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Crystallization and preliminary X-ray crystallographic analysis of two dimeric hyperthermostable thioredoxins isolated from *Sulfolobus solfataricus*

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The thioredoxin system of the archaeon *Sulfolobus solfataricus* involves a number of different proteins: two thioredoxin reductases (*Ss*TrxRB2 and *Ss*TrxRB3), two distinct thioredoxins (*Ss*TrxA1 and *Ss*TrxA2) and a disulfide oxidoreductase (*Ss*PDO). Here, the crystallization and preliminary crystallographic analyses of *Ss*TrxA1 and *Ss*TrxA2, two dimeric proteins endowed with extraordinary thermal stability, are reported. In addition to the functional thioredoxin domain, both *Ss*TrxA1 and *Ss*TrxA2 present an extra N-terminal fragment of approximately 30 residues. Although crystallization trials have been conducted on both forms of the proteins, crystals that were suitable for X-ray crystallographic analyses have only been obtained for their truncated variants. The crystals of *Ss*TrxA2 belonged to space group $P2$, with unit-cell parameters $a = 28.27$, $b = 27.88$, $c = 62.06$ Å, $\beta = 92.34^\circ$, and diffracted to 1.83 Å resolution, whereas the crystals of *Ss*TrxA1 belonged to space group $P2_1$, with unit-cell parameters $a = 51.76$, $b = 75.09$, $c = 55.35$ Å, $\beta = 112.64^\circ$, and diffracted to 1.90 Å resolution. The structures of the two proteins have been solved by molecular replacement.

1. Introduction

The disulfide bridge is a common structural element that provides covalent links between cysteine residues in a large number of proteins (Bulaj, 2005; De Simone *et al.*, 2006; Petersen *et al.*, 1999; Thornton, 1981). Depending on specific functional or structural requirements, proteins in their native state may contain permanent or transient disulfide bridges. Although proteins containing disulfide bridges have traditionally been associated with extracytoplasmic environments, recent investigations have shown that a significant fraction of proteins from hyperthermophilic organisms exhibit disulfide bridges (Ladenstein & Ren, 2006; Mallick *et al.*, 2002). This finding has been related to the contribution that disulfide bridges may provide to the thermostability of these proteins (Ladenstein & Ren, 2006). Despite growing evidence of the importance of disulfide bridges in hyperthermophilic organisms, current knowledge of the enzymes involved in disulfide-bond formation is still limited.

The process of disulfide-bond formation is modulated by complex enzymatic systems. A major role in this process is played by ubiquitous proteins belonging to the thioredoxin superfamily, which includes enzymes such as thioredoxins (Trxs), thioredoxin reductases (TrxRs) and disulfide oxidases/isomerases (PDOs/PDIs). In the hyperthermophilic archaeon *Sulfolobus solfataricus*, several genes of the thioredoxin/thioredoxin reductase system have been identified. Analysis of the *S. solfataricus* genome (She *et al.*, 2001) suggests the coexistence of two distinct thioredoxin (*Ss*TrxA2 and *Ss*TrxA1) and thioredoxin reductase (*Ss*TrxRB2 and *Ss*TrxRB3) genes (Grimaldi *et al.*, 2008; Ruocco *et al.*, 2004). Biochemical characterization of these proteins indicated that both *Ss*TrxA2 and *Ss*TrxA1 are substrates of *Ss*TrxRB3 (Grimaldi *et al.*, 2008). On the other hand, *Ss*TrxRB2 does not display any reductase activity towards either *Ss*TrxA1 or *Ss*TrxA2

(Grimaldi *et al.*, 2008). In order to acquire structural information on this intricate *S. solfataricus* thioredoxin system, we have undertaken structural characterization of the enzymes involved. In this context, we have previously reported structural investigations of *Ss*TrxRB3 (Ruggiero *et al.*, 2005, 2009). Here, we report the crystallization and preliminary crystallographic analyses of the thioredoxins *Ss*TrxA1 and *Ss*TrxA2, two proteins that share 38% sequence identity. The structural characterization of these proteins may provide useful information for the elucidation of the structure–function relationship of these proteins. Indeed, although Trxs isolated from bacteria and eukarya have been extensively investigated, structural information on archaeal Trxs is still rather limited. The first structure of a Trx isolated from an archaeon (*S. tokodaii*) has only recently been reported (Ming *et al.*, 2007). In contrast to this Trx and to the majority of eubacterial and eukaryal Trxs, which are monomeric, both *Ss*TrxA2 and *Ss*TrxA1 are dimeric (Grimaldi *et al.*, 2008). Therefore, structural investigations on these Trxs may provide insights into the role that oligomerization plays in their function. These studies are also important for understanding structure–stability relationships. Indeed, *Ss*TrxA2 and *Ss*TrxA1 show a remarkable thermophilicity and thermostability (Grimaldi *et al.*, 2008). Moreover, far-UV circular-dichroism spectra of these proteins show that they remain in a folded state even when kept at 382 K for several hours (Ruggiero *et al.*, unpublished results).

2. Experimental methods

2.1. Purification and crystallization

Recombinant *Ss*TrxA2 and *Ss*TrxA1 were expressed in *Escherichia coli* BL21 (DE3). After expression, the cells were mechanically disrupted and cell debris was removed by centrifugation at 100 000*g* for 45 min. After heating the supernatant at 343 K, the proteins were purified by FPLC using a MonoQ HR 10/30 anion-exchange column. For both proteins, fractions containing single protein bands on 15% SDS–PAGE were pooled together. As reported previously (Grimaldi *et al.*, 2008), after expression and purification the N-terminal fragments of both proteins were frequently cleaved. Intact *Ss*TrxA1 and *Ss*TrxA2 forms were obtained by adding the protease inhibitor PMSF at 1 m*M* concentration to all purification solutions. Both recombinant proteins were concentrated to 10 mg ml^{-1} using a Centricon-5K concentrator and stored in 20 m*M* Tris–HCl pH 7.8 at 277 K. Further details of the expression and purification of *Ss*TrxA2 and *Ss*TrxA1 have been reported elsewhere (Grimaldi *et al.*, 2008).

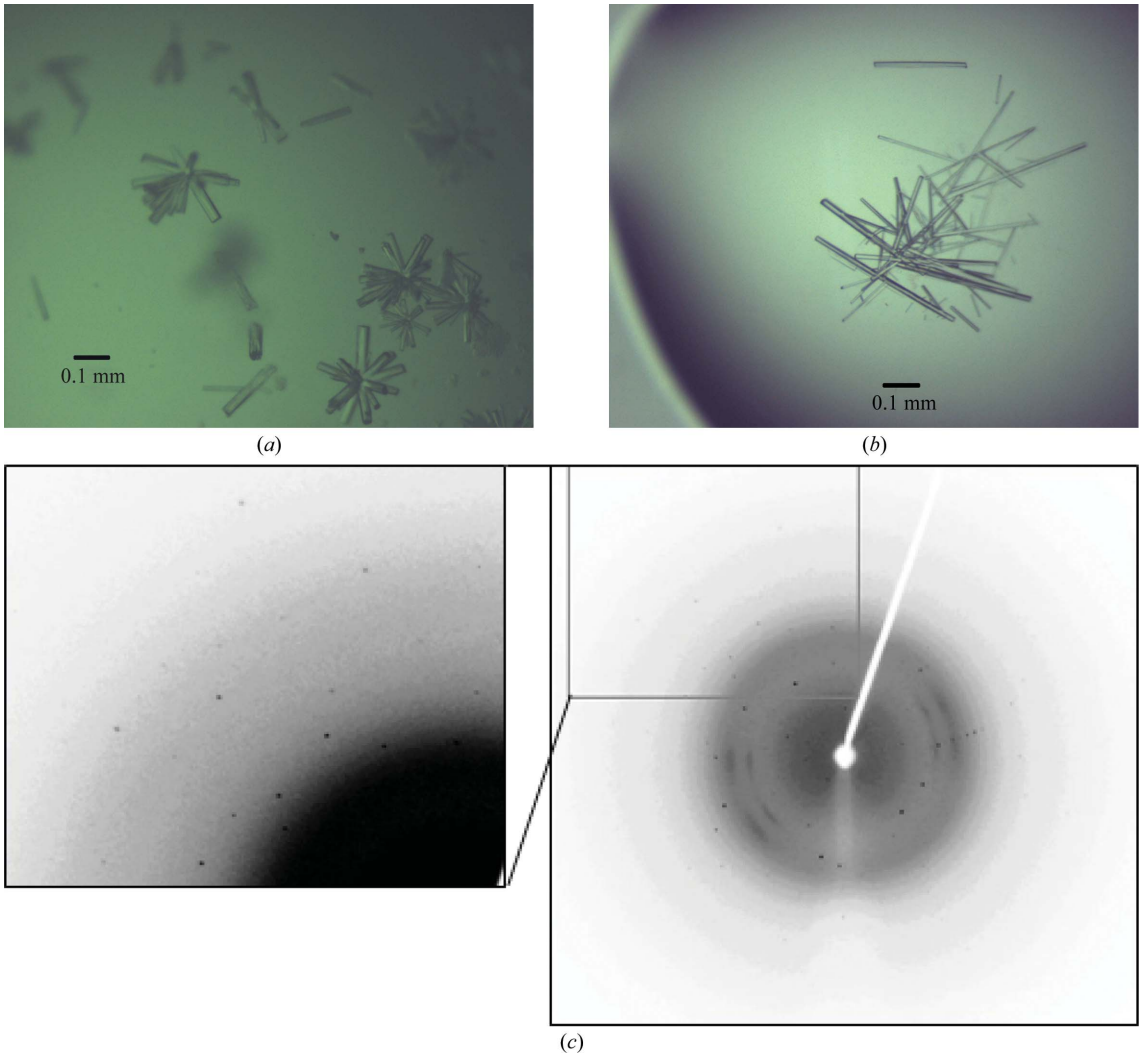


Figure 1
*Ss*TrxA2 crystals and diffraction pattern. (*a*) Crystals of the full-length form, (*b*) crystals of the truncated form and (*c*) diffraction pattern of a typical crystal of the truncated form (the resolution at the edge of the detector is 1.78 Å). The contrast of the snapshot has been increased to highlight the high-resolution data.

Crystallization experiments were performed at 293 K using either the microbatch-under-oil or hanging-drop vapour-diffusion method. Preliminary crystallization trials were carried out using commercially available sparse-matrix screens (Crystal Screens I and II; Hampton Research). Crystallization conditions were then optimized by fine-tuning the protein and/or precipitant concentrations.

2.2. Data collection and processing

For both proteins, diffraction data were collected in-house at 100 K using a Rigaku 007HF X-ray generator equipped with a Saturn 944 CCD detector. Crystals were flash-cooled 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).

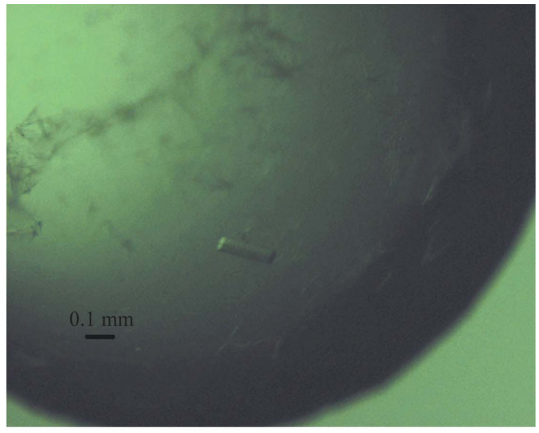
2.3. Structure determination and model building

The structures of *Ss*TrxA2 and *Ss*TrxA1 were solved by molecular replacement. Automatic model building was performed using the *warpNtrace* procedure implemented in the *ARP*/*wARP* package (Perrakis *et al.*, 1999).

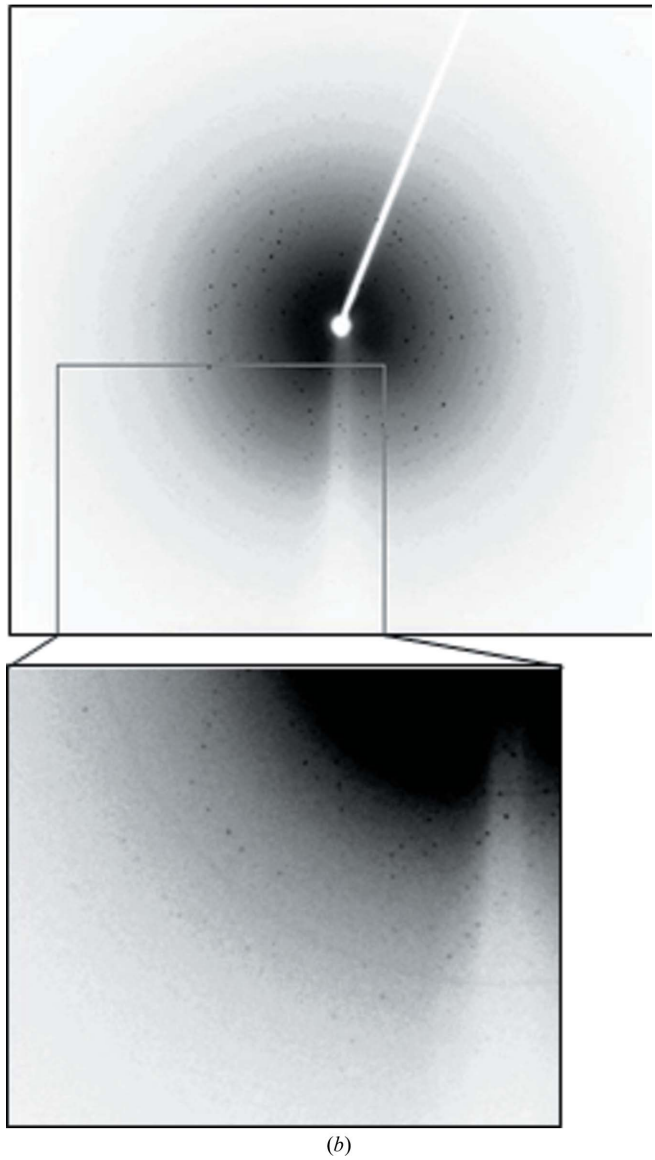
3. Results and discussion

Purified *Ss*TrxA2 and *Ss*TrxA1 were obtained in either full-length or truncated forms lacking an N-terminal fragment, depending on the protein-purification conditions (Grimaldi *et al.*, 2008). Full-length proteins were obtained by adding the protease inhibitor PMSF to the purification solutions. The truncated form of *Ss*TrxA2 shows a molecular mass of 12 522 Da, corresponding to the thioredoxin domain of the protein lacking the first 25 residues at the N-terminal end. Cleavage of *Ss*TrxA1 leads to a mixture of variants with molecular masses of 12 607, 12 580 and 12 421 Da that correspond to the thioredoxin domain of the protein lacking the first 22, 23 and 24 residues, respectively, at the N-terminus (Grimaldi *et al.*, 2008). Functional characterization of these variants of *Ss*TrxA2 and *Ss*TrxA1 indicated that the N-terminal fragments do not influence the thioredoxin activity (Grimaldi *et al.*, 2008).

Crystallization trials were conducted on both the full-length and the truncated forms of the two proteins. Trials conducted on the full-length forms only yielded crystals for *Ss*TrxA2. Large crystals of *Ss*TrxA2 were grown when polyethylene glycol (PEG) 3350 at a concentration of ∼20%(*w*/*v*) was used as precipitating agent (Fig. 1*a*). However, crystallographic analysis of these crystals clearly indicated that they were highly disordered. All attempts to improve the quality of these crystals were unsuccessful. On the other hand, initial screenings carried out using commercially available crystallization kits revealed different conditions for crystal formation for the truncated forms of both proteins. A common feature of all these promising conditions was the presence of PEG as precipitating agent. Starting from these conditions, attempts to improve the quality of the crystals were conducted by fine-tuning the concentration of the protein and of the precipitants. Well diffracting crystals of the thioredoxin domain of *Ss*TrxA2 were grown using 18–22%(*w*/*v*) PEG 3350 and 0.2 *M* ammonium iodide as precipitants at pH 6.2 (Fig. 1*b*) in the protein concentration range 4–9 mg ml^{-1}. These crystals reached their final dimensions (0.05 × 0.05 × 0.5 mm) within 3 d. Diffraction data (Fig. 1*c*) were collected to 1.83 Å resolution using a conventional X-ray source (see Table 1 for further details). Matthews coefficient calculations suggested the presence of a single *Ss*TrxA2 monomer per asymmetric unit (V_M = 1.95 Å^3 Da^{-1}; 37% solvent content). The structure of the thioredoxin domain was solved by molecular replacement using the structure of thioredoxin M from



(*a*)



(*b*)

Figure 2
(*a*) Crystals of truncated *Ss*TrxA1 and (*b*) its diffraction pattern (the resolution at the edge of the detector is 1.85 Å). The contrast of the snapshot has been increased to highlight the high-resolution data.

Table 1
Data-collection parameters and data-processing statistics.

Values in parentheses are for the highest resolution shell (1.90–1.83 Å for *Ss*TrxA2 and 1.97–1.90 Å for *Ss*TrxA1).

	*Ss*TrxA2	*Ss*TrxA1
Crystal-to-detector distance (mm)	40	40
Oscillation angle per frame (°)	0.40	0.25
Exposure time per frame (s)	30	30
No. of frames	470	350
Space group	*P*2	$P2_1$
Unit-cell parameters		
a (Å)	28.27	51.76
b (Å)	27.88	75.09
c (Å)	62.06	55.35
β (°)	92.34	112.64
Resolution (Å)	50.0–1.83	25.0–1.90
Mean redundancy	2.7	2.4
Completeness (%)	98.8 (96.5)	93.1 (93.8)
Total reflections	23445	68990
Unique reflections	8660	28606
$R_{\rm merge}$† (%)	5.8 (17.6)	9.7 (35.7)
Mean $I/\sigma(I)$	29.8 (7.5)	19.0 (4.0)

† $R_{\rm merge} = \sum_{hkl}\sum_i |I_i(hkl) - \langle I(hkl)\rangle| / \sum_{hkl}\sum_i I_i(hkl)$, where $I_i(hkl)$ is the intensity of the *i*th measurement of reflection *hkl* and $\langle I(hkl)\rangle$ is the mean value of the intensity of reflection *hkl*.

spinach chloroplast (PDB code 1fb6; Capitani *et al.*, 2000) as a starting model (sequence identity 45%) and the program *AMoRe* (Navaza, 1994). Initial model building was carried out with the program *ARP*/*wARP* (Perrakis *et al.*, 1999), which was able to automatically trace most of the enzyme structure. Refinement of the crystallographic model is in progress.

Well diffracting crystals of the *Ss*TrxA1 thioredoxin domain were grown using 30%(*w*/*v*) PEG 4000 and 0.2 *M* ammonium sulfate as precipitants with a protein concentration of 2–6 mg ml^{-1} (Fig. 2*a*). The crystals reached their final dimensions (0.2 × 0.15 × 0.05 mm) within one week. Diffraction data were collected to 1.90 Å resolution using a conventional source (Table 1 and Fig. 2*b*). Matthews coefficient calculations suggested the presence of either three ($V_{\rm M}$ = 2.63 Å^3 Da^{-1}; 53.2% solvent content) or four ($V_{\rm M}$ = 1.97 Å^3 Da^{-1}; 39.6% solvent content) *Ss*TrxA1 monomers per asymmetric unit. Molecular-replacement tests carried out using a preliminary structure of *Ss*TrxA2 as a starting model and the program *AMoRe* (Navaza, 1994) did not provide straightforward solutions. On the other hand, the *Ss*TrxA1 structure was rapidly solved by the programs *Phaser* (Read, 2001) and *REMO* implemented in *IL MILIONE* (Caliandro *et al.*, 2006). These analyses unambiguously indicated that the asymmetric unit of the *Ss*TrxA1 crystals contained three monomers. As for *Ss*TrxA2, the program *ARP*/*wARP* (Perrakis *et al.*, 1999) was able to automatically trace most of *Ss*TrxA1 structure. Refinement of the crystallographic model is in progress.

In conclusion, we report the crystallization and preliminary crystallographic analysis of the two distinct dimeric Trxs isolated from *S. solfataricus*. For both proteins the removal of the N-terminal fragment, which is likely to be unfolded, was essential for the growth of crystals suitable for crystallographic investigations.

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