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Crystallization and preliminary X-ray crystallographic analysis of *Sulfolobus solfataricus* thioredoxin reductase

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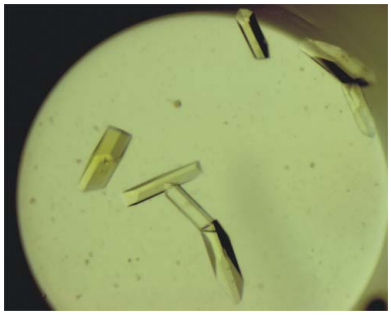

A thermostable thioredoxin reductase isolated from *Sulfolobus solfataricus* (*Ss*TrxR) has been successfully crystallized in the absence and in the presence of NADP. Two different crystal forms have been obtained. Crystals of the form that yields higher resolution data (1.8 Å) belong to space group $P2_12_12_1$, with unit-cell parameters $a = 76.77$, $b = 120.68$, $c = 126.85$ Å. The structure of the enzyme has been solved by MAD methods using the anomalous signal from the Se atoms of selenomethionine-labelled *Ss*TrxR.

1. Introduction

All living organisms have developed efficient systems for scavenging reactive oxygen species produced by metabolism. The thioredoxin system, composed of thioredoxin (Trx), thioredoxin reductase (TrxR) and NADPH, plays a pivotal role in maintaining the redox state of the cell and in protecting the cell against oxidative stress (Arner & Holmgren, 2000; Hirt *et al.*, 2002). Trx is the major ubiquitous disulfide reductase responsible for keeping proteins in their reduced state. Trx is a substrate of TrxR, which provides the electrons necessary to regenerate the reduced state of Trx. These electrons originate from NADPH and are transferred *via* FAD to the disulfide of the active site of TrxR, which subsequently reduces its protein substrate.

TrxRs are homodimeric proteins in which each monomer contains an FAD prosthetic group. Although TrxRs have been isolated from organisms belonging to all three domains of life, archaea, eubacteria and eukarya, differences between TrxRs isolated from different species have emerged (Arner & Holmgren, 2000; Hirt *et al.*, 2002). TrxRs are currently classified into two distinct classes depending on their molecular mass. TrxRs isolated from higher eukaryotes (class i) exhibit a molecular mass of approximately 55 000 Da per subunit. TrxRs extracted from archaea, eubacteria and lower eukaryotes, which include several plants and fungi, are smaller, with a molecular weight of about 35 000 Da per subunit (class II).

Of the class II TrxRs, enzymes isolated from eubacteria and lower eukaryotes have been extensively characterized. Functional and structural investigations have been carried out in depth on *Escherichia coli* TrxR. Indeed, analysis of the structure–function relationships in this enzyme has been made possible by the determination of the structure of the wild-type protein in both the reduced (Lennon *et al.*, 1999) and the oxidized state (Kuriyan *et al.*, 1991; Waksman *et al.*, 1994) and of the complex between TrxR and Trx (Lennon *et al.*, 2000). The latter structure has demonstrated that this protein is endowed with an unusual conformational plasticity that favours a large twist of the enzyme structure during catalysis. Preliminary structural investigations have also been reported for eubacterial *Mycobacterium tubercolosis* TrxR (Akif *et al.*, 2004). The structure of a class II TrxR isolated form the eukaryotic organism *Arabidopsis thaliana* has also been reported (Dai *et al.*, 1996).

On the other hand, limited information is available on class II TrxRs isolated from archaea. The functional characterization of TrxR from *Aeropyrum pernix* (Jeon & Ishikawa, 2002) and *Pyrococcus horikoshii* (Kashima & Ishikawa, 2003) have only recently been reported. Even more recently, the thioredoxin reductase activity of an

enzyme, previously classified as an NAD(P)H oxidase (Masullo *et al.*, 1996), isolated from the hyperthermophilic archaeon *Sulfolobus solfataricus* has been described (Ruocco *et al.*, 2004). Although this thermostable TrxR (*Ss*TrxR), the molecular mass of which is 35 000 Da per subunit, shows significant sequence identity (30–35%) to eubacterial TrxRs, some of its functional properties resemble those of class I eukaryal enzymes (Ruocco *et al.*, 2004). These findings have been correlated (Ruocco *et al.*, 2004) with a previously reported hypothesis that the *Sulfolobus* gene is the putative ancestor of animal mitochondria (Karlin & Campbell, 1994). In order to elucidate the structure–function relationship of this enzyme, a crystallographic investigation has been undertaken. Here, we report the crystallization of wild-type *Ss*TrxR and of its complex with NADP.

2. Experimental methods

2.1. Purification and crystallization

Recombinant *Ss*TrxR was expressed in *E. coli* as previously reported (Ruocco *et al.*, 2004). *E. coli* cells were mechanically disrupted and the cell debris was removed by centrifugation. After heating the supernatant at 343 K, the protein was purified using cation-exchange cromatography (FPLC). The purity and homogeneity of the protein were tested by SDS–PAGE and its redox state was checked by UV–visible spectroscopy. The protein was concentrated to 20 mg ml^{-1} using a Centricon-30 concentrator and stored in a buffer containing 25 m*M* Mes–KOH pH 5.5.

The selenomethionine (SeMet) derivative of *Ss*TrxR was prepared by growing the *E. coli* strain expressing the recombinant enzyme in minimal media containing 1 mg l^{-1} vitamins (riboflavin, niacinamide, pyridoxine monohydrochloride and thiamine), 0.4% glucose, 2 m*M* $MgSO_4$, 0.1 m*M* $CaCl_2$, 50 mg l^{-1} of the amino acids Phe, Thr, Lys, Ile, Leu and Val and 60 mg l^{-1} of seleno-L-methionine.

Crystallization experiments were performed at 293 K using either microbatch-under-oil or hanging-drop vapour-diffusion methods. Preliminary crystallization trials were carried out using commercially available sparse-matrix screens (Crystal Screen kits I and II, Hampton Research).

In order to determine the structure of *Ss*TrxR complexed with NADP, crystals of the enzyme were also grown adding the cofactor to the crystallization conditions. An NADP:protein molar ratio of 100:1 was employed.

Finally, the conditions used for the crystallization of form I were also successfully used to grow crystals of the SeMet derivative. To prevent oxidation of the SeMet derivative, 5 m*M* DTT was added to the crystallization medium.

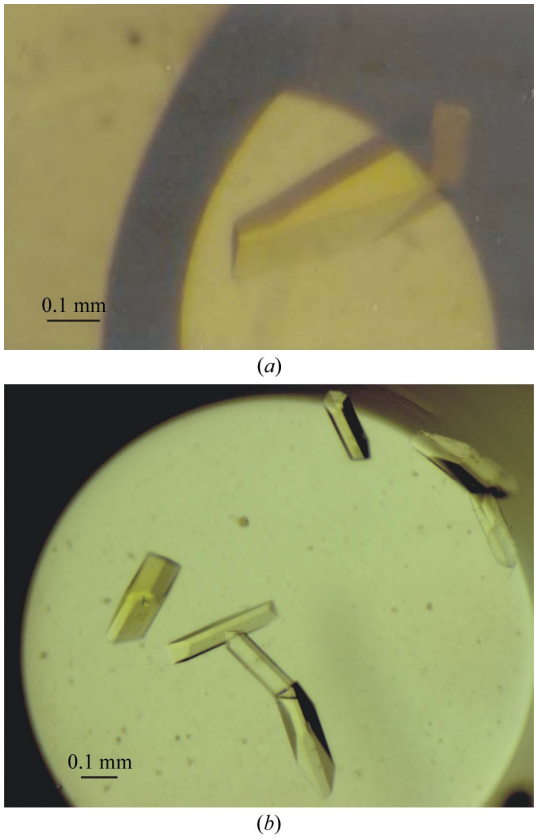

Figure 1
Images of typical *Ss*TrxR crystals: (*a*) crystal form I, (*b*) crystal form II.

2.2. Data collection and processing

Preliminary diffraction data were collected in-house at 298 K using a MacScience DIP2030b imaging plate equipped with a Nonius FR591 generator producing Cu $K\alpha$ radiation of wavelength 1.5418 Å. Higher resolution data were collected at the ESRF (Grenoble, France) at 100 K (beamline ID29). Crystals were frozen after the addition of 22%(*v*/*v*) glycerol to the crystallization buffer. Data processing was performed using the program *DENZO* (Otwinowski & Minor, 1997). The data sets were scaled and merged using the program *SCALEPACK* (Otwinowski & Minor, 1997).

Multiwavelength anomalous diffraction (MAD) data were collected at the synchrotron beamline ID14-4 at the ESRF. Three different data sets were collected from a single crystal using wavelengths determined from the selenium absorption spectrum.

2.3. Structure determination

The structure of the enzyme was solved by MAD methods using the anomalous signal from the Se atoms of selenomethionine-labelled *Ss*TrxR. The program *SOLVE* (Terwilliger & Berendzen, 1999) was used to localize the selenium sites present in the asymmetric unit and to derive the experimental phases. Phases were improved by density modification using the program *DM* (Cowtan & Main, 1998). Automatic model building was performed using *ARP*/*wARP* in warpNtrace mode (Perrakis *et al.*, 1999).

3. Results and discussion

An initial screening carried out using commercially available crystallization kits revealed different conditions for crystal formation of *Ss*TrxR. The quality of the crystals was improved by fine-tuning the concentration of the protein and of the precipitants. Large yellow *Ss*TrxR crystals (form I; Fig. 1*a*) were grown using 12–15%(*w*/*v*) polyethylene glycol 2000 monomethyl ether (PEG MME 2K), 0.1 *M* $(NH_4)_2SO_4$ and 50 m*M* sodium acetate pH 4.6. A different crystal form (form II; Fig. 1*b*) of *Ss*TrxR was obtained in 8–12%(*w*/*v*) PEG 4000, 3–4%(*v*/*v*) propan-2-ol and 50 m*M* Na HEPES pH 7.5 (Fig. 1*b*). In both cases a protein concentration of 4–9 mg ml^{-1} was used.

Matthews coefficient calculations (Matthews, 1968) suggested the presence of two *Ss*TrxR dimers per asymmetric unit in crystal form I (V_M = 2.1 Å^3 Da^{-1}, with 41% solvent content). A single *Ss*TrxR dimer is present in the asymmetric unit of crystal form II (V_M = 2.7 Å^3 Da^{-1}, with 55% solvent content).

The two crystal forms of *Ss*TrxR exhibit large differences in mosaicity and diffraction limit. Indeed, crystal forms I and II have

Table 1
Data-collection statistics.

Values in parentheses refer to the highest resolution shell.

	Wild type			SeMet derivative		
	Form I	Form II	Complex with NADP	Peak	Edge	Remote
Beamline	ESRF ID29	ESRF ID29	ESRF ID29	ESRF ID14-4		
Space group	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$	$P2_12_12_1$		
Unit-cell parameters (Å)						
a	76.77	68.12	76.77	76.66		
b	120.68	75.78	120.68	121.82		
c	126.85	149.37	126.85	127.82		
Resolution (Å)	20–1.8 (1.86–1.80)	20–2.5 (2.59–2.5)	20–1.95 (2.02–1.95)	30–1.75 (1.81–1.75)		
Wavelength (Å)	0.9756	0.9756	0.9756	0.97932	0.97956	0.93931
Mean redundancy	5.9	5.6	2.5	3.0	2.3	2.3
Completeness (%)	99.8 (98.4)	99.3 (97.6)	89.9 (73.5)	99.6 (98.9)	97.1 (93.8)	95.3 (94.5)
Unique reflections	108800	27327	80365	232089	226693	221362
R_{merge}†(%)	5.2 (31.2)	7.6 (23.7)	5.3 (33.9)	7.0 (30.8)	6.0 (21.7)	5.3 (22.4)
Mean $I/\sigma(I)$	22.3 (5.35)	21.3 (6.02)	12.7 (2.8)	22.1 (3.8)	18.8 (3.8)	18.9 (3.7)

† $R_{merge} = \sum_h \sum_i |I(h,i) - \langle I(h)\rangle| / \sum_h \sum_I I(h,i)$, where $I(h,i)$ is the intensity of the ith measurement of reflection h and $\langle I(h)\rangle$ is the mean value of the intensity of reflection h. For the SeMet derivative, R_{merge} was calculated considering the Bijovet pairs individually.

mosaicities of 0.31 and 0.68°, respectively. Using the ESRF synchrotron radiation, crystal forms I and II diffract to 1.8 Å (Fig. 2) and 2.5 Å, respectively (see Table 1 for details).

The better quality of crystal form I prompted us to use these conditions to crystallize *Ss*TrxR in the presence of the NADP cofactor. Despite the use of the same crystallization conditions, the unit-cell parameters of these crystals were slightly different from those determined for the wild-type enzyme (Table 1). This is an indirect indication that NADP is likely to be bound to the enzyme.

Several attempts were made to solve the structure by molecular replacement using the diffraction data derived from crystal form I. In particular, trials were conducted using the structures of *E. coli* (PDB code 1tdf; Waksman *et al.*, 1994) and *A. thaliana* (PDB code 1vdc; Dai *et al.*, 1996) TrxR as starting models. However, all these attempts were unsuccessful. The rather low sequence identity between the search models and *Ss*TrxR (33–35%) and the presence of four monomers of the enzyme in the asymmetric unit of the crystal are likely to be the main factors that led to the failure.

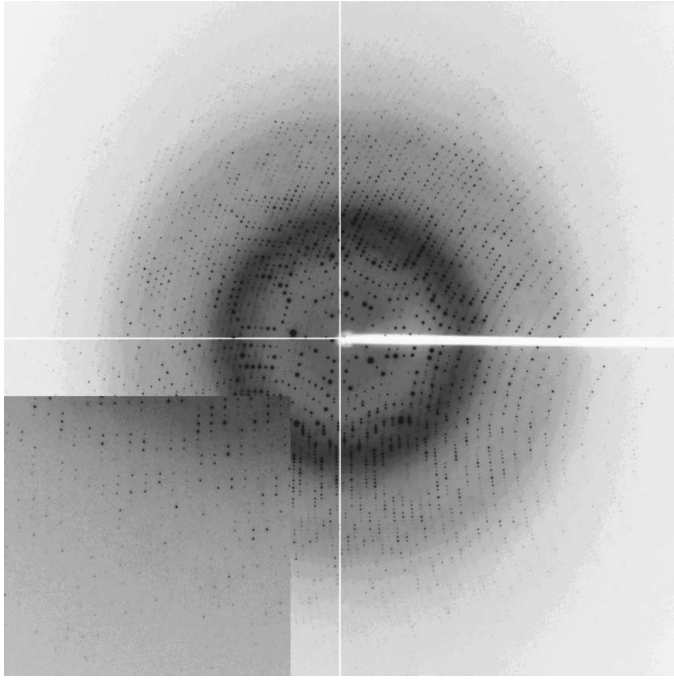

Figure 2
Diffraction pattern of a typical *Ss*TrxR crystal (form I). Diffraction data are detectable to 1.8 Å resolution.

Since molecular replacement proved to be unsuccessful, a three-wavelength MAD experiment was performed to obtain experimental phases. The feasibility of this approach was assured by the presence of three methionines in the 324 residues that constitute each *Ss*TrxR monomer. In order to determine the peak and the inflection wavelengths, a fluorescence scan was recorded on a single SeMet-labelled *Ss*TrxR crystal. Using data sets collected at wavelengths optimized for selenomethionine, the program *SOLVE* identified all 12 selenium sites expected in the asymmetric unit of the enzyme. The arrangement of these sites confirmed the presence of two *Ss*TrxR dimers in the asymmetric unit. The program *SOLVE* (Terwilliger & Berendzen, 1999) also provided a set of initial phases. Subsequent density modification using the program *DM* (Cowtan & Main, 1998) produced experimental electron density of excellent quality. Indeed, the program *ARP/wARP* (Perrakis *et al.*, 1999) was able to automatically trace most of the enzyme structure. The refinement of the crystallographic model is in progress.

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