**Sulfolobus solfataricus** protein disulphide oxidoreductase: insight into the roles of its redox sites

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**Sulfolobus solfataricus** protein disulphide oxidoreductase (**SsPDO**) contains three disulphide bridges linking residues C41XXC44, C155XXC158, C173XXXXC178. To get information on the role played by these cross-links in determining the structural and functional properties of the protein, we performed site-directed mutagenesis on Cys residues and investigated the changes in folding, stability and functional features of the mutants and analysed the results with computational analysis. The reductase activity of **SsPDO** and its mutants was evaluated by insulin and thioredoxin reductase assays also coupled with peroxiredoxin Bcp1 of *S. solfataricus*. The three-dimensional model of **SsPDO** was constructed and correlated with circular dichroism data and functional results. Biochemical analysis indicated a key function for the redox site constituted by Cys155 and Cys158. To discriminate between the role of the two cysteine residues, each cysteine was engineered and the behaviour of the single mutants was investigated elucidating the basis of the electron-shuffling mechanism for **SsPDO**. Finally, cysteine pK values were calculated and the accessible surface for the cysteine side chains in the reduced form was measured, showing higher reactivity and solvent exposure for Cys155.

**Keywords**: Sulfolobus solfataricus/protein disulphide oxidoreductase/redox sites/thioredoxin fold/thioredoxin system

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**Introduction**

Disulphide bonds between cysteine pairs are a major structural feature of many proteins. Proteins capable of catalysing protein disulphide bond formation include a large number of thiol–disulphide oxidoreductases that occur in all living cells, from bacteria to humans. Protein disulphide oxidoreductases fall into several families [thioredoxins (Trxs), glutaredoxins (Grxs), protein disulphide isomerases (PDIs) and disulphide-bond–forming (Db) proteins] and are characterized by an active site containing a CXXC motif and by a Trx-fold (a four-stranded central β-sheet and three-flanking α-helices) (Kadokura et al., 2003; Gilbert, 1990; Berndt et al., 2008; Ito and Inaba, 2008). In thermophilic organisms, a potential key role in disulphide bond formation has recently been ascribed to a new cytosolic protein disulphide oxidoreductase (**PDO**) family (Pedone et al., 2004). The finding that **PDOs** exclusively occur in thermophiles with high disulphide-content cytoplasmic proteins (Pedone et al., 2004; Beeby et al., 2005) is particularly interesting, suggesting a crucial role of **PDOs** in adaptation to extreme conditions. Three members of this family have so far been characterised from both the structural and functional points of view: two **PDOs** from the archaea Pyrococcus furiosus (**P/PDO**) (Guagliardi et al., 1995; Ren et al., 1998; Bartolucci et al., 2001) and Aeropyrum pernix (**A/PDO**) (D’Ambrosio et al., 2005, 2006) and a **PDO** from the thermophilic bacterium Aquifex aeolicus (**AaPDO**) (D’Ambrosio et al., 2004; Pedone et al., 2006a). These proteins have a molecular mass of ~26 kDa, and structural analysis has revealed that they are organized into two Trx folds, each with a CXXC active-site motif.

The analysis of the complete sequenced Sulfolobus solfataricus P2 genome (She et al., 2001) revealed that the ORF Sso0192 (**SsPDO**) is a homologue of **P/PDO** because it has two identical redox sites, C41QYC44 and C155PYC158, respectively, located at the N- and C-terminals and a deduced molecular weight of ~26 kDa. Furthermore, sequence analysis showed that **SsPDO** possesses a third redox site (C173RAGKC178) that predominately occurs in an oxidized form as already previously suggested (Pedone et al., 2006b). As reported for the site at the N-terminal of the **PDO** from Pyrobaculum aerophilum (CAGRETNWC), the insert between the two cysteines does not prevent disulphide formation, thus pointing to a potential function or structural relevance of this disulphide bond in protein folding. The extent to which a third site is an atypical finding is confirmed by the fact that when we compared **SsPDO** with all of the **PDOs** available in the database we only found a third site in the related *S. tokodaii* **PDO**.

In a general research project aimed to provide insight into the functions, structural diversity and evolution of **PDOs**, the protein **SsPDO** was characterized. **SsPDO** proved to be a typical **PDO**, a member of the PDI-like family (Pedone et al., 2006b) whose redox and channeler activities confirm a central role in the biochemistry of cytoplasmic disulphide bonds and suggest a potential role in intracellular protein stabilisation, respectively (Pedone et al., 2006b). Moreover, a new redox system formed of **SsPDO** and the Thioredoxin reductase (**Tr**) from *S. solfataricus* (**Sso2416/Tr**) has been investigated and finally the possible role of the **SsPDO/Tr** system as an *in vivo* partner of *S. solfataricus* peroxiredoxins, Bacterioferritin co-migratory protein 1 (**Bcp**), **Bcp** and Bcp4, in enzyme recycling was evidenced (Limauro et al., 2008).

In order to obtain information about the specific role of each of the three disulphide bridges between Cys41 and Cys44, Cys155 and Cys158, Cys173 and Cys178 on the stability and function of the proteins, we substituted Cys residues...
of each site with Ser residues by site-directed mutagenesis. All mutants were expressed and purified, and their reductase activities and involvement in the redox system were compared with that of the wild-type protein. The physicochemical behaviour of each mutant was compared with the wild type using a computational approach and circular dichroism (CD) measurements following incubation at variable temperatures and in the presence of different chaotropic agents.

Materials and methods

Materials, bacterial strains and plasmids

Bovine insulin, NADPH and all the other reagents used were from Sigma Aldrich. Molecular-mass standards for SDS/PAGE were obtained from Sigma Aldrich.

Escherichia coli Top F'10 was used as a general host for DNA manipulation. For expression of the recombinant proteins, E. coli BL21-Codon Plus (DE3)RIL (Stratagene) was used. This strain was cultivated in Luria–Bertani (LB) medium at 37°C. When necessary 50 μg ml⁻¹ of kanamycin and 33 μg ml⁻¹ of chloramphenicol (Sigma Aldrich) were added to the medium to maintain plasmids as needed.

Construction, expression and purification of SsPDO and its mutants

Gene cloning, overexpression and purification of SsPDO were described previously (Pedone et al., 2006b). Three SsPDO double mutants, namely C41S/C44S, C155S/C158S, C173S/C178S, and the mutant C41S/C44S/C155S/C158S were produced by following the protocol outlined in the Quik Change II site-directed mutagenesis Kit (Qiagen), using primers complementary to the coding and non-coding template sequence (pET28SsPDO) containing a double mismatch. To generate the double mutants C41S/C44S, C155S/C158S and C173S/C178S, the forward primers respectively

| 5′-CACACTCTAATAGGCAATATAGGCGTTGTTACTA-3′ |
| 5′-GTCAACTCTATGTCGGCTGTTACTA-3′ |
| 5′-GCTTTAGGCTAGGAGGCAGGAAGGCAA TGTTATATC-3′ |

with their complementary reverse primers were used (underlined letter indicate the base pair mismatch) using as template pET28SsPDO155S/C158S.

Cells were grown to an OD₆₀₀nm of ~0.6 in LB media supplemented with kanamycin (50 μg ml⁻¹) and chloramphenicol (33 μg ml⁻¹) at 37°C and were induced for 3 h. The optimised overexpression of all the proteins was obtained by exposing the cells to 1 mM isopropyl β-D-thiogalactoside (IPTG). E. coli BL21-Codon Plus (DE3)RIL cells transformed with pET28c(+) represented a negative control. The purification of the mutant proteins was carried out in a similar way as already described for the wild-type protein (Pedone et al., 2006b).

Analytical methods for protein characterisation

Protein concentration was determined using BSA as the standard (Bradford, 1976). Protein homogeneity was estimated by SDS/PAGE (12.5% (w/v) gels), using the silver staining procedure of Rabilloud et al. (Rabilloud et al., 1994). In addition, the proteins were analysed using a non-denaturing electrophoresis (12.5% (w/v) polyacrylamide slab gel). The molecular mass of the proteins was determined by a gel-filtration column on a Superdex 75 PC (0.3 × 3.2 cm) connected to the AKTA system (GE-Healthcare) and eluted with 50 mM Tris–HCl buffer, pH 8.0, 0.2 M KCl at a flow rate of 0.04 ml min⁻¹. As standards of molecular weight BSA (65.4 kDa), Ovalbumin (48.9 kDa), Chymotrypsinogen (22.8 kDa) and the RNase A (15.6 kDa) were used.

A LCQ DCA XP Ion Trap mass spectrometer (ThermoElectron, Milan, Italy) was used. This was equipped with an OPTION ESI source (operating at a needle voltage of 4.2 kV and a temperature of 320°C) and a complete Surveyor HPLC system [including an MS pump, an auto-sampler and a photo diode array (PDA)]. Analyses were performed using a 300 Å narrow bore 250 × 2 mm C₄ Jupiter column (Phenomenex, Torrance, CA, USA) and applying a gradient of solvent B (0.05% TFA in CH₃CN) on solvent A (0.08% TFA in H₂O) from 5 to 70%, over a period of 40 min. Mass spectra were recorded continuously in the mass interval 400–2000 amu, in positive mode (LC-MS). Multicharge spectra were then deconvoluted using the BioMass program implemented in the Bioworks 3.1 package provided by the manufacturer. Mass calibration was performed automatically by means of selected multiple charged ions, in the presence of a calibrant (UltraMark; ThermoElectron, Milan). All masses were reported as average values. LC–MS analyses were performed using 5 μl of 0.1 mg ml⁻¹ sample.

Assays of SsPDO and mutant activities

Insulin reductase activity The insulin reductase activity of SsPDO and its mutants (1.2 μM) was assayed according to Holmgren’s turbidimetric method with a few modifications (Holmgren, 1979). The catalytic reduction of insulin disulphide bonds was measured at 30°C. Proteins were added in...
1 ml of a solution containing 100 mM sodium phosphate buffer, pH 7.0, 2 mM EDTA and 1 mg of bovine insulin. A control cuvette contained only the buffer and insulin. The reaction started by addition of 2 mM dithiothreitol (DTT) to both cuvettes. Increasing turbidity from precipitation of insulin B chain was recorded at OD650nm.

**Thioredoxin reductase activity assays**

Thioredoxin reductase activity was evaluated by both 5-5'-dithiobis-(2-nitrobenzoic acid) (DTNB) and thioredoxin reduction methods (Ruocco et al., 2004). In the DTNB reduction, the formation of the product 2-nitro-5-thiobenzoate (TNB) was followed spectrophotometrically by the increase in absorbance at 412 nm. To calculate the enzymatic activity, a molar extinction coefficient of 19,200 M⁻¹ cm⁻¹ was used. The assay was performed at 60°C in a reaction mixture containing 0.1 M potassium phosphate buffer, pH 7.0, 2 mM EDTA, 0.5 mM DTNB, 0.25 mM NADPH, 0.05 mM flavo adenine dinucleotide (FAD), 200 mM SsTr in the presence of different concentrations of SsPDO and its mutants (0.03–4 μM). In the thioredoxin reduction assay, the reaction mixture contained 30 μM SsPDO or its mutants, 0.05 mM FAD, 2 mM EDTA and 200 mM SsTr in a final volume of 1 ml; the reaction was started by adding 0.25 mM NADPH. The activity was calculated from the decrease in absorbance at 340 nm using a molar extinction coefficient of 6.2 mM⁻¹ cm⁻¹.

**Assays of peroxidase activity**

SsPDO mutants were tested for their ability to function as alternative electron donor coupled with SsTr of one of the Beps in *S. solfataricus*, i.e. Bcp1 (Limauro et al., 2008). Bcp1 was tested for its ability to remove peroxides utilising as reducing system SsPDO or its mutants coupled with SsTr/NADPH. The reaction mixture contained 0.1 M potassium phosphate, pH 7.0, in the presence of 5 μM Bcp1 in a final volume of 0.5 ml. A mixture contained 0.25 mM NADPH, 200 mM SsTr, 30 μM SsPDO or mutants, 0.05 mM FAD, forming reducing cascade to recycle the enzymes, was used. The reaction was incubated at 60°C for 2 min. The reaction was started by adding 0.25 mM NADPH and 0.2 mM H₂O₂. The activity was calculated from the decrease in absorbance at 340 nm using a molar extinction coefficient of 6.2 mM⁻¹ cm⁻¹. Experiments were done in triplicate.

**Circular dichroism spectroscopy**

Circular dichroism spectra were recorded using a Jasco J-810 spectropolarimeter (JASCO Corp) equipped with a Peltier-type temperature control system (Jasco, model PTC-343). Recordings were carried out in the wavelength range of 195–250 nm, at a scanning speed of 20 nm min⁻¹, a bandwidth of 2 nm and a temperature of 20°C, under constant N₂ flow. The recorded spectra were then signal-averaged over at least three scans, and the baseline was corrected by subtracting the spectrum obtained with sample buffer. The value of molar ellipticity per mean residue [Θ, expressed in deg cm² dmol⁻¹] was finally calculated using the following equation: \[ Θ = Θ_{obs} \times \text{Mr/100C} \], where [Θ]_{obs} is the ellipticity (in mdeg), Mr is the mean MW of the residues of the protein, C is the protein concentration (in g l⁻¹) and l is the optical path length of the cell used (in cm).

The protein concentration to be used for acquisition of the CD spectra was optimized in preliminary assays.

A concentration of SsPDO and mutants of 2.5 × 10⁻⁶ M was ultimately chosen for these analyses in 10 mM sodium phosphate, pH 7.0.

**Homology modelling**

A first three-dimensional model of SsPDO was built using the SWISS-MODEL Protein Modeling Server. This model was completed using the ‘Homology’ module of the INSIGHT/DISCOVER package (Accelrys), running on a Silicon Graphics Octane2 workstation. Energy minimisations were carried out using the conjugate gradient algorithm to refine the model and to avoid high-energy conformations of protein backbone and residue side chains with the consistent valence force field (Hagler et al., 1979a, 1979b, 1979c). These procedures were stopped when the maximum derivative was ≤0.001 kcal mol⁻¹. All graphical analyses were also run on an SGI workstation, using the INSIGHT program.

**pK calculations**

The calculation of pK was done on the three-dimensional model of SsPDO in reduced form. This model has been built from that obtained for the oxidized form breaking the disulphide bonds at the CXXC active sites. Then, energy minimisations were carried out using the conjugate gradient algorithm to refine the model and to avoid high-energy conformations. The pK calculation was carried out using the PROPKA method developed by the Jensen Research Group (Li et al., 2005; Bas et al., 2008).

**Results**

**Biochemical characterisation of SsPDO mutants**

Primary structure analysis revealed two sequence putative redox sites, C¹⁶¹QYC²⁴⁶ and C¹⁵⁵PYC¹⁵⁸, and a putative third active site (C¹⁷³RAGKC¹⁷⁸) that had never been found in any other PDOs.

To investigate the contribution of each putative redox site in SsPDO catalytic activity and structure, three mutants were constructed by substituting the two cysteines of each redox site with serine. All the mutants were overexpressed in *E. coli* BL21-Codon Plus (DE3)RII and then purified using the procedure described for the wild-type enzyme (Pedone et al., 2006b). To further characterize protein purity and molecular weight, LC–MS analyses were performed and the molecular mass of the mutants was verified. The values obtained were in perfect agreement with the corresponding theoretical values and with the mutations introduced.

The catalytic activities of the mutated proteins were assayed and compared with those of the native enzyme. In particular, the reductase activity of each mutant was evaluated not only in insulin and thioredoxin reductase activity...
assays but also in reductive recycling of Bcp1, an antioxidant enzyme belonging to the peroxiredoxin family, characterized in *S. solfataricus* (Limauro et al., 2008). This enzyme shows a catalytic motif CXXXXC in the N-terminal region and it is hypothesized that the intra-molecular disulphide bond formed in oxidative conditions is reduced by the SsTr/NADPH/SsPDO system (Limauro et al., 2008).

C41S/C44S and C173S/C178S mutants show reductive activities comparable with wild-type SsPDO in insulin reductase assay while the C155S/C158S mutant and the double mutant proved completely inactive (Fig. 1).

In thioredoxin reductase, the DTNB-coupled assay SsTr was able to reduce C41S/C44S ($K_M = 0.17 \mu M$) and C173S/C178S mutants ($K_M = 0.12 \mu M$) with an affinity comparable with the wild-type SsPDO ($K_M = 0.20 \mu M$). In agreement with the insulin reductase activity, the C155S/C158S mutant and the double mutant were inactive.

The Trx-like system of *S. solfataricus* formed by SsTr/NADPH/ and SsPDO or its mutants was used to understand the role played by the putative active sites of SsPDO to reduce Bcp1. The peroxidase activity of Bcp1 was determined indirectly following NADPH oxidation. Our results show that H$_2$O$_2$-dependent NADPH oxidising activity of Bcp1 is detected only in the presence of SsPDO or C41S/C44S or C173S/C178S mutants (Fig. 2). These results confirm the major catalytical role of C$_{155}$PY$_{158}$ site in redox activity.

**Insight into the role of C$_{155}$PY$_{158}$ site**

To understand the role in redox activity of each cysteine residue in the site C$_{155}$PY$_{158}$, each cysteine was mutagenised in serine. The single mutants were constructed, overexpressed in *E. coli* and purified as already described for the wild-type protein (Pedone et al., 2006b).

The reductase activity of each single mutant was evaluated in thioredoxin reductase assay. The single mutants were compared with wild-type activity and while C$_{155}$PY$_{158}$ was found to preserve the 7% residual activity, the mutant S$_{155}$PY$_{158}$ was completely inactive (data not shown). These data evidence the major role played by the Cys155 in the redox site C$_{155}$PY$_{158}$.

**Structural characterisation of SsPDO and its mutants**

To assess the secondary structure of SsPDO and its mutants and to compare the stabilities of the mutant and wild-type proteins against thermal denaturation, far-UV CD spectra in the 190–260 nm region were recorded and the magnitude of the CD band at 223 nm was monitored at different temperatures. While near-UV CD spectra reflect alterations in tertiary structure, ellipticity at 223 nm is usually correlated with the α-helical content of a protein. The positive increases in the rotation values at 223 nm are assumed as a measure of the decrease in the α-helical content of a polypeptide. As shown in Fig. 3, the CD spectra of native SsPDO and its mutants exhibited two negative peaks at 208 and 222 nm and one positive peak at 195 nm, indicative of a predominantly folded structure with a high α-helical content. The estimated α-helical content is ~50% for wild-type protein. The CD

![Fig. 1. Assay of reductase activity by the reduction of bovine insulin disulphides. Assay of reductase activity by bovine insulin disulphides reduction. The DTT-dependent reduction of bovine insulin disulphides was carried out as described in ‘Materials and methods’. In absence [control (dashed line with two dots)] or in presence of 1.2 μM of pure wild-type SsPDO (straight line), C41S/C44S (dotted line); C173S/C178S (broken line); C155S/C158S (dashed line with single dot).](https://academic.oup.com/peds/article-abstract/22/1/19/1476585/1836182)

![Fig. 2. SsPDO/mutants-dependent peroxidase activity of Bcp1. The peroxidase activity of Bcp1 was detected following NADPH oxidation. Reducing system was constituted by SsTr/NADPH in the presence of SsPDO wt (squares), C41S/C44S (triangles), C155S/C158S (open circles), or C173S/C178S (filled circles).](https://academic.oup.com/peds/article-abstract/22/1/19/1476585/1836182)

![Fig. 3. Far-UV circular dichroism spectra of SsPDO (squares), C41S/C44S (circles), C155S/C158S (inverted triangles), C173S/C178S (triangles). The ellipticity is given as the molar ellipticity per residue.](https://academic.oup.com/peds/article-abstract/22/1/19/1476585/1836182)
spectra of the mutants C41S/C44S and C155S/C158S were indistinguishable, having the same overall shape, showing that the proteins shared the same secondary structure, and in particular displaying only a reduction in \( \alpha \)-helical content of 20% compared with wild-type protein. In contrast, the C173S/C178S mutant proved more structured presenting a further increase of 10% of \( \alpha \)-helical content with respect to wild-type protein. These structures are highly stable, because CD spectra were not significantly affected by the exposure to increasing temperatures. Indeed, no clear unfolding transition occurs. Instead, the increasing temperature slightly, but steadily, reduces the overall helical content (data not shown).

Stability to GuHCl (from 0 to 8 M) and urea (from 0 to 9 M) was analysed at a constant temperature (25°C). The minimal times necessary to complete the unfolding equilibria transitions and the optimal conditions for completely reversible denaturation were previously determined. All the proteins revealed a comparable resistance to GuHCl (3.5 M denaturing concentration) though a different, more marked resistance to urea. In particular, the wild-type and C41S/C44S mutant (7 M urea) proved to be the most resistant. This may indicate that electrostatic interactions, which are weakened more efficiently by GuHCl than urea, have an important role in the stabilisation of these proteins.

### Table I. Melting temperatures in presence of 1 M GuHCl or 2 M urea calculated from sigmoidal curves of wild-type and SsPDO mutants

<table>
<thead>
<tr>
<th>Protein</th>
<th>Temp. + 1 M GuHCl</th>
<th>Temp. + 2 M urea</th>
</tr>
</thead>
<tbody>
<tr>
<td>SsPDO wt</td>
<td>87.2</td>
<td>92.4</td>
</tr>
<tr>
<td>C41S/C44S</td>
<td>79.5</td>
<td>84.9</td>
</tr>
<tr>
<td>C155S/C158S</td>
<td>79</td>
<td>86</td>
</tr>
<tr>
<td>C173S/C178S</td>
<td>83</td>
<td>88.4</td>
</tr>
</tbody>
</table>

The heat stabilities of all the proteins were also determined by the trend of the melting curves obtained from the far UV CD change at 223 nm at various temperatures and in the presence of 1 M GuHCl or 2 M urea (concentrations of denaturant at which proteins are fully structured). The \( T_m \) calculated is reported in Table I. The plateau was reached in all the curves. The CD spectra registered in these conditions revealed a different stability for the mutants with respect to wild-type proteins, where the wild-type protein always proved to be the most resistant. CD data evidence that all the CX\(_n\)C motifs contribute to the structural stability of SsPDO. In particular, C41S/C44S and C155S/C158S seem to be more unstable compared with C173S/C178S, suggesting a key role of the disulphide bridges between Cys41 and Cys44, and Cys155 and Cys158 for the stabilisation of SsPDO. On the other hand, the disulphide bridge between Cys173 and Cys178 seems to play a minor stabilisation role, justifying its absence in other PDOs.

### Three-dimensional model of SsPDO

The SsPDO preliminary model obtained from the SWISS-MODEL Protein Modelling Server Sequence was built using as template the X-ray structure of the PDO from *A. pernix*, ApPDO (pdbcode 2HLS, D’Ambrosio *et al.*, 2006). Subsequently, this model was refined for the alignment and completed for the N- and C-terminal residues with the ‘Homology’ module of INSIGHT/DISCOVER program suite, using the secondary structure prediction tools of ExPASy server. A preliminary energy minimisation was then carried out keeping all backbone atoms fixed to refine the spatial position of side chains, followed by a full minimisation to obtain the final model.

The alignment data between SsPDO and ApPDO (Fig. 4) show that the two structures, on an alignment length of 216 residues, present a 52% of identity. In Fig. 5A, ribbon...
representation of the superimposition of SsPDO and ApPDO is depicted.

The general fold of the SsPDO is similar to that observed for protein members of the PDO family (Katti et al., 1990; Eklund et al., 1992; Kemmink et al., 1996; Ren et al., 1998). The SsPDO folds into seven α-helices and eight β-strands (Fig. 6). The comparative analysis of the SsCEI model with the ApPDO structure evidences the partial defolding of one α-helix in SsPDO between 78 and 85 that it is present in ApPDO (α3, 75–82). This behaviour is due to the insertion of two aminoacid residues in SsPDO with respect to ApPDO in this region.

The SsPDO structure contains two Trx folds. SsPDO presents the two CXXC active site motifs located on the protein surface. The first of these (CQYC) is located in the N-terminal unit at the amino end of the second helix, whereas the second one (CPYC) is positioned in the C-terminal part of the molecule at the beginning of the fifth helix. A careful comparison between the two active sites shows a remarkable degree of structural similarity, revealing that dihedral angle values of the two disulphides are similar to each other. In fact, the dihedral angles for the N-terminal disulphide are $\chi_1 = -179^\circ$, $\chi_2 = -149^\circ$ for Cys41, $\chi_1 = -85^\circ$, $\chi_2 = 87^\circ$ for Cys44, and $\chi_3 = 83$ for the S–S bond. On the other side, the C-terminal disulphide presents similar dihedral angles: $\chi_1 = 179^\circ$, $\chi_2 = -153^\circ$ for Cys155, $\chi_1 = -63^\circ$, $\chi_2 = 83^\circ$ for Cys158, and $\chi_3 = 86$ for the S–S bond. These values are characteristic of stable disulphide bonds (Thornton, 1981) and are observed in most of the PDOs.

SsPDO presents also a third disulphide, located at the amino end of the sixth helix involving Cys173 and Cys177 with dihedral angles $\chi_1 = -54^\circ$, $\chi_2 = -82^\circ$ for Cys173, $\chi_1 = -161^\circ$, $\chi_2 = 60^\circ$ for Cys177, and $\chi_3 = 75$ for the S–S bond. The formation of this disulfide bond produces a light distortion of the sixth helix.

In order to have some insights on the different reactivity of the three disulphide bonds, we have predicted pKs for cysteine residues, using the minimised SsPDO model in the reduced form. The computed pK values are reported in Table II. The data analysis of the sites shows that in these sites the first cysteine has a pK value lower than that of the second one. These results are in agreement with previous experimental and computational pKs studies on members of the Trx family (D’Ambrosio et al., 2006). In SsPDO, the first cysteine (Cys155) of the active site shows a significantly lower pK value (see Table II) with respect to the first cysteine (Cys41) of the other site. This result can be attributed to the low solvent exposure of Cys41, which causes a strong destabilisation for the thiolate. On the contrary, the lower pK value of the Cys155 indicates a higher solvent exposure, with a higher catalytic activity of this site with respect to the other site. The predicted pKs for the third disulphide bond (Cys173 and Cys178) are in agreement with values found for the Cys41 and Cys44, indicating similar solvent exposure.

With the aim to verify these results, we have also computed the accessible surface for the cysteine side chains in the reduced form. The analysis of the accessible surfaces reveals that Cys155 presents the higher value of solvent exposure with respect to the other cyste residues. In fact, the total surface computed with a 1.4 Å probe is 19.1 Å$^2$ for Cys173, 4.2 Å$^2$ for Cys155, 12.2 Å$^2$ for Cys158, 6.7 Å$^2$ for Cys41, 8.6 Å$^2$ for Cys173 and 6.6 Å$^2$ for Cys178. These values underline again a higher solvent exposure for Cys155.

Discussion

The new PDO protein family recently identified is typical of thermophiles, and as it plays a major role as a cytoplasmic system in catalysing the formation of disulphide bonds, it provides a basis for further elucidating protein-folding mechanisms in such microorganisms. In this context, this paper reports the characterisation of the mutants of the three disulphide bridges of the last member discovered of this family of PDOs, SsPDO (Pedone et al., 2006b). These mutants were designed to determine the contribution of each disulphide bridge to the structure and function of the protein.

The effects of mutations on protein structure were examined by CD spectroscopy, and the overall structure of these mutants proved similar to that of native SsPDO, indicating that the loss of the disulphide bridge did not affect the global fold of the molecule. On the other hand, thermal denaturation experiments showed that the structure of the mutants was

Table II. Estimated pKs for the cysteine residues computed on SsPDO

<table>
<thead>
<tr>
<th>Residue number</th>
<th>Cys41</th>
<th>Cys44</th>
<th>Cys155</th>
<th>Cys158</th>
<th>Cys173</th>
<th>Cys178</th>
</tr>
</thead>
<tbody>
<tr>
<td>pK value</td>
<td>8.63</td>
<td>12.26</td>
<td>5.06</td>
<td>9.06</td>
<td>8.41</td>
<td>12.57</td>
</tr>
</tbody>
</table>
less stable than that of wild-type protein as expected from the literature where Trx is usually more stable in its disulphide form compared with the dithiol form (Holmgren, 1972).

Our results indicate that the site C173RAGKC178 has only a structural and no functional relevance, as suggested previously (Pedone et al., 2006b). Destruction of the disulphide bridge in the mutant brings about an increase of secondary elements, i.e. α-helical content, eliminating a distortion of the sixth helix as observed in the model, thereby affecting the structure by a conformational modification but not the function of the protein. Indeed, the C173S/C178S mutant shows less thermostability but the same reductase activity compared with wild-type protein.

Contrasting with structural analysis where C41S/C44S and C155S/C158S mutants were identical, functional data show a different behaviour. In particular, the C41S/C44S mutant has the same reductase activity as the wild-type while the C155S/C158S mutant shows no reductase activity, suggesting that the disulphide bond in this site is essential for the activity of 5S PDO as observed for the mutant of the homologue Pp PDO (Pedone et al., 2004). Site-directed mutagenesis performed on Cys 155 and Cys 158 has elucidated the greater role played by Cys155: the mutant C158S retained partial enzymatic activity with respect to the single-mutant C155S and the double-mutant C155S/C158S that were inactive. This residual activity (7%) of the mutant C158S could be justified by the formation of a disulphide bridge between the two accessible Cys155 of two monomers acting as substrate of 5S Tr.

To shed light on the catalytic properties of each CXXC motif of 5S PDO, we determined the theoretical pK values of the cysteines. In the literature, it had been widely reported that the pK values of the two cysteines were related to the redox potential of the active site, thus playing a critical role in determining the physiological functions of the members of the Trx superfamily (Grauschopf et al., 1995; Kortemme et al., 1996; Lappi et al., 2004). Our results showed that the pK values of the N-terminal cysteines of all the active sites were lower than those observed for the C-terminal cysteines. Interestingly, the pK of Cys155 (5.06) is lower than that of Cys41 (8.63), suggesting that the Cys155 is particularly reactive as demonstrated by functional results, the computed higher value of solvent exposure and in agreement with Pp PDO data (Moutevelis, and Warwicker, 2004; D’Ambrosio et al., 2006). The pK value of the nucleophilic cysteine and the redox potential are the main determinants for the distinct reactivities of the CXXC proteins motif. This value can be as high as 7.2 for E.coli Trx, but strikingly different from that of other members of the Trx family like yeast Grx (3.5) and the periplasmic DsbA (3.5) (Recktenfelderbäumer and Krauth-Siegel, 2002; Wunderlich et al., 1993). The pK value of the nucleophilic cysteine is significantly lower than that of free cysteine (8.7). Low pK values of thiol groups may be due to the close proximity of positive charges. In Trx-like proteins, since positively charged residues are not found in the neighbourhood of the redox active disulphide, the nucleophilic cysteine, localized at the N-terminus of an α-helix, may well be stabilised by the positive partial charge of the helix dipol (Kortemme and Creighton, 1995).

Finally, our results show that the contribution of each active site of 5S PDO to total catalytic activity and to the whole structure can vary enormously. These results were achieved by using a panoply of energy minimisation procedures, protein engineering, bio-informatic, biochemical and molecular dynamics techniques.

**Funding**

This work was supported by grants from CIB (2007), from MIUR (FIRB-RBNN07BMCT) and Regione Campania (N.5 del 28.03.2002).

**References**


Received July 17, 2008; revised October 2, 2008;
accepted October 6, 2008

Edited by David Ollis