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The chaperonin from the archaeon *Sulfolobus solfataricus* promotes correct refolding and prevents thermal denaturation in vitro

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Abstract

We have isolated a chaperonin from the hyperthermophilic archaeon *Sulfolobus solfataricus* based on its ability to inhibit the spontaneous refolding at 50 °C of dimeric *S. solfataricus* malic enzyme. The chaperonin, a 920-kDa oligomer of 57-kDa subunits, displays a potassium-dependent ATPase activity with an optimum temperature at 80 °C. *S. solfataricus* chaperonin promotes correct refoldings of several guanidine hydrochloride-denatured enzymes from thermophilic and mesophilic sources. At a molar ratio of chaperonin oligomer to single polypeptide chain of 1:1, *S. solfataricus* chaperonin completely inhibits spontaneous refoldings and suppresses aggregation upon dilution of the denaturant; refoldings resume upon ATP hydrolysis, with yields of active molecules and rates of folding notably higher than in spontaneous processes. *S. solfataricus* chaperonin prevents the irreversible inactivations at 90 °C of several thermophilic enzymes by the binding of the denaturation intermediate; the time-courses of inactivations are unaffected and most activity is regained upon hydrolysis of ATP. *S. solfataricus* chaperonin completely prevents the formation of aggregates during thermal inactivation of chicken egg white lysozyme at 70 °C, without affecting the rate of activity loss; ATP hydrolysis results in the recovery of most lytic activity. Tryptophan fluorescence measurements provide evidence that *S. solfataricus* chaperonin undergoes a dramatic conformational rearrangement in the presence of ATP/Mg, and that the hydrolysis of ATP is not required for the conformational change. The ATP/Mg-induced conformation of the chaperonin is fully unable to bind the protein substrates, probably due to disappearance or modification of the substrate binding sites. This is the first archaeal chaperonin whose involvement in protein folding has been demonstrated.

Keywords: Archaea; chaperonin; protein aggregation; protein folding

The evidence that protein folding in the cell is assisted by proteins termed molecular chaperones is well documented (see Gething & Sambrook, 1992, for a review). The specialized term "chaperonins" describes a family of molecular chaperones that promote correct folding by binding to non-native structures that are folding intermediates, thus preventing the aggregation of hydrophobic surfaces and coupling their release in a correctly folded form to the binding and/or hydrolysis of ATP (see Ellis & van der Vies, 1991, and Hartl et al., 1992, for reviews). Chaperonins are constitutively abundant proteins essential for cell viability; their cellular levels increase upon stress, constituting an "emergency system" that limits the molecular damage caused by harsh environmental conditions. Chaperonins have a complex quaternary structure: 2 rings of about 60-kDa subunits each are stacked face to face with a central cavity. GroEL, hsp60, and ribulose 1,5-bisphosphate carboxylase oxygenase (RuBisCo)-binding protein—the chaperonins isolated from *Escherichia coli*, mitochondria, and chloroplasts, respectively—consist of heptameric rings and have highly similar primary sequences. Sequence-related co-chaperonins, consisting of a single ring of seven 10-kDa subunits, are required for the full function of GroEL, hsp60, and RuBisCo-binding protein (Laminet et al., 1990; Lubben et al., 1990; Hartman et al., 1992; Langer et al., 1992).

The prokaryotes that belong to the kingdom of Archaea display unique genetic, morphological, and physiological features with respect to Bacteria (Woese et al., 1990; Wheelis et al., 1992). Chaperonins isolated from thermophilic Archaea display the typical double-ring structure with some differences: in each ring, 8 subunits are present in the chaperonin from *Pyrodictium*

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occultum (Phipps et al., 1991, 1993), 9 subunits in the chaperonin, named TF55, from *Sulfolobus shibatae* (Trent et al., 1991), and in the chaperonin from *S. solfataricus* (Marco et al., 1994). Co-chaperonins have not been isolated for archaebacterial chaperonins.

The primary structure of TF55 (Trent et al., 1991) showed no significant relationship to GroEL, hsp60, or RuBisCo-binding protein, but nearly 40% identity with the t-complex polypeptide 1, TCP-1, a eukaryotic protein that plays an important role in vivo and in vitro in tubulin assembly and in the folding of actin (see Horwich & Willison, 1993, for a recent review). Therefore, based on sequence data, archaeal chaperonins and chaperonins from eukaryotic cytosol have been proposed to be members of the same class (Ellis, 1992; Hartl et al., 1994). As concerns the functional role, the demonstration that archaeal chaperonins assist protein folding is lacking.

This paper describes the activity of the *S. solfataricus* chaperonin (which we call Ssocpn) in promoting correct refolding and preventing thermal denaturation of several thermophilic and mesophilic proteins.

Results

Purification of the chaperonin and molecular size determinations

In order to recognize Ssocpn through the purification procedure, we assessed an in vitro assay (the assay of chaperoning activity) by using malic enzyme (ME) from *S. solfataricus* as substrate protein (Bartolucci et al., 1987; Guagliardi et al., 1988). ME was denatured by incubation in excess guanidine hydrochloride and 2-mercaptoethanol and allowed to refold spontaneously at 50 °C upon dilution of the reagents and lowering of the protein concentration. The regain of activity was determined by measuring the ME activity in aliquots drawn from the renaturation mixture at time intervals (see below for details). The assay consisted of 2 steps: first, the dilution of denatured ME into a renaturation mixture containing an aliquot from the chromatographic fraction; second, the determination of the ME activity regained. The fractions able to completely arrest the spontaneous regain of activity were considered active.

The crude extract of *S. solfataricus* was loaded onto a Superose 6 gel filtration chromatography and the entire profile of elution was tested with the chaperoning assay. No regain of ME activity was detected in the renaturation mixtures containing an aliquot from the first peak eluted from the column (active sample): an ME activity regain similar to that of the spontaneous event was obtained in the renaturation mixtures containing an aliquot from all the other peaks (inactive samples). The active sample was fractionated on a Matrex Gel Red A affinity chromatography column whose ligand is a dye that mimics the structure of a nucleotide coenzyme molecule; the flowthrough was found inactive, and the active fractions eluted by a linear salt gradient contained a homogeneous protein, whose subunit molecular weight was 57 kDa (Fig. 1). The native molecular weight of Ssocpn was determined by gel filtration chromatography on an analytical Superose 6 column (Pharmacia, 0.32 × 30 cm) connected to a SMART system (Pharmacia, LKB), eluted with Buffer A supplemented with 0.1 M NaCl at a flow rate of 30 μL/min. A calibration curve was constructed using thyroglobulin (669 kDa), ferritin (440 kDa), catalase (232 kDa), and aldolase (158 kDa) as standards, and a molecular size of 920 kDa was calculated by extrapolation (not shown). This native size was used for the calculations of the molar concentration of Ssocpn.

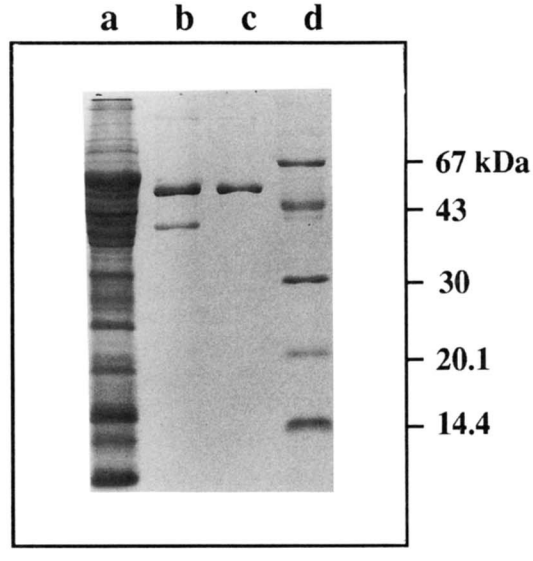

Fig. 1. SDS-PAGE of the active samples at different stages of Ssocpn purification. Lane a, crude extract; lane b, pooled active fractions from Superose 6 column; lane c, pooled active fractions from Matrex Gel Red A column; lane d, molecular weight standards. Samples (6 μg) were heated at 100 °C for 15 min in 2% SDS, 5% 2-mercaptoethanol, 5% glycerol, and the run was performed using 5% stacking gel and 12.5% separating gel. Proteins were revealed by Coomassie Blue staining.

The ATPase activity of the chaperonin

The ATPase activity of Ssocpn was fully dependent on the presence of potassium ions. Figure 2 reports the amount of Pi released from [γ-^{32}P]ATP calculated in assay mixtures containing Ssocpn, in the absence (the squares) or in the presence (the closed

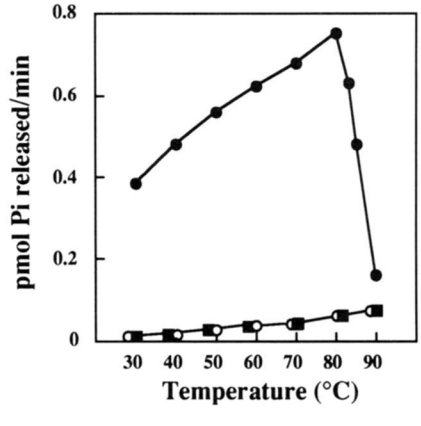

Fig. 2. Potassium and temperature dependence of the ATPase activity of purified Ssocpn. The amount of Pi released was relative to the Ssocpn-mediated hydrolysis in the absence (■) or in the presence (●) of potassium ions and to the spontaneous hydrolysis of ATP in the absence of Ssocpn (○). The experimental details are described under Materials and methods.

circles) of potassium ions, as a function of the assay temperature in the range from 30 °C to 90 °C; the rate of spontaneous hydrolysis of ATP (the open circles) over the same temperature range is depicted. In the absence of K^+ ions, the rate of Ssocpn-mediated hydrolysis, for every temperature value, was comparable to that of the spontaneous, noncatalyzed hydrolysis of the nucleotide at that temperature. In the presence of K^+ ions, Ssocpn catalyzed an effective hydrolysis of ATP and it displayed an optimal ATPase activity at 80 °C; in the rate calculations, the amount of spontaneous hydrolysis has been corrected for.

The chaperonin undergoes a conformational change in the presence of ATP/Mg

A 1-mL solution containing 100 μg pure Ssocpn excited at 295 nm displayed a large emission peak between 310 and 390 nm (Fig. 3, trace a): the maximum centered around 345 nm is typical of tryptophanyl residues. From the ultraviolet absorbance at 280 nm, 29 tryptophan residues per molecule were calculated.

We used tryptophan fluorescence to investigate eventual conformational rearrangements in the Ssocpn molecule upon ATP binding (in the presence of 0.5 mM ATP, 0.5 mM $MgCl_2$, briefly ATP/Mg) or hydrolysis (in the presence of 0.5 mM ATP, 0.5 mM $MgCl_2$, 10 mM KCl, briefly ATP/Mg/K). Two samples of pure Ssocpn were incubated for 10 min at 50 °C with ATP/Mg or ATP/Mg/K and chromatographed on a Superose 6 gel filtration chromatography to remove the nucleotide; each sample (1 mL containing 100 μg) was excited at 295 nm and its emission spectrum was recorded between 310 and 390 nm (Fig. 3, traces b, c). We verified that the emission spectrum of native Ssocpn (trace a) did not vary following incubation of the sample for 10 min at 50 °C. Traces b and c were superimposable and dramatically different from trace a: the decrease in the fluorescence intensity and the blue shift of the maximum at 327 nm could reflect transfer of Trp residues to a less polar environment. In light of these results, we can conclude that Ssocpn undergoes a dramatic conformational rearrangement in the presence of ATP/Mg, and that the hydrolysis of the nucleotide is dispensable for this change.

The chaperonin promotes correct refolding

We reconstituted in vitro refoldings of 3 chemically denatured enzymes from *S. solfataricus*: dimeric 50-kDa subunit ME (Bartolucci et al., 1987; Guagliardi et al., 1988), dimeric 35-kDa subunit ADH (Ammendola et al., 1992), and exameric 45-kDa subunit GDH (Consalvi et al., 1991). The enzymes were incubated for 1 night at 37 °C in 4 M guanidine hydrochloride, except for ME, which also required excess 2-mercaptoethanol to reduce intersubunit disulfide bonds (Guagliardi et al., 1988). Following this treatment, the enzymes were found completely inactive and lacking secondary structures as judged from CD spectra recorded in the far ultraviolet range (not shown). The spontaneous refoldings, negligible at 70 °C, were obtained at 50 °C upon dilution from the denaturants in renaturation mixtures designed for each enzyme as concerns protein amount, buffer, pH, ionic strength, and cofactors. Refolding of zinc-containing ADH (Ammendola et al., 1992) required the presence of 5 μM zinc chloride in the renaturation mixture; no refolding was obtained in the absence or at different concentrations of the salt. The spontaneous refoldings of ME, ADH, and GDH (Fig. 4) were not affected by the presence of bovine serum albumin or ATP/Mg/K in the renaturation mixtures.

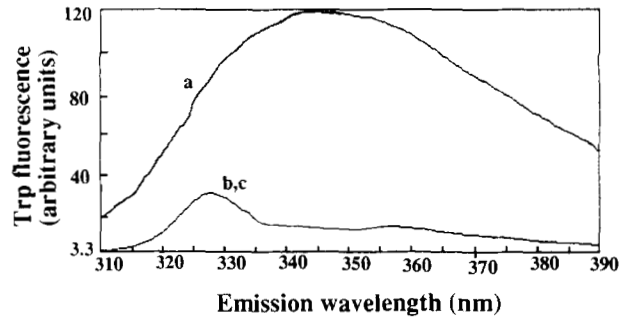

Fig. 3. Tryptophan fluorescence emission spectra of purified Ssocpn (trace a), Ssocpn after incubation in the presence of ATP/Mg (trace b), and Ssocpn after incubation in the presence of ATP/Mg/K (trace c). A Perkin Elmer LS 50B spectrofluorimeter was used.

The effects of purified Ssocpn on spontaneous refoldings at 50 °C of ME, ADH, and GDH were investigated by diluting denatured enzymes into renaturation mixtures containing Ssocpn in a molar ratio of 1 chaperonin oligomer to 1 single polypeptide chain. Complete inhibitions of spontaneous refoldings were observed for each enzyme (Fig. 4) as a consequence of the formation of a complex between the chaperonin and the refolding intermediate. When we tried to remove the inhibitions, it was observed that: (1) the addition of ATP/Mg in the renaturation mixtures had no effect; (2) the refoldings resumed upon the addition of ATP/Mg/K, and optimum yields were achieved within 3 min; and (3) the inhibitions persisted upon the addition of 0.5 mM ADP, 0.5 mM $MgCl_2$, 10 mM KCl. Therefore, hydrolysis, and not simply binding of ATP, is required to dissociate the complex between Ssocpn and the refolding intermediate. The final yields in Ssocpn-assisted refoldings were higher with respect to those of spontaneous events; the same yields are achieved upon addition of ATP/Mg/K at 12 h after starting the incubation at 50 °C, demonstrating that the complex between the chaperonin and the refolding intermediate is very stable.

Does Ssocpn assist refolding of a mesophilic enzyme? To answer this question, we studied the Ssocpn-assisted refolding of chemically denatured ME from chicken liver, a 65-kDa subunit tetramer. The denaturation of chicken ME was irreversible upon dilution from chaotropic agent and incubation at 40 °C (Fig. 5A) or at room temperature; the increase of light scattering monitored at 40 °C (Fig. 5B) showed that the rapid aggregation of the chains upon dilution from denaturants prevented refolding. We diluted denatured ME in the renaturation mixture at 40 °C containing Ssocpn in a molar ratio of 1 chaperonin oligomer to 1 single polypeptide chain; the chaperonin completely suppressed aggregation reactions during refolding (Fig. 5B) and an activity regain of 45% was obtained within 3 min (Fig. 5A) from the addition of ATP/Mg/K in the renaturation mixture. The yield of active enzyme did not exceed 45% when refolding was carried out at lower temperature or protein concentration or when using a molar excess of chaperonin: nonproductive side reactions (i.e., unspecific adsorption to vessels) are likely to occur.

We wondered whether the conformational rearrangement that Ssocpn undergoes in the presence of ATP/Mg influences its ability to bind the refolding intermediate. The denatured enzymes

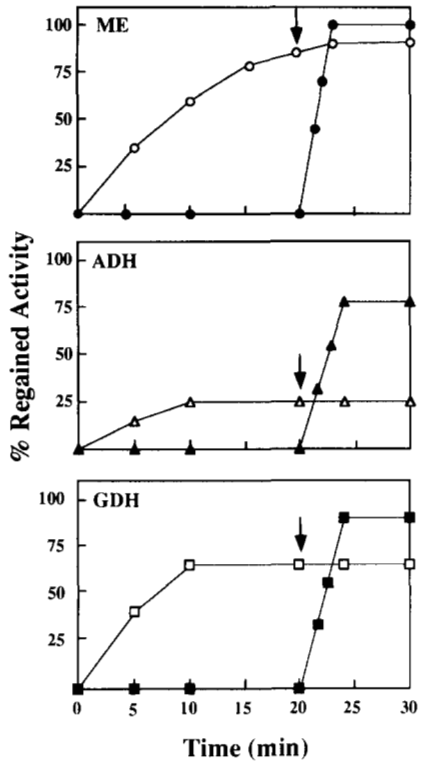

Fig. 4. Refolding of chemically denatured ME, ADH, and GDH from *S. solfataricus* in the absence (○, △, □) and in the presence (●, ▲, ■) of purified Ssocpn. The enzymes were denatured upon incubation for 1 night at 37 °C in mixtures composed as follows: ME, 4 M guanidine hydrochloride, 0.2 M 2-mercaptoethanol (80 μg protein per mL); ADH, 4 M guanidine hydrochloride (600 μg protein per mL); GDH, 4 M guanidine hydrochloride (100 μg protein per mL). Aliquots drawn from the denaturation mixtures were diluted in the following renaturation mixtures (final volume 6 mL) incubated at 50 °C in the absence or in the presence of Ssocpn at an equimolar ratio with the single polypeptide chains (reported in parentheses): ME, 50 mM sodium phosphate, pH 8.0 (32 nM); ADH, 0.1 M sodium phosphate, pH 8.0, 5 μM $ZnCl_2$ (120 nM); GDH, 0.1 M sodium phosphate, pH 8.0 (38 nM). After 20 min, ATP/Mg/K was added to the mixtures (arrows) and the incubations were continued at the same temperature. At time intervals, the enzymatic activities were assayed on aliquots drawn from the mixtures; the activity regains were calculated as percentages with respect to the specific activities of the native enzymes.

(ME, ADH, and GDH from *S. solfataricus*, and chicken ME) were diluted into renaturation mixtures containing Ssocpn previously incubated at 50 °C for 10 min in the presence of ATP/Mg, and the experiments were continued according to the standard protocols. Spontaneous refoldings of thermophilic enzymes were not arrested because their renaturation profiles were identical to spontaneous events; aggregation during refolding of chicken ME was not suppressed, and no enzyme activity was regained upon addition of ATP/Mg/K. We conclude that, upon incubation with ATP/Mg, Ssocpn does not bind the refolding intermediate, which, free in solution, undergoes refolding and/or aggregation. It is conceivable to hypothesize that the ATP/Mg-induced conformation of Ssocpn does not carry the surface(s) responsible for the binding of the protein substrate.

The chaperonin prevents irreversible heat inactivation

ME, ADH, and GDH from *S. solfataricus* were completely inactive after 90 min of incubation at 90 °C, and the inactivations were irreversible upon shifting down the temperature at 50 °C (Fig. 6). Aggregation did not occur during inactivations, as demonstrated by the absence of light-scattering increases (not shown); the enzyme molecules are likely to undergo modification(s) responsible for the irreversibility of the activity loss.

The enzymes were incubated at 90 °C in the presence of Ssocpn at a molar ratio of 1:1 between the chaperonin oligomer and the single polypeptide chain (Fig. 6). The time courses of the inactivation processes in the presence of the chaperonin were comparable to those obtained in its absence; however, these inactivations were not irreversible. Following 90 min at 90 °C, the temperature was shifted to 50 °C and ATP/Mg/K was added into the inactive enzyme solutions; within 3 min of the addition, we calculated activity regains of 100%, 75%, and 80%, respectively, in the mixtures of ME, ADH, and GDH heated in the presence of the chaperonin. We found that the final yields of activities were significantly lower when Ssocpn was omitted from the solutions and supplemented 10 min after starting the incubations at 90 °C (not shown). This finding means that the presence of the chaperonin from the beginning of the incubation at the denaturing temperature was essential for optimum recovery of activity and strongly suggests that the chaperonin binds a denaturation intermediate very likely before the inactivation

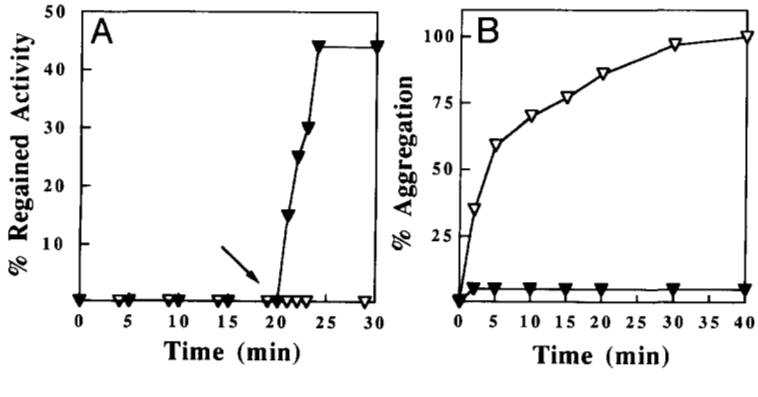

Fig. 5. Refolding (**A**) and aggregation in the time course of refolding (**B**) of chemically denatured ME from chicken liver in the absence (▽) and in the presence (▼) of purified Ssocpn. The enzyme (18 μg/mL) was denatured by a 1.5-h incubation at room temperature in 1.5 M guanidine hydrochloride, 0.2 M 2-mercaptoethanol; an aliquot from the denaturation mixture was diluted (final concentration of single polypeptide chain, 22 nM) in a renaturation mixture (6 mL) of 10 mM Tris/HCl buffer, pH 8.0, in the absence or in the presence of Ssocpn at an equimolar ratio with the single polypeptide chain and incubated at 40 °C. After 20 min, ATP/Mg/K was added to both the mixtures (arrow), and the incubations were continued at the same temperature. The activity regains were calculated as described in the legend to Figure 4. Protein aggregation during refolding was followed by measuring the turbidity at 450 nm using a Varian DMS-100 spectrophotometer; the maximal turbidity was taken as 100%.

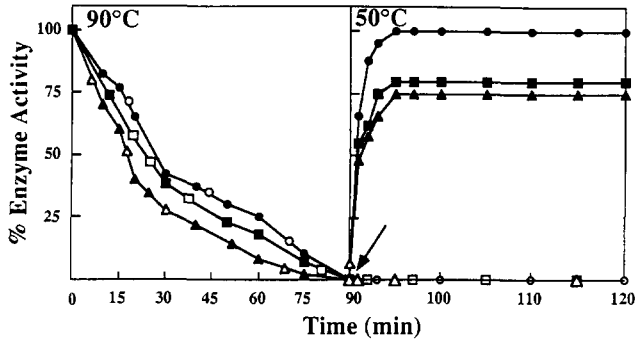

Fig. 6. Thermal inactivation of ME, ADH, and GDH from *S. solfataricus* in the absence (○, △, □) and in the presence (●, ▲, ■) of purified Ssocpn. The enzymes were incubated at the final concentrations of single polypeptide chains of 32 nM (ME), 120 nM (ADH), and 38 nM (GDH) at 90 °C in 10 mM Tris/HCl buffer, pH 8.0, in the absence or in the presence of Ssocpn at an equimolar ratio with the single polypeptide chain. After 90 min, the temperature was shifted to 50 °C, ATP/Mg/K was added to the mixtures (arrow), and the incubations were continued. Percentages of enzyme activities were calculated as described in the legend for Figure 4. Note that the scale of the *x*-axis changes at the time indicated by the arrow.

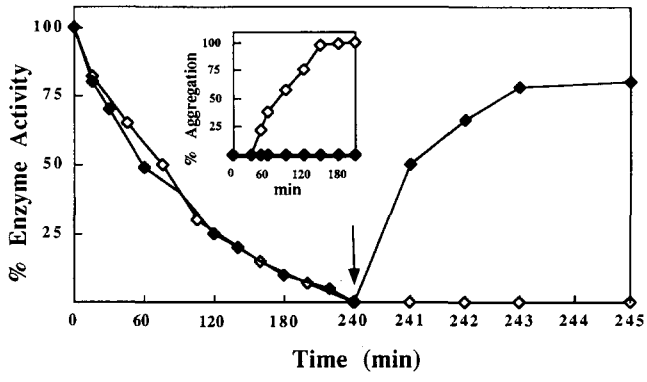

Fig. 7. Inactivation and, **inset**, aggregation during heating of chicken egg white lysozyme in the absence (◇) and in the presence (◆) of purified Ssocpn. Lysozyme (final concentration of 5 nM) was incubated at 70 °C in 10 mM Tris/HCl buffer, pH 8.0, in the absence or in the presence of Ssocpn at an equimolar ratio with the single polypeptide chain; after 4 h, ATP/Mg/K was added to the mixtures (arrow), and the incubations were continued at the same temperature. Percentages of lytic activity were calculated as described in the legend for Figure 4. Protein aggregation was determined as described in the legend to Figure 5. Note that the scale of the *x* axis changes at the time indicated by the arrow.

process drops into irreversibility; the chaperonin–intermediate complex requires the hydrolysis of ATP to be dissociated.

We incubated Ssocpn for 10 min at 50 °C in the presence of ATP/Mg before its addition into the enzymatic solutions subjected to heating at 90 °C. The result was that the inactivation processes occurred as expected, but addition of ATP/Mg/K into the inactive enzyme solutions at 50 °C did not lead to any activity regain; therefore, upon incubation with ATP/Mg, Ssocpn did not bind the denaturation intermediate, and the enzymes inactivated irreversibly. The incubation with ATP/Mg prevents Ssocpn from binding the refolding intermediate of chemically denatured ME, ADH, and GDH, and the final activity yields are exactly the same in Ssocpn-assisted refoldings as well as in Ssocpn-assisted thermal inactivations; these considerations prompt us to believe that the protein structures bound by Ssocpn during refolding and during thermal denaturation of the used enzymes are similar or closely related.

The chaperonin prevents thermal aggregation

The effect of Ssocpn on protein thermal aggregation was investigated by using chicken egg white lysozyme (14.4 kDa) as a model system. The heating of a solution of lysozyme at 70 °C led to an activity loss (Fig. 7), which is accompanied by aggregation (Fig. 7, inset) independently of the presence of an excess of bovine serum albumin in the lysozyme solution (not shown). The addition of Ssocpn and ATP/Mg/K in the inactive lysozyme solution did not cause a decrease of the light scattering, demonstrating that the chaperonin cannot dissolve aggregates.

We incubated native lysozyme at 70 °C in the presence of Ssocpn at an equimolar ratio with the polypeptide chain; the chaperonin had no effect on the time course of the inactivation process (Fig. 7), but it completely suppressed the aggregation (Fig. 7, inset). When lysozyme was completely inactive, ATP/Mg/K was added into the enzyme solutions; within 3 min of the addition, we calculated 80% activity regain in the solution heated in the presence of Ssocpn and no regain in the solution heated in the absence of Ssocpn. When Ssocpn was included in the lysozyme solution after a 10-min incubation at 50 °C in the presence of ATP/Mg, the lysozyme aggregated as in the absence of the chaperonin. The results can be reasonably interpreted as follows. Upon heating, lysozyme generates a partially unfolded protein structure, which is responsible for precipitation, as pointed out by the rapid decrease of lytic activity with respect to the increase of light scattering; the chaperonin captures this structure and suppresses aggregation; ATP hydrolysis promotes the dissociation of the complex and gives active enzyme; Ssocpn becomes unable to bind the structure responsible for aggregation following the conformational change driven by ATP/Mg.

We wondered what the yield of activity was in Ssocpn-assisted refolding of chemically denatured lysozyme. The enzyme (26 μg/mL) was denatured by a 2-h incubation at room temperature in the presence of 3 M guanidine hydrochloride and 0.2 M 2-mercaptoethanol. Upon dilution from denaturants and incubation at 50 °C, lysozyme was unable to spontaneously regain its activity because the refolding chain aggregated rapidly, as indicated by the increase in light scattering (not shown). When denatured lysozyme (final concentration of polypeptide chain, 25 nM) was diluted into a renaturation mixture containing Ssocpn in a molar ratio of 1:1 with the polypeptide chain, the increase of light scattering was completely suppressed and an activity regain of 76% was obtained upon ATP/Mg/K addition; this yield, within the limits of an experimental error, is comparable to that obtained in Ssocpn-assisted thermal denaturation of lysozyme. This result could imply that Ssocpn binds in a comparable way the protein structure, which, during heating or during refolding of guanidine hydrochloride-denatured lysozyme, is responsible for the formation of aggregates.

Discussion

In order to purify the chaperonin from the hyperthermophilic archaeon *S. solfataricus*, an in vitro assay of chaperoning activity was assessed, taking advantage of the knowledge about

GroEL-assisted refoldings in vitro of chemically denatured proteins (Goloubinoff et al., 1989; Laminet et al., 1990; Badcoe et al., 1991; Buchner et al., 1991; Holl-Neugebauer et al., 1991; Martin et al., 1991; Mendoza et al., 1991a, 1991b; Viitanen et al., 1991; Baneyx & Gatenby, 1992; Fisher, 1992; Schmidt & Buchner, 1992; van der Vies et al., 1992; Brunschier et al., 1993; Jackson et al., 1993). GroEL forms a stable complex with an early folding intermediate that occurs upon dilution from denaturant by interacting with its transiently exposed hydrophobic surfaces; as a consequence, spontaneous folding to the native state is inhibited. Starting from a crude extract of *S. solfataricus*, we considered active the chromatographic fractions able to inhibit the spontaneous refolding at 50 °C of dimeric ME from *S. solfataricus* from its guanidine hydrochloride-denatured form. Ssocpn, purified to homogeneity by molecular exclusion chromatography and affinity chromatography, represents 5% of the cytosolic protein amount; it has a native molecular mass of 920 kDa and a subunit size of 57 kDa.

Ssocpn promotes correct refolding of several thermophilic and mesophilic enzymes from their chemically denatured forms. At a molar ratio of chaperonin oligomer to single polypeptide chain of 1:1, Ssocpn completely arrests the spontaneous refoldings of oligomeric ME (dimer), ADH (dimer), and GDH (examer) from *S. solfataricus* and suppresses aggregation during refoldings of tetrameric chicken liver ME and monomeric chicken egg white lysozyme. At a molar ratio of Ssocpn oligomer to single chain of 0.5:1, incomplete arrests of spontaneous refoldings of thermophilic enzymes and partial increases of light scattering during refoldings of chicken ME and lysozyme were observed (not shown); in no case does the final yield of active enzyme improve at a molar excess of chaperonin over the single chain.

Like GroEL (Viitanen et al., 1990), Ssocpn displays an ATPase activity that is fully dependent on the presence of potassium ions. Upon hydrolysis of the nucleotide, the bound protein chain is released and immediate regains of activity are obtained. The rate enhancement could result from the presence of refolding intermediate on the chaperonin molecule, which immediately precedes its transition state.

Assuming that chaperonins are thought to play a cellular role not only in assisting folding of nascent proteins but also in protecting mature proteins against denaturation, we investigated the effects of Ssocpn on thermal denaturation of several thermophilic and mesophilic proteins. Upon heating at 90 °C, ME, ADH, and GDH from *S. solfataricus* inactivate irreversibly without aggregating. Ssocpn present in the enzymatic solutions at an equimolar ratio with the single chains forms a complex with the denaturation intermediates that occur upon heating; as a consequence, the loss of enzyme activity is not prevented, but the process is not irreversible because the intermediate is protected from undergoing chemical modifications while bound to the chaperonin. The dissociation of the complex upon ATP hydrolysis results in catalytically active enzyme.

We chose chicken egg white lysozyme as a model system to investigate the effects of Ssocpn on protein thermal aggregation. Once formed, lysozyme aggregates cannot be dissolved by Ssocpn, in agreement with the knowledge that chaperonins are not able to rescue aggregated proteins. Ssocpn present in the enzymatic solution of lysozyme at an equimolar ratio with the substrate chain does not affect the rate of inactivation at 70 °C, but it completely suppresses the aggregation by the formation of a complex with the protein structure responsible for precipitation in the absence of the chaperonin; most lytic activity is regained following dissociation of the complex upon ATP hydrolysis. The final yield does not significantly vary when the chaperonin and the protein chain are present at a molar ratio of 0.5:1 (A. Guagliardi & L. Cerchia, pers. comm.). We are currently detailing this issue in an attempt to determine the minimum amount of Ssocpn able to afford full protection to proteins against thermal denaturation.

A large body of literature indicates that chaperonins bind unfolded or partially folded protein molecules with a rather low specificity. The structural motifs that are essential for the recognition are unknown; on the basis of investigations performed on the GroEL–rhodanese and GroEL–dihydrofolate reductase complexes, some authors (Holl-Neugebauer et al., 1991; Martin et al., 1991; Langer et al., 1992) suggested that the conformation of the bound protein could be that of a "molten globule," an early folding intermediate that has native-like secondary structures but hydrophobic surfaces still exposed to the solvent (Ptitsyn, 1992). Ssocpn binds several different thermophilic and mesophilic proteins, demonstrating its low substrate specificity; because it recognizes protein structures that have a tendency to aggregate during refolding or during heating, a "recognition motif" seems to be the occurrence of exposed hydrophobic patches. Whether or not the structure of the protein substrates bound by the archaeal chaperonin corresponds to the molten globule remains to be seen.

Unlike GroEL and related chaperonins, but like TF55 from *S. shibatae* (Trent et al., 1991), Ssocpn contains tryptophan residues. Fluorescence measurements demonstrated that Ssocpn undergoes a dramatic conformational rearrangement in the presence of ATP/Mg and that the hydrolysis of the nucleotide is not required for this change. We obtained clear evidence that the conformational rearrangement induced by ATP/Mg strongly influences the Ssocpn ability to bind its protein substrates: the complexes between Ssocpn and the protein structures that occur during refolding or during heating do not occur upon incubation of the chaperonin in the presence of ATP/Mg, probably due to the modification or disappearance of the substrate binding site(s) in the ATP/Mg-induced conformation. Electron microscopy reveals that the oligomeric structure of the chaperonin from *Rhodobacter sphaeroides* undergoes large conformational changes upon ATP binding (Saibil et al., 1993); GroEL becomes more susceptible to cleavage by trypsin upon ATP binding (Baneyx & Gatenby, 1992); ATP binding drives a structural change in GroEL, which was labeled with a fluorescent probe (Jackson et al., 1993). There is compelling evidence in the literature that binding of ATP weakens the affinity of GroEL for several chemically denatured protein substrates (Badcoe et al., 1991; Martin et al., 1991; Mendoza et al., 1991b; Viitanen et al., 1991, 1992; Baneyx & Gatenby, 1992; Fisher, 1992; Jackson et al., 1993), and the existence of 2 GroEL conformers with different affinity for ATP and unfolded proteins has been reported (Badcoe et al., 1991; Fisher, 1992; Jackson et al., 1993). The ability of GroEL to hydrolyze ATP and bind protein substrates with positive cooperativity (Bochkareva et al., 1992) is well suited to a protein molecule whose conformational changes are very important for its functionality. In light of these considerations, the interpretation of molecular consequences of ATP binding and hydrolysis, and the analysis of the conformational changes, represent central tasks to be solved for a molecular understanding of the chaperonin mechanism of action.

The in vitro demonstration that the chaperonin from the hyperthermophilic archaeon *S. solfataricus* promotes correct refolding and prevents irreversible heat denaturation and thermal aggregation is consistent with the roles played in vivo by chaperonins: to facilitate folding and assembly of nascent polypeptides and to protect folded proteins against denaturation. Our findings provide the functional evidence that chaperonins from thermophilic Archaea do not require co-chaperonins for their activity; the future challenge will be to understand whether archaeal chaperonins contain a domain that performs the function of the co-chaperonin or whether this function is dispensable in the archaeal molecules.

Materials and methods

Materials

ATP, NAD, NADP, ME from chicken liver (29 U/mg), lysozyme from chicken egg white (183 U/mg), and guanidine hydrochloride were purchased from Sigma; [γ-^{32}P]ATP (3,000 Ci/mmol) was from Amersham. The calibration kit for SDS-PAGE and the molecular standards for the gel filtration chromatography were supplied by Pharmacia. The other chemicals were of the highest grade available. The enzymes from *S. solfataricus* were purified according to Bartolucci et al. (1987) (ME), Ammendola et al. (1992) (ADH), and Consalvi et al. (1991) (GDH); the enzyme preparations were homogeneous as judged from SDS-PAGE performed according to Laemmli (1970).

Protein concentration

Protein concentration was determined by the Bradford (1976) assay using bovine serum albumin as the standard.

ATPase activity of the chaperonin

The assay mixture consisted of 50 mM sodium phosphate buffer, pH 8.0, 0.5 mM ATP, 30 μCi of [γ-^{32}P]ATP, 0.5 mM $MgCl_2$, 10 mM KCl, 8 μg pure Ssocpn, in a total volume of 150 μL. After 5 min of incubation at the desired temperature, a 25-μL aliquot was drawn from each mixture, added to 0.5 mL of a suspension containing 50 mM HCl, 5 mM H_3PO_4, 7% activated charcoal, and centrifuged at 4,000 × *g* for 20 min; the radioactivity of the supernatant was counted on a 100-μL aliquot by a liquid scintillation counter (Beckman System model LS1701). In rate calculations, the amount of spontaneous ATP hydrolysis, calculated in assay mixtures without Ssocpn, has been corrected for.

Enzyme activity assays

The enzymes were assayed at 50 °C (the enzymes from *S. solfataricus*) or at 25 °C (the enzymes from mesophilic sources) by a Varian DMS-100 recording spectrophotometer provided with a thermostated cell compartment. Absorbance variations were always linear within 2 min; each activity assay was performed in duplicate. The assay mixtures for the enzymes from *S. solfataricus* were: ME, 40 mM glycine/NaOH buffer, pH 8.0, 0.05 mM NADP, 1 mM $MgCl_2$, 1 mM L-malate; ADH, 25 mM barbital/HCl buffer, pH 8.0, 2 mM NAD, 5 mM benzyl alcohol; GDH, 0.1 M sodium phosphate buffer, pH 8.0, 1 mM NADP, 0.1 mM EDTA, 25 mM L-glutamate. ME from chicken liver was assayed in 20 mM Tris/HCl buffer, pH 7.5, 0.05 mM NADP, 1 mM $MgCl_2$, 1 mM L-malate. The assay mixture for chicken egg white lysozyme consisted of 1 mL of a fresh suspension 0.1 mg/mL of lyophilized *E. coli* cells in 50 mM Tris/HCl buffer, pH 7.4; following the addition of lysozyme, the time required for an absorbance decrease of 0.1 OD at 350 nm was measured and the specific activity was calculated by the formula time^{-1} mg^{-1} of protein, according to Tsugita and Inouye (1968).

Chaperoning activity assay

ME from *S. solfataricus* was denatured upon incubation for 1 night at 37 °C in 4 M guanidine hydrochloride, 0.2 M 2-mercaptoethanol (80 μg protein per mL). An aliquot was diluted (final concentration of the single polypeptide chain, 32 nM) in a 6-mL renaturation mixture of 50 mM sodium phosphate buffer, pH 8.0, in the absence (spontaneous refolding) or in the presence of an aliquot from the chromatographic fraction, and the mixtures were incubated at 50 °C. After 20 min, the ME activity was assayed on aliquots drawn from the renaturation mixtures; the chromatographic fractions able to inhibit the spontaneous regain of ME activity were considered active.

Chaperonin purification

Hyperthermophilic archaeon *S. solfataricus* strain MT-4 was grown aerobically at 87 °C and pH 3.0 as described (Bartolucci et al., 1987). The cells were harvested in the exponential growth phase by continuous-flow centrifugation in an Alfa Laval model LAB 102B 20 separator; the biomass was washed twice in 10 mM Tris/HCl buffer, pH 7.5, and collected by centrifugation at 10,000 × *g* for 30 min. Bacteria were stored at −20 °C.

Step 1: Preparation of the crude extract

Bacterial cells (10 g) underwent freeze-thawing twice, were added to 10 g sand and 10 mL 50 mM Tris/HCl buffer, pH 8.0, containing 0.2 M NaCl, 5% glycerol, and homogenized in a Homni Mixer. The homogenate was centrifuged at 4,000 × *g* for 20 min to remove the sand; the supernatant solution was centrifuged at 160,000 × *g* for 90 min at 4 °C, and the residue was discarded. From a typical preparation, about 0.5 g of protein was obtained starting from 10 g of bacteria. The crude extract was aliquoted and stored at −20 °C.

Step 2: Gel filtration chromatography

The crude extract (20 mg) was dialyzed against 10 mM Tris/HCl buffer, pH 8.4 (Buffer A), and loaded onto a Superose 6 column (Pharmacia, 2.6 × 60 cm) eluted with the same buffer, supplemented with 0.1 M NaCl, at a flow rate of 0.5 mL/min and room temperature. The active sample was concentrated by a vacuum centrifuge.

Step 3: Affinity chromatography

The sample from the gel filtration column (1.4 mg) was dialyzed against Buffer A and loaded onto a Matrex Gel Red A column (Amicon, 1 × 3.5 cm), which was equilibrated and eluted in Buffer A at a flow rate of 0.25 mL/min at room temperature. The column was washed with the same buffer until the absor-

bance returned to the baseline and the bound proteins were eluted with a linear 0–0.4 M NaCl gradient in Buffer A. The active sample (0.9 mg) was concentrated as described above, dialyzed against Buffer A, and stored at 4 °C.

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References

Ammendola S, Raia CA, Caruso C, Camardella L, D'Auria S, De Rosa M, Rossi M. 1992. Thermostable NAD^+-dependent alcohol dehydrogenase from *Sulfolobus solfataricus*: Gene and protein sequence determination and relationship to other alcohol dehydrogenases. *Biochemistry 31*: 12514–12523.

Badcoe IG, Smith CJ, Wood S, Halsall DJ, Holbrook JJ, Lund P, Clarke AR. 1991. Binding of a chaperonin to the folding intermediate of lactate dehydrogenase. *Biochemistry 30*:9195–9200.

Baneyx F, Gatenby AA. 1992. A mutation in GroEL interferes with protein folding by reducing the rate of discharge of sequestered polypeptides. *J Biol Chem 267*:11637–11644.

Bartolucci S, Rella R, Guagliardi A, Raia CA, Gambacorta A, De Rosa M, Rossi M. 1987. Malic enzyme from archaebacterium *Sulfolobus solfataricus*: Purification, structure and kinetic properties. *J Biol Chem 262*:7725–7731.

Bochkareva ES, Lissin N, Flynn GC, Rothman JE, Girshovich AS. 1992. Positive cooperativity in the functioning of molecular chaperone GroEL. *J Biol Chem 267*:6796–6800.

Bradford MM. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Anal Biochem 72*:248–254.

Brunschier R, Danner M, Seckler R. 1993. Interactions of phage P22 tailspike protein with GroE molecular chaperones during refolding in vitro. *J Biol Chem 268*:2767–2772.

Buchner J, Schmidt M, Fuchs M, Jaenicke R, Rudolph R, Schmid F, Kiefhaber T. 1991. GroE facilitates refolding of citrate synthase by suppressing aggregation. *Biochemistry 30*:1586–1591.

Consalvi V, Chiaraluce R, Politi L, Gambacorta A, De Rosa M, Scandurra R. 1991. The protein sequence of glutamate dehydrogenase from *Sulfolobus solfataricus*, a thermoacidophilic archaebacterium. *Eur J Biochem 196*:459–467.

Ellis J. 1992. Cytosolic chaperonin confirmed. *Nature 358*:191–192.

Ellis RJ, van der Vies SM. 1991. Molecular chaperones. *Annu Rev Biochem 60*:321–347.

Fisher MT. 1992. Promotion of the in vitro renaturation of dodecameric glutamine synthetase from *Escherichia coli* in the presence of GroEL (chaperonin-60) and ATP. *Biochemistry 31*:3955–3963.

Gething M, Sambrook J. 1992. Protein folding in the cell. *Nature 355*:33–45.

Goloubinoff P, Christeller JT, Gatenby AA, Lorimer GH. 1989. Reconstitution of active dimeric ribulose biphosphate carboxylase from an unfolded state depends on two chaperonin proteins and Mg-ATP. *Nature 342*:884–889.

Guagliardi A, Moracci M, Manco G, Rossi M, Bartolucci S. 1988. Oxalacetate decarboxylase and pyruvate carboxylase activities, and effect of sulfhydryl reagents in malic enzyme from *Sulfolobus solfataricus*. *Biochim Biophys Acta 957*:301–311.

Hartl FU, Hlodan R, Langer T. 1994. Molecular chaperones in protein folding: The art of avoiding sticky situations. *Trends Biochem Sci 19*:20–25.

Hartl FU, Martin J, Neupert W. 1992. Protein folding in the cell: The role of molecular chaperones hsp 70 and hsp 60. *Annu Rev Biophys Biomol Struct 21*:293–322.

Hartman DJ, Hoogenraad NJ, Condron R, Hoj PB. 1992. Identification of a mammalian 10-kDa heat shock protein, a mitochondrial chaperonin 10 homologue essential for assisted folding of trimeric ornithine transcarbamoylase. *Proc Natl Acad Sci USA 89*:3394–3398.

Holl-Neugebauer B, Rudolph R, Schmidt M, Buchner J. 1991. Reconstitution of a heat shock effect in vitro: Influence of GroE on the thermal aggregation of α-glucosidase from yeast. *Biochemistry 30*:11609–11614.

Horwich AL, Willison KR. 1993. Protein folding in the cell: Functions of two families of molecular chaperones, hsp60 and TF55-TCP1. *Philos Trans R Soc Lond 339*:313–326.

Jackson GS, Staniforth RA, Halsall DJ, Atkinson T, Holbrook JJ, Clarke AR, Burston SG. 1993. Binding and hydrolysis of nucleotides in the chaperonin catalytic cycle: Implications for the mechanism of assisted protein folding. *Biochemistry 32*:2554–2563.

Laemmli UK. 1970. Cleavage of structural proteins during the assembly of the head of bacteriophage T4. *Nature 227*:680–685.

Laminet AA, Ziegelhoffer T, Georgopoulos C, Pluckthun A. 1990. GroES modulate the folding of the β-lactamase precursor. *EMBO J 9*:2315–2319.

Langer T, Pfeifer G, Martin J, Baumeister W, Hartl FU. 1992. Chaperonin-mediated protein folding: GroES binds to one end of the GroEL cylinder, which accommodates the protein substrate within its central cavity. *EMBO J 11*:4757–4765.

Lubben TH, Gatenby AA, Donaldson GK, Lorimer GH, Viitanen PV. 1990. Identification of a GroES-like chaperonin in mitochondria that facilitates protein folding. *Proc Natl Acad Sci USA 87*:7683–7687.

Marco S, Urena D, Carrascosa JL, Waldmann T, Peters J, Hegerl R, Pfeifer G, Sack-Konehl H, Baumeister W. 1994. The molecular chaperone TF55. Assessment of symmetry. *FEBS Lett 341*:152–155.

Martin J, Langer T, Boteva R, Schramel A, Horwich AL, Hartl FU. 1991. Chaperonin-mediated protein folding at the surface of GroEL through a "molten globule"-like intermediate. *Nature 352*:36–42.

Mendoza JA, Lorimer GH, Horowitz PM. 1991a. Intermediates in the chaperonin-assisted refolding of rhodanese are trapped at low temperature and show a small stoichiometry. *J Biol Chem 266*:16973–16976.

Mendoza JA, Rogers E, Lorimer GH, Horowitz PM. 1991b. Chaperonins facilitate the in vitro folding of monomeric mitochondrial rhodanese. *J Biol Chem 266*:13044–13049.

Phipps BM, Hoffmann A, Stetter KO, Baumeister W. 1991. A novel ATPase complex selectively accumulated upon heat shock is a major cellular component of thermophilic archaebacteria. *EMBO J 10*:1711–1722.

Phipps BM, Typke D, Hegerl R, Volker S, Hoffmann A, Stetter KO, Baumeister W. 1993. Structure of a molecular chaperone from a thermophilic archaebacterium. *Nature 361*:475–477.

Ptitsyn OB. 1992. The molten globule state. In: Creighton TE, ed. *Protein folding*. New York: Freeman and Company. pp 243–300.

Saibil HR, Zheng D, Roseman AM, Hunter AS, Watson GMF, Chen S, auf der Mauer A, O'Hara BP, Wood SP, Mann NH, Barnett LK, Ellis RJ. 1993. ATP induces large quaternary rearrangements in a cage-like chaperonin structure. *Curr Biol 3*:265–273.

Schmidt M, Buchner J. 1992. Interaction of GroE with an all-β-protein. *J Biol Chem 267*:16829–16833.

Trent JD, Nimmesgern E, Wall JS, Hartl FU, Horwich AL. 1991. A molecular chaperone from a thermophilic archaebacterium is related to the eukaryotic protein t-complex polypeptide-1. *Nature 354*:490–493.

Tsugita A, Inouye M. 1968. Purification of bacteriophage T4 lysozyme. *J Biol Chem 243*:391–397.

van der Vies S, Viitanen PV, Gatenby AA, Lorimer GH, Jaenicke R. 1992. Conformational states of ribulosebisphosphate carboxylase and their interaction with chaperonin 60. *Biochemistry 31*:3635–3644.

Viitanen PV, Donaldson GK, Lorimer GH, Lubben TH, Gatenby AA. 1991. Complex interactions between the chaperonin 60 molecular chaperone and dihydrofolate reductase. *Biochemistry 30*:9716–9723.

Viitanen PV, Gatenby AA, Lorimer GH. 1992. Purified chaperonin 60 (groEL) interacts with the nonnative states of a multitude of *Escherichia coli* proteins. *Protein Sci 1*:363–369.

Viitanen PV, Lubben TH, Reed J, Goloubinoff P, O'Keefe DP, Lorimer GH. 1990. Chaperonin-dependent refolding of ribulose bisphosphate carboxylase and ATP hydrolysis by chaperonin 60 (GroEL) are K^+ dependent. *Biochemistry 29*:5665–5671.

Wheelis ML, Kandler O, Woese CR. 1992. On the nature of global classification. *Proc Natl Acad Sci USA 89*:2930–2934.

Woese CR, Kandler O, Wheelis ML. 1990. Towards a natural system of organisms: Proposal for the domains Archaea, Bacteria and Eucarya. *Proc Natl Acad Sci USA 87*:4576–4579.