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ORIGINAL PAPER

Characterisation of the components of the thioredoxin system in the archaeon *Sulfolobus solfataricus*

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Abstract The thioredoxin system is a redox machinery widely distributed in nature and involved in several cellular functions. It is constituted of thioredoxin reductase (Trx-B), its protein substrate thioredoxin (Trx-A) and NADPH. We have previously characterised a Trx-B from the hyperthermophile *Sulfolobus solfataricus* (SsTrx-B3) (Ruocco et al. in Biochimie 86:883–892, 2004). As in the genome of this archaeon, the gene coding for another Trx-B (SsTrx-B2) and for two Trx-A (SsTrx-A1, SsTrx-A2) have been putatively identified, these proteins were obtained as recombinant forms and characterised. SsTrx-B2, different from SsTrx-B3, did not elicit a thioredoxin reductase activity. *S. solfataricus* possessed only one Trx-B (SsTrx-B3), which had two thioredoxins (SsTrx-A1 and SsTrx-A2) as substrates. These latter showed a homodimeric structure and catalysed insulin reduction using either DTT or NADPH/SsTrx-B3 as electron donors. In addition, the electron transfer between SsTrx-B3 and either SsTrx-A1 or SsTrx-A2 was fully reversible, thus allowing the determination of the redox potential of the thioredoxin system in *S. solfataricus*. Among the two thioredoxins, SsTrx-A2 appeared slightly more active and stable than SsTrx-A1. These data, besides shedding light on thioredoxin system in *S. solfataricus*, will contribute to add further information on this key enzyme system in Archaea.

Keywords *Sulfolobus solfataricus* · Thioredoxin system · Active site disulfide · Archaea · Redox potential

Abbreviations

Trx-A	Thioredoxin
Trx-B	Thioredoxin reductase
Ss	*Sulfolobus solfataricus*
DTNB	5,5'-Dithio-*bis*(2-nitrobenzoic acid)

Introduction

The thioredoxin system is a powerful redox enzymatic machinery widely distributed in nature and involved in several cellular functions. It represents, together with glutathione reductase, the major ubiquitous disulfide

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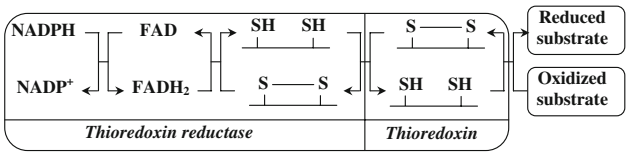
reductase responsible for maintaining proteins in their reduced state, preserving redox homeostasis in the cytoplasm of Archaea, Bacteria and Eukarya (Hirt et al. 2002; Arner and Holmgren 2000). The thiol groups of numerous proteins can often represent a target of protein function regulation, and therefore, their oxidation or reduction state can influence the biological function. Indeed, the thioredoxin system is involved in regulating DNA synthesis, gene transcription, immunomodulation, cell growth, signal transduction and apoptosis (Arner and Holmgren 2000). It is not surprising that alterations in this system could play a role in the genesis of many diseases (Becker et al. 2000; Lillig and Holmgren 2007). Therefore, the involvement of this system in many aspects of cellular metabolism has stimulated a great number of studies even in different sources. The thioredoxin system is constituted by the flavoenzyme thioredoxin reductase (Trx-B), NADPH and its protein substrate thioredoxin (Trx-A). Trx-B catalyses the NADPH-dependent electrons transfer to the active site disulfide of oxidised Trx-A to form a dithiol according to the reaction mechanism reported in Scheme 1.



The reducing equivalents associated to the dithiol present on the reduced thioredoxin are then transferred to several cellular substrates to keep them in a reduced state. Proteins containing disulfide bridges have been associated for long time to extra-cytoplasmatic environments. Indeed, it was assumed that the reducing conditions of the cytoplasm were not compatible with the presence of proteins with cysteine residues in their oxidised form (Kadokura et al. 2003). This common belief has been recently undermined by the discovery that a large fraction of proteins of several hyperthermophilic organisms exhibits disulfide bridges (Mallick et al. 2002; Ladenstein and Ren 2006). Indeed, it has been shown that this structural element may provide a significant contribution to the thermostability of these proteins by compensating an insufficient level of electrostatic charge optimisation in these proteins (Arner and Holmgren 2000; Ladenstein and Ren 2006; Spassov et al. 1994). Despite the growing evidence of the importance of disulfide bridges in hyperthermophilic Archaea and Bacteria, the current knowledge on the enzymes involved in disulfide bond formation is still limited (Ladenstein and Ren 2006; Jeon and Ishikawa 2002; Kadokura 2006; Sevier and Kaiser 2006). In the hyperthermophilic archaeon *Sulfolobus solfataricus*, several components of the thioredoxin/thioredoxin reductase system have been identified (Guagliardi et al. 1994; Ruocco et al. 2004; Pedone et al. 2006). The analysis of the *S. solfataricus* genome (She et al. 2001) reveals the coexistence of two distinct thioredoxin (SsTrx-A1—sequence entry Q980E5, and SsTrx-A2—sequence entry Q97WI4) and thioredoxin reductase (SsTrx-B2—sequence entry Q97V69, and SsTrx-B3—sequence entry Q97W27) genes, all harboring the canonical CXXC motif. Furthermore, a different protein (SsTrx-B1—sequence entry Q97WJ5) lacking the CXXC motif but with a significant sequence identity with other Trx-Bs has also been identified as a NADH oxidase (Arcari et al. 2000). This scenario is further complicated by the discovery that *S. solfataricus* protein disulfide oxido-reductase is a substrate for SsTrx-B3 (Pedone et al. 2006). Along this line, the presence of different putative thioredoxins and thioredoxin reductases in the genome seems to be present even in the archaeal genomes sequenced so far.

We have recently isolated and characterised a Trx-B from the hyperthermophilic archaeon *Sulfolobus solfataricus* (SsTrx-B3). The gene coding for this enzyme was also cloned in a prokaryotic expression vector that allowed the purification of recombinant SsTrx-B3 together with its modified forms (Ruocco et al. 2004). This protein was also successfully crystallised (Ruggiero et al. 2005) and the resolution of its 3D-structure has been started. To shed light on the role and the partnerships of the intricate *S. solfataricus* thioredoxin system and to verify whether the sequence redundancies are reflected also at a functional level, here, we report the expression and the characterisation of the two thioredoxins (SsTrx-A1 and SsTrx-A2) and the thioredoxin reductase SsTrx-B2 from this archaeon.

Materials and methods

Materials

Restriction and modifying enzymes, labeled compounds and chromatographic media were obtained from GE Healthcare; PMSF, DTNB, NADPH, FAD and recombinant human insulin solutions (10 mg/ml) were from Sigma. Cloning vectors were obtained from Novagen or Promega. Plasmids DNA, genomic DNA and labeled probes were prepared as described (Ruocco et al. 2004). rSsTrx-B3 was expressed and purified as reported (Ruocco et al. 2004). The following buffers were used: buffer A, 20 mM Tris/HCl, pH 7.8; buffer B, 20 mM Tris/HCl, pH 7.8, 50% (v/v) glycerol; buffer C, 100 mM potassium phosphate, pH 7.0,

2 mM EDTA; buffer D, 25 mM MES/KOH, pH 5.5, 2 mM EDTA.

Plasmid construction and sequencing

DNA from *S. solfataricus* strain MT-4 (DSM 5833) was used as template to amplify by PCR (Perkin-Elmer) the genes coding for SsTrx-B2, SsTrx-A1 and SsTrx-A2 using as primers the oligonucleotides listed below, which were derived from the genome sequence of *S. solfataricus* (She et al. 2001).

Gene	Forward primer		Reverse primer	
SsTrx-B2	TrxR-F	5′-GAGATATA**CC**ATG**G**CGTTAAAGAC-3′	TrxR-R	5′-ATAAAG**CTT**AAAAGTTAGAGTCTAA-3′
SsTrx-A1	TrxA1-F	5′-TGGTAGATAG**CCA**TG**G**GCGAAATT-3′	TrxA1-R	5′-CAATAACGTTTAATAATGTACTT-3′
SsTrx-A2	TrxA2-F	5′-TATAATCTC**A**TATGAATGACGAA-3′	TrxA2-R	5′-CATT**GA**TA**T**CACTTGTTACTCTAAT-3′

These primers were designed with the aim of inserting specific cloning sites (underlined) also by inserting point mutations (in bold). In particular, those used to amplify the genes encoding SsTrx-B2 and SsTrx-A1 contained a *Nco*I and a *Hind*III restriction sites, to insert a start codon and a cloning site, respectively. In addition, the cloning strategy led to the substitution of the first two residues in the amino acid sequence of SsTrx-A1 ($Leu_1Ser \rightarrow MetGly$) and of the second one in that of SsTrx-B2 ($Pro_2 \rightarrow Ala$). Vice versa, in the primers designed to amplify the gene encoding SsTrx-A2, a *Nde*I and a *EcoR*V cloning sites were inserted without modification of the amino acid sequence. The amplified DNA fragments were digested with appropriate restriction enzymes and inserted in the polylinker region of the prokaryotic expression vector pET-28, for SsTrx-B2 and *Ss*Trx-A1 or in pET-22 for SsTrx-A2, previously digested with the same enzymes. After transformation of the *E. coli* TG1 strain, recombinant clones were selected only if they contained inserts of the correct size. These inserts were sequenced to control the PCR reaction and in the case of SsTrx-A1 we found a difference, namely $A_{128} \rightarrow G$ (the numbering included the starting codon), which led to the $R_{43} \rightarrow K$ difference. This behavior was found even when a different DNA polymerase and/or primers were used instead. Therefore, this finding could represent a polymorphism present in the genome of the strain used in this work. The resulting plasmids were designed as pSsTrx-B2, pSsTrx-A1 and pSsTrx-A2, respectively.

Expression and purification of recombinant proteins

To express SsTrx-B2, SsTrx-A1 or SsTrx-A2, *E. coli* BL21 (DE3) competent cells were transformed with pSsTrx-B2, pSsTrx-A1 or pSsTrx-A2, respectively, and grown in Luria–Bertani medium at 37°C; when OD_{600} reached 0.6, 1 mM IPTG was added for induction and the cultures were grown for further 3 h for pSsTrx-B2 and pSsTrx-A2 and overnight for pSsTrx-A1. Cells were harvested by centrifugation at 3,000×*g* for 15 min at 4°C, and resuspended in 20 ml of buffer A, supplemented with 1 mM PMSF and disrupted by a constant cell disruption system (Constant Systems Ltd., UK). The suspension was centrifuged at 100,000×*g* for 2 h to remove cellular debris and the supernatant was incubated at 70°C for 30 min to denaturate most of the *E. coli* proteins that were then removed by centrifugation at 22,000×*g* for 30 min. The supernatants were then dialyzed against buffer A and loaded separately on a MonoQ HR 10/30 anion exchange column, connected to an FPLC system (Pharmacia), equilibrated at room temperature with buffer A at 3 ml/min. Under these conditions, either rSsTrx-B2, rSsTrx-A1 or rSsTrx-A2 bound to the column and their elution was achieved by a linear 0–200 mM KCl gradient in buffer A. In particular, rSsTrx-B2, rSsTrx-A1 and rSsTrx-A2 were eluted at around 80, 60 and 25 mM KCl, respectively. Fractions containing single protein bands from each chromatography were pooled together, concentrated by Aquacide II (Calbiochem), dialyzed against buffer B and stored at −20°C. Under these conditions, the yield from 1-liter culture was 5–15 mg of purified recombinant rSsTrx-B2, rSsTrx-A1 or rSsTrx-A2.

Enzymatic assays

Thioredoxin activity was determined by the turbidimetric insulin reduction method, using either DTT or thioredoxin reductase/NADPH as reducing equivalent donors (Holmgren 1979a, b). The initial velocity of the insulin reduction was derived from the linear part of the increase in absorbance at 650 nm in the interval 0.1–0.4.

Thioredoxin reductase activity was determined at 60°C spectrophotometrically by both the DTNB- and the thioredoxin-methods as already reported (Ruocco et al. 2004). NADH oxidase and glutathione reductase activities were measured as reported (Masullo et al. 1996; Gromer et al. 2002).

The kinetic parameters K_m and maximum reductase activity were calculated from Lineweaver–Burk plots

obtained by measuring the initial velocity of the enzymatic reaction determined at different substrate concentrations that have been chosen with the aim of guaranteeing the same weight in the linear regression.

Determination of the redox potential

The redox potential of the thioredoxin/thioredoxin reductase system was determined at 60°C as reported (Jeon and Ishikawa 2002) by measuring the absorbance change at 340 nm. To this aim, 10–50 μM rSsTrx-A1 or rSsTrx-A2 were mixed with 50 μM NADPH in a final volume of 1 ml buffer D, and the reaction was started by adding 2 μM rSsTrx-B3. When the absorbance reached a constant low level, excess $NADP^+$ (1,200 μM) was added, and the reaction was allowed to proceed until a constant higher level of absorbance at 340 nm was reached. Redox potentials were obtained by the Nernst equation in which the concentration of reduced and oxidised forms of both thioredoxin and electron donors were employed. A calculated value of −271 mV for the redox potential of $NADP^+$ at pH 5.5 was used. Blank runs in the absence of thioredoxins were carried out in parallel and subtracted. The values reported represented the mean over three to four determinations carried out at different concentrations of thioredoxins and the variations never exceeded 4%.

Heat inactivation

Samples of rSsTrx-A1 or rSsTrx-A2 at 50 μM final concentration in buffer C were treated at different temperatures. At selected time intervals, aliquots were withdrawn and assayed for the residual activity in the insulin reduction method, using DTT as electron donor. The rate constants of the heat inactivation process were calculated according to the first-order kinetics equation $\ln A_t/A_0 = -kt$, where A_0 corresponds to the activity of an untreated sample, A_t the activity after the time t of the heat treatment and k is kinetic constants of the heat inactivation process. The energy of activation of the inactivation process was calculated from Arrhenius plots in which the natural logarithm of k was plotted against the reciprocal of absolute temperature.

Other methods

SDS-PAGE was carried out by the method of Laemmli (1970). The concentration of protein solutions was estimated by the method of Bradford (1976) using bovine serum albumin as the standard.

The M_r in nondenaturing condition was determined by gel filtration on a Superdex 10/300 GL column connected to a FPLC system and equilibrated at 0.5 ml/min with buffer A supplemented with 100 mM KCl, in the absence or in the presence of 7 mM β-mercaptoethanol. The column was calibrated using 10 μg in 100 μl of the elution buffer of the following protein markers, all isolated from *S. solfataricus*: elongation factors 2 (78 kDa; Raimo et al. 1992), 1α (49 kDa; Masullo et al. 1991), 1β (20 kDa; Raimo et al. 1996) and ribonuclease P2 (14 kDa, Fusi et al. 1993).

Results

Molecular properties of rSsTrx-B2, rSsTrx-A1 and rSsTrx-A2

Purified rSsTrx-B2, rSsTrx-A1 and rSsTrx-A2 appeared homogeneous on 15% SDS-PAGE (Fig. 1), exhibiting a molecular mass of 35, 15 and 15 kDa, respectively. However, when the purification procedure was carried out in the absence of PMSF, both rSsTrx-A1 and rSsTrx-A2 were purified also as truncated forms of about 12 kDa. These observations were confirmed by mass spectrometry experiments on truncated and full-length forms of the two proteins. Indeed, for rSsTrx-A2, the full-length form presents a molecular mass of 15,517 Da, a value nearly coincident with that expected on the basis of the protein sequence (15,518 Da). rSsTrx-A2 truncated form shows a molecular mass of 12,522 Da coincident with that expected for the region Lys26–Glu135. The largest fragment of rSsTrx-A1 shows a molecular mass of 15,037 Da, corresponding to the mass of the full-length protein lacking the initial methionine. On the other hand, three distinct peaks were detected for the truncated form of rSsTrx-A1 with masses of 12,607, 12,580 and 12,421 Da, which

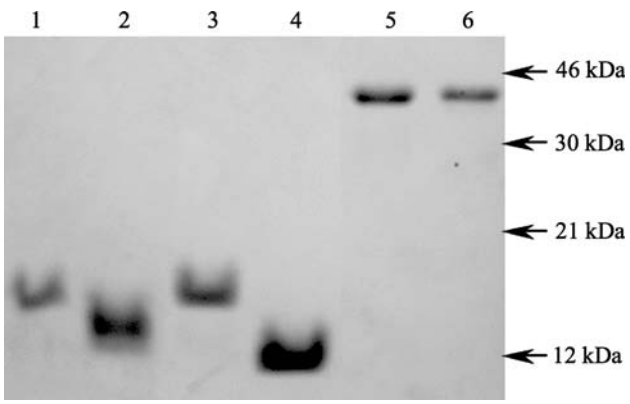


Fig. 1 SDS-PAGE of recombinant SsTrx-A1, SsTrx-A2 and SsTrx-B2. Ten micrograms each of intact (*lane 1*), truncated (*lane 2*) rSsTrx-A1, intact (*lane 3*), or truncated (*lane 4*) rSsTrx-A2, SsTrx-B3 (*lane 5*) and SsTrx-B2 (*lane 6*) were separated by SDS-PAGE and stained with Coomassie Brilliant Blue R-250. The migration of molecular mass standards is reported on the right

correspond to the masses expected for the fragments Gly23–Lys133, Lys24–Lys133 and Lys25–Lys133, respectively (including the initial methionine). These observations were confirmed by crystallographic analyses of both rSsTrx-A1 and rSsTrx-A2, which are currently in progress (Vitagliano et al., unpublished results).

The molecular mass determined by gel-filtration under native conditions on Superdex 75 gave values of 70, 32 and 33 kDa for rSsTrx-B2 and unnicked rSsTrx-A1 and rSsTrx-A2, respectively (Fig. 2a). In addition, the molecular masses of truncated rSsTrx-A1 and rSsTrx-A2 were 21 and 19 kDa, respectively. These results were obtained even when the gel-filtration was carried out in the presence of reducing agents, such as β-mercaptoethanol (Fig. 2b). These observations indicate that rSsTrx-B2, rSsTrx-A1 or rSsTrx-A2 were organised as homodimers not linked with disulfide bonds. Furthermore, the truncation of both rSsTrx-A1 and rSsTrx-A2 did not affect the oligomerisation state of the proteins. Therefore, unless otherwise indicated, the characterisation of the biochemical properties will be carried on intact rSsTrx-A1 and rSsTrx-A2.

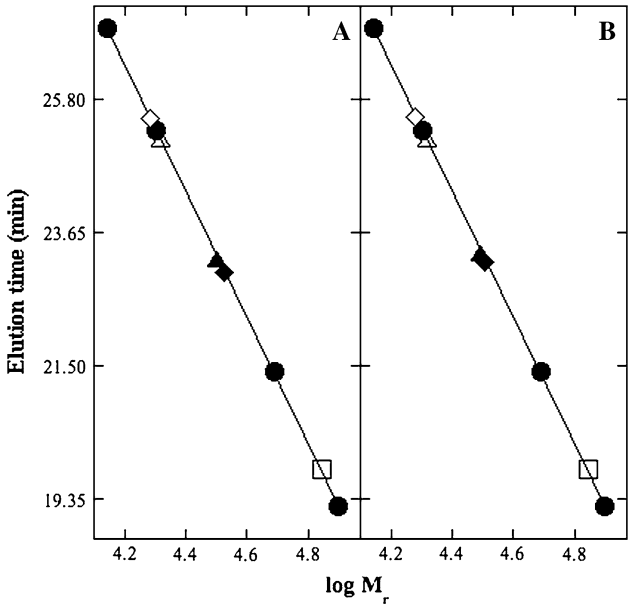


Fig. 2 Determination of the molecular mass under non-denaturing conditions of recombinants SsTrx-A1, SsTrx-A2 and SsTrx-B2. Ten micrograms in 100 µl of elution buffer of intact (*filled triangle*) or truncated (*open triangle*) SsTrx-A1, intact (*filled diamond*) or truncated (*open diamond*) SsTrx-A2 and SsTrx-B2 (*open square*) were analysed on a Superdex 10/300 GL gel-filtration column as reported in "Material and methods" in the absence (**a**) or in the presence (**b**) of 7 mM β-mercaptoethanol. The elution position of each protein was reported in comparison to that of protein molecular mass standards (*filled circle*) anlalysed under identical experimental conditions

Biochemical properties of rSsTrx-A1 and rSsTrx-A2

The activity of rSsTrx-A1 and rSsTrx-A2 was assayed by either the DTT or the thioredoxin reductase insulin reduction nephelometric method (Holmgren 1979a, b). Either rSsTrx-A1 or rSsTrx-A2 was able to catalyse electron transfer from DTT to insulin, causing its reduction and then precipitation (Fig. 3). The time at which the phenomenon started depended on the pH for both thioredoxins. At 10 µM rSsTrx-A1, the reduction at 25°C of insulin started at around 3.5 and 7.0 min at pH 7.0 and 5.5, respectively. Under identical experimental conditions, rSsTrx-A2 exhibited shorter starting times (2.2 and 3.9 min at pH 7.0 and 5.5, respectively), thus indicating that rSsTrx-A2 displayed a higher activity than rSsTrx-A1 in transferring electrons from DTT to the disulfide of insulin. This behavior was also confirmed by the values of the initial velocity of insulin reduction that was faster for rSsTrx-A2 and a pH 7.0. It has to be pointed out that the reductase activity of the truncated form of both thioredoxins was undistinguishable from that of the intact ones (not shown).

To investigate on the thioredoxin system in *S. solfataricus*, rSsTrx-A1 or rSsTrx-A2 has been used as homologous substrates for the thioredoxin reductase insulin reduction catalysed by rSsTrx-B3 or rSsTrx-B2 using

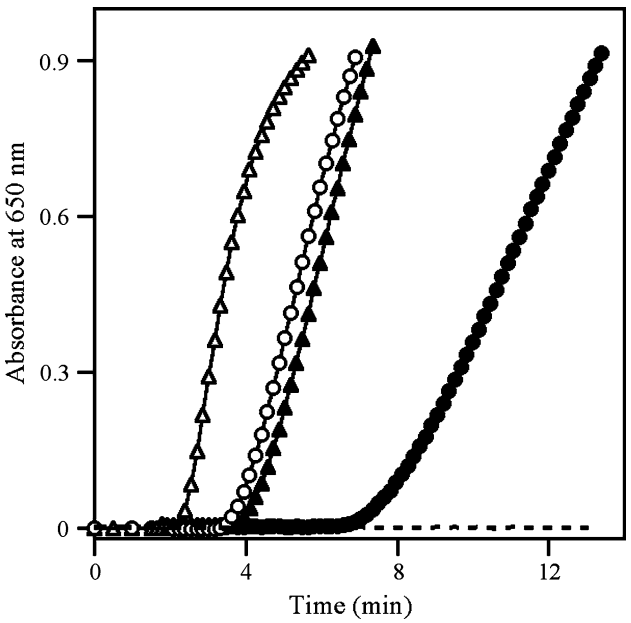


Fig. 3 DTT reduction of insulin catalysed by rSsTrx-A1 or rSsTrx-A2. Five-hundred microliter of reaction mixture contained 0.13 mM human insulin in the absence (*dashed line*) or in the presence of 10 µM rSsTrx-A1 (*circles*) or rSsTrx-A2 (*triangles*) in buffer C (*empty symbols*) or buffer D (*filled symbols*), respectively. The reaction was carried out at 25°C, started by adding 0.5 mM DTT and the absorbance at 650 nm was recorded kinetically

NADPH as electron donor. As shown in Fig. 4a, in the presence of rSsTrx-B3, both rSsTrx-A1 and rSsTrx-A2 were able to transfer electrons from NADPH to insulin, even though the reaction started at times significantly longer than that found using DTT. Also in this case, rSsTrx-A2 exhibited a higher activity in electron transfer than rSsTrx-A1, but a different behavior was observed regarding the effect of pH. In fact, the highest activity was measured for both thioredoxins at pH 5.5.

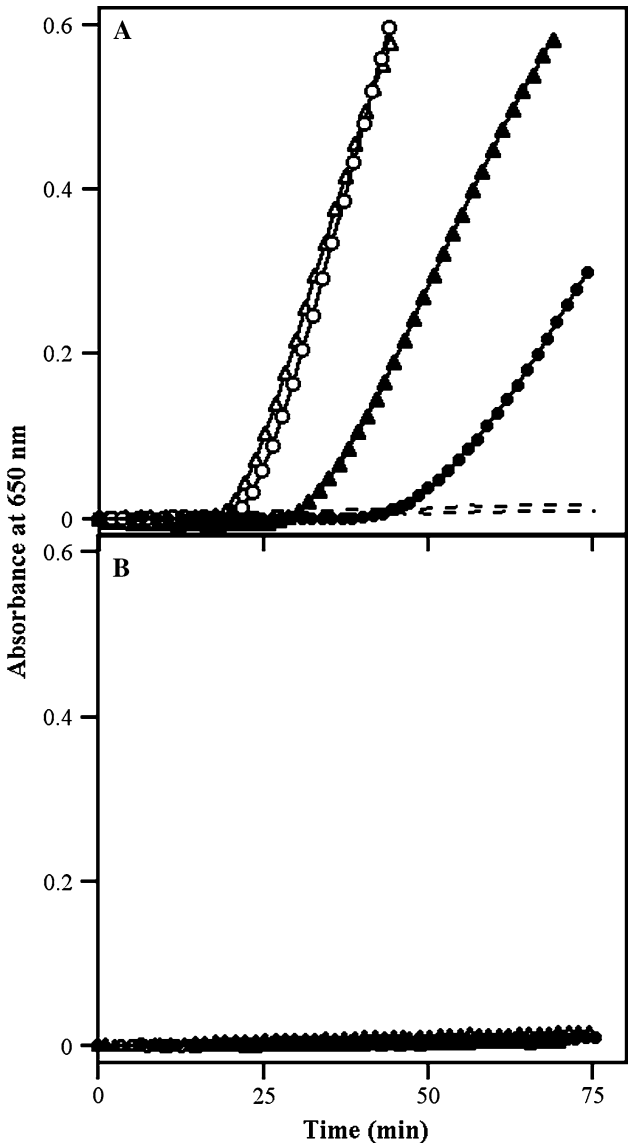


Fig. 4 rSsTrx-A1 or rSsTrx-A2 reduction of insulin catalysed by rSsTrx-B3 in the presence of NADPH. Five-hundred microliter of reaction mixture contained 0.13 mM human insulin in the absence (*dashed line*) or in the presence of 1 μM rSsTrx-B3 (**a**) or rSsTrx-B2 (**b**). The reaction was started by adding 200 μM NADPH in the presence of 10 μM rSsTrx-A1 in buffer C (*filled circle*) or D (*open circle*) or 10 μM rSsTrx-A2 in buffer C (*filled triangle*) or D (*open triangle*), respectively. The reaction was carried out at 25°C and the absorbance at 650 nm was recorded kinetically

The insulin nephelometric method was then used to determine the kinetic parameters of the activity of rSsTrx-A1 and rSsTrx-A2, using either DTT or rSsTrx-B3/NADPH as electron donor. The results reported in Table 1 indicate that the highest catalytic efficiency was observed using DTT as electron donor and at pH 7.0. It is interesting to note that for both rSsTrx-A1 and rSsTrx-A2 the almost identical catalytic efficiency was reached through a different way for the two proteins. In fact, while rSsTrx-A1 exhibited a higher affinity than rSsTrx-A2, the latter displayed a higher catalytic rate. Using the NADPH/rSsTrx-B3 as electron donor, the highest catalytic efficiency was observed for rSsTrx-A2 and at pH 5.5, and the value was similar to that measured using DTT as electron donor. However, the value of the catalytic efficiency depended on a lower catalytic rate with an increased affinity for the substrate rSsTrx-A2. Concerning rSsTrx-A1, although the catalytic rate was lower compared to that measured using DTT, the affinity for the protein substrate was higher.

Redox potential of *S. solfataricus* thioredoxins

The fully reversibility of electron transfer between thioredoxin reductase and thioredoxin was demonstrated by adding excess $NADP^+$ upon the complete oxidation of NADPH at 60°C and pH 5.5, catalysed by rSsTrx-B3 (Fig. 5). This procedure allowed the determination of the redox potential through the Nernst equation at the equilibrium. The values obtained were −153 and −166 mV for unnicked and nicked rSsTrx-A1, respectively; whereas, slightly more negative values of −173 and −174 mV for intact or truncated rSsTrx-A2, respectively, were found.

Heat stability of rSsTrx-A1 and rSsTrx-A2

The heat inactivation of rSsTrx-A1 and rSsTrx-A2 was studied by measuring their residual DTT insulin reduction activity after exposure of purified proteins at different temperatures. Both rSsTrx-A1 and rSsTrx-A2 were very stable proteins; in fact, significant loss of activity can be observed after several hours of treatment in the interval 92–99°C. The results reported in Fig. 6 indicate that rSsTrx-A1 (Fig. 6a) was slightly less stable than rSsTrx-A2 (Fig. 6b); for instance, at 92°C, the half inactivation time was 20.5 h and 56.6 h for of rSsTrx-A1 and rSsTrx-A2, respectively. However, this difference became less evident at higher temperatures. At each temperature tested, the inactivation process can be described as a first-order kinetics (Fig. 6c, d). This behavior allowed the analysis of the data according to the Arrhenius equation, whose plot is reported in Fig. 7. The data indicated that the heat inactivation process was characterised by different energy of

Table 1 Kinetic parameters of the reductase activity of rSsTrx-A1 and rSsTrx-A2

rSsTrx	Electron donor	pH	K_m^a (mM)	Maximum reductase activity[a] (ΔE/min)	Catalytic efficiency (ΔE/min/mM)
A1	DTT	5.5	0.270	0.092	0.341
	DTT	7.0	0.089	0.130	1.461
A2	DTT	5.5	0.337	0.347	1.030
	DTT	7.0	0.571	0.830	1.454
A1	NADPH/rSsTrx-B3	5.5	0.042	0.041	0.976
	NADPH/rSsTrx-B3	7.0	0.082	0.025	0.305
A2	NADPH/rSsTrx-B3	5.5	0.024	0.032	1.333
	NADPH/rSsTrx-B3	7.0	0.055	0.030	0.545

[a] These values represent the mean values of four to six determinations and the standard deviation never exceeded 8%

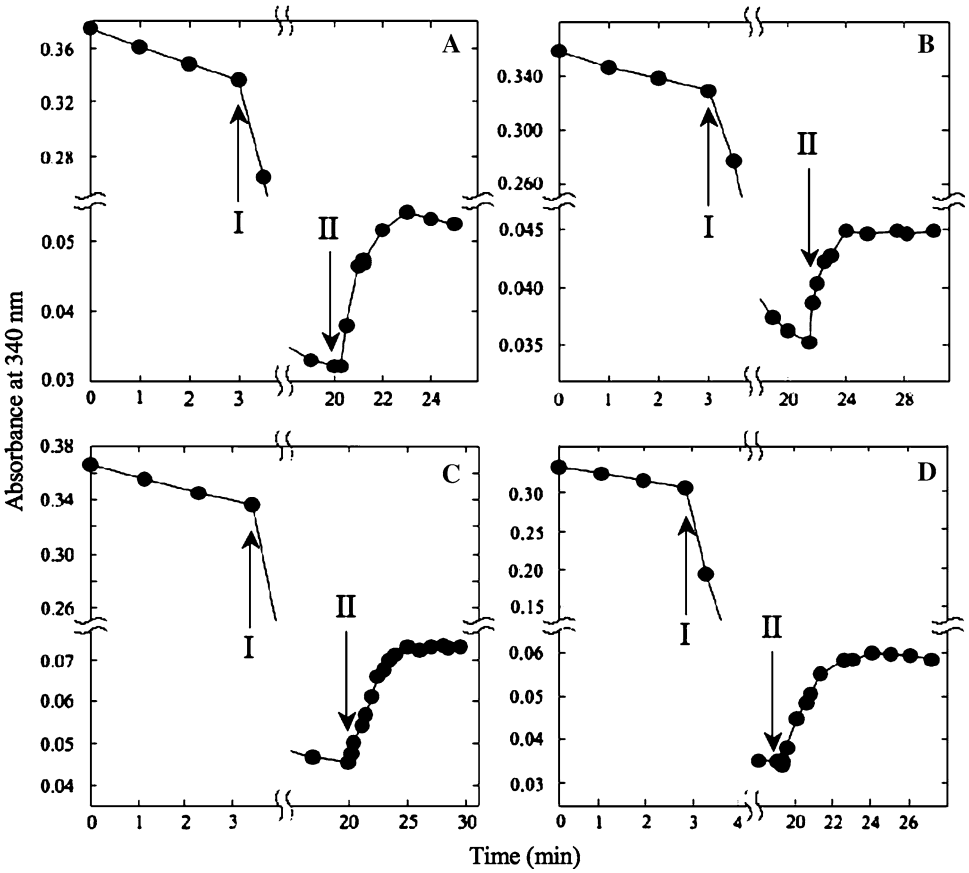


Fig. 5 Determination of the redox potential. Fifty micromolar intact (**a**, **c**) or truncated (**b**, **d**) rSsTrx-A1 (**a**, **b**) or rSsTrx-A2 (**c**, **d**) were mixed with 60 μM NADPH at 60°C. The NADPH oxidation/thioredoxin reduction was started by adding 2 μM rSsTrx-B3 at the time indicated by I. When the absorbance reached a constant low level, 1.2 mM $NADP^+$ was added at the time II, and the reaction was allowed to proceed until a constant higher level of absorbance at 340 nm was reached. The redox potential was calculated as reported in "Materials and methods"

activation, being 227 and 292 kJ/mol for rSsTrx-A1 and rSsTrx-A2, respectively.

Biochemical properties of rSsTrx-B2

Purified rSsTrx-B2, different from SsTrx-B3, was not isolated as a flavoprotein, as revealed from its fluorescence spectrum (not shown). rSsTrx-B2 was not able to transfer electrons from NADPH to insulin, in the thioredoxin reductase insulin reduction assay (Fig. 4b). We have then measured other NAD(P)H-dependent reductase/oxidase activity of the recombinant enzyme. In particular, no significant level of activity was observed in the DTNB-reduction, NAD(P)H oxidase, glutathione reductase and disulfide isomerase assays.

Discussion

In this paper, we have studied the biochemical properties of components of the thioredoxin system in *Sulfolobus solfataricus*, a hyperthermophilic and acidophilic archaeon

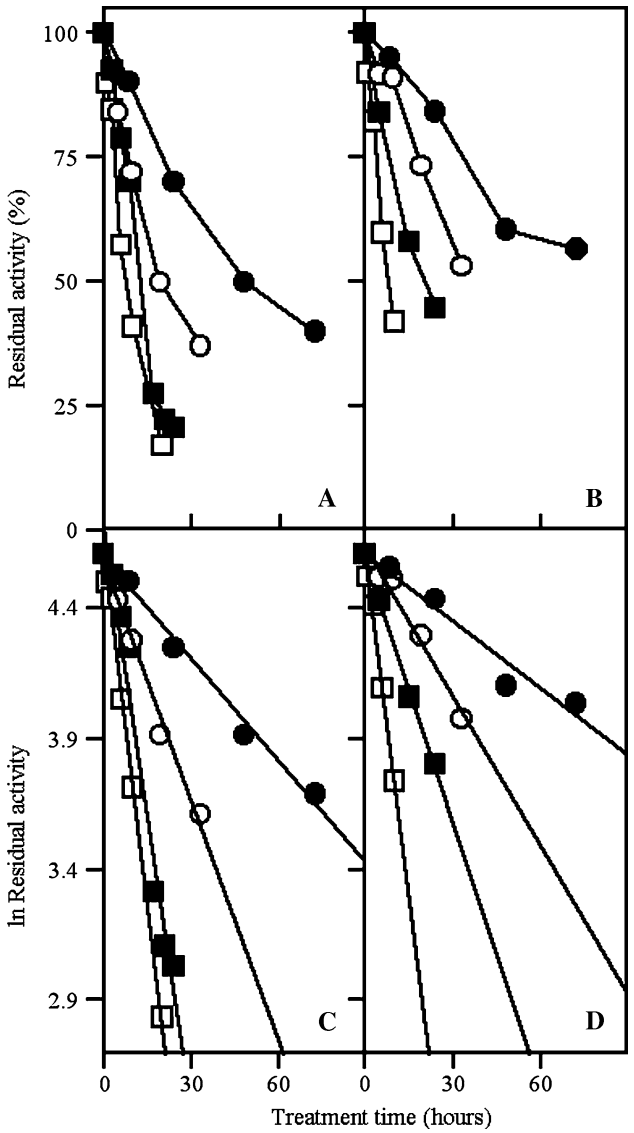


Fig. 6 Heat stability of rSsTrx-A1 or rSsTrx-A2. Fifty micromolar of rSsTrx-A1 (**a**) or rSsTrx-A2 (**b**) was incubated at 90 (*filled circle*), 94 (*open circle*), 96 (*filled square*) or 99°C (*open square*) in buffer A and at the times indicated, aliquots were withdrawn and analyzed for the residual insulin reduction activity using DTT as electron donor as reported in the text. A sample kept at 0°C was taken as untreated control. The data were treated as first-order kinetics and reported in (**c**) for rSsTrx-A1 and in (**d**) for rSsTrx-A2

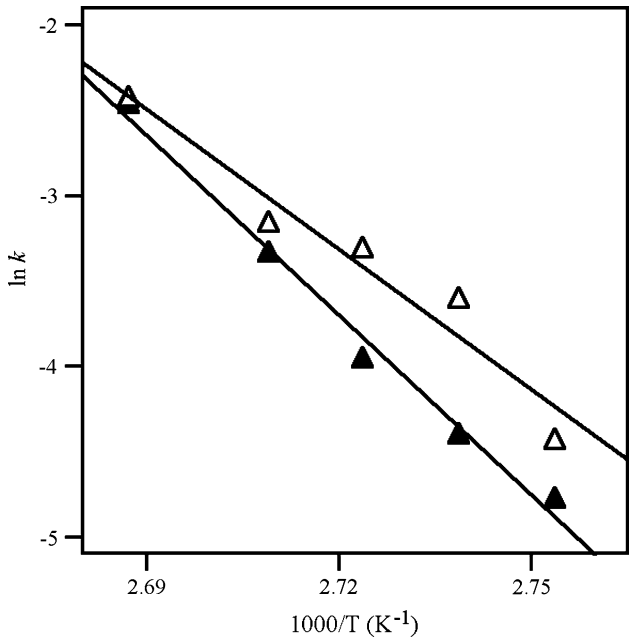


Fig. 7 Arrhenius analysis of the heat inactivation process. The kinetic constants of the heat inactivation process derived from Fig. 6a, b for rSsTrx-A1 (*open triangle*) and rSsTrx-A2 (*filled triangle*), respectively, were plotted according to the Arrhenius equation

growing optimally at 87°C and at pH 3.5 (De Rosa et al. 1986). In the genome of this archaeon, two thioredoxins (SsTrx-A1 and SsTrx-A2) and three thioredoxin reductases (SsTrx-B1, SsTrx-B2 and SsTrx-B3) have been putatively identified on the basis of sequence homology. We have previously characterised a thioredoxin reductase (SsTrx-B3) for which the determination of the 3D structure has been undertaken (Ruocco et al. 2004; Ruggiero et al. 2005). Here, we show that the thioredoxin system in *S. solfataricus* is constituted by a single thioredoxin reductase (SsTrx-B3), and two thioredoxins, SsTrx-A1 or SsTrx-A2, as homologous substrates. As expected, the specific reductase activity of SsTrx-B3 was higher than that elicited towards heterologous thioredoxin from *E. coli* (Ruocco et al. 2004). Moreover, the biological function of SsTrx-B2 remains still unclear, but considering the finding that it was not able to catalyse electron transfer from NADPH to thioredoxins from different sources, it did not belong to a thioredoxin system. Furthermore, SsTrx-B1 cannot be classified as a thioredoxin reductase, as it does not harbor the canonical CXXC motif even though it elicits only a FAD-dependent NAD(P)H oxidase activity (Arcari et al. 2000). As done previously for SsTrx-B1 (unpublished results) and SsTrx-B3 (Ruocco et al. 2004), SsTrx-A1, SsTrx-A2 and SsTrx-B2 have been obtained through the heterologous expression of the corresponding encoding gene in *E. coli*.

Purified recombinants rSsTrx-A1 and rSsTrx-A2 were obtained in either intact (15 kDa) or truncated forms (12 kDa) lacking an N-terminal fragment; all these thioredoxin forms displayed a homodimeric structure on gel-filtration under nondenaturing conditions, even in the presence of reducing agents. The loss of the N-terminal fragment on both rSsTrx-A1 and rSsTrx-A2 could be derived from an (auto)proteolytic cleavage, which is faster when the purification procedure was carried out in the absence of protease inhibitors. In fact, in the presence of these inhibitors, the formation of the truncated rSsTrx-A1

and rSsTrx-A2 takes place in a longer time. The data here reported, demonstrating that truncated and full-length forms have virtually identical activity, do not provide any indication of the role played by the residues present in the N-terminal region of rSsTrx-A1 and rSsTrx-A2. Although these regions might represent a signal peptide, they do not show any sequence similarity with known canonical localisation regions. However, the C-terminal truncation of human cytosolic thioredoxin has been reported as involved in its secretion (Pekkari and Holmgren 2004). Regarding the quaternary structure of both rSsTrx-A1 and rSsTrx-A2, a dimeric form was already reported for other thioredoxins belonging to both eubacterial and eukaryal sources, and different hypotheses have been proposed to explain the meaning of the dimerisation. For instance, in human thioredoxin, the dimerisation was involved in the regulation of its biological function (Jin et al. 2002); in *Thermus thermophilus* dimer formation was postulated to be important for the activity (Rehse et al. 2005); and finally in *Saccharomyces cerevisiae* dimeric thioredoxin 2 was obtained only at the millimolar concentration used during crystallisation trials (Bao et al. 2007).

As expected, rSsTrx-A1 and rSsTrx-A2 were very stable proteins, as inactivation can be detected only after several hours of treatment at temperatures beyond 90°C. The inactivation process of both rSsTrx-A1 and rSsTrx-A2 followed a first-order kinetics behavior at all the temperatures investigated, showing that rSsTrx-A2 was slightly more stable than rSsTrx-A1. This finding was also confirmed from the analysis of the energy of activation of the heat inactivation process that was lower for rSsTrx-A1 (227 kJ/mol) compared to that determined for rSsTrx-A2 (292 kJ/mol). These values were similar to those reported for other *S. solfataricus* enzymes (Masullo et al. 1993; Dello Russo et al. 1997).

Purified rSsTrx-A1 and rSsTrx-A2 were able to reduce human insulin in the presence of DTT as electron donor. This result was opposite to that recently published (Pedone et al. 2006), in which heterologously expressed His-tagged rSsTrx-A1 and rSsTrx-A2 were inactive in electron transfer activity to bovine insulin; it cannot be excluded that the presence of the histidine tag may affect the mechanism of the electrons transfer in that case. To verify whether rSsTrx-A1 and rSsTrx-A2 belonged to a thioredoxin system, they were also used as substrate of rSsTrx-B3 in the presence of NADPH as electron donor, in the insulin precipitation assay. The results here reported confirmed this hypothesis, which is reinforced by the finding that the maximum reductase activity was observed at pH 7 when DTT was used as electron donor, and at pH 5.5 when rSsTrx-B3 and NADPH were used instead. In fact, in our one of our previous work, we have reported that SsTrx-B3, purified from *S. solfataricus* cells, exhibited maximum NAD(P)H oxidase activity at acidic pH values (Masullo et al. 1996). The redox potential determination indicated that rSsTrx-A1 was slightly more oxidant than rSsTrx-A2. In addition, the truncated forms of both thioredoxins were slightly less oxidant than the intact ones, even though they showed identical reductase activity towards human insulin, an heterologous protein substrate. However, the data on the redox potential determination indicated that the electron transfer between SsTrx-B3 and SsTrx-A1 or SsTrx-A2 was fully reversible and then associable to a cyclic process, thus confirming both the functionality and the composition of the thioredoxin system in *S. solfataricus*.

The data described in this paper provide clear evidence that the thioredoxin/thioredoxin reductase system of *S. solfataricus* is rather intricate. Moreover, these results indicate that the assignment of the role and the partnerships of the protein involved in this system are not trivial and certainly cannot be accomplished by simple sequence homology analyses. Indeed, of the three putative TrxRs present in the *S. solfataricus* genome, only SsTrx-B3 plays a role in this archaeal thioredoxin system. On the other hand, the coexistence in the genome of two genes encoding for two functional thioredoxins indicates that some redundancy at functional level may exist. A deeper insight on the role played by these proteins in the *S. solfataricus* thioredoxin system likely requires 3D-strucutre characterisation of the individual components as well as of their complexes. Not only will the structural analysis of these proteins provide interesting insights into the molecular bases of the specificity of *S. solfataricus* thioredoxin system, but it will also provide clues on the structure–stability relationships in these hyperthermostable proteins. Investigation in this direction are in progress.

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