The EMBO Journal vol.9 no.7 pp.2315–2319, 1990

The *Escherichia coli* heat shock proteins GroEL and GroES modulate the folding of the β-lactamase precursor

Axel A.Laminet[1], Thomas Ziegelhoffer[2], Costa Georgopoulos[2] and Andreas Plückthun[1,3]

[1]Genzentrum der Universität München, Max-Planck-Institut für Biochemie, D-8033 Martinsried, FRG and [2]Department of Cellular, Viral and Molecular Biology, University of Utah Medical Center, Salt Lake City, UT 84132, USA

[3]To whom correspondence should be addressed

Communicated by E.-L.Winnacker

One of the fundamental problems in biochemistry is the role of accessory proteins in the process of protein folding. The *Escherichia coli* heat shock protein complex GroEL/ES has been suggested to be a 'chaperonin' and be involved in both oligomer assembly as well as protein transport through the membrane. We show here that the folding of the purified precursor of β-lactamase is inhibited by purified GroEL or the GroEL/ES complex with a stoichiometry of one particle per molecule of pre-β-lactamase. Purified GroES alone has no effect on folding. After Mg^{2+}ATP addition folding resumes and the yield of active enzyme is higher than in the absence of GroEL or GroEL/ES. Unexpectedly, GroEL or GroEL/ES, when added to folded pre-β-lactamase, lead to an apparent net 'unfolding', probably to a collapsed state of the protein, which can be reversed by the addition of Mg^{2+}ATP. The reversible and Mg^{2+}ATP-dependent association of GroEL/ES with non-native proteins might explain its postulated role in both protein transport and oligomer assembly.

Key words: protein folding/protein transport/β-lactamase/GroEL/ES/chaperonins

Introduction

While it has been demonstrated that a large number of proteins spontaneously fold *in vitro* to their native structure (reviewed e.g. in Anfinsen, 1973; Creighton, 1978; Jaenicke, 1987) and thus apparently need no specific helper factor, it is possible that within the cell accessory proteins guide the folding process. Proteins that have been discussed as being involved in the folding process of other proteins include protein disulfide-isomerase (Bulleid and Freedman, 1988) and proline *cis-trans* isomerase (Lang *et al.*, 1987). Two specific situations have been discussed in which the involvement of additional protein factors, among them heat-shock related proteins (Pelham, 1986; Ellis, 1987; Bochkareva *et al.*, 1988; Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Hemmingsen *et al.*, 1988; Zimmermann *et al.*, 1988; Flynn *et al.*, 1989; Goloubinoff *et al.*, 1989a,b), is likely: the correct assembly of some oligomeric proteins and the stabilization of a non-native state for membrane transport. Such factors may prevent incorrect interactions within and between polypeptides (Ellis and Hemmingsen, 1989; Ellis *et al.*, 1989).

In vivo studies of protein export in *Escherichia coli* (Randall and Hardy, 1986; Cover *et al.*, 1987) as well as *in vitro* studies of mitochondrial import (Eilers and Schatz, 1986) have indicated that the passage of a protein through a membrane occurs in a non-native state. In *E.coli*, three protein factors have been identified that may play a role in securing a transport competent conformation of the precursor protein: SecB (Collier *et al.*, 1988; Kumamoto and Gannon, 1988), trigger factor (Crooke and Wickner, 1987; Crooke *et al.*, 1988) and the GroEL/ES complex (Bochkareva *et al.*, 1988; Lecker *et al.*, 1989; Georgopoulos and Ang, 1990). Additionally, a factor homologous to the 54 kd subunit of the eukaryotic signal recognition particle (SRP) (Walter *et al.*, 1984) has been discovered in *E.coli* (Bernstein *et al.*, 1989; Römisch *et al.*, 1989). Yet, while this eukaryotic SRP subunit can be crosslinked to a nascent precursor in a eukaryotic translation system (Kurzchalia *et al.*, 1986), recent crosslinking studies in an *E.coli* translation system demonstrated instead an interaction of nascent pre-β-lactamase with GroEL (Bochkareva *et al.*, 1988).

GroEL is a ring-shaped oligomer made up of 14 subunits (Hendrix, 1979; Hohn *et al.*, 1979) and has homologs in mitochondria (McMullin and Hallberg, 1988; Ostermann *et al.*, 1989) and chloroplasts (Hemmingsen *et al.*, 1988), while GroES is probably a 7-mer (Chandrasekhar *et al.*, 1986; Hemmingsen *et al.*, 1988) for which no eukaryotic homologs have been identified. The two GroE proteins interact only in the presence of hydrolyzable Mg^{2+}ATP (Chandrasekhar *et al.*, 1986; Fayet *et al.*, 1989; Georgopoulos and Ang, 1990). The importance of the GroE complex is reflected in the fact that they are absolutely essential for *E.coli* growth (Fayet *et al.*, 1989) and must serve an important function in addition to that in the heat shock response.

We have recently developed a procedure for purifying the precursor of β-lactamase to homogeneity and have conducted folding studies with it (Laminet and Plückthun, 1989). In the experiments described here we investigated the effect of GroEL/ES on the folding of β-lactamase and its precursor and studied the mechanism involved.

Results and discussion

To investigate the folding reaction, β-lactamase and pre-β-lactamase were diluted from a urea solution into a refolding cocktail containing the factor to be tested. The rate of folding was determined by directly measuring the increase in enzymatic activity as described in Materials and methods.

The addition of GroEL (at a molar ratio of ~14 subunits per molecule of pre-β-lactamase) completely inhibited any folding of the precursor in the absence of Mg^{2+}ATP

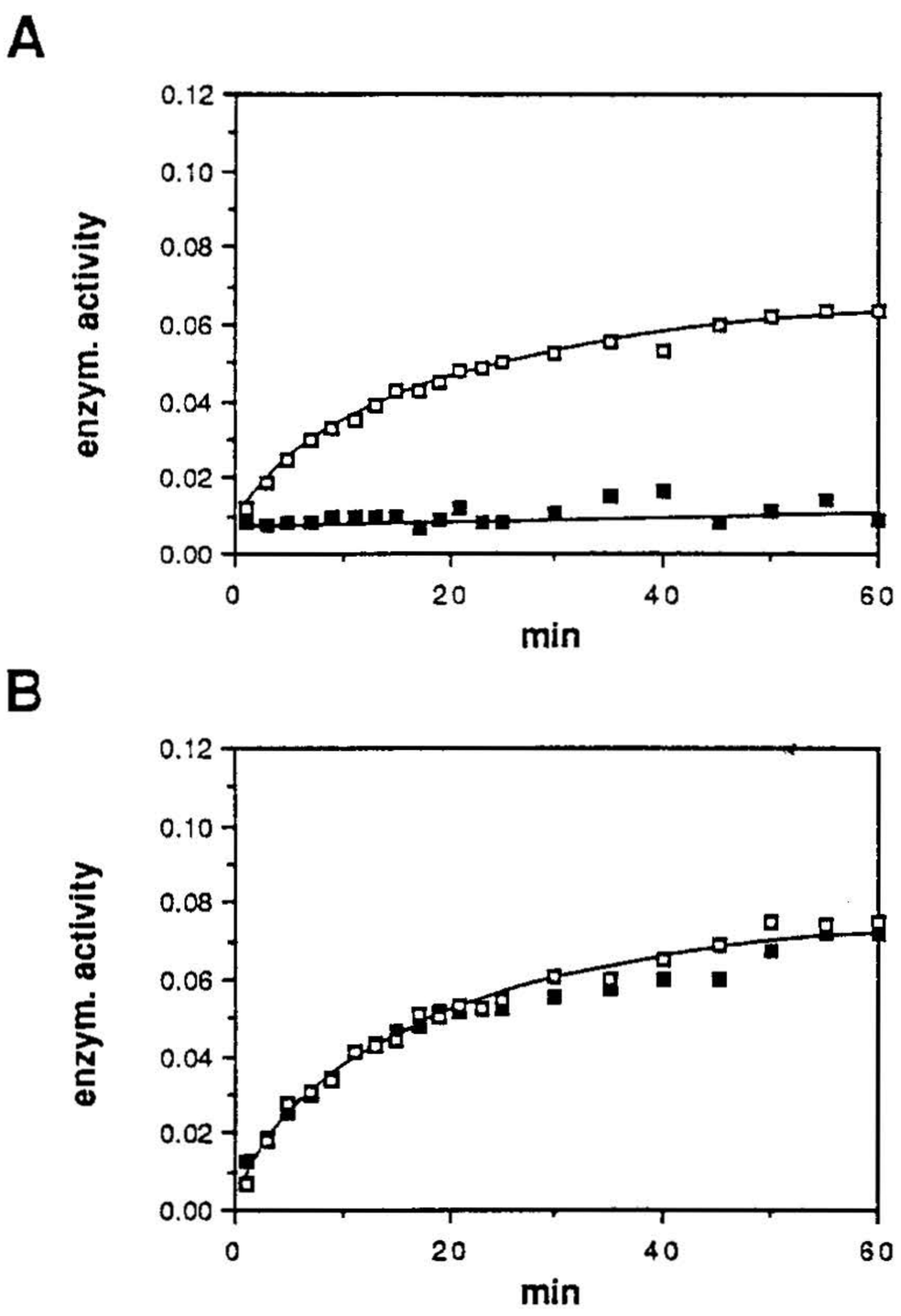

Fig. 1. (**A**) Effect of GroEL on the folding of pre-β-lactamase (□, no GroEL; ■, with GroEL). (**B**) Effect of GroES on the folding of pre-β-lactamase (□, no GroES, ■, with GroES). (**C**) Stoichiometry of interaction of GroEL with pre-β-lactamase (□, no GroEL; ⊡, 25 mol% GroEL; ◘, 50 mol% GroEL; ■, 100 mol% GroEL). Pre-β-lactamase was diluted from a urea containing stock solution into the refolding cocktail. The reaction was followed by withdrawing aliquots that were immediately assayed spectrophotometrically for β-lactamase activity. The enzymatic activity is given in arbitrary units. The refolding cocktail contained the folding modulator to be tested before pre-β-lactamase was added. The final concentrations in the refolding cocktail were 0.16 μM pre-β-lactamase and either 0.16 μM GroEL particles (calculated assuming a 14-mer as the particle size, in **A**) or 0.16 μM GroES particles (calculated assuming a 7-mer as the particle size, in **B**). In **C**, 100 mol% refers to 14 subunits of GroEL per molecule of pre-β-lactamase (0.16 μM).

(Figure 1A). This retardation of folding is reversed by the addition of Mg^{2+}ATP (Figure 2B). In the latter case, the yield of folded protein is higher than that obtained in the absence of GroEL. This increase in folding yield is also observed when GroEL and Mg^{2+}ATP are present from the beginning of the folding reaction (data not shown). In contrast, the addition of GroES alone has no measurable

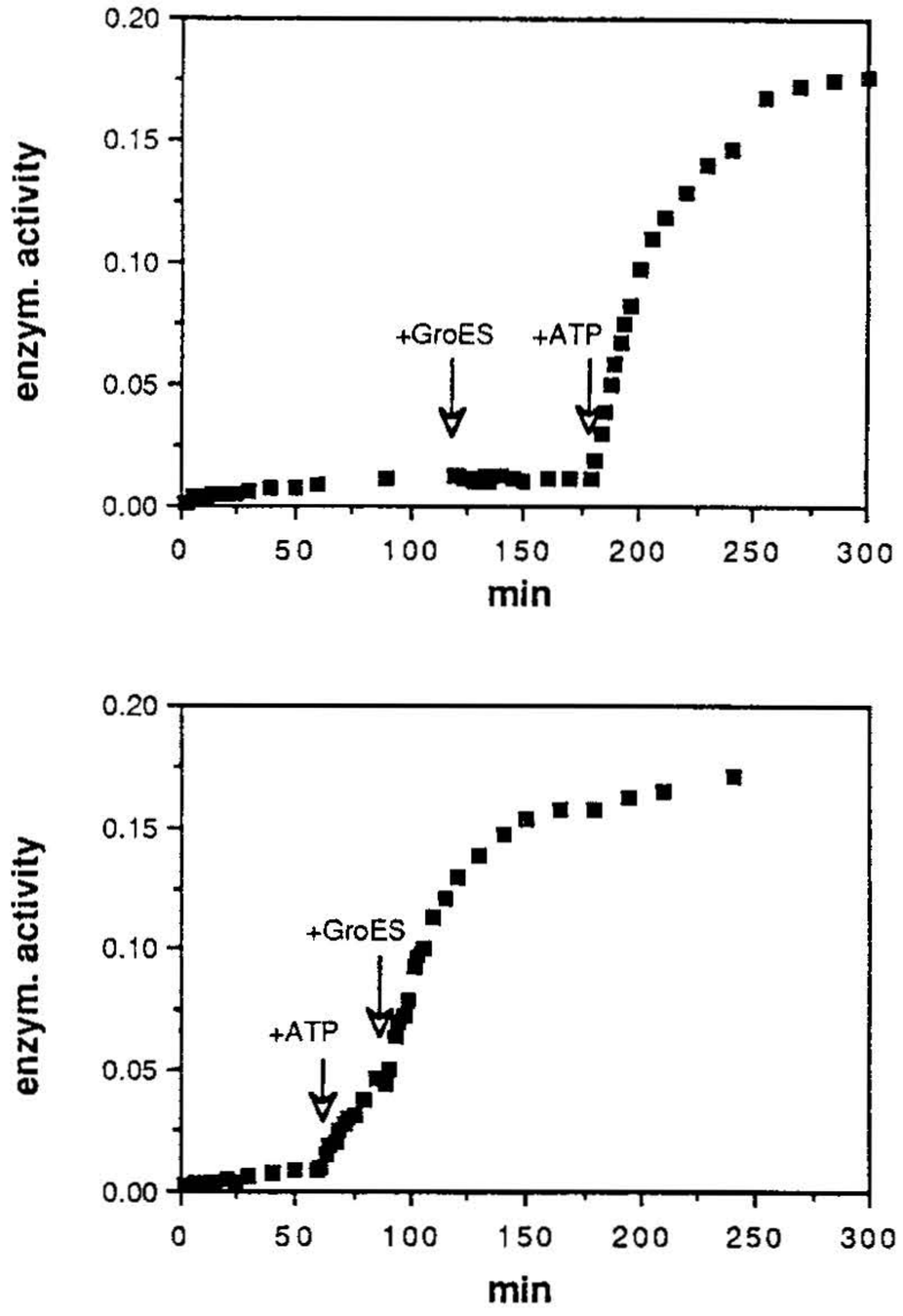

Fig. 2. Effect of the addition of GroES and Mg^{2+}ATP to a GroEL-arrested folding reaction of pre-β-lactamase. Pre-β-lactamase was diluted into a refolding cocktail containing an equimolar amount of GroEL particles (Final concentrations: 0.12 μM). At the indicated times, Mg^{2+}ATP (5 mM) and GroES (in equimolar amounts, related to the number of particles) was added to the refolding solution. All assay conditions were as in Figure 1 and given in Materials and methods. (**A**) GroES added before Mg^{2+}ATP; (**B**) Mg^{2+}ATP added before GroES.

effect on the folding reaction either in the absence of Mg^{2+}ATP (Figure 1B) or in its presence (data not shown). The inhibition of folding was measured in the presence of various amounts of GroEL and it appears that a molar ratio of ~14 GroEL subunits per pre-β-lactamase molecule is required for complete folding inhibition (Figure 1C). This indicates that only one pre-β-lactamase molecule binds to a single GroEL complex, consistent with observations on other precursors (Lecker *et al.*, 1989).

When the folding reaction is inhibited by GroEL in the absence of Mg^{2+}ATP, the further addition of GroES to a GroEL-pre-β-lactamase mixture has no measurable effect: there is still no folding of pre-β-lactamase (Figure 2A). If, however, the 'folding arrest' is released first by Mg^{2+}ATP and GroES is added later, a small acceleration in the initial rate of release or folding over that observed in the presence of GroEL and Mg^{2+}ATP alone is seen (Figure 2B). It thus appears as if the folding arrest is mediated by GroEL alone, as is the Mg^{2+}ATP dependent release, but the presence of GroES makes the release reaction more efficient. In no experiment with GroEL or GroES, however, was the half-time of increase in β-lactamase activity dramatically changed compared to that observed in the absence of any factor. The simplest explanation of this observation is that the nature of the rate limiting step in folding of pre-β-lactamase has not been changed by GroEL/ES.

The folding inhibition can also be demonstrated with

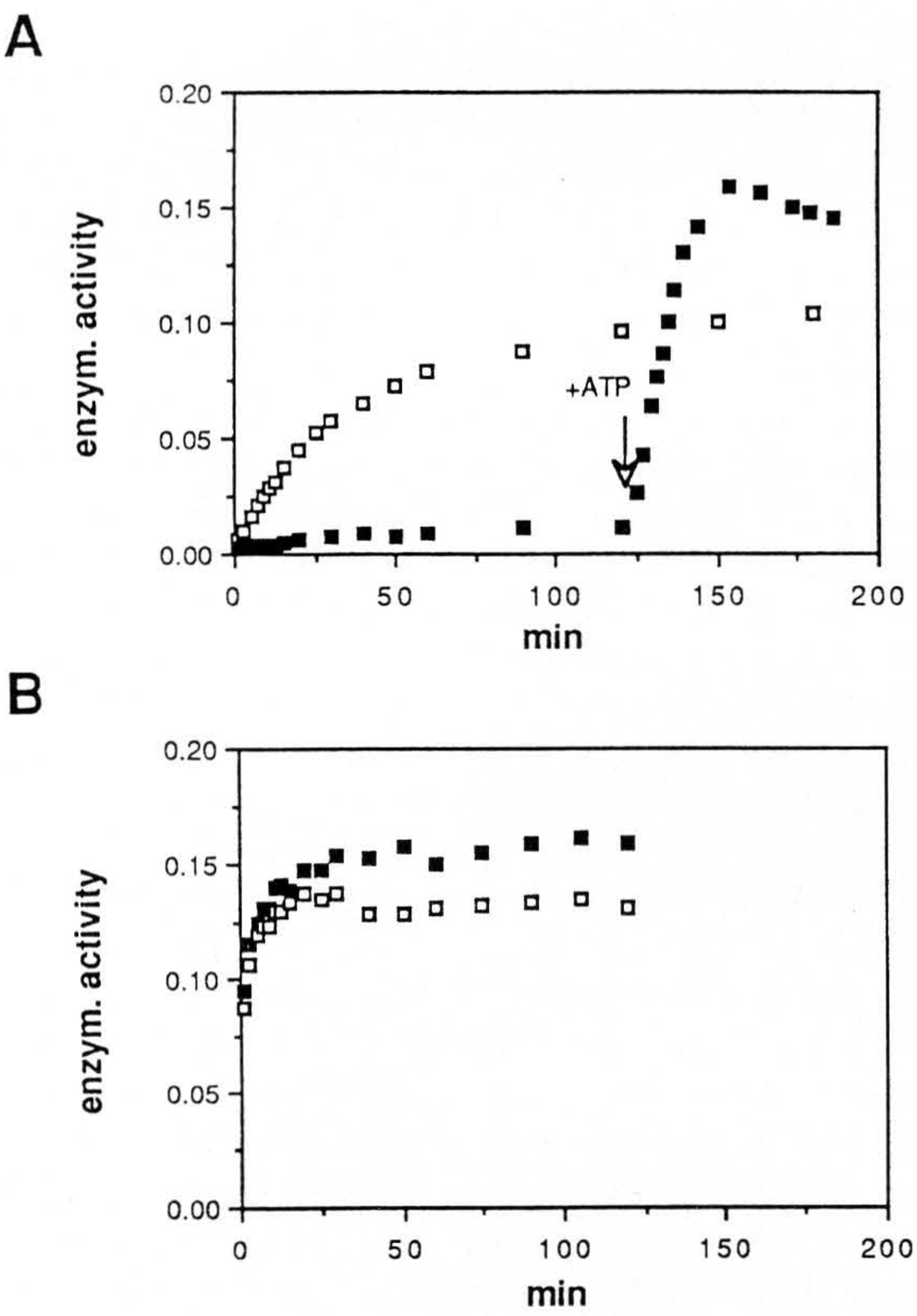

Fig. 3. Effect of a GroEL/ES mixture on the folding of the precursor and mature β-lactamase (□ no GroEL/ES, ■ with GroEL/ES). GroEL and GroES were added to final concentrations identical in particle molarity to the molarity of β-lactamase. All assay conditions were as in Figure 1 and given in Materials and methods. (**A**) Pre-β-lactamase and GroEL/ES complex (all components 0.12 μM, particle molarity) were used. At the time indicated by the arrow, Mg^{2+}ATP was added to a final concentration of 5 mM. (**B**) Reduced unfolded β-lactamase and GroEL/ES complex (all components 0.08 μM, particle molarity) were employed. Native mature β-lactamase was unfolded and reduced in 8 M urea containing 5% mercaptoethanol for 1 h before dilution into the refolding cocktail. Note that mature β-lactamase has reached ~65% of the final activity in the first measurement after 1 min.

premixed GroEL/GroES, and the subsequent Mg^{2+} ATP-dependent release shows the same behavior as with GroEL alone (Figure 3A). When an identical experiment is carried out with mature β-lactamase (Figure 3B), there is no measurable effect on the folding rate either in the presence or the absence of Mg^{2+}ATP. The folding rates of the mature and precursor β-lactamase are very different under the same experimental conditions (Laminet and Plückthun, 1989) and, most likely, also in the cell. Either this difference in folding rates or a direct recognition of the signal sequence by GroEL may be responsible for the different behavior of mature and precursor β-lactamase.

We then examined whether the GroEL/ES complex could interact with refolded pre-β-lactamase. The precursor was refolded in the absence of protein factors and Mg^{2+}ATP, until a plateau of constant enzymatic activity was reached. Then, the GroEL/ES complex was added. Surprisingly, a very slow disappearance of enzymatic activity is observed. The activity reappears after addition of Mg^{2+}ATP (Figure 4A). The identical result is observed with GroEL alone (data not shown).

The GroEL mediated reaction is specific for the precursor

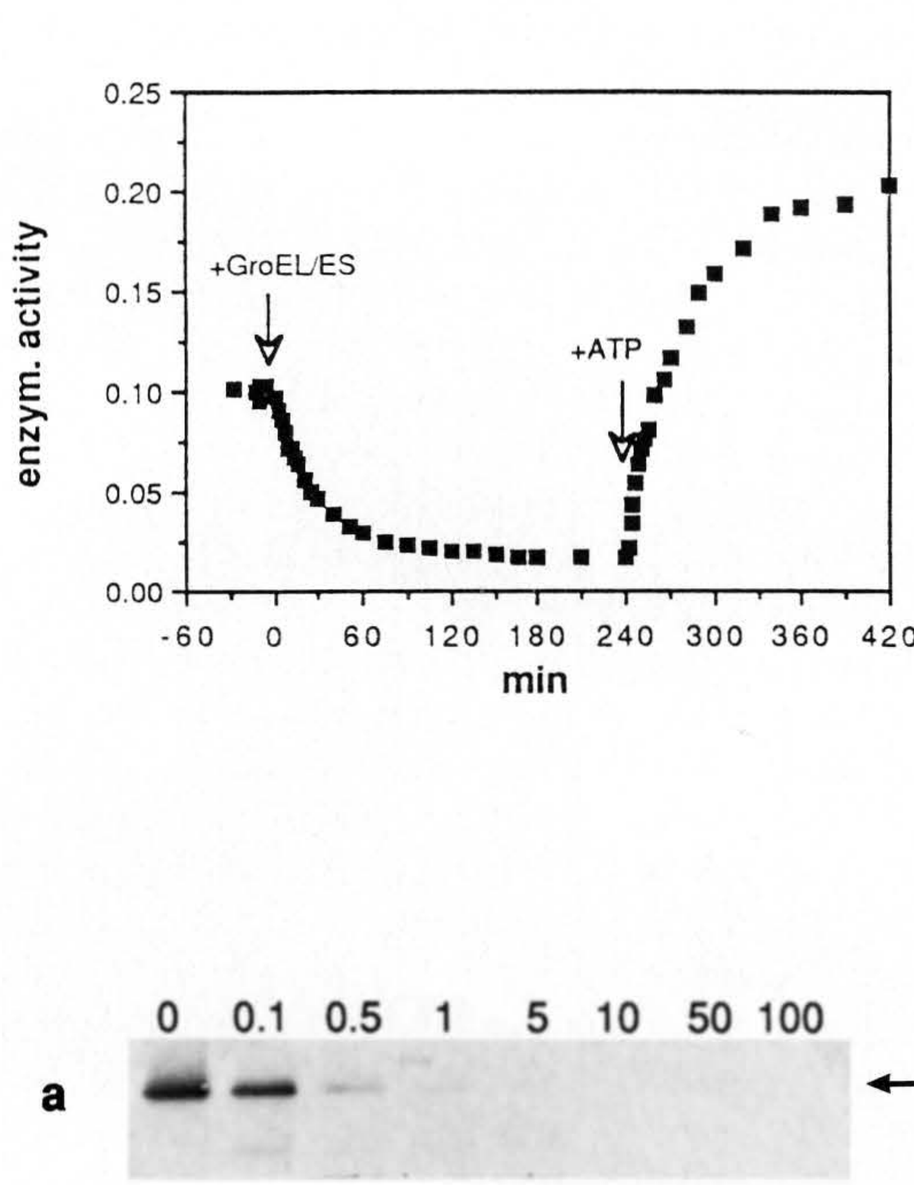

Fig. 4. (**A**) Apparent net unfolding of refolded pre-β-lactamase in the presence of GroEL/ES. Pre-β-lactamase was refolded in the standard refolding cocktail (see Materials and methods) not containing GroEL/ES or Mg^{2+}ATP. The precursor was refolded for 4 h and reached a plateau value of enzymatic activity before, at time 0, an equivalent amount (by particle molarity, all final concentrations: 0.12 μM) of GroEL/ES was added. At the time indicated by the second arrow, Mg^{2+}ATP (final concentration: 5 mM) was added. All assay conditions were as in Figure 1 and given in Materials and methods. Under the assumption that the refolded precursor has the same specific activity as the mature reduced β-lactamase (Laminet and Plückthun, 1989) the first plateau corresponds to 8%, the second plateau to 16% folding yield. The true specific activity of the precursor may be lower than this estimate, and the true folding yield would then be higher. (**B**) Trypsin sensitivity of pre-β-lactamase. An immunostain after polyacrylamide gel electrophoresis is shown. The numbers indicate the amount of trypsin in mg/ml, *p* denotes the precursor of β-lactamase and *i* denotes a proteolytic intermediate of refolded pre-β-lactamase. (**a**) Unfolded pre-β-lactamase taken directly from 8 M urea; (**b**) pre-β-lactamase refolded for 4 h; (**c**) pre-β-lactamase incubated with GroEL/ES without Mg^{2+}ATP; (**d**) pre-β-lactamase incubated with GroEL/ES and Mg^{2+}ATP for 4 h; (**e**) pre-β-lactamase refolded for 4 h and incubated for another 4 h in the presence of GroEL/ES without Mg^{2+}ATP. The concentration of pre-β-lactamase was identical in all samples shown in **a**–**e**.

of β-lactamase under the experimental conditions used here. Neither GroEL, nor GroEL/ES, leads to a net 'unfolding' of mature β-lactamase to a non-native state, after the enzyme has been previously reduced, denatured and subsequently refolded in the reduced state (data not shown).

There are several noteworthy observations about these experiments. First, the disappearance of activity would be extraordinarily slow for a mere binding reaction. Second, more enzymatic activity is obtained after Mg^{2+}ATP addition than was present during the first plateau phase (Figure 4A). This strongly suggests that the folding yield is higher in the presence of GroEL/ES than in its absence. Third, the half-time of reappearance of enzymatic activity is of the same order of magnitude with or without any addition of factors. These observations make it highly unlikely that the enzymatic activity of folded pre-β-lactamase is merely inhibited by GroEL/ES and the molecule is simply released after Mg^{2+}ATP addition. In such a case, additional unfolded precursor molecules would have to be bound and then released to account for the higher activity, giving rise to two kinetic phases of reappearance of enzymatic activity. This is not observed, however. Rather, GroEL seems to recognize and stabilize some non-native conformation of pre-β-lactamase, regardless of the initially presented folding state.

The trypsin sensitivity of pre-β-lactamase was also examined. The reaction with trypsin was examined in the absence (Figure 4B,a,b) and in the presence (Figure 4B, c−e) of GroEL/ES. Since GroEL/ES comprises ~96% of all protein in the experiment, it influences the trypsin reaction as a competing substrate. Therefore, meaningful comparisons can only be made between Figure 4B a and b (GroEL/ES absent) or between parts c,d and e (GroEL/ES present). Refolded pre-β-lactamase is much more resistant than denatured pre-β-lactamase (Figure 4B, a,b) and gives rise to a characteristic intermediate of proteolysis. This intermediate is not seen if either the unfolded or the folded precursor is incubated with GroEL/ES in the absence of Mg^{2+}ATP (Figure 4B, c,e) but is seen again when Mg^{2+}ATP is added (Figure 4B, d). The crucial observation in this experiment is that the 'folding arrest' (Figure 4B, c) and the apparent net 'unfolding reaction' (Figure 4B, e) give rise to identical proteolytic patterns. These data and the absence of enzymatic activity are consistent with pre-β-lactamase having a non-native conformation when complexed to GroEL/ES in the absence of Mg^{2+}ATP, no matter whether native or denatured precursor was initially presented.

The fairly high protease resistance of pre-β-lactamase in the complex might be due to steric protection and/or partial folding in the complex to a collapsed state (see below). Only few deductions about the nature of pre-β-lactamase in the complex can be made. Since after Mg^{2+}ATP driven release from GroEL/ES, the rate of folding is insignificantly faster than in the absence of any factor, the highest transition state in folding must still be crossed. A likely candidate for the state that pre-β-lactamase assumes in the complex would therefore be a collapsed state (Ptitsyn, 1987; Bychkova *et al.*, 1988; Kuwajima, 1989), which is native-like in many aspects. When urea-denatured pre-β-lactamase is added to GroEL/ES, few molecules escape to a native form indicating that the interacting conformation is available almost immediately from the urea-denatured state, without a previous slow rearrangement. In contrast, when native pre-β-lactamase is added to GroEL/ES, an exceedingly slow binding reaction occurs with a half-time of about an hour. This is consistent with the crossing of an energy barrier, either in the complex or in solution, from a native to a non-native state. A model consistent with these data is therefore a collapsed state of pre-β-lactamase in complex with GroEL/ES.

In contrast to GroEL, neither SecB (A.Laminet, C.Kumamoto and A. Plückthun, unpublished data) nor trigger factor (A.Laminet, W.Wickner and A.Plückthun, unpublished data) have any measurable effect on the folding of pre-β-lactamase. Of all the combinations measured between putative folding factors and either the precursor or the mature β-lactamase, only the combination GroEL/ES or GroEL alone with pre-β-lactamase has an effect on folding. This observation complements and extends previous experiments on the relative affinity of GroEL, SecB and trigger factor for different precursor proteins (Lecker *et al.*, 1989).

In a recent investigation of the transport of proteins in *gro*EL/ES temperature sensitive mutants, only the transport of β-lactamase was affected at the non-permissive temperature (Kusukawa *et al.*, 1989). In contrast, in *sec*B mutants, the transport of β-lactamase was not hampered (A.Laminet and A.Plückthun, unpublished data). No such *in vivo* data are yet available for trigger factor. These results, together with the crosslinking results during *in vitro* translation (Bochkareva *et al.*, 1988), suggest that GroEL/ES, SecB and trigger factor may have some specificity for different transported proteins.

How can GroEL/ES be involved in both protein transport and oligomer assembly? The apparent stoichiometry of interaction of one pre-β-lactamase molecule per complex suggests that the interaction might take place at a very reactive surface in the center of the ring-shaped GroEL molecule. Such a reactive surface would be ideally protected from self interaction by its location in the middle of a ring-shaped particle. The nature of this interaction may allow tight binding to non-native proteins. At present we cannot distinguish whether GroEL can only passively trap spontaneously appearing non-native molecules out of solution or is actually capable of catalyzing a partial 'unfolding reaction' to a collapsed state of certain proteins on its surface. ATP hydrolysis may then cause a conformational change and displace the bound protein. In the course of protein transport, the particle might act as a stoichiometric 'folding preventase' as long as the Mg^{2+}ATP dependent protein release is somehow blocked. Alternatively, GroEL/ES may work as a sub-stoichiometric catalyst facilitating the equilibration between native and non-native conformations, since non-native precursors may be constantly removed from the equilibrium by translocation. Folded precursors might thus be rescued for transport. The state of GroEL in the absence of Mg^{2+}ATP may correspond to an arrested intermediate of the normal catalytic cycle of GroEL/ES. In the assembly of oligomeric proteins on the other hand, a premature mis-association of early folding intermediates must be prevented by the same molecular mechanism. Folding may occur up to a certain point (e.g. to a collapsed state) while the protein is still bound to GroEL/ES, which may be a 'polypeptide chain binding (PCB-) protein' (Rothman, 1989) or 'molecular chaperone' (Ellis *et al.*, 1989). Such

a mechanism may also explain why the folding yield of monomeric pre-β-lactamase is increased by GroEL/ES *in vitro*, since the precursor may be prevented from reaching alternative 'dead-end' conformations. Our studies with purified components may begin to clarify the mechanism of action of this folding modulator.

Materials and methods

Protein purification

Pre-β-lactamase was purified as described previously (Laminet and Plückthun, 1989). GroES and GroEL were purified using slight modifications of the procedures described previously (Fayet *et al.*, 1989).

Folding assay

The folding assay and conditions have been described previously (Laminet and Plückthun, 1989). Briefly, pre-β-lactamase was diluted from a urea containing stock solution into the refolding cocktail. The reaction was followed by withdrawing aliquots that were immediately assayed spectrophotometrically for β-lactamase activity. The refolding cocktail contained the folding modulator to be tested before pre-β-lactamase was added. The β-lactamase activity was determined spectrophotometrically at 486 nm with the chromogenic substrate nitrocefin (O'Callaghan *et al.*, 1972; Laminet and Plückthun 1989) at 25°C.

When native mature β-lactamase was used in the refolding assay, it was first unfolded and reduced in 8 M urea containing 5% mercaptoethanol for 1 h as described previously (Laminet and Plückthun, 1989), before dilution into the refolding cocktail.

Trypsin sensitivity

The sample to be tested was taken directly from 8 M urea, from a refolding cocktail or from a GroEL/ES containing solution as described in the legend of Figure 4B. The concentration of pre-β-lactamase was identical in all samples. After this reaction, pre-β-lactamase was digested with the indicated amount of trypsin TPCK (Cooper Biomedical) for 5 min at 4°C. The reaction was stopped with 10 mM PMSF and incubated for another 5 min at 4°C. PAGE-loading buffer was added, the samples were boiled for 10 min and separated on a 12.5% polyacrylamide gel (Fling and Gregerson, 1986). The gel was blotted onto nitrocellulose and the filter incubated with anti-β-lactamase serum from rabbit. The pre-β-lactamase was detected after incubation with anti-rabbit-immunoglobulin antibody from pig conjugated with alkaline phosphatase (Dakopatts) and immunostaining according to Blake *et al.* (1984).

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Received March 15, 1990; revised April 17, 1990