Reversion of protein aggregation mediated by Sso7d in cell extracts of *Sulfolobus solfataricus*

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In eukaryotic cells and in *Escherichia coli*, reversion of protein aggregation is mediated by the network of chaperones belonging to Hsp70 and Hsp100 families [Weibezahn, Bukau and Mogk (2004) *Microb. Cell Fact.* 3, 1–12]. The thermophilic prokaryotes of the archaea domain lack homologues of these chaperone families, and the mechanisms they use to rescue aggregated proteins are unknown [Macario, Malz and Conway de Macario (2004) *Front. Biosci.* 9, 1318–1332]. In the present study, we show that stable protein aggregates can be detected in extracts of starved cells of the thermophilic archaeon *Sulfolobus solfataricus*, and that the protein Sso7d interacts with the aggregates and mediates

the disassembly of the aggregates and the re-activation of insolubilized β -glycosidase in the presence of ATP hydrolysis. Furthermore, we report that heat-induced protein aggregates in extracts of exponential cells of *S. solfataricus* contain Sso7d that rescues insolubilized proteins in the presence of ATP hydrolysis. Results of these experiments performed in cell extracts are consistent with an *in vivo* role of Sso7d in reverting protein aggregation.

Key words: archaeon, chaperone, protein aggregation, Sso7d, *Sulfolobus*.

INTRODUCTION

Protein aggregation, a cause for irreversible loss of biological activity, results from the interaction of hydrophobic surfaces and generally involves non-native protein states which, unlike the native ones, do not have such surfaces buried. During physiological growth, protein aggregates are mainly formed as a consequence of interactions among the intermediates of the folding process and the non-native proteins generated by the occasional unfolding of native proteins. The accumulation of aggregated material is high during stress situations (e.g. heat shock and exposure to protein-denaturing agents), which increase the intracellular amount of hydrophobic protein surfaces. The proteins known as chaperones constitute the cellular defence against protein aggregation. Chaperones are a ubiquitous and heterogeneous group of proteins, most of which are stress-inducible, capable of reversible interaction with non-native proteins and, in some cases, endowed with an intrinsic ATPase activity, which is required for their 'chaperoning activity' [1]. The concept of chaperone does not contradict the *in vitro* renaturation of RNase A obtained by Anfinsen in the absence of any other component (this result led to the conclusion that the sequence of a protein dictates its three-dimensional structure); in fact, it is known that small one-domain proteins do not need assistance from chaperones in the cell to fold correctly, whereas a long polypeptide with more than one domain undergoes chaperone-assisted folding [2].

In bacteria and eukaryotes, the main families of ATP-dependent chaperones are Hsp60 (the so-called chaperonins), Hsp70 (very conserved proteins, considered to be the cellular thermometer) and Hsp100; they have housekeeping functions under physiological conditions and important damage-control functions during and after stress (Table 1). Among all the chaperones known, only those of the Hsp70 and Hsp100 families rescue aggregated proteins in an ATP hydrolysis-dependent reaction whose molecular details are still far from clear [3,4].

Archaea are prokaryotes that are distinct from bacteria and eukaryotes in the tree of life and mostly thrive in extreme environments [5]. Thermophilic archaea have a peculiar chaperone machinery [6]. The protein hallmark of the heat-shock response in these microorganisms is a chaperonin that has the overall structural architecture of all chaperonins (two superimposed multi-subunit rings that delimit a central chamber) but shares sequence homology with chaperonins of the eukaryotic cytosol (termed CCT, chaperonin-containing T-complex polypeptide-1); together with these proteins, it constitutes the Group II chaperonins, whereas the sequence-related chaperonins from bacteria and eukaryotic organelles constitute the Group I chaperonins. Group I chaperonins (GroEL of *Escherichia coli* is the archetype of this group) have seven identical subunits/ring; in archaeal chaperonins, more than seven subunits occur in each ring, and the existence of two or three non-identical subunits has been described [6]. Furthermore, GroEL requires a co-chaperonin GroES for its function; in contrast, no GroES homologue or other partner has been identified for Group II chaperonins. Strikingly, genome sequence analyses showed that thermophilic archaea lack homologues to the chaperones of the Hsp70 and Hsp100 families, and the mechanisms they use to revert protein aggregation are unknown.

The thermophilic archaeon *Sulfolobus solfataricus* contains a chaperonin that, similar to other chaperones of this family, prevents protein aggregation among non-native proteins but is unable to rescue proteins once they aggregate [7–10]. In the present study, we describe protein aggregates in cell extracts of *S. solfataricus* and show that the protein Sso7d interacts with the aggregates and promotes the disassembly of the aggregates and the re-activation of insolubilized proteins in the presence of ATP hydrolysis. The present study widens the knowledge of the chaperone machinery in thermophilic archaea and describes for the first time an activity of Sso7d, a protein whose physiological role is still unclear, in extracts of *S. solfataricus*.

Abbreviations used: AMP-PNP, adenosine 5'-[β , γ -imido]triphosphate; HMM, high molecular mass.

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Table 1 The main families of ATP-dependent chaperones in bacteria and eukaryotes

Chaperone family	Cellular function
Hsp60 (chaperonins)	
Bacteria and eukaryotic organelles	Protein folding; prevention of protein aggregation
Eukaryotic cytosol	Folding of actin and tubulin; cytoskeleton organization
Hsp70	Protein folding; prevention of protein aggregation; disassembly of protein aggregates; control of heat-shock response
Hsp100	Disassembly of protein aggregates; unfolding for degradation; thermotolerance

EXPERIMENTAL

Materials and analytic methods

[γ - 32 P]ATP (3000 Ci/mmol) was obtained from Amersham Biosciences. Nucleotides and Protein A–Sepharose were from Sigma. Other chemicals were of the highest grade available.

Protein concentration was determined by the Bradford assay [11] using BSA as the standard. For SDS/PAGE analysis, the samples were incubated for 15 min at 100 °C in the presence of 3 % (w/v) SDS and 5 % (w/v) 2-mercaptoethanol and then the proteins (20 μ g/lane) were separated on 15 % (w/v) polyacrylamide gels by the method of Laemmli [12] and stained with Coomassie Brilliant Blue. N-terminal in-gel microsequence analysis was performed by automated Edman degradation on an Applied Biosystems model no. 473A sequencer using the manufacturer's standard instructions and chemicals.

Cell growth and purification of Sso7d

Cells of *S. solfataricus* strain P2 were grown aerobically at 80 °C in DSM 182 culture medium supplemented with 50 mM glucose at the exponential phase (absorbance A_{600} 0.6); alternatively, cells were grown to the stationary phase (A_{600} approx. 1.0) and kept in culture for a further period of 4 days (starved cells). Cells harvested by centrifugation at 4000 *g* for 15 min were added to sand and 50 mM Tris/HCl (pH 7.5), containing 0.2 M NaCl, 5 % (w/v) glycerol and 3 mM PMSF and then homogenized in a Homni mixer. The homogenate was centrifuged at 4000 *g* for 20 min at 4 °C to remove the sand; the supernatant obtained after ultracentrifugation at 160 000 *g* for 90 min at 4 °C represented the extract (approx. 100 mg of protein/g of cell).

Sso7d was purified from the extract of exponential cells by the following procedure. The extract was loaded on to a Superdex 75 High Load column (2.6 cm $\times$ 60 cm; Amersham Biosciences) connected to an FPLC system, and eluted with buffer A (10 mM Tris/HCl, pH 7.5) containing 0.2 M NaCl at a flow rate of 2 ml/min; the fractions containing Sso7d were pooled, concentrated by poly(ethylene glycol) 6000 and re-chromatographed on a Superdex 75 High Load column (1.6 cm $\times$ 60 cm; Amersham Biosciences), which was eluted with buffer A containing 0.2 M NaCl at a flow rate of 0.8 ml/min.

Sso7d was purified from the extract of cells kept in culture over the stationary phase by the following procedure. The extract was dialysed against 100 vol. of 0.2 M sulphuric acid overnight at 4 °C and then ultracentrifuged at 140 000 *g* for 45 min at 4 °C; the supernatant was extensively dialysed against 200 vol. of buffer A. The dialysed sample was loaded on to a Mono S column (0.5 cm $\times$ 5 cm; Amersham Biosciences) equilibrated in buffer A and connected to an FPLC system; the bound proteins were eluted by a linear 0–0.4 M NaCl gradient in buffer A at a

flow rate of 0.5 ml/min in 40 min, and the fractions containing Sso7d (eluted at 0.34 M NaCl) were pooled.

Co-immunoprecipitation

A polyclonal serum directed against Sso7d was prepared in rabbit (Primm, Milan, Italy). ELISA tests were performed by standard techniques using 1:100 to 1:72 900 dilutions of the antiserum and the preimmune serum, and the antigen–antibody complexes were visualized by the peroxidase assay.

Cross-linking of antibodies to Protein A–Sepharose

Protein A–Sepharose beads (30 μ l) were incubated with excess preimmune serum (beads-1) or anti-Sso7d serum (beads-2) for 2 h at room temperature (25 °C); the beads were recovered by centrifugation at 2000 *g* for 10 min and washed twice with ice-cold buffer B (50 mM triethanolamine, pH 8.5); the antibodies were covalently linked to beads by reaction with 100 mM dimethyl-suberimidate in buffer B for 3 h at room temperature; the beads were recovered by centrifugation and washed with buffer B.

Co-immunoprecipitation and visualization of the co-precipitated proteins

Samples (3 mg) were incubated for 2 h at 4 °C with gentle mixing using beads-1 or beads-2; the beads were recovered by centrifugation at 2000 *g* for 10 min and washed first with buffer B and then with buffer A; the beads were incubated with 10 μ l of Laemmli buffer for 10 min at 100 °C and then centrifuged at 8000 *g* for 10 min, and the supernatant was analysed by SDS/PAGE (15 % gel).

Enzyme assays

The standard reaction mixture for the ATPase activity contained 2 mM ATP, 15 μ Ci of [γ - 32 P]ATP, 5 mM MgCl₂ and 10 μ g of sample in buffer A (final volume 150 μ l). After 10 min incubation at 70 °C, a 25 μ l aliquot was drawn from the assay mixture, added to 0.5 ml of a suspension containing 50 mM HCl, 5 mM H₃PO₄ and 7 % activated charcoal. It was centrifuged at 4000 *g* for 20 min and the radioactivity of the reaction product [γ - 32 P] was counted on a 100 μ l aliquot of the supernatant. The amount of spontaneous ATP hydrolysis has been corrected for in rate calculations.

Enzymes of *S. solfataricus* were assayed at 70 °C by a Cary 1E Varian recording spectrophotometer equipped with a thermostatically controlled cell compartment. The reaction mixture for β -glycosidase contained 5 mM *p*-nitrophenyl- β -D-galactopyranoside in buffer C (final volume 1 ml); the increase in A_{410} was followed, and the activity was calculated based on a molar absorption coefficient of 9.34×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ for *p*-nitrophenol. The reaction mixture for glutamate dehydrogenase contained 25 mM L-glutamate, 1 mM NADP and 0.1 mM EDTA in buffer C (50 mM sodium phosphate, pH 7.5) (final volume 1 ml); the increase in A_{340} was followed, and the activity was calculated based on a molar absorption coefficient of 6.3×10^3 litre $\cdot$ mol⁻¹ $\cdot$ cm⁻¹ for NADH. The activities of these enzymes were unaffected by the presence of excess anti-Sso7d serum or 10 mM ATP in the reaction mixtures.

Analysis of ATP content

ATP standards were loaded on to a C₁₈ reverse-phase column (Vydac, Hesperia, CA, U.S.A.; 0.46 $\times$ 25 cm; eluent, buffer C; flow rate, 0.5 ml/min; detection at 254 nm) connected to an HPLC system equipped with an area integrator, and the nucleotide peak areas versus μ mol of ATP (in the range 0.05–0.4) was plotted. The extract (0.5 mg) was incubated in 1 M HClO₄ for 30 min at

Figure 1 Isolation of the aggregated sample from the starved extract, protein composition of the aggregated sample and association of Sso7d with the aggregated proteins

(A) Superose 6 column (Amersham Biosciences; 2.6 cm $\times$ 60 cm; flow rate, 1 ml/min; eluent, buffer A containing 0.1 M NaCl) was separately loaded with 30 mg aliquots of the extracts prepared from starved (thick line) and exponential (thin line) cells of *S. solfataricus*. The shaded fraction in the profile relative to the starved extract indicates the elution of the HMM sample containing the aggregated proteins. Arrows indicate elution volumes of the protein standards (mass in kDa). (B) SDS/PAGE analysis (20 μ g/lane) of the aggregated sample from the Superose 6 column (lane 2), of the control of the co-immunoprecipitation (lane 3) and of aggregated proteins that were co-precipitated with anti-Sso7d serum (lane 4); the migration of molecular-mass standards is reported (lane 1). The proteins indicated were identified by N-terminal in-gel microsequencing analyses and by queries in the *Sulfolobus* genome bank. The following amino acid residues were determined: ATKFKYKGEEKE for Sso7d; YSFNPSFRF for β -glycosidase; SETQLSEGTRIKL for the transcriptional regulator; and AKKRSKLEIQ for the protease.

–20 °C and then centrifuged at 20000 *g* for 30 min. The supernatant was neutralized with NaOH, clarified by further centrifugation and analysed for ATP content as described above.

RESULTS AND DISCUSSION

Detection of stable protein aggregates in extracts of starved *S. solfataricus* cells

Cells of *S. solfataricus* were maintained in the stationary phase for 4 days; enzymes (alcohol dehydrogenase, malic enzyme, glutamate dehydrogenase and β -glycosidase) could not be detected in the total extract of these cells (referred to as the 'starved extract'); this result can be taken as an indicator of protein damage. Stable protein aggregates are resistant to dilution and they can be separated from the soluble proteins by gel-filtration chromatography [10]. The starved extract was chromatographed on a Superose 6 gel-filtration column (Figure 1A); in a comparison of the profile relative to the extract of exponential non-starved cells of *S. solfataricus* (thin line), the profile relative to the starved extract (thick line) featured a large amount of protein in the exclusion volume of the column [referred to as the HMM (high molecular mass) sample]. The HMM sample was reloaded on to the Superose 6 column after 1 h incubation at 37 °C in the presence of 4 M guanidinium chloride, 1 % SDS, 1 % Triton X-100

or 0.5 M NaCl; in any case, the column yielded one peak in the exclusion volume, showing that the sample withstood the dissociation by the compounds used (results not shown). Stable protein aggregates resist the dissociation by mild treatments with protein-denaturing agents [3]; therefore, the HMM sample will be referred to as the 'aggregated sample'. To the best of our knowledge, this is the first report on the identification of aggregated protein material in a hyperthermophilic microorganism. Electron microscopy analyses excluded the presence of ordered aggregates in the sample; our results do not rule out the possibility that ordered aggregated structures, such as amyloid plaques and prions, can be detected in hyperthermophilic cells under growth conditions different from those described here.

A search for stable protein aggregates in the extracts of heat-shocked or thermotolerant cells (i.e. cells capable of surviving exposure to a normally lethal temperature following a previous exposure to a high, non-lethal temperature) from *S. solfataricus* revealed that these extracts contain large protein assemblies that quickly dissociate on dilution (results not shown).

Association of Sso7d with the aggregated proteins

SDS/PAGE analysis of the aggregated sample after 15 min at 100 °C in the presence of 3 % SDS and 5 % 2-mercaptoethanol yielded the pattern shown in Figure 1(B, lane 2). As can be seen, the protein species present in the aggregated sample greatly varied in mass: the abundant small-sized band was identified as the monomeric 7 kDa protein Sso7d on the basis of in-gel N-terminal microsequencing.

Sso7d belongs to a family of constitutively abundant proteins present uniquely in Sulfolobales whose physiological role is still puzzling. The structure of Sso7d, consisting of a five-stranded incomplete β -barrel capped at the opening by a C-terminal α -helix [13], in the absence of sequence similarity with any known protein, was found to be similar to the chromo domain [14] and the eukaryotic SH3 domains (Src homology 3 domains) [15]. SH3 domains are small globular modules that mediate protein–protein interactions [16]. Sso7d has different biochemical activities *in vitro*. Sso7d displays non-specific DNA-binding activity (see [17] and references therein), and affects DNA topology by promoting the annealing of complementary DNA strands [18] and by inducing negative supercoiling [19,20]; these results allowed us to hypothesize that Sso7d is involved in protecting DNA against heat-induced damages. Sso7d has an intrinsic ATPase activity whose features are known [21]; we assume that the debated 'RNase activity' of Sso7d (see [22] and references therein) results from the ability of the protein to cleave the phosphodiester bond (e.g. during ATP hydrolysis). In the presence of ATP hydrolysis, pure Sso7d promotes the renaturation of preformed aggregates of purified proteins; Sso7d is the only molecule among those present in an *S. solfataricus* crude extract that rescues aggregated proteins *in vitro* [21].

We decided to investigate the association of Sso7d with the proteins in the aggregated sample by co-immunoprecipitation (see the Experimental section for details). A polyclonal serum directed against Sso7d was prepared; the results of ELISA tests showed that the anti-Sso7d serum, but not the preimmune serum, recognized Sso7d (results not shown). A method was established to obtain the covalent link of the IgG present in the preimmune serum (beads-1) and in the anti-Sso7d serum (beads-2) to Protein A–Sepharose beads by reaction with the bifunctional cross-linking agent, dimethylsuberimidate. We verified that pure Sso7d was precipitated by beads-2 but not by beads-1, and no appreciable precipitates were detected when beads-2 or beads-1 were incubated with a cellular extract from *E. coli* (results not shown).

Figure 2 ATPase activity of the aggregated sample

The assays were performed using 10 μ g of aggregated sample as described in the Experimental section except for the pH value (10 mM sodium acetate at pH 5.5, 10 mM Tris/HCl at pH 7–9) (**A**) and temperature (**B**). The activity measured after 10 min incubation at 70 °C in buffer A was set at 100 %.

The results showed that the antiserum was specific for Sso7d and it could be used for co-immunoprecipitation.

Incubation of the aggregated sample with beads-1 (the control) did not precipitate Sso7d or other proteins in the sample (Figure 1B, lane 3), whereas incubation with beads-2 precipitated Sso7d and several other proteins (Figure 1B, lane 4). We performed in-gel N-terminal microsequence analyses of the most abundant co-precipitating proteins; search in the *Sulfolobus* genome bank (www-archbac.u-psud.fr/projects/sulfolobus) identified a putative 14 kDa transcriptional regulator, the 10 kDa novel protease [23] and the β -glycosidase, a 240 kDa tetramer of identical subunits [24]. It is known that β -glycosidase of *S. solfataricus* undergoes *in vitro* irreversible thermal denaturation by aggregation [21].

Sso7d-dependent disassembly of the protein aggregates and re-activation of insolubilized β -glycosidase

We found that the aggregated sample displayed ATPase activity. When assayed over the pH range 5.5–9, maximum ATP hydrolysis was detected around neutral pH (Figure 2A); assays performed over the temperature range 50–90 °C showed that the optimal temperature was 70 °C (Figure 2B); ATPase activity of the aggregated sample was inhibited by vanadate and was unaffected by azide (results not shown). The ATPase activity assayed in the aggregated sample and that displayed by pure Sso7d [21] have the same optimum pH and optimum temperature and the same inhibitor sensitivity. Since the ATPase activity of Sso7d is independent of its binding to protein molecules [25], we hypothesized that Sso7d in complex with aggregated proteins hydrolysed ATP. Incubation of the aggregated sample with anti-Sso7d serum before the assay abolished the activity, implying that Sso7d was responsible for ATP hydrolysis.

The aggregated sample was incubated at 70 °C for 30 min either alone or in the presence of hydrolysable ATP (ATP/Mg consisting of 10 mM ATP and 5 mM MgCl_2) and was then loaded on to a Superose 6 column (Figure 3); a 'disassembly' was detected after

Figure 3 Sso7d-dependent disassembly of the aggregated sample and re-activation of insolubilized β -glycosidase

Superose 6 column [2.6 cm $\times$ 60 cm; flow rate, 1 ml/min; eluent, 0.1 M sodium phosphate (pH 7.0) containing 0.1 M NaCl] was separately loaded with the aggregated sample (10 mg), which was incubated for 30 min at 70 °C either alone (run a) or in the presence of ATP/Mg (run b) or AMP-PNP/Mg (run c). Arrows indicate elution volumes of the protein standards (masses in kDa). Peaks of the nucleotides are omitted. β -Glycosidase activity was assayed as described in the Experimental section.

ATP hydrolysis (run b) but not after heat treatment (run a). If the area of the peak relative to run a was set at 100 %, the area of the peak relative to run a was reduced by approx. 60 % in the presence of ATP hydrolysis; the same result was observed on extending the incubation time. The results obtained raised the question whether binding or hydrolysis of ATP was required for the disassembly of the sample. To address this point, the aggregated sample was incubated at 70 °C for 30 min in the presence of a non-hydrolysable ATP analogue AMP-PNP/Mg [consisting of 10 mM AMP-PNP (adenosine 5'-[β , γ -imido]triphosphate) and 5 mM MgCl_2], and was loaded on to the Superose 6 column; in this case, no disassembly was detected (run c). Furthermore, no disassembly was obtained when the aggregated sample was incubated in the presence of GTP, ADP or dATP (results not shown); it is known that Sso7d does not hydrolyse these nucleotides [19].

We speculated whether the disassembly of the aggregated sample was associated with re-activation of inactive proteins. No β -glycosidase activity was detected in the aggregated sample using *p*-nitrophenyl- β -D-galactopyranoside as the substrate (β -glycosidase is the only protein of *Sulfolobus* which hydrolyses this compound). Enzyme assays performed on the fractions relative to the three chromatographic runs detected a peak with β -glycosidase activity in run b (native β -glycosidase had the same elution volume), whereas no activity was detected in runs a and c (Figure 3). No β -glycosidase activity was regained when the

aggregated sample was incubated with excess anti-Sso7d serum before incubation with ATP/Mg.

Taken together, these results show that Sso7d mediates the disassembly of the aggregated sample and the re-activation of an insolubilized enzyme in the presence of ATP hydrolysis.

Sso7d-dependent rescue of heat-induced protein aggregates in extracts of exponential *S. solfataricus* cells

To summarize the results presented above, we showed that (i) stable protein aggregates exist in starved cells of *S. solfataricus*, (ii) Sso7d interacts with the aggregated proteins and (iii) Sso7d mediates the disassembly of aggregated material and the re-activation of an insolubilized protein in a process which requires the hydrolysis of ATP.

Unlike the starved extract, the total extract of exponential cells of *S. solfataricus* (referred to as the 'exponential extract') contained various enzymes whose specific activities were in the expected ranges, and it did not contain stable protein aggregates (results not shown). The starved extract and the exponential extract contained the same amount of Sso7d (approx. 3% of the total protein content). However, in the starved extract, Sso7d was strongly associated with the proteins, and its purification required a procedure that disrupts the protein–protein interactions; in contrast, Sso7d could be purified from the exponential extract by a mild procedure (see the Experimental section). We found that the ATP concentration in the exponential extract was in the range 1.5–1.8 mM, which averages that of endogenous ATP in mammalian cells; the nucleotide concentration in the starved extract decreased to approx. 0.05 mM, which is an indicator of cellular stress (see the Experimental section). Since the K_m value of Sso7d for ATP is 0.2 mM [19], it is possible that the low ATP concentration in starved cells does not favour the release of Sso7d from the aggregates.

We dialysed the exponential extract to lower the concentration of ATP and studied the effects of Sso7d on protein aggregation induced in this extract by heat treatment. A 1 ml solution of dialysed 38 mg/ml extract was kept at 80 °C, followed by centrifugation to separate the aggregated proteins (the pellet fraction) from the soluble proteins (the supernatant fraction); after 60 min incubation, the formation of protein aggregates was negligible. Therefore we incubated the extract at 90 °C; after heating for 60 min, the solution showed visible aggregates and the relative pellet fraction contained 65% of the total protein. Enzyme assays showed that the supernatant fraction was devoid of β -glycosidase and glutamate dehydrogenase (a hexamer of 45 kDa sub-units) activities; it is reasonable to assume that these enzymes underwent thermal denaturation under the described experimental conditions. On the other hand, the dialysed extract and the supernatant fraction contained the same units of malic enzyme and alcohol dehydrogenase, demonstrating that these enzymes withstood the exposure to heat.

We performed SDS/PAGE analysis of the pellet fraction and found that Sso7d was present in the sample (Figure 4A, lane 2); since Sso7d is monomeric in solution over a wide range of protein concentrations [17], we concluded that Sso7d established an interaction with the aggregated proteins. Pellet fractions were resuspended in buffer alone or in buffer containing ATP/Mg or in buffer containing AMP-PNP/Mg and then incubated at 70 °C; after 30 min incubation, the samples were centrifuged and the supernatant fractions were analysed for protein content. The incubations in buffer alone and in buffer containing AMP-PNP/Mg did not reveal detectable amounts of protein in the solution, whereas the incubation in buffer containing ATP/Mg resulted in the resolubilization of approx. 10% of protein of the pellet fraction. SDS/

Figure 4 Sso7d-dependent rescue of heat-induced protein aggregates in the exponential extract

(A) The exponential extract of *S. solfataricus* was dialysed against 20 vol. of buffer A overnight at 4 °C. A 38 mg/ml solution of dialysed extract (1 ml, containing 5.0 units of β -glycosidase and 0.4 unit of glutamate dehydrogenase) was incubated at 90 °C for 60 min. Centrifugation at 4000 g for 15 min yielded the aggregated proteins in the pellet fraction, whose SDS/PAGE analysis (20 µg/lane) is shown in lane 2 (the migration of molecular-mass standards is shown in lane 1). The pellet fraction (10 mg) was resuspended in buffer A containing ATP/Mg, and centrifugation at 4000 g for 15 min yielded the resolubilized proteins in the supernatant fraction, whose SDS/PAGE analysis (20 µg/lane) is shown in lane 3. (B) Sso7d-dependent re-activations of aggregated glutamate dehydrogenase and β -glycosidase in the presence of ATP hydrolysis. Pellet fractions (10 mg) were resuspended in buffer C with or without nucleotides, excess anti-Sso7d serum and pure Sso7d as indicated and incubated at 70 °C for 30 min; the samples were then centrifuged at 4000 g for 15 min, and the supernatant fractions were assayed for glutamate dehydrogenase and β -glycosidase activities as described in the Experimental section. The activity regains were calculated as percentages with respect to the number of units of native enzymes present in the dialysed extract.

PAGE analysis showed that different aggregated proteins were resolubilized (Figure 4A, lane 3). The enzyme assays showed that β -glycosidase and glutamate dehydrogenase were among the resolubilized proteins; regains of 35 and 10% respectively of β -glycosidase activity and glutamate dehydrogenase activity were calculated; the inclusion of excess anti-Sso7d serum in the incubation mixture containing ATP/Mg abrogated the activity regains (Figure 4B). We verified that the percentage of resolubilized proteins and the regains of β -glycosidase and glutamate dehydrogenase activities did not increase after incubating at 70 °C for

60 min instead of 30 min in buffer alone or in buffer containing the nucleotides. We hypothesized that the amount of Sso7d present in the pellet fraction could be limiting for the rescue of the enzymic activities. Indeed, the regains of β -glycosidase and glutamate dehydrogenase activities were increased in a dose-dependent manner by the addition of pure Sso7d into the pellet fractions to be incubated in the presence of ATP/Mg (Figure 4B).

Conclusions

The present study indicates that Sso7d is a protein of *S. solfataricus* involved in the reversal of protein aggregation and describes for the first time an activity of Sso7d in extracts of the archaeon. Sso7d is a constitutively abundant protein of *Sulfolobus* whose physiological role is still unclear. The definition of the biological role(s) of Sso7d is hampered by the poor knowledge of many DNA-related events in Sulfolobales and by the lack of molecular tools to obtain targeted mutants in these microorganisms. Many *in vitro* studies clearly show that Sso7d remodels DNA for the sake of the stability of the nucleic acid at high temperatures. Since the DNA- and protein-binding activities of Sso7d are *in vitro* mutually exclusive events due to the occurrence of the same or overlapping surfaces for interacting with DNA or protein aggregates [25], we hypothesize that Sso7d could be a multifunctional protein with protective functions towards both DNA and proteins. It is worth noting that the binding of Sso7d to protein aggregates is reversed by ATP hydrolysis *in vitro* [21] as well as in cell extracts (the present study), whereas the binding of the protein to DNA is independent of pH and temperature values and ATP binding/hydrolysis [18]. The competition between protein aggregates and DNA for the interaction with Sso7d [25] could mirror the physiological mechanism of regulation of the Sso7d activities; alternatively, protein factors, yet to be identified, which regulate the binding of Sso7d to the nucleic acid could exist.

Based on experiments performed using pure Sso7d and performed aggregates of purified proteins [21] and the experiments performed in cell extracts (the present study), Sso7d displays a 'chaperone' behaviour in mediating the disassembly of protein aggregates. Sso7d is not obviously related to any of the known chaperone molecules. Many chaperones are active as oligomeric structures, except members of the Hsp70/DnaK family, which function as monomers; there is no evidence in the literature that Sso7d forms polymers and, in our experiments, Sso7d is in monomeric status. However, Sso7d shares some functional features with conventional chaperones: (i) it binds to non-native proteins; (ii) it has an intrinsic ATPase activity (the turnover number for ATP hydrolysis falls within the range of values reported for chaperones of the Hsp70 family), which is required for the renaturation of non-native proteins; and (iii) it cycles between a conformation, in the absence of nucleotide, which binds to non-native proteins and an ATP hydrolysis-induced conformation that is incapable of this interaction [21].

The mechanism by which chaperones of the hsp70 and hsp100 families rescue protein aggregates is still poorly defined [3,4]. Since Sso7d does not renature fully unfolded conformations of proteins (A. Guagliardi, unpublished work), the aggregated proteins probably do not undergo complete unfolding in the pathway towards their native conformations. We hypothesize that Sso7d can destabilize the aggregate through repeated interactions with the hydrophobic surfaces of the aggregate. Experiments are currently being performed with the purpose of obtaining information about the mechanism of Sso7d-mediated protein disassembly.

Given their intrinsic resistance to heat treatment and protein denaturants and their 'chaperoning activity' in the absence of other molecules, the chaperones of thermophilic archaea are useful tools to renature inactive protein materials [26]. Inclusion bodies are inactive aggregates, which accumulate in host cells after the overexpression of foreign proteins; current methods to regain biologically active proteins consist of solubilization of the protein aggregate in denaturants and dilution in media containing 'folding enhancers', such as detergents, arginine, poly(ethylene glycol) and thiol reagents [27]. However, the efficiency of the process may be very low, mainly with multisubunit and disulphide bond-containing proteins.

Finally, exploring the mechanisms of chaperone-mediated protein disaggregation in different organisms could suggest strategies to protect and cure organisms from the diseases termed amyloidoses (including Alzheimer's disease, cystic fibrosis, Type II diabetes, the transmissible spongiform encephalopathies, Creutzfeldt-Jakob disease and Huntington's disease), which are associated with the deposition in tissues of proteinaceous aggregates [28].

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