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An archaeal chaperonin-based reactor for renaturation of denatured proteins

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Abstract We describe an original chaperonin-based reactor that yields folded and active proteins from denatured materials. We used the 920-kDa chaperonin of the archaeon *Sulfolobus solfataricus*, which does not require any protein partner for its full activity and assists in vitro folding with low substrate specificity. The reactor consists of an ultrafiltration cell equipped with a membrane that retains the chaperonin in a functional state for folding in solution and permits the flowthrough of the folded substrates. By studying the ATP-dependent functional cycle of the chaperonin, we were able to use the reactor for repeated refolding processes. The scale-up of the reactor is made possible by the overproduction of chaperonin in *Sulfolobus solfataricus* cells that acquired thermotolerance upon appropriate heat shock.

Key words Archaea · Chaperonin · Protein folding · Heat shock · Thermotolerance

Introduction

In all cells, the heat-shock proteins endowed with ATPase activity known as chaperonins play an essential role in promoting the functional conformation of proteins during growth on the ribosome, following translocation across organellar membranes, and upon environmental damage (reviewed in Bukau and Horwich 1998; Klumpp and Baumeister 1998; Ellis and Hartl 1999). Chaperonins are complexes of two stacked rings each containing 7 to 9 approximately 60-kDa subunits delimiting a central cavity, and this accounts for a native size averaging 1×10^6 daltons. Two sequence-related classes of chaperonins exist: class I comprises the chaperonins found in Bacteria, mitochondria, and chloroplasts, whose ATPase activity is regulated by proteins known as co-chaperonins; the chaperonins of Archaea and eukaryotic cytoplasm belong to class II, and they do not require co-chaperonins.

It is known that chaperonins bind nonnative states of proteins via the recognition of hydrophobic surfaces in the context of globular, nonextended conformations; thus, they prevent protein aggregation by shielding the interactive surfaces on the substrate protein, being unable to dissolve protein aggregates once they form. Generally speaking, chaperonins promote correct folding by binding to the folding intermediates and by releasing them in a folded or folding-competent status upon the binding or hydrolysis of ATP. Although the molecular mechanisms underlying this amazing activity are not yet completely clear, some concepts are well established for class I chaperonins. The affinity of the chaperonin for the substrate protein is allosterically regulated by the nucleotide: in the absence of nucleotide or in the presence of ATP binding, the chaperonin exists in the "high-affinity" conformation for the protein; upon hydrolysis of ATP, the chaperonin adopts the "low-affinity" conformation for the protein that is released; the binding of the nucleotide drives the chaperone to the conformation able to enter another folding cycle; several rounds of binding and release of the protein upon expenditure of energy could be required to reach the folded state. The functional cycling of class II chaperonins is much less known.

Protein aggregation places a limitation on the availability of active molecules in vitro as well as in vivo. Chaperonins that prevent aggregative damage in the cells can be usefully employed to rescue active proteins from aggregated materials. Renaturation from inclusion bodies of a recombinant immunotoxin has been obtained in the presence of the *E.*

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In memory of Professor Giacomino Randazzo

coli holochaperonin system consisting of chaperonin GroEL plus co-chaperonin GroES (Buchner et al. 1992). The solubility of dihydrofolate reductase (Dale et al. 1994) and of various vertebrate proteins (Yasukawa et al. 1995) has been enhanced in *E. coli* cells that overproduce the holochaperonin system. Active fragments of GroEL linked to an agarose gel have been used for protein renaturation but have proved effective only with proteins that refold in the presence of the holochaperonin system (Altamirano et al. 1997).

In this article we describe an original refolding reactor equipped with the chaperonin of the archaeon *Sulfolobus solfataricus* (Ssocpn) whose in vitro folding activity is known (Guagliardi et al. 1994, 1995, 1997). The reactor consists of an ultrafiltration cell that retains Ssocpn and permits the flowthrough of the folded substrates. The ATP-dependent functional cycle of Ssocpn was elucidated, thus enabling the use of the reactor for consecutive refolding processes. With a view to scaling up the method, we also describe a growth protocol that greatly increases the production of Ssocpn (up to 30% of total protein amount) in *S. solfataricus*.

Materials and methods

Materials

ATP, NADH, NADP, chicken lysozyme (Lys, 183 units/mg), pig heart malate dehydrogenase (MDH, 282 units/mg), and bovine alkaline phosphatase (AP, 11 units/mg) were purchased from Sigma. *Sulfolobus solfataricus* glutamate dehydrogenase (GDH, 1.2 units/mg) was purified according to Consalvi et al. (1991). The calibration kit for SDS-PAGE was supplied by Pharmacia. The other chemicals were of the highest grade available.

Cell growth, preparation of the crude extract, and chaperonin purification

Cells of *S. solfataricus* strain Gθ were grown at 75°C as described in Cannio et al. (1998) to the late exponential phase (about 0.6 OD at 600 nm), maintained at 85°C for 2 h, then at 90°C for 6 h, and harvested by centrifugation at 4000 $\times$ *g* for 15 min. The cells (about 2.5 g wet weight from a 1.5-l culture) were lysed according to Hudepohl et al. (1990), and the crude extract (about 30 mg) was obtained by ultracentrifugation at 166000 $\times$ *g* for 40 min. The crude extract was loaded onto a Superose 6 column (Pharmacia Biotech; 2.6 $\times$ 60 cm) and eluted with 10 mM Tris-HCl, pH 8.4, plus 0.1 M NaCl; the fractions containing the chaperonin (about 9 mg) were pooled, dialyzed against 10 mM Tris-HCl, pH 8.4, and stored in aliquots at 4°C.

Enzyme activity assays

The enzymes were assayed at 25°C (enzymes from mesophilic sources) or at 60°C (GDH from *S. solfataricus*) in a Cary 1E Varian thermostated spectrophotometer. Lys and GDH were assayed as described by Guagliardi et al. (1994); MDH and AP were assayed according to Boehringer Mannheim Biochemica information.

Miscellaneous

SDS-PAGE analysis was carried out according to Laemmli (1970); proteins were detected by Coomassie blue staining. Protein concentration was determined by the Bradford (1976) assay using bovine serum albumin as the standard. Protein aggregation was monitored as an increase of absorbance at 450 nm. In tryptophan fluorescence analyses, samples were excited at 295 nm and the emission was recorded between 310 and 390 nm at 25°C using a Perkin Elmer LS 50B spectrofluorimeter.

Results

The overproduction of Ssocpn in thermotolerant cells

Ssocpn, which has been purified to homogeneity from crude extracts of *S. solfataricus* by gel filtration chromatography and affinity chromatography, accounts for about 5% of the total cytosolic protein amount (Guagliardi et al. 1994). We decided to study the heat-shock response in *S. solfataricus* with the ultimate goal of assessing a growth protocol for the overproduction of Ssocpn.

S. solfataricus cells were grown aerobically up to the late exponential phase at the optimal temperature of 75°C; one of four equal samples was maintained at the same temperature (the control culture) and the others were variously heat shocked. The growth profile after shifting from 75°C to 85°C (Fig. 1A, curve b) was similar to that of the unshocked culture (curve a); the shock at 90°C dramatically affected cell survival (curve c), indicating that this is the lethal temperature for the archaeon; cells did survive when the shock at 90°C was preceded by an exposure to 85°C for 2 h (curve d). The latter result agrees with the knowledge that all organisms acquire thermotolerance, i.e., the capacity to survive exposure to a normally lethal temperature, following a previous exposure to a high, nonlethal temperature (Conway de Macario and Macario 1994; Trent et al. 1994).

Crude extracts were prepared from cells harvested at 4 and 6 h after shifting to 85°C and from thermotolerant cells harvested at 6 h after shifting to 90°C and were each subjected to SDS-PAGE analysis (Fig. 1B). The electrophoretic pattern of the extract from the 4-h growth at 85°C (lane c) was similar to the pattern of the extract from the control culture (lane b); the protein pattern of the extract from the 6-h growth at 85°C (lane d) revealed some qualitative and quantitative differences in comparison with the pattern of the extract from the control culture; the pattern of the extract from thermotolerant cells (lane e) showed an increased level of Ssocpn (as judged from the intensity of the 60-kDa subunit) and decreased levels of most of the cytosolic proteins in comparison with the pattern of control

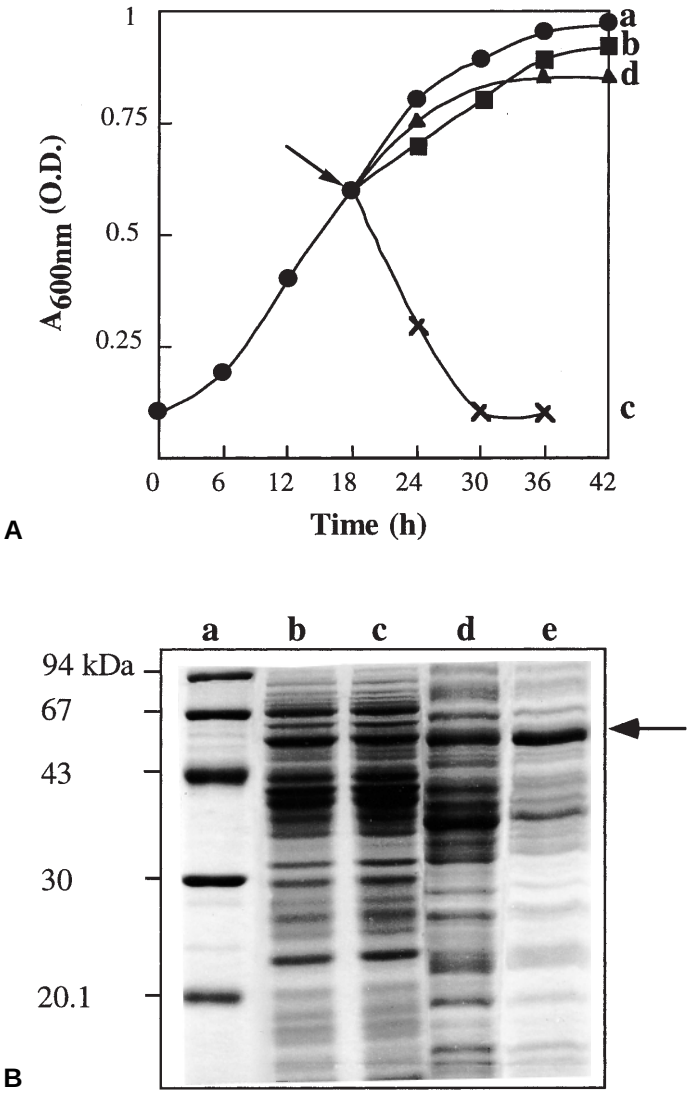

Fig. 1A,B. Heat-shock response in *Sulfolobus solfataricus*. **A** Growth profiles of a culture of *S. solfataricus* at different temperatures. Cells of the archaeon were grown at 75°C; at the late exponential phase (*arrow*), one of four equal samples was maintained at the same temperature (*control, curve a*), and the others were shifted to 85°C (*curve b*), 90°C (*curve c*), or 85°C for 2h and then to 90°C (*curve d*). **B** SDS-PAGE analysis (12.5%) (15μg/lane) of the crude extracts from unshocked cells (*lane b*), at 85°C for 4h (*lane c*), at 85°C for 6h (*lane d*), at 85°C for 2h, and then at 90°C for 6h (*lane e*). Molecular weight standards, *lane a*. *Arrow*, Ssocpn subunit band

cells. It is known that in most thermophilic Archaea the chaperonin is the main protein expressed under heat shock and that it is involved in the establishment of thermotolerance (Conway de Macario and Macario 1994; Trent et al. 1994). Ssocpn was purified to homogeneity from thermotolerant cells only by gel filtration chromatography; pure Ssocpn accounted for about 30% of total soluble protein (3.6mg pure chaperonin/g cell mass).

To the best of our knowledge, a protocol for the overproduction of a chaperonin in thermotolerant cells has never been described. The chaperonin was purified from heat-shocked *S. solfataricus* cells according to the protocol described by Knapp et al. (1994) with a yield of 0.25mg protein/g cell mass.

The Ssocpn-based refolding reactor

First we reconstituted the spontaneous refoldings of lysozyme (Lys, a monomer of 14.4kDa containing four disulfide bonds), malate dehydrogenase (MDH, a homodimer of 35-kDa subunits containing two catalytic thiol groups per subunit), and alkaline phosphatase (AP, a homodimer of 70-kDa subunits containing two catalytic zinc ions per subunit). Solutions of 10mg/ml Lys, MDH, and AP were denatured by overnight incubation at RT (room temperature) in 4M guanidine hydrochloride, plus 0.2M 2-mercaptoethanol for Lys. Each denatured protein was allowed to spontaneously renature at RT upon dilution to a protein concentration of 50μg/ml in the following renaturation buffers: 10mM Tris-HCl, pH 8.4 (Lys); 10mM Tris-HCl, pH 8.4, 10μM dithiothreitol (MDH); 10mM Tris-HCl, pH 8.4, 5μM $ZnCl_2$ (AP). The enzyme activity was assayed on aliquots withdrawn at time intervals from each renaturation mixture; as a result, none of the proteins regained activity (Fig. 2; open symbols). Light scattering monitoring (insets) showed that aggregation prevented productive refoldings.

The effects of Ssocpn on spontaneous refoldings of Lys, MDH, and AP were investigated by diluting the denatured proteins into the renaturation buffers, which contained Ssocpn in a molar ratio of one chaperonin oligomer to one single polypeptide chain. In the presence of Ssocpn, the aggregation reactions during refoldings were completely suppressed as a consequence of the capture by the chaperonin of the refolding intermediates responsible for aggregation. Ssocpn releases the bound substrate on the hydrolysis (not the binding) of ATP, which is strictly dependent on potassium ions (Guagliardi et al. 1994). Thus, ATP–Mg–K (0.5mM ATP, 0.5mM $MgCl_2$, 10mM KCl) was added to the renaturation mixtures (Fig. 2). Full regains of enzyme activity were calculated within a few minutes from the addition.

It is worth noting that MDH and AP did not regain any activity in the Ssocpn-assisted refolding events if their renaturation buffers were devoid of dithiothreitol and zinc ions, respectively. In fact, MDH has essential cysteine residues, while the chaotropic agent-mediated denaturation of AP might cause the loss of the essential zinc ions. The presence of correct disulfide bonds greatly influences the biological function of a protein; thus, the employ of redox compounds during classical refolding experiments in the absence of a chaperone molecule is a common practice to enhance the regain of activity. As already observed with other disulfide bond-containing proteins (Guagliardi et al. 1994), the Ssocpn-promoted renaturation of lysozyme did not require the presence of any redox system. It is possible to hypothesize that the chaperonins release the substrate protein in a conformation already committed to a correct folding in solution.

The reactor used for quantitative Ssocpn-assisted refoldings of Lys, MDH, and AP consisted of an ultrafiltra-

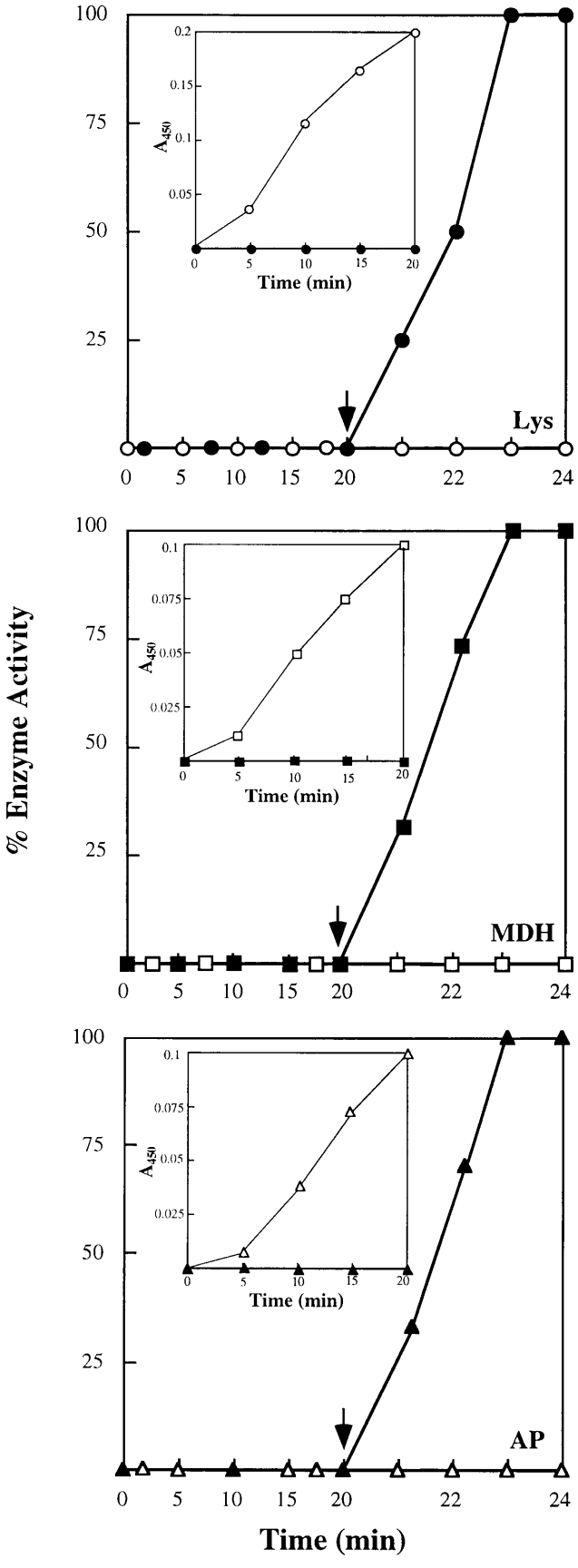

Fig. 2. Renaturation and aggregation in the time-course of renaturation (*insets*) of chemically unfolded Lys, MDH, and AP in the absence (*open symbols*) and presence (*closed symbols*) of Ssocpn. *Arrow*, addition of ATP/Mg/K. Note that the scale of the *x*-axis changes at the time indicated by the *arrow*

tion cell (Amicon; 3-ml volume) equipped with an XM500 membrane (Amicon; 500-kDa cutoff). The procedure for the use of the reactor consists in the following steps: (1) denaturation of the substrate protein; (2) mixing of the denatured protein and Ssocpn in the reactor at various molar ratios, and incubation at RT for 10–20 min with gentle stirring; (3) addition of ATP/Mg/K to the reactor and, 5 min after the addition, connection of the stirred reactor to nitrogen flow, rinsing with about 3 volumes of renaturation buffer, which separated an "upstream" solution (the solution retained in the reactor) from a "downstream" solution (the filtrate of the reactor) that was concentrated by vacuum centrifuge before being analyzed.

At any molar ratio of Ssocpn : protein, the upstream solution always contained only the chaperonin molecule, as demonstrated by SDS-PAGE analyses (not shown); protein concentration assays proved that the chaperonin was fully retained in the reactor. Protein concentration analyses and enzymatic assays were performed on the downstream solutions; the results are summarized in Table 1. The protein yields were always very high (>80%); some loss of protein is likely to occurr for nonspecific adsorption to test tubes during concentration. The enzymatic assays showed that the downstream solutions relative to the refoldings carried out at an equimolar ratio of Ssocpn : protein contained a catalyst with the same specific activity as the native one. When the refoldings were performed at the molar ratios 0.5 : 1 and 0.25 : 1 between Ssocpn and the substrate protein, the downstream solutions displayed lower specific activity values because the chaperonin was unable to assist the folding of all the species in solution, which therefore are recovered as inactive molecules.

The ATP-dependent cycle of Ssocpn and the regeneration of the refolding reactor

In the described reactor, Ssocpn was unable to enter a new folding cycle after the ATP hydrolysis-promoted release of the substrates in folded state. From an applied point of view, it is extremely important that the Ssocpn-based reactor could be used repeatedly, without the need to feed the chaperonin. Therefore, we addressed the functional cycling of Ssocpn.

The presence of tryptophan residues in Ssocpn allows performing experiments of intrinsic fluorescence to investigate the conformational changes of chaperonin molecule. Samples (5 μg each) of the upstream solution after one refolding cycle and of freshly prepared Ssocpn were excited at 295 nm and their emission spectra were recorded between 310 and 390 nm; the traces strongly differed in fluorescence intensity and emission maximum (Fig. 3). There is previous evidence that upon ATP hydrolysis pure Ssocpn displays a tryptophan fluorescence spectrum almost identical to that relative to the chaperonin present in the upstream solution after one refolding cycle (Guagliardi et al. 1994). Thus, we assumed that the ATP hydrolysis-induced conformation of Ssocpn (which we termed Ssocpn*) does not bind the refolding intermediate.

Table 1. Performance of the Ssocpn-based reactor

Protein	Ssocpn:protein (molar ratio)	Protein yield in the downstream solution (%)	Specific activity in the downstream solution	
			Units/mg	% relative to native[a]
Lys	1:1	88	183	100
	0.5:1	87	98.8	54
	0.25:1	85	21.9	12
MDH	1:1	85	282	100
	0.5:1	85	191.8	68
	0.25:1	84	93.1	33
AP	1:1	87	11	100
	0.5:1	84	8	73
	0.25:1	84	2.2	20

[a] The specific activity values of the native proteins are Lys, 183 units/mg; MDH, 282 units/mg; AP, 11 units/mg

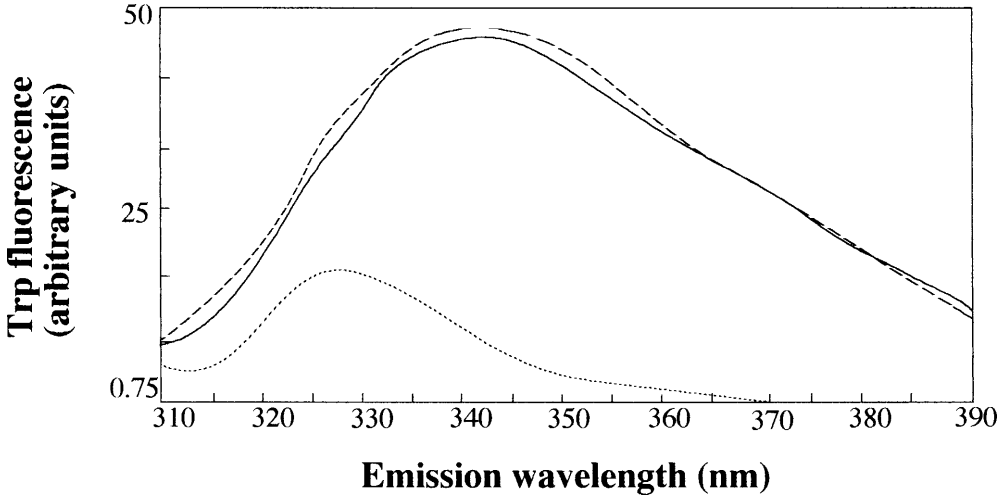

Fig. 3. Tryptophan fluorescence emission spectra of different chaperonin samples. The traces refer to the following samples (5 μg each): Ssocpn in the upstream solution at the end of a refolding process, termed Ssocpn* (*dotted line*); freshly prepared Ssocpn (*solid line*); Ssocpn* following the addition of ATP/Mg in the refolding reactor and the removal of the nucleotide (*dashed line*)

We found that ATP binding triggers Ssocpn* again to the native conformation. At the end of a refolding cycle, ATP–Mg (0.5 mM ATP, 0.5 mM $MgCl_2$) was added to the upstream solution containing Ssocpn*; after 5 min at RT, the reactor was connected to the nitrogen flow and rinsed thoroughly with 10 mM Tris-HCl, pH 8.4. Fluorescence emission spectrum of the upstream solution yielded a trace almost identical to that of freshly prepared Ssocpn (Fig. 3). The Ssocpn contained in the upstream solution after conditioning by ATP binding was able to enter a new refolding process, and the reactor was reused without significant loss of its efficiency.

Discussion

The archaeal chaperonin Ssocpn has some features that make it an ideal tool for practical use: it does not require any other protein for its function, unlike the chaperonins of Bacteria and eukaryotic organelles; it has a very low substrate specificity, as it is active on monomeric and oligomeric proteins from mesophilic and thermophilic sources; and it works over a wide temperature range.

To develop a Ssocpn-based folding protocol we exploited the large size of the archaeal chaperonin (920 kDa) to retain it on an ultrafiltration membrane that is permeable to most if not all protein molecules. The reactor designed appears to be a very efficient and simple means of keeping the chaperonin in a functional state for folding in solution and of recovering the active product with good yield. The refolding reactor was successfully used at room temperature to renature different substrate proteins whose chemical denaturation was spontaneously irreversible.

The protocol described was scaled up to 0.5 mg substrate protein/ml of renaturation buffer without any loss of regained activity. The availability of Ssocpn does not restrict the large-scale use of the reactor because the overproduction of Ssocpn was obtained in thermotolerant cells of the thermophilic archaeon without the need to express the recombinant protein.

The regeneration of the reactor for its repeated use required an understanding of Ssocpn functional cycling. According to previous (Guagliardi et al. 1994, 1995) and

current results, we identified a conformation of the chaperonin molecule that has high affinity for the protein substrate (Ssocpn) and a conformation which has low affinity for the protein (Ssocpn*). In the absence of nucleotide, Ssocpn forms a complex with the refolding intermediate, which prevents aggregation and misfolding; ATP hydrolysis on the complex drives the rearrangement of the chaperonin molecule to the form Ssocpn* and the release of the bound protein in an active form; ATP binding triggers Ssocpn* to its original state (Ssocpn), which is able to begin a new refolding process. Conformational changes induced by ATP have been observed for cytoplasmic CCT chaperonin (Melki and Cowan 1994; Llorca et al. 1998) and for the chaperonin from the archaeon *Thermoplasma acidophilum* (Ditzel et al. 1998); our current results contribute to elucidating the functional implications of nucleotide binding or hydrolysis for a class II chaperonin.

The refolding reactor, illustrated in Fig. 4, could easily be extended to the renaturation of different inactive protein materials. A number of proteins in solution (membrane proteins and, in general, proteins with hydrophobic patches) undergo inactivation by aggregative events. Many foreign proteins are expressed in host cells as inclusion bodies, which are inactive aggregates. Current methods to regain biologically active proteins consist of solubilization of the inclusion bodies in denaturants and dilution in media containing "folding enhancers," i.e., substances preventing aggregation (detergents, arginine, polyethylene glycol, thiol reagents). However, the efficiency of the process can be very low, mainly with multisubunit and disulfide bond-containing proteins.

As an example, we describe the case of the *S. solfataricus* GDH (a homoexamer of 45-kDa subunits). A pure GDH solution of 1 mg/ml lost all activity on storage at −20°C for 1 year; analytical gel filtration chromatography showed the presence of large aggregates (>700 kDa) in the solution. After dissolving aggregates by overnight incubation at 50°C in 6 M guanidine hydrochloride, renaturation of the protein was performed in the refolding reactor at 50°C at a molar ratio of one chaperonin oligomer to one single GDH polypeptide chain. As a result, the downstream solution had the same specific activity as native GDH, and it proved homogeneous for a 270-kDa protein as analyzed by gel filtration chromatography. When the renaturation was performed in the absence of chaperonin, the enzyme regained about 50% of initial activity; this finding is in agreement with the published value relative to the spontaneous renaturation of this enzyme in comparable experimental conditions (Guagliardi et al. 1994).

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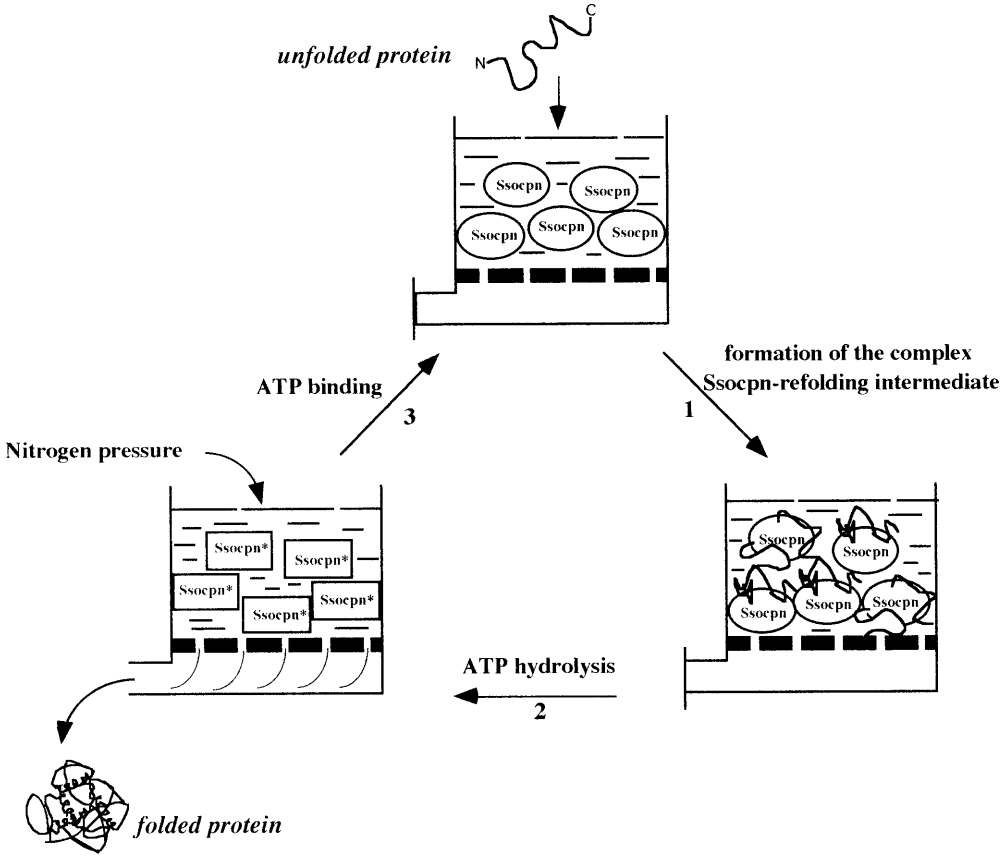

Fig. 4. Diagram of the refolding reactor

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