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Folding *in vitro* and transport *in vivo* of pre-β-lactamase are SecB independent

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Summary

The rate of folding of the precursor of β-lactamase is not influenced by the presence of SecB under conditions in which GroEL/ES retards the folding. Wild-type β-lactamase and several mutants in the signal or the mature protein, affecting either transport or enzyme kinetics and probably folding, were examined for total expression, total enzymatic activity, and transported β-lactamase (*in vivo* resistance) in *secB*⁻ and *secB*⁺ strains. We conclude that there is no indication of any relevant interaction between SecB and pre-β-lactamase *in vitro*, nor did the *secB*⁻ mutation affect the transport of wild-type β-lactamase or any of the mutants *in vivo*. Thus, putative *Escherichia coli* 'folding modulators' must be of limited specificity.

Introduction

In vivo (Randall and Hardy, 1986) as well as *in vitro* (Eilers and Schatz, 1986) experiments have strongly suggested that the passage of a protein through the membrane occurs in a folding state that is distinct from native. Proteins destined for transport in bacteria are initially synthesized as precursors containing an amino-terminal signal sequence that is cleaved off by a membrane-bound signal peptidase (for a recent review, see Randall and Hardy, 1989). The examination of two completely unrelated periplasmic proteins from *Escherichia coli*, maltose-binding protein (MBP, the product of the *malE* gene; Park *et al.*, 1988) and β-lactamase (Laminet and Plückthun, 1989), has demonstrated that the presence of the signal sequence retards the folding reaction without significantly altering the native structure.

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The question thus arose as to whether additional factors in *E. coli* might further promote this folding retardation, e.g. by an interaction with the unfolded precursor protein. Three such factors have been proposed so far in *E. coli*: SecB (Collier *et al.*, 1988; Kumamoto and Gannon, 1988), trigger factor (Crooke *et al.*, 1988a,b), and GroE (Bochkareva *et al.*, 1988). We have recently developed the methodology to measure the folding kinetics of purified pre-β-lactamase (Laminet and Plückthun, 1989) and could demonstrate a reversible and Mg^{2+} ATP-dependent folding retardation by GroE (Laminet *et al.*, 1990). Since a defect in *groES* or *groEL* has been shown to interfere specifically with β-lactamase transport (Kusukawa *et al.*, 1989), the relevance of the *in vitro* folding assay to interactions occurring *in vivo* was demonstrated. We have now investigated, with the same methodology, the effects of purified SecB on *in vitro* folding of pre-β-lactamase and of a *secB* null mutation on *in vivo* transport in *E. coli*.

SecB was first identified using a mutant selection for defects in protein secretion (Kumamoto and Beckwith, 1983). It is a cytosolic factor with a subunit molecular weight of 16.6 kDa (Kumamoto and Nault, 1989), was described as an oligomer of about 90 kDa on gel filtration columns (Weiss *et al.*, 1988; Kumamoto *et al.*, 1989), and was proposed to constitute a part of a 150 kDa export factor found in crude post-ribosomal supernatants in the form of a homotetramer of 64 kDa (Watanabe and Blobel, 1989a). The *secB* null mutation *secB*::Tn*5* is not lethal to *E. coli*, but the cells do not grow on rich media (Kumamoto and Beckwith, 1985). SecB protein was found to play a role in transport of the periplasmic protein MBP as well as the outer-membrane proteins LamB, OmpA, OmpF and PhoE. SecB associates with these exported proteins and, in addition, enhances the rate of protein export (Kumamoto, 1989). Outer-membrane lipoprotein (Lpp) and ribose-binding protein (RBP) were found to be transported *in vivo* in a SecB-independent way (Kumamoto and Beckwith, 1985; Watanabe *et al.*, 1988), although a weak interaction of SecB with pre-RBP can be demonstrated (C. A. Kumamoto, unpublished results). Thus, the absence of *in vivo* effects on transport does not necessarily rule out interactions between precursor and SecB. SecB-independent transport was also observed with slow-folding mutants of MBP (Collier and Bassford, 1989).

We therefore wished to investigate whether an interaction between the purified and well-characterized RTEM

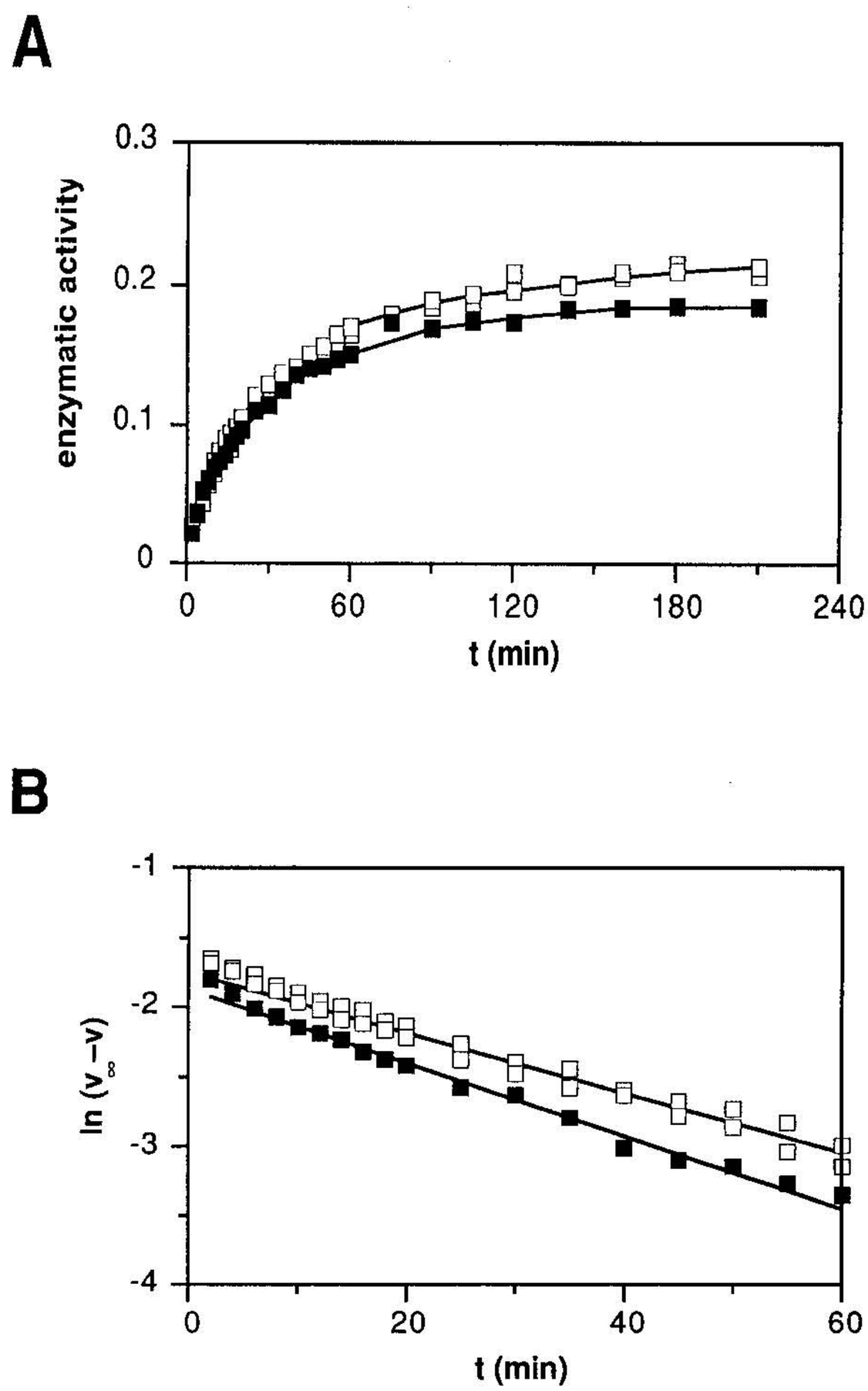

Fig. 1. Effect of SecB on the folding of pre-β-lactamase (□, no SecB; ■, with SecB).
A. The enzyme activity is shown in arbitrary units as a function of time after dilution into the refolding cocktail.
B. Replot of the data for a reversible first-order process.

Pre-β-lactamase was diluted from a urea stock solution into the refolding cocktail containing either SecB protein or SecB buffer alone. The reaction was followed by withdrawing aliquots that were immediately assayed spectrophotometrically for β-lactamase activity. The final concentrations in the refolding cocktail were 0.2 μM pre-β-lactamase and 1.1 μM SecB protomers. Assuming SecB hexamers as the oligomerization state, this would correspond to about 1 SecB particle per molecule of pre-β-lactamase.

pre-β-lactamase and SecB can be demonstrated *in vitro* and whether a mutant β-lactamase might acquire SecB dependence *in vivo*.

Results and Discussion

Purified pre-β-lactamase from a urea-containing stock solution was refolded by dilution into a buffer containing purified SecB protein. SecB was present at the same molarity as pre-β-lactamase, calculated for SecB particles under the assumption of an M_r of 96 kDa for the SecB oligomer (Kumamoto *et al.*, 1989). No folding inhibition could be observed and an identical folding rate was measured as in the control experiment with only the buffer components of the SecB experiments present (Fig. 1). Thus, there is no indication of any *in vitro* interaction between pre-β-lactamase and SecB under conditions in which an interaction between pre-β-lactamase and GroEL can be detected (Laminet *et al.*, 1990).

Aliquots of the refolding assay were then used in cross-linking experiments with glutaraldehyde (Jaenicke and Rudolph, 1986). The cross-linking samples showed no additional bands of higher molecular weight in the protein pattern when compared to non-cross-linked controls after separation by sodium dodecyl sulphate/polyacrylamide gel electrophoresis (SDS–PAGE) and silver staining, even when pre-β-lactamase and SecB were each present at five-fold higher concentrations (data not shown). The absence of cross-linking does not conclusively rule out a molecular interaction, since we do not know whether there are lysine residues in sufficient proximity. Yet neither a functional test (folding inhibition) nor a physical one (cross-linking) can detect any interaction of SecB with pre-β-lactamase *in vitro*.

To test whether the absence of SecB has any influence on the transport of β-lactamase *in vivo*, several plasmids carring either wild-type or mutant β-lactamases were transformed into a *secB*$^-$ strain (CK1953) or the isogenic *secB*$^+$ strain (MC4100). The rationale of the mutants was as follows. The deletion of the signal sequence (mutant Δ(−20, −1); Plückthun and Knowles, 1987) leads to a

Table 1. Effect of the *secB* null mutation (*secB*::Tn*5*) on ampicillin resistance *in vivo* (LD_{50}).

	Ampicillin Concentration (μg ml^{-1})	
β-lactamase	MC4100(*secB*$^+$)	CK1953(*secB*$^-$)
None	<50	<50
w.t.	3000	2500
Cys→Ala	2000	1500
Δ(−20, −1)	<50	<50
P82A	800	600
P82G	300	300
C −6A	400	400
C −6S	3000	2500

The value given is the highest ampicillin concentration (in μg ml^{-1}) at which the cells did not grow after incubation in minimal M9/glycerol-medium at 37°C for 50 h. Tetracycline at a concentration of 10 μg ml^{-1} was added to all cultures harbouring plasmids to maintain the plasmid independently of ampicillin concentration. The LD_{50} values for the mutants obtained in the *secB*$^-$ strain (CK1953) were slightly lower than those in the *secB*$^+$ strain (MC4100) after 50 h, but both strains reached the same values after longer incubation times. The *secB*$^-$ strain seems to display slight growth inhibition under these conditions, which is a property of the *secB*$^-$ strain and independent of the presence of a plasmid, making exact comparisons difficult. The mutations are described in the *Experimental procedures*.

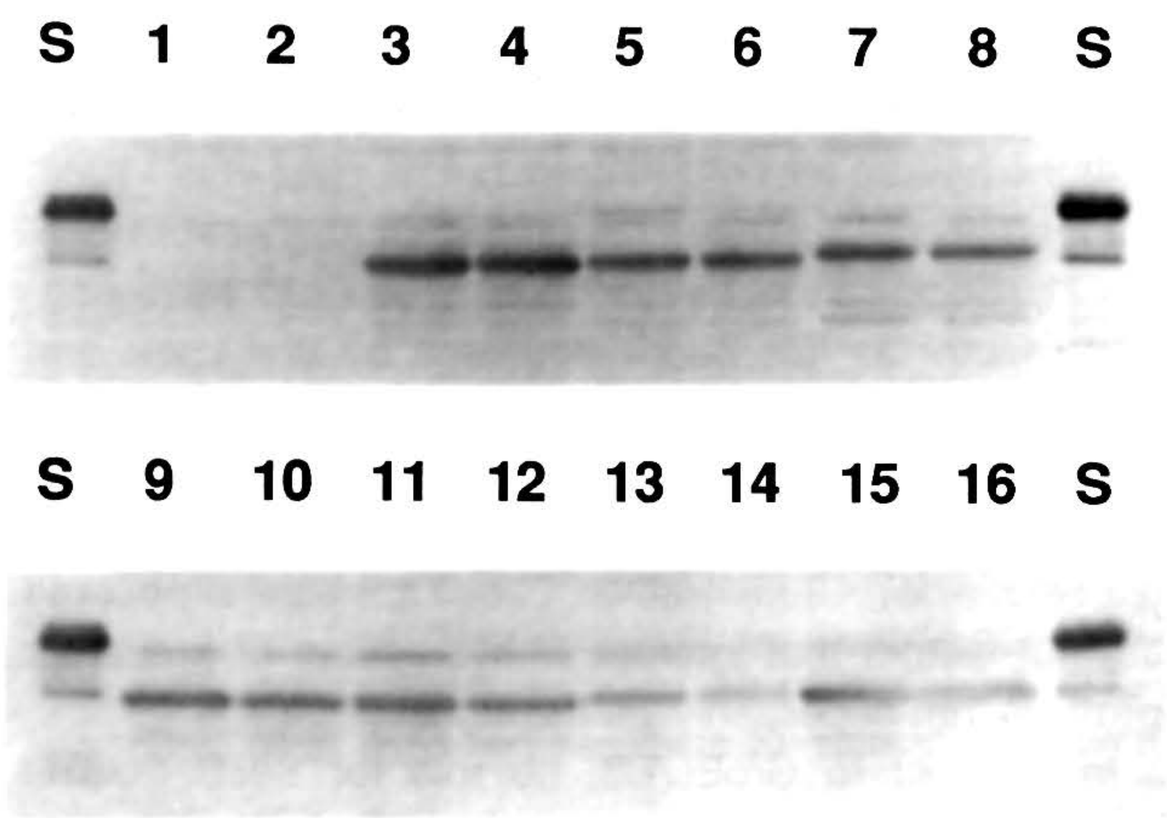

Fig. 2. Immunoblot of β-lactamase. Lane 1, MC4100; lane 2, CK1953; lane 3, MC4100/pTG2; lane 4, CK1953/pTG2; lane 5, MC4100/pTG2(Cys→Ala); lane 6, CK1953/pTG2(Cys→Ala); lane 7, MC4100/pTG2Δ(−20, −1); lane 8, CK1953/pTG2Δ(−20, −1); lane 9, MC4100/pTG2(P82A); lane 10, CK1953/pTG2(P82A); lane 11, MC4100/pTG2(P82G); lane 12, CK1953/pTG2(P82G); lane 13, MC4100/pTG2 (C−6A); lane 14, CK1953/pTG2(C −6A); lane 15, MC4100/pTG2(C −6S); lane 16, CK1953/pTG2(C −6S); lane S, molecular weight standard (pre-β-lactamase and β-lactamase). Aliquots, which were equivalent to 1 ml of cell suspension at an OD_{550nm} of 0.5, were centrifuged and the pellets were resuspended in 50 μl of loading buffer. The suspension was boiled for 10 min, separated on a 12.5% SDS–polyacrylamide gel and immunostained as described in the *Experimental procedures*. The samples are aliquots of the same cultures as those used for Table 2.

functional, enzymatically active, cytoplasmically localized enzyme and provides a control for transport-specific effects. The mutants P82G and P82A remove the only *cis*-proline in the molecule. This statement is based on the assumption that the folding of the peptide chain of the TEM2 enzyme is essentially identical to that of the closely related enzyme from *Staphylococcus aureus* PC1, for which the crystal structure has been determined (Herzberg and Moult, 1987). The isomerization of peptide bonds located amino-terminal to proline may be a slow step in the folding of some proteins (for reviews, see Fischer and Schmid, 1990; Jaenicke, 1987) and the removal of a proline will almost certainly lead to an altered loop with a *trans* peptide bond, and will possibly alter folding kinetics (Kelley and Richards, 1987). These mutants might therefore lead to interactions that only occur when the protein has different folding kinetics. The removal of the disulphide bond (mutant Cys→Ala, in which both cysteines of the mature β-lactamase have been changed to alanine residues; Laminet and Plückthun, 1989) should affect only the stability of the periplasmic product and serves as an internal control. Finally, the removal of the cysteine residue in the signal sequence (mutants C −6A and C −6S) affects only the precursor, but not the mature enzyme. While C −6S is a neutral mutation (see below), the mutation C −6A leads to a lower amount of periplasmic β-lactamase.

The *in vivo* resistance of all these plasmids in both strains was tested in a 50% lethal dose (LD_{50}) assay (Table 1). In the *secB*$^-$ strain, no significant decrease of resistance to ampicillin is observed for any plasmid compared to the *secB*$^+$ strain. Thus, it appears that for all mutant proteins tested the amount of functional and exported β-lactamase is roughly identical in the *secB*$^-$ or *secB*$^+$ strain.

To determine the total β-lactamase activity present in these cells, identical amounts of cells in exponential growth phase were subjected to French Press lysis and enzymatic assays were carried out (O'Callaghan *et al.*, 1972). The β-lactamase activities measured in the *secB*$^-$ and the *secB*$^+$ strain were very similar (Table 2). Additionally, in the immunostaining of aliquots of lysed culture samples, essentially equal amounts of total β-lactamase in the *secB*$^-$ or *secB*$^+$ strain were detected (Fig. 2).

When the mutants were compared to each other, generally similar trends were found in the measurements of total activity (Table 2) and of *in vivo* resistance (Table 1). The exception was the mutation Δ(−20, −1), which led to a functional enzyme, but no protection against the β-lactam antibiotic, since *in vivo* resistance requires a periplasmic localization of the enzyme (Kadonaga *et al.*, 1984; Plückthun and Knowles, 1987). The two mutations, in which the putative *cis*-proline was exchanged (P82A and P82G), increased the K_m value and decreased the k_{cat} value for nitrocefin as measured for the purified enzymes (A. A. Laminet and A. Plückthun, unpublished data). Therefore these two mutations affected the measured activity in both assays, but part of the loss of total enzymatic activity was due to a lower number of active and transported enzyme molecules (Fig. 2). The signal sequence was identical to wild-type in these mutants and the low amounts found in the periplasm may have to do with altered folding kinetics

Table 2. Relative β-lactamase activity in *secB*$^-$ and *secB*$^+$ cells.

	Relative Activity (%)	
β-lactamase	MC4100(*secB*$^+$)	CK1953(*secB*$^-$)
None	0.1	0.1
w.t.	100.0	64.2
Cys→Ala	20.3	16.7
Δ(−20, −1)	22.0	20.7
P82A	25.8	24.1
P82G	13.6	11.0
C −6A	15.6	16.2
C −6S	91.4	75.1

The bacterial cultures were incubated in minimal M9/glycerol-medium containing 20 μg ml^{-1} streptomycin to an OD_{550nm} of about 0.5. Tetracycline (10 μg ml^{-1}) was added to all cultures harbouring plasmids. The enzymatic activity was determined with nitrocefin after French Press lysis as described in the *Experimental procedures*. The values have been corrected for the exact OD_{550nm} value. The slightly lower values of β-lactamase activity in *secB*$^-$ cells may be due to a higher scattering of these cells, which is suggested by a different colony morphology of the two strains on minimal M9/glycerol-agar plates, and therefore a slightly overestimated cell number.

and/or proteolytic degradation. The kinetic parameters of the Cys→Ala mutant protein have been found to be very similar to the wild-type (Laminet and Plückthun, 1989). This mutation mostly affects the stability of β-lactamase in the periplasm. Additionally, two signal mutations are reported here: one (C −6S) is essentially neutral, and the other (C −6A) leads to a reduction of transported enzyme, and probably to partial degradation of the precursor, since no precursor was found in the steady state (Fig. 2).

In summary, neither the total amounts of β-lactamase (as measured by Western blotting), the amounts of total folded β-lactamase (as measured by total activity) nor the amounts of transported β-lactamase (as measured by *in vivo* resistance) of any mutant protein tested showed any significant difference between the *secB*$^-$ strain and the *secB*$^+$ strain within the error limits of the measurements. These *in vitro* and *in vivo* data lead us to the conclusion that transport of β-lactamase and of all variants tested is SecB-independent under all conditions examined. This extends the observation reported by Kusukawa *et al.* (1989) of a qualitative SecB independence of wild-type β-lactamase.

A mutation that leads to altered folding kinetics of MBP has been reported (Liu *et al.*, 1988). This mutation, termed MBP2261, improves MBP export in the absence of SecB (Collier *et al.*, 1988) and enhances SecB binding *in vivo* and *in vitro* (Liu *et al.*, 1989). In the case of β-lactamase, however, no differential SecB requirement was found for the mutant proteins tested. The simplest interpretation of these data is that wild-type β-lactamase and the mutants described here do not react with SecB.

The *in vitro* folding assay clearly reflects, therefore, the *in vivo* dependence of β-lactamase on folding modulators. While no folding modulation can be demonstrated with SecB, GroEL/ES does retard the folding of pre-β-lactamase *in vitro* (Laminet *et al.*, 1990). This result is complemented by the observation that *groEL* and *groES* mutants show a defect in β-lactamase transport *in vivo* (Kusukawa *et al.*, 1989). Trigger factor does not influence the folding of pre-β-lactamase folding either (A. A. Laminet, W. Wickner and A. Plückthun, unpublished data), but *in vivo* data are not yet known. It therefore appears that the folding modulators have a defined specificity and that this specificity can be measured by the present assay. A narrow specificity of folding modulators is consistent with previous examinations of pro-OmpA and pre-PhoE (Lecker *et al.*, 1989). However, it remains completely unknown which structural features determine which precursor might interact with which folding modulator.

The mechanism by which SecB may retard the folding of susceptible precursor proteins has remained obscure. After the initial demonstration of the effect (Collier *et al.*, 1988; Kumamoto and Gannon, 1988; Weiss *et al.*, 1988), some investigators have proposed an interaction of SecB with the signal peptide (Watanabe and Blobel, 1989b), while others have provided evidence for an interaction with the mature part of the precursor (Collier *et al.*, 1988; Gannon *et al.*, 1989; Liu *et al.*, 1989; Randall *et al.*, 1990). Clearly, SecB cannot be a general cytosolic signal-recognition factor as has been suggested by Watanabe and Blobel (1989b). Our present work shows the absence of any interaction with the precursor of β-lactamase under conditions where such interactions can be detected (Laminet *et al.*, 1990). Rather, *E. coli* appears to possess several factors that interact with a subset of precursor proteins, not necessarily via the signal.

In contrast to GroEL/ES, no role for SecB outside protein translocation has been identified. Thus it may constitute a specialized factor retarding the folding of a subset of precursor proteins. Pre-β-lactamase might have taken advantage of the folding modulator GroE whose predominant role may be in the assembly of oligomeric proteins. Future studies will have to determine whether these folding modulators share a common mechanistic basis.

Experimental procedures

Bacterial strains

All strains are derivatives of *E. coli* K12. The strains MC4100 (F$^-$, *araD139*, Δ*lacU169*, *relA*, *rbsR*, *rpsL*, *thi*) and CK1953 (MC4100 *secB*::Tn*5*) (Kumamoto and Beckwith, 1985) were used for the *in vivo* investigations. The strains N4830 (F$^-$, *su*$^-$, *his*, *ilv*, *rpsL*, Δ*8*(λ cI_{857}, Δ*BAM*, Δ*H1*)) harbouring the plasmid pAL2 (Laminet and Plückthun, 1989) and CK2001 (MC4100 *recA1*, (λ cI_{857} $S_{am}7$) harbouring pCK57) (Kumamoto *et al.*, 1989) were used for purification of pre-β-lactamase and SecB, respectively, as described previously.

Plasmids

All β-lactamase mutants are derivatives of the TEM2 enzyme as modified in the plasmid pTG2 (Kadonaga *et al.*, 1984). This pTG2-encoded protein is referred to as the wild type in this work. The signal is numbered from −23 to −1, and the mature protein from +1 to +263. The Cys→Ala double mutant (Laminet and Plückthun, 1989) and the cytoplasmic β-lactamase mutant, not coding for a signal (denoted Δ(−20, −1); Plückthun and Knowles, 1987), have been described previously. The plasmids pAL11 to pAL14 are derivatives of the plasmid pT62. The plasmids pAL11 (Pro82→Ala, referred to as P82A), pAL12 (Pro82→Gly, referred to as P82G), pAL13 (Cys −6→Ala, referred to as C −6A) and pAL14 (Cys −6→Ser, referred to as C −6S) were constructed by site-directed mutagenesis employing the method of Kunkel *et al.* (1987) with synthetic oligonucleotides and an M13mp11 derivative with the *Eco*RI–*Pst*I fragment of β-lactamase from pTG2. The mutated and sequenced DNA fragments were religated into the vector pTG2.

Growth of bacterial strains

Bacterial growth was performed according to Miller (1972). The strains MC4100 and CK1953 were grown in minimal M9-medium (Miller, 1972) supplemented with 0.2% glycerol. Streptomycin (20 μg ml^{-1}) and tetracycline (10 μg ml^{-1}) were added where indicated.

Protein purification

Pre-β-lactamase was purified from *E. coli* strain N4830 harbouring the plasmid pAL2 as described previously (Laminet and Plückthun, 1989). SecB protein was purified from *E. coli* strain CK2001 as described elsewhere (Kumamoto *et al.*, 1989).

β-lactamase assays

The β-lactamase activity was determined spectrophotometrically at 486 nm with 0.2 mM nitrocefin at 25°C (O'Callaghan *et al.*, 1972). The standardized pre-β-lactamase folding assay was described previously (Laminet and Plückthun, 1989). In the experiments described here, the folding mix contained 0.2 μM pre-β-lactamase and, additionally, either a six-fold molar excess of SecB protomer (160 μl of a 0.12 mg ml^{-1} SecB solution in 50 mM Tris/HCl, 100 mM NaCl, pH 7.6) or only the buffer (160 μl of 50 mM Tris/HCl, 100 mM NaCl, pH 7.6). For determining the total activity of mutant β-lactamase in MC4100 or CK1953 cells, cells were harvested at an OD_{550nm} of 0.5, passed twice through a French Pressure Cell at 16000 p.s.i. (110 MPa) and assayed, if necessary after dilution with the growth medium.

Chemical cross-linking

After incubation of 100 μl of β-lactamase refolding mix with 1% glutaraldehyde for 1 min, $NaBH_4$ (1 M in 0.1 M NaOH) was added to a final concentration of 0.1 M and incubated for another 20 min (Jaenicke and Rudolph, 1986). The reaction was stopped by the addition of sodium desoxycholate and phosphoric acid to final concentrations of 0.1% (w/v) and 8.5% (v/v), respectively. The protein was pelleted for 5 min by centrifugation and the pellet was washed twice with cold acetone and dried in a Speed Vac Concentrator. The dried pellet was resuspended in PAGE-loading buffer, the proteins were separated on a 10% SDS–polyacrylamide gel (Fling and Gregerson, 1986) and stained with the commercial silver staining kit 'Quick-silver' from Amersham.

LD_{50} assay

Fresh cultures of cells, grown for 20 h at 37°C in minimal M9-medium containing glycerol (0.2%) as carbon source, were diluted by a factor of 10^6 into 5 ml fresh medium containing ampicillin. Tetracycline at a concentration of 10 μg ml^{-1} was added to all cultures harbouring plasmids to maintain the plasmid independently of the ampicillin concentration. To eliminate artefacts caused by the spontaneous hydrolysis of ampicillin or cell lysis, the signal sequence deletion mutant Δ(−20, −1) was included as a control. The ampicillin concentrations tested were 0, 50, 100, 200, 300, 400, 500, 600, 800, 1000, 1500, 2000, 2500 and 3000 μg ml^{-1}. The cells were grown for 60 h at 37°C and checked periodically; the results for 50 h are shown in Table 1.

Immunoblotting

The cells were pelleted by centrifugation, resuspended and boiled in PAGE-loading buffer, and the proteins were separated on a 12.5% SDS–polyacrylamide gel (Fling and Gregerson, 1986). The gel was blotted onto nitrocellulose and the filter was 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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