GroE Prevents the Accumulation of Early Folding Intermediates of Pre-β-lactamase without Changing the Folding Pathway†

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ABSTRACT: In folding studies of pre-β-lactamase in the presence of GroE, we investigated the pH dependence of the folding reaction. Two critical intermediates in the folding pathway were defined kinetically. I₁ is an early folding intermediate recognized by GroE; the misfolding of I₁ leads to aggregation, and this is prevented by GroE. A second intermediate I₂ is released from GroE after ATP hydrolysis. Its pH-dependent misfolding to a non-native form, which is not an aggregate, is not prevented by GroE. From these results, a model is proposed, in which the crucial role of GroE consists of allowing the change from I₁ to I₂ to take place in the complex. Fluorescence spectra of the pre-β-lactamase complexed to GroE are very similar to those of the native state. The pathway of pre-β-lactamase folding is not changed by GroE as evidenced by the same half-time and pH dependence of the folding reaction. GroE probably recognizes the signal sequence and some portion of the mature protein since mature β-lactamase does not interact with GroE even under conditions of slow folding.

While the central dogma in protein folding of the sequence determining the structure (Anfinsen, 1973; Creighton, 1978; Jaenicke, 1987) stands unchallenged, the involvement of cellular factors in the folding process is now emerging (Pelham, 1986; Ellis, 1987; Rothman, 1989; Fischer & Schmid, 1990; Schmid, 1991; Jaenicke, 1991). A number of such putative protein folding modulators have been proposed, but mechanistic folding experiments on any of those are still sparse. Some factors have been described, where at least the chemical reaction catalyzed is understood (if not their physiological role), such as peptidyl-prolyl cis-trans isomerase (Lang et al., 1987) or disulfide isomerase (Bulleid & Freedman, 1988; Freedman, 1989). Other proteins, sometimes termed "molecular chaperones" (Ellis, 1987) have been described whose functions involve the noncovalent and transient association with folding intermediates. These include heat-shock proteins and their homologues of the Hsp70, Hsp60, and Hsp10 class, and they have been demonstrated to be involved in protein folding [see, e.g., Laskey et al. (1978), Bochkareva et al. (1988), Chirico et al. (1988), Deshaies et al. (1988), Hemmingsen et al. (1988), Flynn et al. (1989), Goloubinoff et al. (1989a,b), Laminet et al. (1990), Buchner et al. (1991), and Martin et al. (1991)]. The extent of this involvement, the mechanisms of action, and their specificities are largely unknown. Roles in the assembly of oligomeric proteins (Goloubinoff et al., 1989a,b) and in preventing transported proteins from folding before crossing a membrane (Bochkareva
et al., 1988; Ostermann et al., 1989; Laminet et al., 1990) have been demonstrated, and more general roles in folding are possible.

This study describes folding experiments with the Escherichia coli Hsp60-Hsp10 complex GroEL/GroES and the precursor of β-lactamase as the "substrate" (Laminet & Pliickthun, 1989; Laminet et al., 1990). GroEL is a complex of 14 subunits (MW 57 000), arranged in two rings of 7 subunits (Hendrix, 1979; Hohn et al., 1979), and GroES is probably a heptamer. GroEL and GroES only interact in the presence of Mg²⁺ATP, probably with 1:1 stoichiometry (Chandrasekhar et al., 1986). The physiological importance of the protein complex is reflected by the fact that it is absolutely essential for E. coli growth (Fayet et al., 1989).

We wished to investigate whether GroE can alter and divert the pathway of folding of a monomeric substrate and by what mechanism it exerts an effect on the folding reaction. The experiments were carried out with the purified precursor of β-lactamase (Laminet & Pliickthun, 1989) and purified GroE (Viitanen et al., 1990) using kinetic assay procedures previously developed (Laminet & Pliickthun, 1989; Laminet et al., 1990) and fluorescence spectroscopy.

**EXPERIMENTAL PROCEDURES**

**Protein Purification.** Pre-β-lactamase and β-lactamase were purified as described previously (Laminet & Pliickthun, 1989). GroEL and GroES were purified from lysates of cells harboring the multicopy plasmid pOF39 (Fayet et al., 1989). The purification method was adapted from Viitanen et al. (1990).

**Folding Assay.** The folding of β-lactamase and pre-β-lactamase was measured at 25 °C (unless indicated otherwise) by diluting the reduced and urea-denatured enzyme into an optimized folding buffer (100 mM urea, 100 mM (NH₄)₂SO₄, 0.01% Tween, 100 mM potassium phosphate, pH 7.0, 10 mM DTT) as described previously (Laminet & Pliickthun, 1989) or into a suboptimal folding buffer (100 mM potassium phosphate, pH 7.0, 10 mM DTT). For the pH-dependent folding reactions a citrate/sodium phosphate wide-range buffer with constant ionic strength (I = 0.2) containing 6.5 mM potassium phosphate was used (Elving et al., 1956). Potassium is required by GroEL (Viitanen et al., 1990). The enzymatic assay was always carried out at pH 7.0.

**Assay of Pre-β-lactamase Activity.** The folding reaction of β-lactamase and pre-β-lactamase was followed by determining their enzymatic activities. The enzymatic activity was assayed spectrophotometrically at 486 nm and 25 °C (unless indicated otherwise) with the chromogenic substrate nitrocefin at pH 7.0 (O’Callaghan et al., 1972) as described previously (Laminet & Pliickthun, 1989).

**ATPase Assay.** The ATPase activity of GroEL was determined in optimized folding buffer (see above). To measure the decrease of ATP in a folding buffer containing 0.13 μM GroEL (14-mer) and 5 mM Mg²⁺ATP, the ATP kit from Sigma Diagnostics (Procedure No. 366 UV) was used. In this assay, phosphoglycerate kinase (PGK) and glyceraldehyde phosphate dehydrogenase (GAPDH) catalyze the conversion of ATP, 3-phosphoglycerate, and NADH to ADP, P₃, glyceraldehyde-3-P, and NAD⁺. The decrease in absorbance at 340 nm was measured.

**Light Scattering.** Experiments were carried out at 25 °C with a Shimadzu RF-5000 fluorescence spectrometer at 500 nm or at 650 nm. The spectral bandwidth was 1.5 nm (at 500 nm) or 5 nm (at 650 nm) respectively for both excitation and emission.

**Fluorescence.** Experiments were carried out at 25 °C with a Shimadzu RF-5000 fluorescence spectrometer. The spectral bandwidth was 5 nm for both excitation and emission.

**RESULTS**

Previous results (Laminet & Pliickthun, 1989) have demonstrated that the precursor of β-lactamase and the mature form fold to a similar native state, albeit with much slowed folding kinetics of the precursor compared to those of the mature enzyme. The reduced rate might be due either to a change in the rate-determining step, to a reduction in the rate of the same rate-determining step for the precursor, or to the depletion of a critical productive folding intermediate. For instance, unproductive side products involving interactions of the signal sequence with the hydrophobic core of the enzyme might transiently accumulate and therefore lower the steady-state concentration of a productive intermediate, slowing down the overall rate.

To gain information about this question, we investigated the pH dependence of the yield of the folding reaction of both precursor and mature enzyme. This was achieved by measuring the enzymatic activity at constant pH of samples that had reached the plateau phase of folding at various pH values. From independent kinetic experiments (Laminet & Pliickthun, 1989), it had been determined that no further change of activity was observed for the precursor after 2 h and for the mature enzyme after 20 min.

The precursor shows a decrease in folding yield at high pH with a titration midpoint of about 6.5, whereas the yield of the mature enzyme is independent of pH in this region (Figure 1A,B). This suggests that a pH-dependent diversion of the folding pathway to a misfolded or an aggregated form occurs for the precursor. This does not happen for the mature enzyme. The signal sequence contains a histidine residue at position -19, and the mature enzyme contains one at +1. It is conceivable that the protonation state of one or both of these is crucial for correct folding. In the mature enzyme, the residue at position +1 would be a terminal residue, whose protonation state may be uncritical, but in the precursor it is preceded by another 23 amino acids and this residue and/or His -19 may require protonation to prevent a diversion from the desired folding pathway.

At the acidic side, neither the precursor nor the mature enzyme can assume the native structure (with an apparent pK of about pH 4-4.5). The "background" activities seen for the mature enzyme at low pH are due to folding during the spectrophotometric assay carried out at pH 7.0 (demonstrated by concave kinetic time courses). All other kinetic traces were linear, excluding significant folding during the assay.

The higher folding yield at pH 5 than at pH 7 for the precursor is not directly paralleled by a dramatic change in kinetics (Figure 2). Whereas the rate of folding can be described by single first-order kinetics at pH 7.0 for the precursor (as demonstrated previously; Laminet & Pliickthun, 1989), at pH 5.0 biphasic kinetics are apparent. This is consistent with the existence of two species with different folding rates. While the X-ray structure of RTEM β-lactamase is not known, it is highly homologous to the enzyme from Staphylococcus aureus, whose structure has been determined (Herzberg & Moult, 1987) and which contains a cis-proline at the position corresponding to Pro82 in RTEM β-lactamase.

We have, however, no direct evidence yet for or against the involvement of a proline cis-trans isomerization. The slower of the rate constants at pH 5.0 is very similar or identical to that at pH 7.0. The diversion of the folding pathway to nonnative forms at higher pH is thus not paralleled by large changes in folding kinetics. Instead, a new pathway leading to a nonnative form must become available and even be pre-
FIGURE 1: pH-dependent folding of pre-β-lactamase (A) and β-lactamase (B). The folding yield was measured as the enzymatic activity at 2 h (A) or at 20 min (B) after addition of the protein 1:10 into optimized folding buffer. The final concentrations of the mature and the precursor β-lactamase were 0.13 μM. β-Lactamase activity is given in arbitrary units. As the true specific activity of the precursor is unknown, we cannot distinguish a lower intrinsic specific activity of the precursor from less than quantitative refolding, compared to the mature enzyme.

FIGURE 2: Refolding kinetics of pre-β-lactamase at pH 7.0 and pH 5.0. Pre-β-lactamase was diluted 1:200 into optimized folding buffer at pH 7.0 (D) and at pH 5.0 (•). The final concentration of pre-β-lactamase was 0.13 μM. V is the final enzymatic activity on the plateau phase of folding, and V is the enzymatic activity at time t, measured as the hydrolysis rate of nitrocefin.

The effect of GroE on this reaction was then examined as function of pH (Figure 3A,B). In the absence of Mg2+ATP, there is an inhibition of the folding of pre-β-lactamase by GroE in the pH range from 4 to 7. In the presence of GroE and Mg2+ATP, the decrease in folding yield at pH greater than 5.5 is unchanged by the presence of GroE (Figures 3B and 4A). While we cannot rigorously prove that the identical pH dependence of folding with and without GroE is due to a common folding mechanism, it is the simplest deduction, and it is strengthened by the observation that the folding kinetics with and without GroE are identical, too (see below). This finding suggests that GroE is not able to prevent the misfolding responsible for this decrease in folding yield. The pH-dependent diversion of the folding pathway is thus not influenced by GroE. This cannot be due to the fact that GroE does not react with the substrate, since in the absence of Mg2+ATP there is a strong folding inhibition in the same pH range. Rather, the pH-dependent diversion of the folding pathway must occur after the release of pre-β-lactamase from GroE. At low pH (lower than 4), there seems to be an irreversible association between GroE and pre-β-lactamase, since the folding yield is actually lower in the presence of GroE and Mg2+ATP than in its absence (Figures 3B and 4A). This finding suggests that Mg2+ATP can no longer reverse the association between substrate and GroE. Indeed, direct measurements of the ATPase activity as a function of pH (Figure 4B) and light scattering experiments (Figure 4C) suggest the presence of an inactive, aggregated form of GroE, which still seems to be able to associate irreversibly with the substrate protein.

It should be noted that there is no evidence for any significant change of the ATP hydrolysis rate by the presence
The stoichiometry of the folding inhibition was investigated for the GroE-substrate reaction (Figure 5). The suggested stoichiometry is 1 pre-β-lactamase molecule per 7-mer of GroEL. The discrepancy from our previously reported ratio of 1 pre-β-lactamase per 14-mer GroEL is due to a previous inaccuracy in the molarity of GroEL. The reinvestigation with quantitative amino acid analysis is consistent with the reported OD$_{280}$ of 2.38 × 10$^4$ M$^{-1}$ cm$^{-1}$ (Viitanen et al., 1990) and leads to the hypothesis that each 7-mer can bind 1 substrate.

The quantitative analysis of the stoichiometry (data not shown) indicates that the absolute concentration of GroEL in the range from 0.033 to 0.52 μM (at constant substrate:GroEL ratio) does not influence the amount of pre-β-lactamase that escapes the association by folding. The titration experiment in Figure 5 demonstrates that the amount escaping by folding is not due to the fact of GroEL being present in substoichiometric amounts. The amount of precursor escaping by folding is not diverted to the complexed state by higher amounts of GroE. Therefore, the failure of a small fraction of the precursor to form a complex is not simply due to a kinetic competition of a second-order association rate with a first-order folding rate nor due to an equilibrium effect of GroE binding to the precursor.

If the folding is studied in a suboptimal buffer favoring misfolding (see Experimental Procedures), then the addition of GroE and Mg$^{2+}$ATP increases the folding yield by about 60% to give the same level as under optimized folding conditions (data not shown). In the optimized folding buffer, no further increase by the addition of GroE is observed. The increase in folding yield in suboptimal folding buffer is a specific reaction between GroE and pre-β-lactamase, since, e.g., bovine serum albumin (used as a control) actually decreases the folding yield under the same conditions. Therefore, GroE prevents the accumulation of certain types of misfolded pre-β-lactamase species in a more specific fashion, probably by transient association. As the reaction is extremely sensitive to pH, even small deviations lead to differences in folding yield, and some further yield enhancements observed in optimized

### Table I: ATP Hydrolysis Rates of GroE in the Absence and Presence of Substrate

<table>
<thead>
<tr>
<th>components present</th>
<th>hydrolysis rate (min$^{-1}$)</th>
<th>components present</th>
<th>hydrolysis rate (min$^{-1}$)</th>
</tr>
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<tbody>
<tr>
<td>GroEL</td>
<td>9.6</td>
<td>GroEL/ES</td>
<td>4.4</td>
</tr>
<tr>
<td>GroEL + pre-β-lactamase</td>
<td>10.9</td>
<td>GroEL/ES + pre-β-lactamase</td>
<td>4.5</td>
</tr>
</tbody>
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*ATP hydrolysis of 0.26 μM GroEL (14-mer) and 0.26 μM GroEL/ES (14-mer/7-mer) was measured in optimized folding buffer in the absence or presence of pre-β-lactamase, which was refolded by diluting 1:100 to a final concentration of 0.26 μM. ATPase activity of GroE is given in moles of ATP per mole of GroEL (monomer) per minute.
folding buffer and ascribed to GroE (Laminet et al., 1990) may be due to very slight pH shifts by the addition of compounds in the experiment.

The action of GroE is to prevent aggregation (Figure 6). Light scattering shows that folding in the presence of GroEL/ES and Mg\(^{2+}\)ATP leads to significantly lower aggregate formation than in the absence of GroE. The time course of the aggregation reaction is consistent with second-order kinetics (data not shown). The background of the complex in the absence of Mg\(^{2+}\)ATP is much lower.

If Mg\(^{2+}\)ATP is present with the GroEL/ES complex from the beginning of the folding reaction, the reaction kinetics are indistinguishable from the folding reaction of pre-\(\beta\)-lactamase in the absence of any factor (Figure 7). GroEL alone in the presence of Mg\(^{2+}\)ATP, however, does not give rise to the same kinetics, possibly because GroES is needed for a more efficient release of the substrate from the GroEL–complex. Note, however, that GroES is not a mandatory component in the release reaction in this system (Laminet et al., 1990). Only Mg\(^{2+}\)ATP, but not unhydrolyzable analogues (AMP–PCP and AMP–PNP at concentrations of 2 mM), was able to allow folding of the precursor, since the nonhydrolyzable analogues did not reverse the folding inhibition seen in the absence of Mg\(^{2+}\)ATP (data not shown).

To characterize the state of pre-\(\beta\)-lactamase bound to GroE further, we obtained fluorescence spectra, from which the GroE contribution was subtracted. The spectrum is remarkably similar to that of refolded precursor but is different both in intensity and in emission maximum from that of the denatured state (Figure 9). This shows that it is not the denatured state that is accumulated in the complex, although it may be recognized initially. It cannot be identical to the native state either, since the gain in enzymatic activity follows the same kinetics after Mg\(^{2+}\)ATP addition to the complex as in refolding without GroE (Laminet et al., 1990). We deduce, therefore, that a folding intermediate accumulates in the complex, which is still separated from the native state by the highest transition state of folding, i.e., the rate-determining step. Even after short mixing times, the fluorescence spectrum
of pre-β-lactamase in the complex looks like that of the folded state, in both the presence and absence of GroE. Therefore, the intermediates must be reached within seconds (and, perhaps, much faster) from the unfolded state.

We then investigated why pre-β-lactamase, but not β-lactamase, is recognized by GroE. This cannot be due simply to the faster kinetics of the latter (thus escaping the complex formation) since at low temperature, where overall rates as slow as the precursor can be observed, there is still no interaction with GroE (Figure 8). While it cannot be rigorously excluded that the first intermediate in the folding pathway no longer able to interact with GroE builds up still too fast in the case of the mature enzyme at lower temperature (and its formation might not be slowed by the same factor as the formation of the native state being observed), a more economical hypothesis is that specific features of the precursor are recognized by GroE. The simplest corollary is that GroE recognizes the signal sequence of pre-β-lactamase.

**Discussion**

The kinetic data measured in this system can be accommodated by a scheme as shown in Figure 10. Central to this scheme is the observation that the folding kinetics of pre-β-lactamase are not changed in the presence of GroE. The pH dependence and the half-time of the folding reaction are the same as in the absence of this folding modulator. This suggests that the folding modulator does not influence the rate-determining step, which presumably is late in the folding pathway (Mitchinson & Pain, 1985; Kim & Baldwin, 1990). Therefore, the GroEL/ES complex must act on early folding intermediates that occur before the rate-determining step.

We postulate two critical intermediates in the pathway of folding of pre-β-lactamase, I₁ and I₂. The precursor may be bound in the form of I₁ and be released in the form of I₂. The rate-determining step lies between I₂ and N and is thus identical for the folding in the presence of GroE and in its absence, as it occurs after the release of I₂.

The intermediate I₁ can be diverted to the aggregated form I₃ (Figure 6). The choice of folding buffer clearly influences this partitioning. In the presence of GroE, this unproductive pathway is mostly blocked, leading to higher folding yields in buffers which otherwise favor misfolding. Other unproductive intermediates, such as I₄, however, occur in a pH-dependent manner, and their occurrence is not prevented by the presence of GroE. They must therefore appear after the release from this particle. I₄ does not seem to consist of aggregates, as there is much less aggregation in the presence of GroE (Figure 6), but the pH-dependent accumulation of I₄ is not changed by GroE (Figure 3B).

In the presence of GroEL or GroEL/ES and in the absence of Mg²⁺ATP, the intermediate I₁ is bound but may convert to I₂ in the complex. I₂ is then released by Mg²⁺ATP and unable to react to the misfolded intermediate I₃. This may be the main action of GroE for this substrate protein. The fluorescence spectra (Figure 9) are consistent with I₂ being native-like in many aspects but being separated from N by the major transition state (the slow step of folding).

More generally, the main role of GroE will be the prevention of early diversion in the folding pathway, which otherwise would lead to aggregates (Figure 6; see also Buchner et al. (1991)). The prevalence of the I₁ → I₂ reaction will of course differ widely for different substrate proteins. In the case of pre-β-lactamase, it is seen in buffers favoring aggregate formation.

Although there is no direct evidence for two intermediates I₁ and I₃, they can accommodate the experimental data better than a one-intermediate model would. If GroE transiently

![FIGURE 10: Model of GroE action. In the absence of GroE, pre-β-lactamase folds to its native form and partly, dependent on the conditions of the folding buffer, to misfolded intermediates (I₃, I₄). In the presence of GroE, the pathway of folding is the same (U → I₁ → I₂ → N) but now GroE prevents the building up of the misfolded intermediate I₁ by associating with the intermediate I₁, which then converts to I₃ in the complex. If the folded form of pre-β-lactamase is added to GroE in the absence of ATP, spontaneously unfolded molecules (N → I₃ → I₄) can be trapped (Laminet et al., 1990). After ATP hydrolysis, GroE releases the intermediate I₄, which now folds in a pH-dependent manner to the native state (N) or an unproductive intermediate I₄. The dotted arrow denotes the minor fraction of pre-β-lactamase that escapes interaction with GroE.](http://example.com/figure10.png)
(Flynn et al., 1989), the situation with the Hsp60 class is not yet clear. The present data are consistent with a direct recognition of the signal peptide of the I₁ intermediate, but other parts of the protein must be bound as well, since folding is prevented. Studies with peptides are now underway to clarify this point.

A very slow turnover of Mg²⁺/ATP on a time scale of seconds (Table I), coupled with a conformational change, might "open" or "close" and "discharge" the binding site, meanwhile (during the short interval of substrate association) allowing the critical transition from GroE⁻I₁ to GroE⁻I₂ to occur. This might then prevent the intermediate I₁ from misfolding to I₁ in solution.

It is likely that the I₁ → I₂ reaction is fast. Both intermediates are early intermediates, and a turnover of GroE on a time scale of seconds may be sufficient to allow this conversion to occur in the complex. Interestingly, GroES slows down the Mg²⁺/ATP turnover by about a factor of 2 [Table I; see also Chandrasekhar et al. (1986)], possibly by taking part in the conformational change coupled to the hydrolysis. We find no evidence for any significant change of hydrolysis rate by the presence of substrate, either in the presence of absence of GroES.

The kinetic characterization of GroE reported here must now be correlated to structural data to further clarify the action of this protein folding modulator.

**Registry No.** Pre-β-lactamase, 83889-99-2.

**References**


