0273-2289/97/6201—0037$10.50

The Chaperonin of the Archaeon *Sulfolobus solfataricus*

A Tool for Applied Biochemistry

ANNAMARIA GUAGLIARDI,* LAURA CERCHIA, AND MOSÈ ROSSI

Dipartimento di Chimica Organica e Biologica, Università di Napoli "Federico II," Via Mezzocannone 16, 80134 Napoli, Italy

Received March 4, 1996; Accepted July 9, 1996

ABSTRACT

The chaperonin of the hyperthermophilic archaeon *Sulfolobus solfataricus*, briefly Ssocpn, was purified by a fast and high-yield procedure. Ssocpn, a 920 kDa-complex of two different subunits, displays a potassium-dependent ATPase activity with a temperature optimum at 80°C. The ability of Ssocpn to function in vitro was investigated using different protein substrates. Ssocpn promotes correct refolding of thermophilic and mesophilic enzymes from their chemically unfolded state; moreover, Ssocpn prevents the irreversible inactivation of native proteins by suppressing their precipitation upon heating. Both the activity in assisting refolding of unfolded proteins and that in preventing heat denaturation of native proteins require the hydrolysis of ATP.

The chaperone-based strategies in different technological fields are discussed, and the advantages in using archaeal chaperonins are underlined.

Index Entries: Chaperonin; protein folding; archaea.

INTRODUCTION

The folding of newly synthesized polypeptide chains is assisted in vivo by two classes of proteins: the enzymes (namely, protein disulfide isomerase and peptidyl prolyl *cis-trans* isomerase) catalyze the covalent slow steps of

*Author to whom all correspondence and reprint requests should be addressed. E-mail: guaglia@ds.unina.it

the process, whereas the "chaperone proteins" suppress the aggregation among the folding intermediates, which is responsible for misfolding *(1)*.

The specialized term "chaperonins" describes a family of chaperone proteins *(2)*. Chaperonins are ubiquitous, essential, and stress-induced proteins endowed with an intrinsic ATPase activity; their quaternary structure consists of two rings of about 60 kDa-subunits each, stacked face-to-face with a central cavity. The sequence-related chaperonins from Eubacteria and eukaryotic organula have seven identical subunits per ring, and their activity is regulated by smaller co-chaperonins. In contrast, the chaperonins isolated from the hyperthermophilic bacteria that belong to the kingdom of Archaea and the chaperonins of the eukaryotic cytosol share similarity in sequence, the presence of 8–9 nonidentical subunits per ring, and the absence of any co-operating proteins. The activity of the chaperonin from the hyperthermophilic archaeon *Sulfolobus solfataricus* (Ssocpn[3]) in promoting correct refolding in vitro has been described *(3)*, and its nine-fold oligomeric organization elucidated *(4)*. Herein we focus on the functionality of this chaperonin, and discuss the potential use in biotechnology of the chaperone proteins.

MATERIALS AND METHODS

Chemicals and Enzymes

ATP, guanidinium hydrochloride (GdmCl), and lysozyme from chicken egg white (183 U/mg) were purchased from Sigma (St. Louis, MO); [γ-^{32}P]ATP (3000 Ci/mmol) was from Amersham (Arlington Heights, IL). α-Glucosidase from yeast (133 U/mg) and p-nitrophenyl-α-D-maltoside were from Boehringer Mannheim (Germany). The enzymes from *S. solfataricus* (malic enzyme [ME], alcohol dehydrogenase [ADH], and glutamate dehydrogenase [GDH]) were purified as reported in refs. 5, 6, and 7, respectively. The other chemicals were of the highest grade available.

Purification of the Chaperonin

S. solfataricus strain MT-4 was grown aerobically at 87°C and pH 3.0 as described in ref. *5*, and the cells were harvested in the exponential growth phase. The crude extract was prepared as described in ref. *3*, and the chaperonin was purified by the following procedure. The crude extract (20 mg) was ultrafiltered in a stirred cell equipped with a XM300 membrane (Amicon); the sample was rinsed with 10 m*M* Tris-HCl buffer, pH 8.4 (Buffer A) until the absorbance of the filtrate solution at 280 nm was zero. The retentate solution (2 mg) was loaded onto a Matrex Gel Red A affinity chromatography column (Amicon, 1 × 3.5 cm) equilibrated in Buffer A and eluted with a linear 0–0.4*M* NaCl gradient in Buffer A at a flow rate of 15 mL/h; the fractions containing the chaperonin (1 mg total protein) were pooled, dialyzed against Buffer A, and stored at 4°C.

Enzyme Activity Assays

The enzymes were assayed at 50°C (enzymes from *S. solfataricus*), at 30°C (α-glucosidase), or at 25°C (lysozyme) by a Varian DMS-100 recording spectrophotometer equipped with a thermostated cell compartment.

The assay mixtures for the enzymes from *S. solfataricus* were: ME, 40 m*M* glycine/NaOH buffer, pH 8.0, 0.05 m*M* NADP, 1 m*M* $MgCl_2$, 1 m*M* L-malate; ADH, 25 m*M* barbital/HCl buffer, pH 8.0, 2 m*M* NAD, 5 m*M* benzyl alcohol; GDH, 0.1 m*M* sodium phosphate buffer, pH 8.0, 1 m*M* NADP, 0.1 m*M* EDTA, 25 m*M* L-glutamate.

The activity of α-glucosidase was assayed in 0.1*M* sodium phosphate buffer, pH 7.0, 0.3 m*M* p-nitrophenyl-α-D-maltoside (1 mL final vol) as described in ref. 8, the continuous increase of absorbance at 400 nm was monitored, and an extinction coefficient for p-nitrophenol was 9.6 m*M*. The assay mixture for lysozyme consisted of 1 mL of a fresh suspension 0.1 mg/mL of lyophilized *Escherichia coli* cells in 50 m*M* Tris/HCl, pH 7.4; following the addition of lysozyme, the time required for an absorbance decrease of 0.1 O.D. at 350 nm was measured, and the specific activity was calculated by the formula $time^{-1}$ mg^{-1} of protein *(9)*.

Miscellaneous

Protein concentration was determined by the Bio Rad assay *(10)*, using bovine serum albumin as the standard. SDS-PAGE analysis was carried out according to Laemmli *(11)* in a 10% or 12.5% polyacrylamide gel. Protein aggregation was monitored as turbidity at 450 nm; the maximal turbidity was taken as 100% aggregation.

RESULTS

Purification of Ssocpn, Molecular size, and ATPase Activity

The chaperonin was purified from crude extracts of *S. solfataricus* by a fast and high-yield procedure. The ultrafiltration of the cytosol through a 300 kDa cut-off membrane, in place of the size exclusion chromatography in the procedure previously described *(3)*, allows a rapid and effective fractionation of the proteins; the knowledge that chaperonins have binding sites for ATP suggested the use of Matrex Gel Red A affinity chromatography whose ligand mimicks the nucleotide coenzyme structure.

The native molecular weight of Ssocpn was 920 kDa. SDS-PAGE analysis of the sample carried out at in a 12.5% polyacrylamide gel showed one band at 57 kDa (Fig. 1A); two bands, at 57 and 60 kDa, were distinguishable when SDS-PAGE analysis of the sample was carried out in a 10% polyacrylamide gel (Fig. 1B). The hetero-oligomeric composition of the *S. solfataricus* chaperonin has been reported by other authors *(4)*.

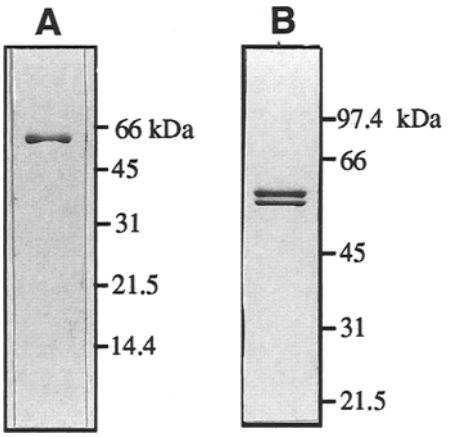

Fig. 1. SDS-PAGE analysis of a pure sample of Ssocpn in a 12.5% **(A)** and in a 10% **(B)** polyacrylamide gel. Molecular weight standards are reported.

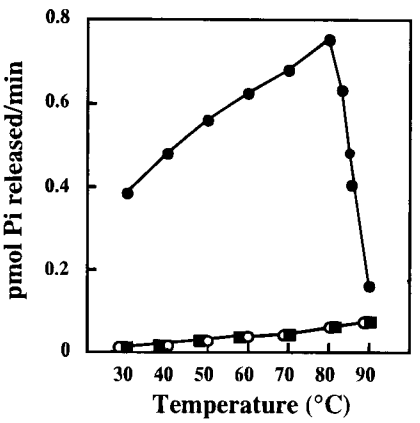

Fig. 2. Potassium and temperature dependence of the ATPase activity of Ssocpn. The amount of Pi released was relative to the Ssocpn-mediated hydrolysis in the absence (open circles) or in the presence (closed circles) or potassium ions, and to the spontaneous hydrolysis of ATP in the absence of Ssocpn (closed squares). The assay mixture consisted of 50 m*M* sodium phosphate buffer, pH 8.0, 0.5 m*M* ATP, 30 μCi of [γ-^{32}P]ATP, 0.5 m*M* $MgCl_2$, 10 m*M* KCl, 8 μg Ssocpn, in a total volume of 150 μL. After 5 min of incubation at the desired temperature, 25 μL-aliquot were drawn from each mixture, added to 0.5 mL of a suspension containing 50 m*M* HCl, 5 m*M* H_3PO_4, 7% activated charcoal, and were centrifuged at 4000*g* for 20 min; the radioactivity of the supernatant was counted on a 100 μL-aliquot by a liquid scintillation counter. Reproduced from ref. 3 with kind permission from Cambridge University Press.

Figure 2 illustrates the features of the intrinsic ATPase activity of Ssocpn. The archaeal chaperonin displays an ATPase activity that is fully dependent on the presence of potassium ions; *Escherichia coli* chaperonin GroEL also hydrolyzes ATP in a potassium-dependent manner *(12)*. The

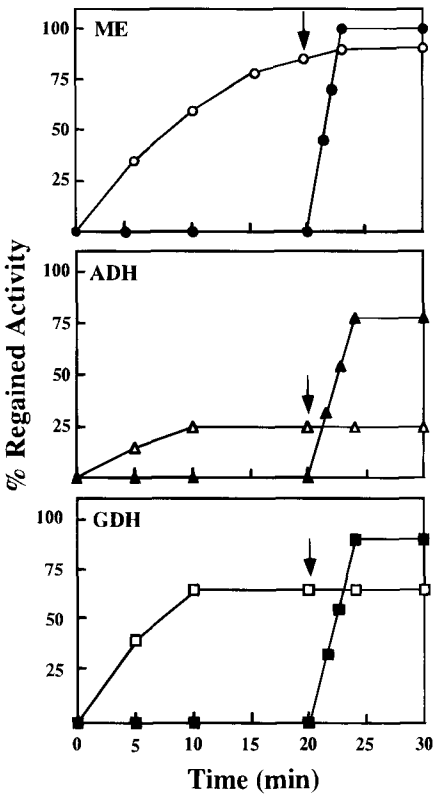

Fig. 3. Refoldings of chemically denatured ME, ADH, and GDH from *S.solfataricus* in the absence (open symbols) and in the presence (closed symbols) of Ssocpn. The enzymes were denatured upon incubation in 4*M* GdmCl for one night at 37°C (only ME in the presence of 0.2*M* 2-mercaptoethanol) at the following protein amounts: ME, 80 μg/mL; ADH, 600 μg/mL; GDH, 100 μg/mL. Aliquots drawn from the denaturation mixtures were diluted in the following refolding mixtures (final vol 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 m*M* sodium phosphate pH 8.0 (32 n*M*); ADH, 0.1*M* sodium phosphate pH 8.0, 5 μ*M* $ZnCl_2$ (120 n*M*); GDH, 0.1*M* sodium phosphate pH 8.0 (38 n*M*). After 20 min, ATP/Mg/K was added to the mixtures (arrows), and the incubations were continued at the same temperature. Reproduced from ref 3 with kind permission from Cambridge University Press.

optimum of ATPase activity for the chaperonin from the hyperthermophilic bacterium is 80°C, in good accordance with the microorganism growth temperature of 87°C.

The Activity of Ssocpn in Promoting Correct Refolding

Ssocpn assists the refolding at high temperature of three thermophilic enzymes (Fig. 3). Dimeric ME (50 kDa-subunit) and ADH (35 kDa-subunit), and exameric GDH (45 kDa-subunit) from *S. solfataricus* were unfolded by incubation in 4*M* GdmCl for one night at 37°C. The sponta-

neous refoldings were obtained at 50°C upon dilution from the denaturant in refolding mixtures designed for each enzyme as concerns protein amount, buffer, pH, ionic strength, and cofactors. The presence in the refolding mixtures of Ssocpn in a molar ratio of one chaperonin oligomer to one single polypeptide chain caused the complete inhibition of spontaneous refoldings; the refoldings resumed upon ATP hydrolysis by Ssocpn (obtained by the addition of ATP/Mg/K [0.5 m*M* ATP, 0.5 m*M* $MgCl_2$, 10 m*M* KCl] in the mixture), and the final yields in Ssocpn-assisted refoldings were always higher with respect to those of spontaneous events.

Yeast α-glucosidase (one 68.5 kDa-chain) was completely unable to spontaneously regain its activity when diluted from 4*M* GdmCl in a refolding mixture at 30°C; the light scattering increase showed that aggregation occurred quickly upon dilution from the denaturant (Fig. 4). We diluted denatured α-glucosidase in a refolding mixture containing Ssocpn in an equimolar ratio with the protein chain; the chaperonin completely suppressed aggregation reactions during refolding, and an activity regain of 10% was calculated upon ATP hydrolysis. It is noteworthy that the presence of dithiothreitol in the refolding mixture was essential for regaining the activity; the reducing agent is likely to prevent the formation of incorrect disulfide bonds. The yield of active α-glucosidase did not exceed 10% when refolding was carried out at lower temperature or protein concentration.

The aforementioned results can be reasonably interpreted as follows. Ssocpn interacts with the hydrophobic surfaces on the refolding intermediates; as a consequence of this binding, the spontaneous refolding of the thermophilic enzymes is arrested, and the aggregation during refolding of α-glucosidase is suppressed. Upon hydrolysis of ATP by Ssocpn (and not simply upon the binding of the nucleotide to Ssocpn) the bound molecule is released in solution in a folded (or folding-competent) state. The role played by ATP in the action mechanism of chaperonins is crucial, even if the interpretation of the molecular consequences of ATP binding and/or hydrolysis remain to be clarified.

The Activity of Ssocpn in Preventing Thermal Aggregation of Native Proteins

Chaperonins are thought to play a cellular role not only in assisting folding of nascent proteins, but also in protecting mature proteins against denaturation. Thermal denaturation of some native proteins is accompanied by the formation of precipitates as a consequence of hydrophobic intermolecular interactions; protein thermal aggregation is responsible for an irreversible loss of biological activity.

Native chicken egg white lysozyme (one 14.4 kDa-chain) and yeast α-glucosidase form visible aggregates upon heating at 70 and 40°C, respectively (Fig. 5). The addition of excess Ssocpn to the solutions after they had precipitated did not exert any effect. When identical solutions of

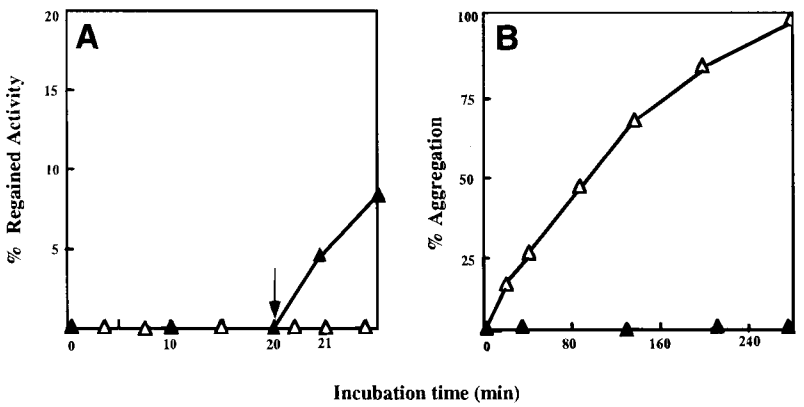

Fig. 4. Refolding **(A)** and aggregation in the time course of refolding **(B)** of chemically denatured yeast α-glucosidase in the absence (open symbols) and in the presence (closed symbols) of Ssocpn. The enzyme (1 mg/mL) was denatured by a 5-h incubation at room temperature in 4*M* GdmCl. Refolding was started by diluting 100-fold the unfolded protein in a refolding mixture consisting of 50 m*M* sodium phosphate pH 8.0, 10 μ*M* dithiothreitol in the absence or in the presence of Ssocpn at an equimolar ratio with the polypeptide chain, and incubated at 30°C. After the addition of ATP/Mg/K (arrow) the incubations were continued at 40°C. Note the scale change at the time indicated by the arrow.

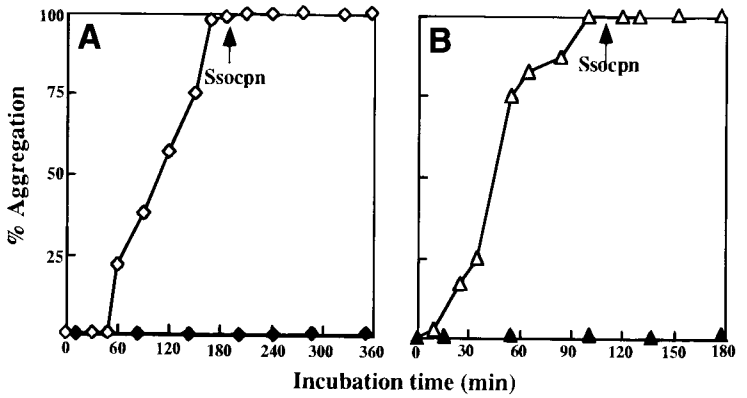

Fig. 5. Thermal aggregation of chicken egg white lysozyme **(A)** and yeast α-glucosidase **(B)** in the absence (open symbols) and in the presence (closed symbols) of Ssocpn. Lysozyme (80 μg in 1 mL) was incubated at 70°C in 10 m*M* Tris-HCl buffer, pH 8.0, in the absence or in the presence of Ssocpn at an equimolar ratio with the polypeptide chain. α-Glucosidase (200 μg in 1 mL) was incubated at 40°C in 50 m*M* sodium phosphate buffer, pH 8.0, in the absence or in the presence of Ssocpn at an equimolar ratio with the polypeptide chain.

lysozyme and α-glucosidase were heated in the presence of Ssocpn at an equimolar ratio between chaperonin oligomer and the protein chain, light scattering did not increase. The effect of Ssocpn in preventing thermal

aggregation was specific: the presence of an excess of bovine serum albumin in the place of Ssocpn in the solutions did not prevent the occurrence of precipitates.

Figure 6 shows the loss of activity of the two proteins at the designated temperatures, in the absence and in the presence of Ssocpn. Ssocpn did not modify the kinetics of inactivation; that is, Ssocpn did not prevent the loss of enzyme activity. Following the hydrolysis of ATP by the chaperonin, final activity regains of 78 and 55% were calculated in the solutions of lysozyme and α-glucosidase, respectively.

The results show that Ssocpn did not rescue the protein aggregates, but rather it interacted with lysozyme or α-glucosidase before they started to precipitate, maintaining them in solution. The chaperonin probably interacted with the hydrophobic protein surfaces that became exposed to the solvent during thermal denaturation. The molecules remained in an inactive but folding-competent conformation while bound to the chaperonin, and correctly folded molecules were released in solution upon ATP hydrolysis.

DISCUSSION

A large body of literature indicates that chaperonins bind unfolded or partially folded protein molecules with a rather low specificity. *S. solfataricus* chaperonin recognizes several different thermophilic and mesophilic proteins, demonstrating its wide substrate specificity; since 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.

The Ssocpn-assisted experiments described were carried out at an equimolar ratio between the chaperonin oligomer and the protein substrate chain. In no case does the final yield of active enzyme improve at a molar excess of Ssocpn over the single chain. We are currently investigating the important issue of the binding stoichiometry, in an attempt to determine the minimum amount of Ssocpn able to promote the correct refolding of unfolded proteins and to prevent thermal denaturation of native proteins.

Thanks to the knowledge concerning the proteins that assist protein folding (enzymes and chaperones) accumulated for the academic research, protein folding now opens the door to biotechnology. Let us dwell on this issue.

The production of recombinant proteins is often accompanied by the formation of inclusion bodies, which are insoluble, incorrectly folded aggregates of the protein. A common method to regain biologically active molecules involves the recovery of the inclusion body, solubilization of the aggregates in a strong denaturant, dilution, and rearrangement of the disulfide bonds through the use of oxidizing reagents; however, the effi-

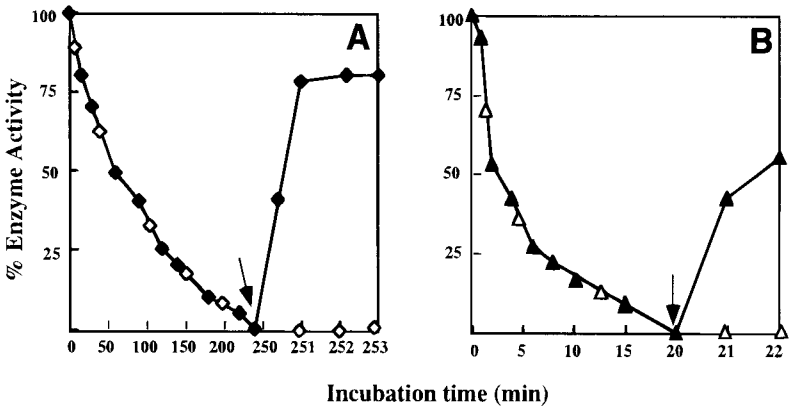

Fig. 6. Thermal inactivation of chicken egg white lysozyme **(A)** and yeast α-glucosidase **(B)** in the absence (open symbols) and in the presence (closed symbols) of Ssocpn. Lysozyme and α-glucosidase were heated exactly as described in the legend to Fig. 5. After the addition of ATP/Mg/K (arrow) the incubations were continued at the same temperatures. Note the scale change at the time indicated by the arrow.

ciency of the method is often very low, mainly with multi-subunit and disulfide bond-containing proteins (see ref. 13 and references therein). Molecular chaperones can aid in producing biologically active foreign proteins. First, chaperones increase the activity regain yield during refolding of the recombinant denatured protein: a successful "chaperone-based" protocol for in vitro refolding of an immunotoxin has been reported *(14)*. Second, the strain of the host can be engineered to overproduce chaperones: the solubility and the activity of a type of dihydrofolate reductase are enhanced in *E. coli* cells that co-overproduce the enzyme, the chaperonin GroEL, and the co-chaperonin GroES *(15)*.

The exploitation of the chaperone molecules in medicine also seems a real possibility (reviewed in ref. 16). Since the expression of these molecules in the cell increases as a response to an environmental insult, their role as marker of cell injury and autoimmune diseases is under investigation. Moreover, protein folding defects are implicated with some inherited diseases, such as cystic fibrosis, and emphysema that is associated with a folding defective mutation of α1-antitrypsin *(17)*.

The chaperonin of *S. solfataricus* does not require a co-chaperonin for its function (unlike the chaperonins from Eubacteria and eukaryotic organula); it is active over a wide range of temperatures (from 30 to 80°C); it displays a very low substrate specificity; and it is an extremely stable molecule, given the source from which it has been isolated. These features make Ssocpn an interesting tool for chaperonin-based strategies, and the study of its activity is therefore extremely timely.

ACKNOWLEDGMENTS

This work was supported by MURST 40%, by C.N.R. Progetto Strategico Molecular Manufacturing, and by EEC Project Biotechnology of Extremophiles, contract BIO2-CT93-0274.

REFERENCES

1. Gething, M. and Sambrook, J. (1992), *Nature* **355**, 33–45.
2. Horwich, A. L. and Willison, K. R. (1993), *Phil. Trans. R. Soc. Lond.* **339**, 313–326
3. Guagliardi, A., Cerchia, L., Bartolucci, S., and Rossi, M. (1994), *Protein Sci.* **3**, 1436–1443.
4. Knapp, S., Schmidt-Krey, I., Hebert, H., Berman, T., Jornvall, H., and Ladenstein, R. (1994), *J. Mol. Biol.* **242**, 397–407.
5. Bartolucci, S., Rella, R., Guagliardi, A., Raia, C. A., Gambacorta, A., De Rosa, M., and Rossi, M. (1987), *J. Biol. Chem.* **262**, 7725–7731.
6. Ammendola, S., Raia, C. A., Caruso, C., Camardella, L., D'Auria, S., De Rosa, M., and Rossi, M. (1992), *Biochemistry* **31**, 12,514–12,523.
7. Consalvi, V., Chiaraluce, R., Politi, L., Gambacorta, A., De Rosa, M., and Scandurra, R. (1991), *Eur. J. Biochem.* **196**, 459–467.
8. Halvorson, H. (1966), *Methods Enzymol.* **8**, 559–562.
9. Tsugita, A., Inouye, M., Terzaghi, E., and Streisinger, G. (1968), *J. Biol. Chem.* **243**, 391–397.
10. Bradford, M. M. (1976), *Anal. Biochem.* **72**, 248–254.
11. Laemmli, U. K. (1970), *Nature* **227**, 680–685.
12. Viitanen, P. V., Lubben, T. H., Reed, J., Goloubinoff, P., O'Keefe, D. P., and Lorimer, G. H. (1990), *Biochemistry* **29**, 5665–5671.
13. Thatcher, D. R. and Hitchcock, A. (1994), in *Mechanisms of Protein Folding*, Pain, R. H., ed., Oxford University, pp. 229–261.
14. Buchner, J., Brinkmann, U., and Pastan, I. (1992), *Bio/Technology* **10**, 682–685.
15. Dale, G. E., Schonfeld, H.-J., Langen, H., and Stieger, M. (1994), *Protein Engng.* **7**, 925–931.
16. Welch, W. J. (1992), *Physiol. Rev.* **72**, 1063–1081.
17. Yu, M.-H., Lee, K. N. and Kim, J. (1995), *Nature Struct. Biol.* **2**, 363–367.