorption at 463 nm using an extinction coefficient of 11.3 mM^{-1} cm^{-1} for the FAD of each subunit. In diluted enzyme samples, the concentration was determined by measuring absorption at 280 nm using the value of 1.625 for a solution containing 1 mg ml^{-1} rat SeCys498Cys TrxR.

2.3. TrxR activity assay

A DTNB reduction assay (Luthman & Holmgren, 1982) was used during preparation of the enzyme. A thioredoxin-coupled insulin reduction assay was used to measure the activity of the pure enzyme, as described elsewhere (Zhong & Holmgren, 2000).

2.4. Crystallization

The frozen enzyme was thawed in an icebath, then washed three times with 50 m*M* potassium phosphate, 1 m*M* EDTA pH 7.5 in Ultrafree MC Centrifugal Filter Units (cutoff 30 kDa) to remove glycerol and concentrate the enzyme.

To determine preliminary crystallization conditions, the standard sparse-matrix crystal screening solutions (Jancarik & Kim, 1991) and the hanging-drop vapour-diffusion technique (Gilliland & Davies, 1984) were used. The drops were prepared by mixing 2–4 µl of the enzyme solution (60 mg ml^{-1}) with the corresponding reservoir solution. Optimization of the crystallization conditions was made by adjusting the pH and protein concentration and using additives.

2.5. Crystallographic data collection

X-ray data were collected at a temperature of 100 K and a wavelength of 1.104 Å at the synchrotron beamline BL711, MAX Laboratory, Lund University, Sweden. Prior to data collection, crystals were transferred to mother liquor containing 20% ethylene glycol as cryoprotectant and then mounted in the cryo-gas stream. The data set was collected using a MAR345 image plate with a crystal-to-detector distance of 370 mm and an oscillation angle of 1°. 180° of data were collected, processed and scaled using the *HKL* suite of programs (Otwinowski, 1993). The space group was determined using the autoindexing routine in *DENZO* and by the analysis of pseudo-precession images generated with the program *PATTERN* (Lu, 1999).

3. Results and discussion

A summary of the purification of rat SeCys498Cys TrxR from *E. coli* cells is shown in Table 1. The enzyme was purified 18-fold in one step to homogeneity as indicated by SDS–PAGE, with an overall yield of 43%. For purifying the enzyme from more than 1 l of cell cultures, it is convenient to separate TrxR from large amounts of positively charged proteins in a DEAE step before using the 2′,5′-ADP Sepharose column. The TrxR purified partially through the DEAE step binds to the 2′,5′-ADP Sepharose more efficiently than the enzyme in crude extracts, which may result from the presence of other molecules preventing binding of the enzyme to the immobilized pyridine nucleotide analogue. The peak thioredoxin reductase activity appeared between 0.18 and 0.28 *M* NaCl in the DEAE-Sephacel chromatography and eluted between 0.06 and 0.14 *M* phosphate in the 2′,5′-ADP Sepharose chromatography. The two methods gave a similar yield. The purified cysteine-containing and wild-type selenocysteine-containing TrxRs were indistinguishable on Coomassie blue-

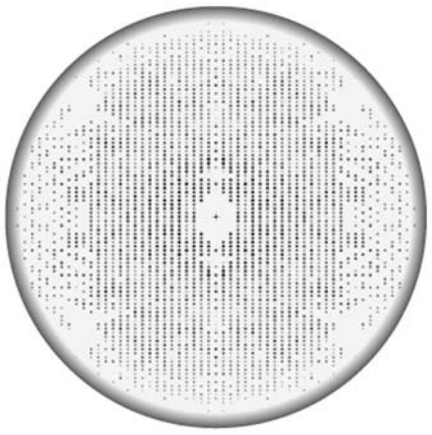

Figure 2
A pseudo-precession picture of the $l = 0$ section generated with the program *PATTERN* (Lu, 1999) using data to 3.00 Å resolution. The crystal belongs to space group $P2_1$. Systematic absences along the k axis, vertical in the figure, verify the presence of a screw axis in the crystal.

Table 2
Data-collection statistics.

Values in parentheses are for the highest resolution shell.

Resolution (Å)	30.0–3.0 (3.05–3.00)
Mosaicity	0.77
No. of observations with $I > \sigma(I)$	212337
No. of unique reflections	69351
$\langle I/\sigma(I)\rangle$	11.0 (7.0)
Completeness (%)	92.7 (77.6)
R_{sym} (%)	9.5 (44.8)

stained SDS–PAGE or by immunoblot analysis using rat TrxR polyclonal antibodies (Zhong & Holmgren, 2000). In the standard thioredoxin-coupled assay for thioredoxin reductase activity, the pure mutant enzyme showed 1% of the wild-type TrxR activity, with a major decrease in k_{cat} (Zhong & Holmgren, 2000).

The mutated enzyme contains FAD as a prosthetic group. Its flavin absorbance spectrum in 0.1 *M* potassium phosphate pH 7.5, 1 m*M* EDTA closely resembles that of rat TrxR (Luthman & Holmgren, 1982). The ratio between A_{276nm} and A_{463nm} for both wild-type and mutant rat TrxR is around 8, demonstrating that replacement of selenocysteine with cysteine does not change the UV–visible spectrum.

Yellow crystals appeared with a reservoir solution containing 20% PEG 8000 in 50 m*M* potassium phosphate pH 4.2–5.0 (well volume 0.7 ml). The crystals appeared after 1–3 d at room temperature at around 60 mg ml^{-1} enzyme concentration and grew as stars. Crystallization experiments were also carried out at 277 K, but were not successful. In 50 m*M* potassium phosphate and 16–20% PEG 8000 crystals occurred between pH 4.2 and 9.2. Large crystals of the oxidized enzyme grew easily at weak acidic pH, whereas large crystals of the 2-mercaptoethanol-reduced enzyme preferred neutral pH. However, these crystals did not diffract well and had a tendency towards twinning. The best diffracting crystals (Fig. 1) appeared in drops consisting of 2 µl of the enzyme solution (25–30 mg ml^{-1}), 1 µl of reservoir solution (50 m*M* potassium phosphate pH 4.2–5 and 20% PEG 8000) and 1 µl of 10 m*M* NADP$^+$.

A complete data set to this resolution was collected from such a crystal and the statistics are given in Table 2. The Laue symmetry indicated that the crystals belong to the monoclinic space group $P2$ or $P2_1$, with unit-cell dimensions a = 78.9, b = 140.5, c = 170.8 Å, β = 94.6°. A pseudo-precession image generated with the program *PATTERN* (Lu, 1999) for the h = 0 layer is shown in Fig. 2. Systematic absences for $k = 2n + 1$ along 0k0 suggest that the space group is $P2_1$.

The enzyme is a homodimer with a subunit of 499 amino acids (molecular mass 54.452 kDa). In order to estimate the number of molecules in the asymmetric unit, the Matthews coefficient (Matthews, 1968) V_m was calculated. With the assumption of three or four dimers in the asymmetric unit, V_m values of 2.89 and 2.17 Å^3 Da^{-1}, respectively, were obtained. Both are within the range typical for protein crystals. A search for suitable heavy-atom derivatives and for well shaped crystals, as well as attempts to solve the structure using the known structure of glutathione reductase, are in progress.

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References

Arnér, E. S. J., Björnstedt, M. & Holmgren, A. (1995). *J. Biol. Chem.* **270**, 3479–3482.
Arnér, E. S. J., Zhong, L. & Holmgren, A. (1999). *Methods Enzymol.* **300**, 226–239.
Gilliland, G. L. & Davies, D. R. (1984). *Methods Enzymol.* **104**, 370–381.
Gromer, S., Schirmer, H. & Becker, K. (1997). *FEBS Lett.* **412**, 318–320.
Holmgren, A. (1989). *J. Biol. Chem.* **264**, 13963–13966.
Jancarik, J. & Kim, S.-H. (1991). *J. Appl. Cryst.* **24**, 409–411.
Kuriyan, J., Krishna, T. S. R., Wong, L., Guenther, B., Pahler, A., Williams, C. H. Jr & Model, P. (1991). *Nature (London)*, **352,** 172–174.
Lee, S. R., Kim, J. R., Kwon, K. S., Yoon, H. W., Levine, R. L., Ginsburg, A. & Rhee, S. G. (1999). *J. Biol. Chem.* **274**, 4722–4734.
Lu, G. (1999). *J. Appl. Cryst.* **32**, 375–376.
Luthman, M. & Holmgren, A. (1982). *Biochemistry*, **21**, 6628–6633.
Matthews, B. W. (1968). *J. Mol. Biol.* **33**, 491–497.
Nordberg, J., Zhong, L., Holmgren, A. & Arnér, E. S. J. (1998). *J. Biol. Chem.* **273**, 10835–10842.
Otwinowski, Z., (1993). *Proceedings of the CCP4 Study Weekend. Data Collection and Processing*, edited by L. Sawyer, N. Isaacs & S. Bailey, pp. 56–62. Warrington: Daresbury Laboratory.
Rigobello, M. P., Callegaro, M. T., Barzon, E., Benetti, M. & Bindoli, A. (1998). *Free Radic. Biol. Med.* 24, 370–376.
Tamura, T. & Stadtman, T. C. (1996). *Proc. Natl Acad. Sci. USA*, **93**, 1006–1011.
Watable, S., Makino, Y., Ogawa, K., Hiroi, T., Yamamoto, Y. & Takahashi, S. Y. (1999). *Eur. J. Biochem.* **264**, 74–84.
Williams, C. H. Jr (1992). *Chemistry and Biochemistry of Flavoenzymes*, Vol. III, edited by F. Müller, pp. 121–211. Boca Raton, FL: CRC Press.
Zhong, L., Arnér, E. S. J., Ljung, J., Åslund, F. & Holmgren, A. (1998). *J. Biol. Chem.* **273**, 8581–8591.
Zhong, L. & Holmgren, A. (2000). *J. Biol. Chem.* **275**, 18121–18128.