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The precursor of β-lactamase: purification, properties and folding kinetics

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The precursor of *Escherichia coli* RTEM β-lactamase was purified to homogeneity on a milligram scale by a procedure independent of the binding properties of the protein and refolded to an active, reduced form. For comparing the folding kinetics, the wild-type enzyme was reduced and a mutant was constructed, in which the two cysteines that form a very stable disulfide bond in the RTEM enzyme were both changed into alanines. The rate of folding was determined by directly measuring the increase in enzymatic activity. The reduced precursor folds at least 15 times more slowly than either the reduced mature enzyme or the mature Cys→Ala double mutant under identical conditions. The wild-type enzyme, the Cys→Ala double mutant and the precursor protein all had similar K_M values, demonstrating a very similar native state. The slow folding of the precursor compared with the mature form may be an essential and general feature to secure a transport competent conformation necessary for the translocation through a membrane in protein export. This folding assay of a precursor by directly following its enzymatic activity may facilitate the characterization of putative folding modulators in bacterial membrane transport.

Key words: β-Lactamase/protein transport/protein secretion/protein folding

Introduction

It is generally believed that proteins fold to their native three-dimensional structure during and/or immediately after their synthesis on the ribosome. The process is generally assumed to occur spontaneously, and the native form is thermodynamically more stable by a few kilocalories/mol (see for example Anfinsen, 1973; Creighton, 1978; Jaenicke, 1987). When a protein is destined for a location that is separated by a membrane from the cytoplasm where protein synthesis occurs, it must traverse this membrane, most likely in a folding state which is distinctly different from the native, compact form. It appears that there must be a mechanism to control cytoplasmic folding or unfolding.

The ways proteins are translocated through the bacterial plasma membrane, the endoplasmic reticulum (ER) membrane and the mitochondrial and chloroplast membranes share several common features [for reviews see, for example, Walter *et al.* (1984), Benson *et al.* (1985), Duffaud *et al.* (1985), Oliver (1985), Wickner and Lodish (1985), Briggs and Gierasch (1986) and Zimmermann and Meyer (1986)]. Translocation may be concurrent with protein synthesis *in vivo*, but is probably not mechanistically coupled to translation in any transport system. Metabolic energy is required for the translocation step.

Proteins that are transported through the bacterial cytoplasmic membrane carry an N-terminal signal sequence that is characterized by a positively charged N-terminus, a hydrophobic core region and a polar C-terminal region that precedes the signal cleavage site (Watson, 1984; von Heijne, 1985a,b, 1986; Sjöström *et al.*, 1987). The eukaryotic signal sequences of proteins transported through the ER membrane are similar to the bacterial ones. A pathway for the transport through the membrane of the eukaryotic ER has been suggested (Walter *et al.*, 1984). During the synthesis of the protein destined for export, a signal recognition particle (SRP), consisting of six polypeptides and one RNA molecule, associates with the emerging protein and translation is arrested. This complex binds to a membrane-bound docking protein, synthesis resumes and the protein is translocated through the membrane by an as yet unknown mechanism. While post-translational transport is unusual in eukaryotes (see, for example, Meyer, 1985; Siegel and Walter, 1985; Ainger and Meyer, 1986; Rothblatt and Meyer, 1986), it is commonly found in bacterial transport (Wickner, 1988). In bacteria, mechanisms similar to the transport into the lumen of the ER have been proposed, but no general analog of the eukaryotic SRP has yet been found. Several factors that might play a similar role have been suggested (Müller and Blobel, 1984; Crooke and Wickner, 1987; Crooke *et al.*, 1988a,b; Lill *et al.*, 1988; Collier *et al.*, 1988), but their functions and specificities remain unknown.

In vivo experiments in *Escherichia coli* (Randall and Hardy, 1986; Cover *et al.*, 1987) as well as *in vitro* experiments on the import of an artificial hybrid protein into mitochondria (Eilers and Schatz, 1986) suggested that a non-native conformation is required for the translocation of a protein. While this work was in progress, folding experiments with the purified precursors of maltose binding protein and ribose binding protein were reported (Park *et al.*, 1988) that suggested that a signal sequence may retard folding. Earlier experiments (Scheele and Jacoby, 1982, 1893) had shown that several precursor proteins translated *in vitro* did not reach a correctly folded active state. It is still unclear whether there are protein components that prevent the folding of the precursors or even actively unfold them—a role that has been proposed by some investigators for the 70 kd heat shock proteins of eukaryotes (Chirico *et al.*, 1988; Deshaies, 1988). Alternatively, the presence of the signal sequence might by itself slow the folding process or divert the folding pathway of the precursor. Stabilizing factors may then still bind to folding intermediates but no actual unfolding step would have to occur.

To address these questions experimentally, we have developed a method to purify milligram amounts of the precursor of RTEM β-lactamase (Ambler, 1980) and, as a

first step, compared its properties and folding kinetics with processed β-lactamase. Of the enzymes that are transported to the periplasm of *E.coli*, β-lactamase is one of the best characterized. It is soluble, monomeric and does not require any co-factors. Its folding state can be assayed by its enzymatic activity *in vitro* and *in vivo*. This fact may make β-lactamase a very convenient model in the search of putative factors influencing or controlling folding rates. Furthermore, its transport is post-translational (Koshland and Botstein, 1982) and the folding problem is therefore not simply solved by a co-translational vectorial translocation. The single disulfide bond is a convenient experimental tool, with which the conformational space may be restricted when desired. Numerous mutants have been constructed (Kadonaga *et al.*, 1985; Plückthun and Knowles, 1987; Plückthun and Pfitzinger, 1988) and it has been shown that the protein can be transported in Gram-positive bacteria and eukaryotic cells as well (Palva *et al.*,1982; Wiedmann *et al.*, 1984; Roggenkamp *et al.*, 1985), thus permitting a direct comparison of the effect of mutations. The *in vitro* folding pathway of the closely related (mature) β-lactamase of *Staphylococcus aureus* PC1 has been studied in much detail (see, for example, Mitchinson and Pain, 1985; Craig *et al.*, 1985). The structures of the homologous enzymes from *S.aureus* PC1 (Herzberg and Moult, 1987) and *Streptomyces albus* G (Dideberg *et al.*, 1987) have been reported, and the crystal structure of the RTEM enzyme has been solved to low resolution (De Lucia *et al.*, 1980).

We can now directly demonstrate and quantitate the refolding of a precursor to an active form since we follow the folding directly by assaying enzymatic activity. By other methods, a folding to compact, but not necessarily native forms, would be difficult to exclude. We can directly quantitate the retardation of folding caused by the signal sequence and we can furthermore show that the reduced cysteines in the precursor do not prevent the folding to the native form. The assay system described here is especially suited to quantitating the effect of additives, such as putative cytoplasmic folding modulators, since it is accurate, specific and not disturbed by the presence of other proteins in the assay.

Results

Overexpression of the precursor

Since the precursor of β-lactamase is not accumulated in detectable amounts under normal conditions, an expression system had first to be designed that would produce the precursor at a high enough level. Our strategy was to express the β-lactamase under conditions where the translocation system is overloaded. The consequence of this strategy was the formation of inclusion bodies from which the precursor could be rapidly purified to homogeneity.

β-Lactamase was first put under the control of the *tac* promoter because the natural constitutive β-lactamase promoter is weak. However, this construct did not result in a large enough overexpression. Using the new restriction sites created in the plasmid, the gene was put under the control of the phage λ P_L-promoter as described in Materials and methods. In the resulting construct, significantly more protein was made. Under these conditions, precursor was mostly accumulated as inclusion bodies. Other investigators reported periplasmic inclusion bodies of processed β-lactamase, when the expression level was increased with the *tac* promoter (Georgiou *et al.*, 1986). Differences in the vector, strain and growth or induction conditions may all be responsible for the formation of precursor inclusion bodies in the present study, but their location has not been determined by electron microscopy. Inclusion bodies were also found, in addition to soluble β-lactamase, when a genetic construct (−20,−1) (Plückthun and Knowles, 1987) not coding for a signal and thus producing cytoplasmic β-lactamase was used with the λ P_L-promoter, while only soluble, cytoplasmic β-lactamase was obtained with this deletion construct under the control of the *bla* and *tac* promoters (data not shown).

Purification of the precursor

The formation of insoluble inclusion bodies greatly facilitated the first steps of purification but required work under denaturing conditions throughout the purification. A considerable enrichment of the β-lactamase precursor (Figure 1) was obtained by a series of centrifugation steps and a resolubilization with a urea solution containing β-mercaptoethanol. Several chromatographic procedures including affinity chromatography proved unsuccessful in purifying the precursor protein to homogeneity either in the presence or absence of denaturants. However, a very efficient separation was achieved by preparative isoelectric focussing in the presence of 8 M urea and β-mercaptoethanol. Under these conditions, other proteins did not adhere to the partially folded precursor and intra- or intermolecular disulfide formation was suppressed. Most importantly, residual mature form of β-lactamase could easily be separated from the precursor since the isoelectric points differ by almost 0.8 pH units (see Materials and methods). By carrying out this procedure twice, completely pure pre-β-lactamase could be obtained (Figure 1). This method should be generally applicable to a wide range of precursor proteins,

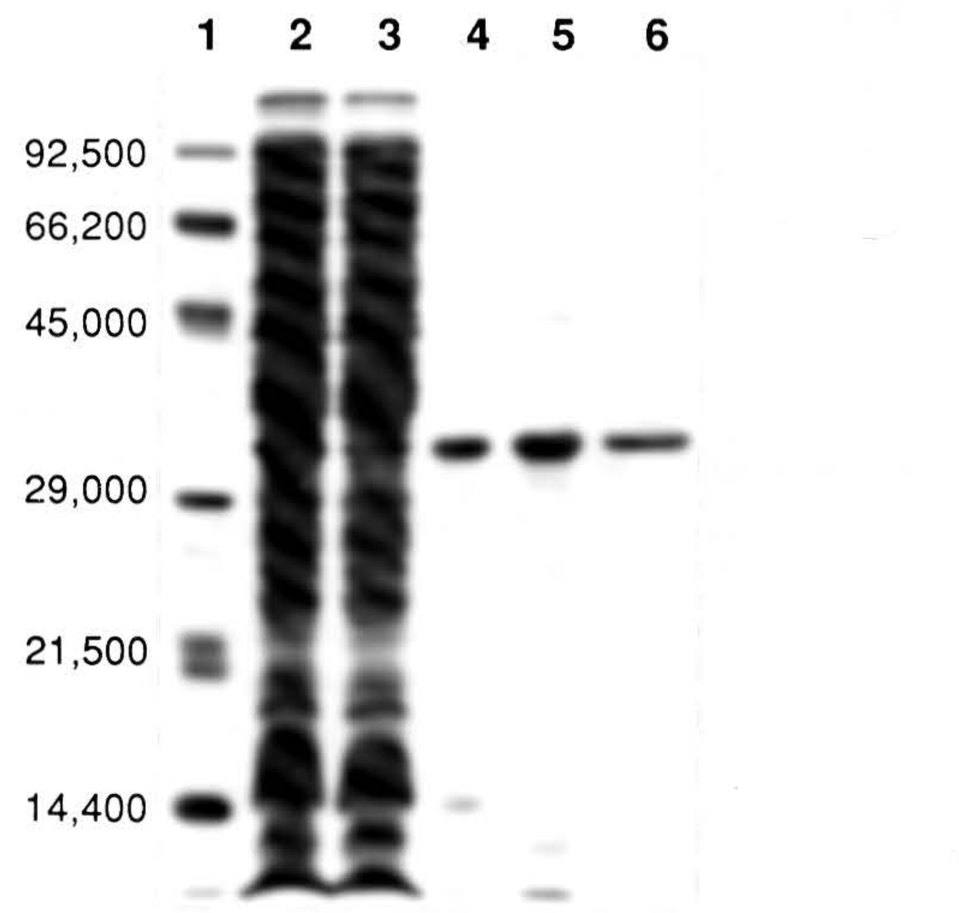

Fig. 1. Purification of pre-β-lactamase. An SDS−PAGE (12.5%) stained with Coomassie brilliant blue is shown. **Lane 1**, mol. wt standard; **lane 2**, total cell protein from a French press lysate; **lane 3**, total soluble protein (supernatant after centrifugation of French press lysate); **lane 4**, total insoluble protein (resuspended pellet after centrifugation of French press lyate); **lane 5**, urea-solubilized protein (pellet of lane 4 solubilized in 8 M urea containing 5% β-mercaptoethanol, cleared by centrifugation); **lane 6**, purified pre-β-lactamase.

even if little is known about their properties and even if no convenient assay or affinity chromatography exists.

We determined the N-terminal sequence of the precursor protein by gas phase sequencing of the purified pre-β-lactamase to be Met-Arg-Ile-Gln, the sequence encoded by pTG2 (Kadonaga *et al.*, 1984). The amount of the N-terminal methionine found in the sequencing was, within experimental error, that expected from a quantitative amino acid analysis of the same sample. Therefore, most if not all of the methionine must be deformylated, but, as expected (Sherman *et al.*, 1984), this residue is not cleaved off. Thus, there is no degradation of the signal sequence in the purified protein.

Construction, expression and purification of a Cys→Ala double mutant of β-lactamase

Since RTEM β-lactamase contains one disulfide bridge, but the precursor is reduced (Pollitt and Zalkin, 1983), a direct comparison of folding rates would be problematic. We have taken two strategies to overcome this problem. First, the wild-type enzyme was reduced with dithiothreitol (DTT) in the presence of urea and the quantitative reduction was verified.

As a second approach, we eliminated the disulfide bond by mutation of both cysteine residues (Cys +52 and Cys +98) to alanine residues using site-directed mutagenesis. The double mutant has the advantage of not allowing any intermolecular disulfide bond formation and can be studied in the absence of reducing agents as well. The resulting protein was found to be normally translated, translocated and processed, when under the control of the *bla* promoter. When the expression of the mutant was maximally induced in the *tac* promoter vector pAP5, material of lower specific activity was obtained from which inactive protein was separated by affinity chromatography. This suggests that a significant amount of processed, but inactive, mutant protein accumulated in the cell at high expression levels. Almost no inactive protein was found for the wild-type protein from the same vector under these conditions, and also not for the mutant protein at lower expression levels.

The purification of wild-type and mutant protein consisted of ion exchange chromatography followed by preparative isoelectric focussing. To obtain absolute specific activites, it was necessary to remove any inactive protein. For this reason, an affinity chromatography step was carried out (see Materials and methods).

Refolding and characterization of the precursor

Initial experiments indicated that when the denaturant was simply diluted out or dialyzed, very low enzymatic activities were recovered from the precursor. Therefore, the conditions of the refolding procedure had to be optimized. The protein concentration was kept low to prevent bimolecular aggregation reactions as much as possible. To prevent oxidation of the cysteines and a possible trapping in a wrong disulfide bond, DTT was added to the refolding reaction. The formation of an incorrect disulfide bond is conceivable in the precursor protein, since the signal sequence contains another cysteine (at position −6), in addition to the two cysteines present in the mature protein. The refolding cocktail also contained a small amount of urea which may increase the folding yield. Several detergents were tested and improvements of refolding yield were obtained with the non-

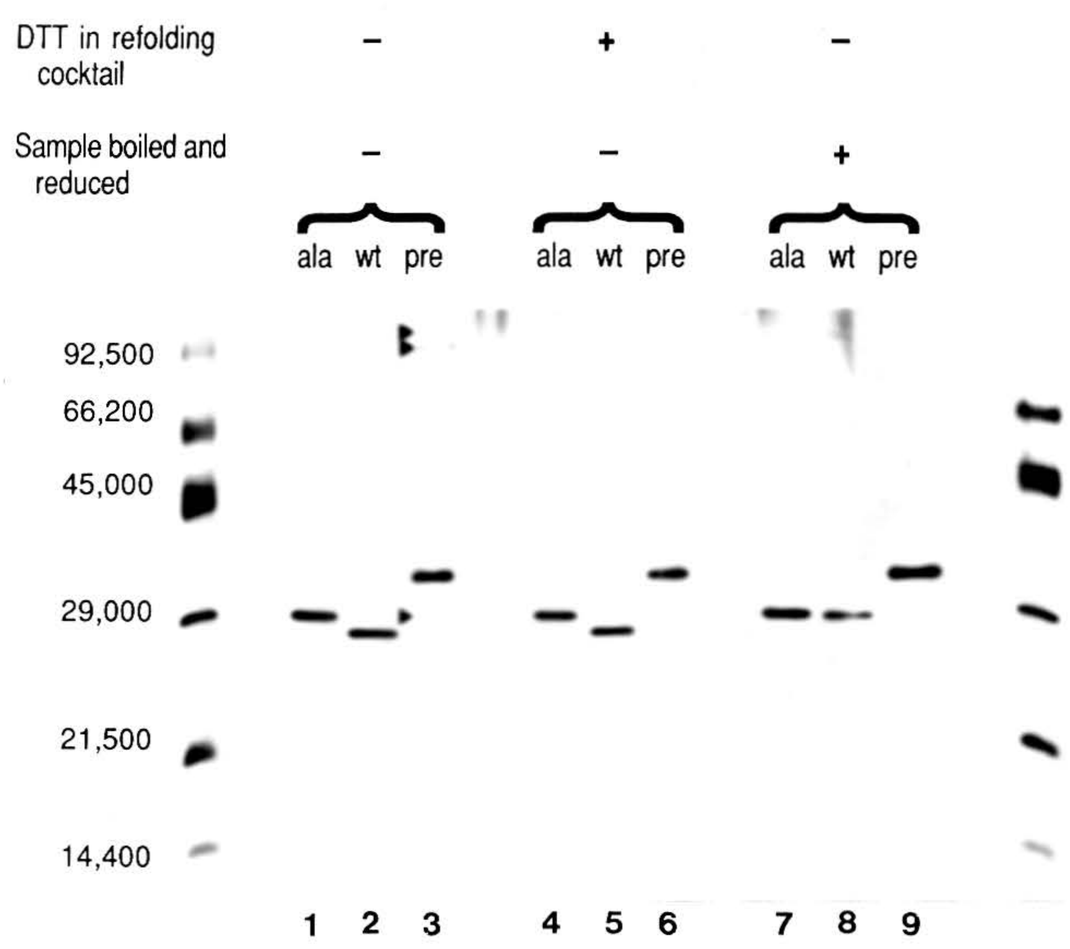

Fig. 2. Oxidation state of β-lactamase and pre-β-lactamase as used in enzyme activity assays. An SDS−PAGE (12.5%) stained with silver is shown. Native wild-type β-lactamase, native Cys→Ala double mutant and pre-β-lactamase were incubated in the cocktail for 1 h. Ala denotes the Cys→Ala double mutant, w.t. the wild-type RTEM enzyme and pre the β-lactamase precursor. The top line above the figure indicates whether reducing agent (10 mM DTT) was present in the incubation, the second line indicates whether the sample was reduced with 5% β-mercaptoethanol and boiled before electophoresis.

ionic polyoxyethylene-containing detergents (Tween 20, Triton X-100, Brij 56). Other surfactants (glucosides, ionic and zwitterionic ones) reduced the final enzymatic activity of the refolded precursor. A further increase in recovery was obtained by the inclusion of ammonium sulfate in the dilution buffer, an addition which had been found to be beneficial for a related mature β-lactamase and which may stabilize the final structure (Mitchinson and Pain, 1985). The refolding cocktail resulting from these experiments, which was then used in the kinetic folding studies, is listed in Materials and methods. The refolding yields were at least 15-fold higher than with phosphate buffer alone and they were sufficient to determine folding rates with small amounts of material.

The effect of this refolding cocktail on the integrity and oxidation state of the wild-type with the intact disulfide bond, the precursor and the Cys→Ala double mutant protein was investigated. All three proteins were incubated with the refolding cocktail for an identical amount of time. The wild-type β-lactamase and the Cys→Ala double mutant were added as native proteins, while the precursor underwent refolding in this solution. In no case was protein degradation observed (Figure 2). The oxidation state of the protein can be examined since the mobility of oxidized and reduced β-lactamase is distinctly different (Figure 2, lanes 2, 5 and 8). The mobility of the Cys→Ala double mutant is identical under all conditions (Figure 2, lanes 1, 4 and 7). The presence of DTT in the cocktail prevented a slight oxidation of the precursor to a more compact form as well as intermolecular disulfide bond formation (Figure 2, lanes 3 and 6). The band corresponding to the faster migrating precursor (Figure 2, lane 3) is not a degradation product since this species is eliminated by boiling the same sample with β-mercaptoethanol (Figure 2, lane 9). Other investigations

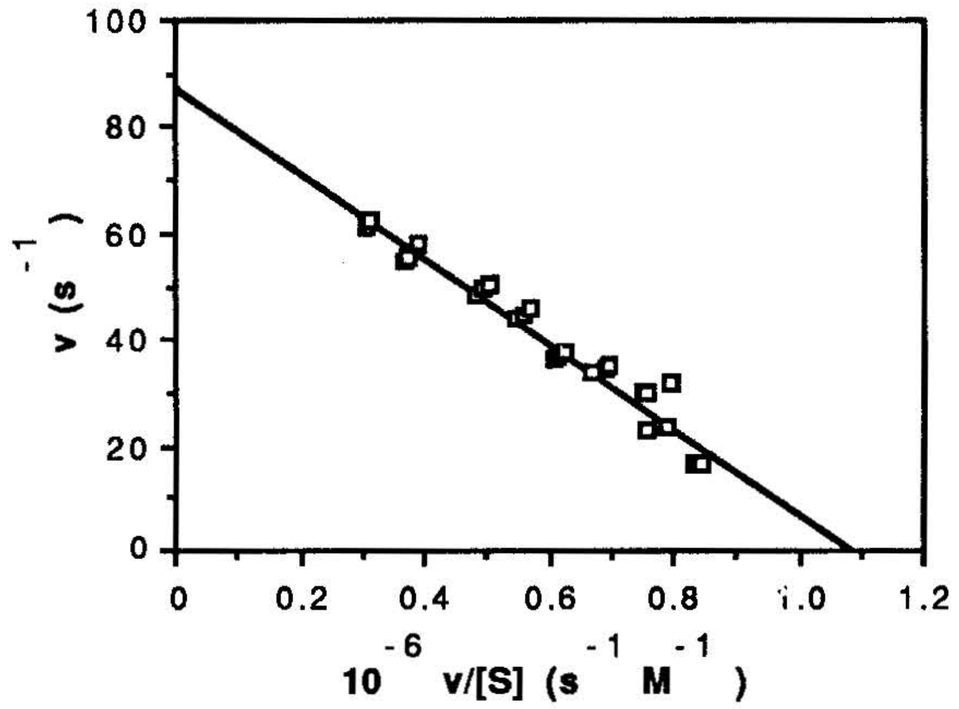

Fig. 3. Eadie–Hofstee plot of pre-β-lactamase refolded in the 'refolding cocktail' and assayed as described in Materials and methods.

Table I. Kinetic constants of β-lactamase and pre-β-lactamase

Enzyme[a]	K_M (μM)	k_{cat} (s^{-1})
Wild-type	49 ± 2	806 ± 13
Cys→Ala	85 ± 3	742 ± 14
Precursor	80 ± 3	[88 ± 3][b]

[a]The enzymes are the wild-type RTEM enzyme with intact disulfide bond, the Cys→Ala double mutant of RTEM β-lactamase (Cys→Ala) and the reduced precursor protein of the wild-type (precursor). The values are averages from replicate measurements with fresh stock solutions.

[b]The k_{cat} value of the precursor protein was related to the total amount of protein and is therefore a lower limit of the turnover number. Since we have no independent measurement of the refolding yield, we cannot determine the true specific activity. This number may, therefore, reflect a low folding yield and/or a low specific activity.

have also shown that reduced and oxidized precursors have different mobilities (Pollitt and Zalkin, 1983). The reduction of mature β-lactamase is negligible under these conditions demonstrating the stability of the disulfide bond (Figure 2, lanes 2 and 5).

We deduce, therefore, that there is no oxidation of the precursor under the refolding conditions in the presence of DTT nor any degradation to mature form and that the activities measured truly arise from a folded, but reduced form of the precursor.

Enzyme kinetics

The kinetic parameters K_M and k_{cat} were determined for the wild-type β-lactamase with the intact disulfide bond, the Cys→Ala double mutant and the refolded precursor (Figure 3, Table I) with nitrocefin as the substrate. Similar values were obtained for all three proteins. The kinetic data demonstrate that the removal of the disulfide bond has a negligible effect on k_{cat} and leads to only a small increase of K_M for this substrate. The refolded precursor with two reduced cysteines shows a K_M almost identical to the mature Cys→Ala double mutant protein. The overall similarity of all K_M values demands that the precursor protein folds to a completely native structure.

The k_{cat} value of the precursor reported in Table I had to be related to the total amount of precursor protein present, since the refolded, active pre-β-lactamase could not be purified by affinity chromatography or quantitated by other means. The number given is therefore only a lower limit of specific activity and may reflect a less than quantitative yield of refolding. The possibility cannot be excluded, therefore, that the presence of the signal sequence has both an effect on the yield of folding as well as on the specific activity. The activity far exceeds the rate that any breakdown product at levels that might have escaped detection (Figure 2) might contribute. The observed activity thus clearly arises from the precursor.

The kinetic properties of the affinity-purified Cys→Ala double mutant were found to be very similar to those of the wild-type β-lactamase (Table I). These findings are consistent with experiments with a mutant in which only Cys +52 was changed to Ser (Schultz *et al.*, 1987). We had established previously that a cytoplasmic form of β-lactamase is enzymatically active in cell lysates (Kadonaga *et al.*, 1984; Plückthun and Knowles, 1987) suggesting that the disulfide bond is not necessary for activity and that the reduced cysteines do not prevent correct folding. Such a reduced form can be obtained in the cytoplasm when the codons of the signal sequence are deleted.

Kinetics of refolding

The rate of refolding was measured as follows. A sample of the protein incubated with denaturant (urea) was diluted into a refolding cocktail (see Materials and methods). Aliquots were withdrawn, added to the assay mix and the enzymatic activity was immediately measured with nitrocefin as the substrate. The resulting kinetics of folding reproducibly showed apparent half-times in the order of 12 min for the precursor (based on several replicate experiments), and the activity was found to plateau for several hours, indicating that an equilibrium state was reached (Figure 4A). The linearity of a plot of $\ln(v_\infty - v)$ versus t indicated that the observed slow reaction was of first order (Figure 4B). Here, v denotes the activity at time t and v_∞ the activity reached on the plateau. The refolding rate was also directly determined to be independent of protein concentration in the range investigated (0.3–9.0 μg/ml) (data not shown). A comparison of the extrapolated zero time point of the measured linear phase with the zero time point calculated from the height of the plateau would accommodate the presence of a rapid kinetic phase with an amplitude in the order of ~10%. The accuracy of the measurements is not sufficient, however, to determine whether another phase is present.

The rate of folding of the precursor was then compared with that of the reduced mature wild-type enzyme and the mature Cys→Ala double mutant. Both the reduced wild-type enzyme and the mutant protein can be reversibly folded and unfolded and are not restrained by a disulfide bond. The reduced wild-type and the mutant enzyme, which had been shown to behave kinetically very similarly to wild-type β-lactamase in the enzyme assays (Table I), were denatured and renatured under conditions identical to those used with the precursor, each in three replicate experiments. A substantially faster (>15-fold) folding rate was observed (Figure 5A and B). This result was obtained independent of whether the Cys→Ala mutant protein was exposed to 8 M urea for 1 min, 1 h or 18 h (data not shown). For the wild-type protein, a complete reduction was verified by SDS–PAGE. The folding rate is too fast to allow a more detailed kinetic analysis with the activity assays and we have

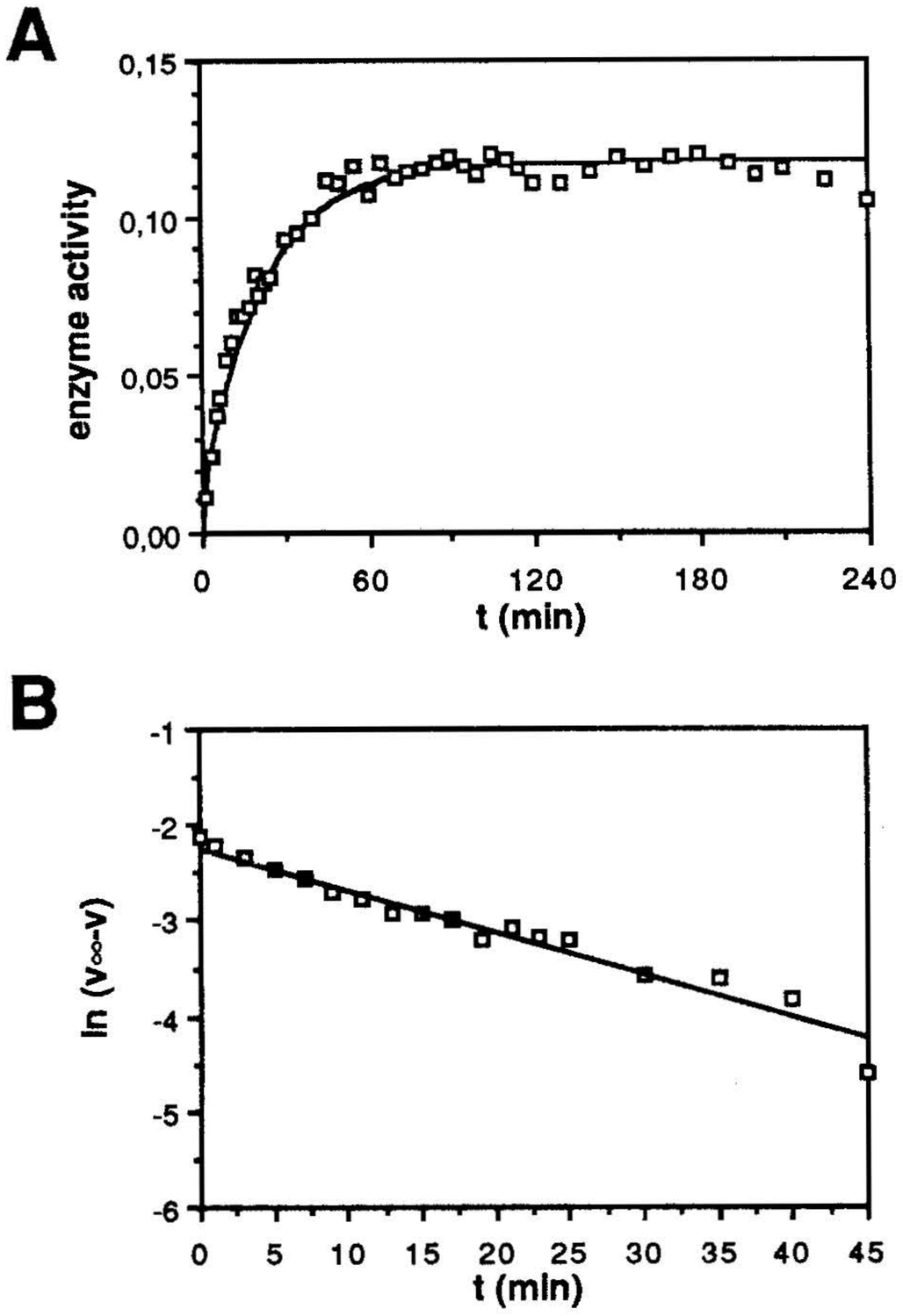

Fig. 4. Refolding kinetics of pre-β-lactamase. (**A**) The enzyme activity is shown (in arbitrary units) as a function of time after dilution into the refolding cocktail. A time course typical of several independent refolding experiments in shown. (**B**) Replot of the data for a reversible first-order process. The zero point corresponds to the plateau in (A), but it was not used in the calculation of the least squares fitted line. The line, together with the small rapid phase implied by (B) was used to generate the curve in (A).

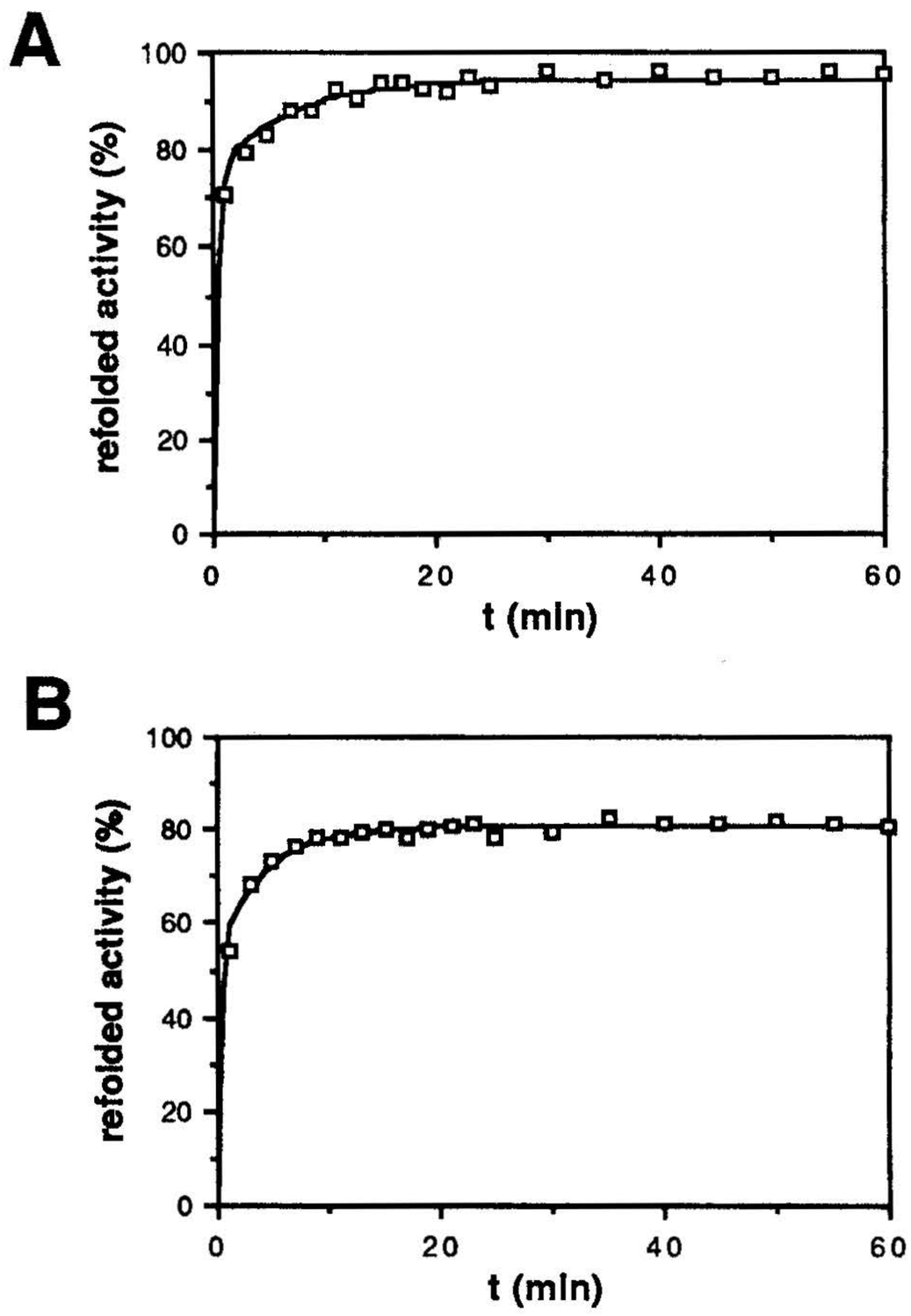

Fig. 5. Refolding kinetics of reduced mature β-lactamase and the β-lactamase Cys→Ala double mutant protein. Typical time courses of several independent refolding experiments are shown. (**A**) The wild-type enzyme was reduced and denatured in 8 M urea containing 10 mM DTT for 1 h and then diluted into the refolding cocktail. (**B**) The Cys→Ala double mutant β-lactamase was denatured in 8 M urea for 1 h and then diluted into the refolding cocktail. The ordinates indicate the refolding yield measured by enzymatic activity.

as yet no information on the presence of different kinetic phases.

Therefore, we can conclude that it is the presence of the signal sequence that causes a slow folding rate. The reduced cysteines have, in contrast, no global effect since the folding rates of the reduced wild-type protein and the Cys→Ala mutant protein appear identical.

Discussion

We have developed a method to purify the precursor of β-lactamase to homogeneity in milligram quantities. This method does not depend on any affinity property of β-lactamase, involves only denatured forms of the protein and should, with few modifications, be applicable to other proteins. We can thus directly demonstrate that pre-β-lactamase can fold to an enzymatically active form, which must be thermodynamically stable. Our experiments show that, at least under the conditions tested, a simple dilution of the denaturant produces only a low yield of enzymatically active protein. The folding intermediates of the precursor may be especially prone to aggregation and a refolding cocktail had to be developed to increase the yield. Conceivably, detergent molecules present in the cocktail bind to the signal and facilitate the folding of the mature domain by preventing the signal from interacting with a hydrophobic region of a folding intermediate. In addition, the final structure might be stabilized by shielding the exposed hydrophobic signal sequence with detergent molecules.

Other investigators (Roggenkamp *et al.*, 1981, 1983, 1985; Tai *et al.*, 1985) have reached the conclusion that either the β-lactamase precursor cannot fold to an active form at all or that the folded form may be inactive. Our results show unambiguously that a conformation can be reached in which pre-β-lactamase is enzymatically active. By developing proper folding conditions, we could demonstrate that the absence of activity of the precursor is not a thermodynamic but a kinetic problem or one of irreversible modification during purification in the published literature. We have measured the K_M values of the mature β-lactamase, a Cys→Ala double mutant and the precursor protein and find very similar values, indicating at most very slight differences in substrate binding. This demands a nearly identical conformation of the protein in all cases.

The rate, however, with which the precursor can be refolded is at least a factor of 15 slower than the rate with which the reduced mature protein or the Cys→Ala double

mutant is refolded under identical conditions. The higher kinetic barrier may slow down the *in vivo* folding of the precursor to permit membrane transport and prevent a premature folding to a compact, native structure. It is, however, difficult to estimate the folding rate of the mature form and of the precursor under *in vivo* conditions. Since we now know that the folding of the precursor can be followed by an enzymatic assay, such measurements might in principle become feasible.

While these experiments were carried out on a variant of β-lactamase that is different in two amino acids in the signal from the form that occurs in nature (Ala→Gly at position −1, Ser→Arg at position −22), it was demonstrated previously that this variant behaves like authentic RTEM β-lactamase in every respect investigated (Kadonaga *et al.*, 1984). Both of these substitutions are commonly observed in signal sequences. We have therefore no reason to suspect that the effect of this signal sequence on the folding and transport of the preprotein should be any different from that of the naturally occurring RTEM signal sequence.

The signal sequence appears to have the effect of preventing a premature folding of the protein by kinetically modulating the folding pathway. It is unclear whether particular structural features in signal sequences are needed for this observed 'folding retardation' effect. Bacterial signal sequences have no primary sequence homology (Watson, 1984). Possibly, both the hydrophobic character and a sufficient length of the signal are necessary to create non-productive folding intermediates of significant lifetimes so that the overall folding rate would be slowed. The cleavage of the signal might then trigger a much faster folding of the mature protein to the native structure on the periplasmic side.

In vivo experiments with shortened (non-functional) signal sequences of β-lactamase (Plückthun and Knowles, 1987) demonstrated that, in all cases, an enzymatically active cytoplasmic β-lactamase is produced. Interestingly, mutated uncleavable signal sequences also lead to a functional membrane-bound (Plückthun and Pfitzinger, 1988) or released (Kadonaga *et al.*, 1985) enzyme. However we have not yet investigated the rate of folding for these mutants. In these cases, the uncleavable signal may be anchored in the membrane during the folding process and would then not be accessible to the rest of the domain to disturb the folding pathway. The mature domain might then conceivably fold to the native form with faster kinetics. For proteins with cleavable signal sequences, the rate of signal cleavage determines whether the periplasmic folding of a precursor or the folding of the mature protein is the relevant process *in vivo*. Detailed investigations of β-lactamase suggest that, under the conditions tested, signal cleavage precedes periplasmic folding, and folding occurs with the processed form (Minsky *et al.*, 1986). A non-native, but processed form of β-lactamase that is bound to the outer face of the inner membrane was shown to be an intermediate in the transport and folding pathway of this enzyme. Recently, mutants were isolated that are blocked in folding and may accumulate in a state similar to this intermediate (Fitts *et al.*, 1987).

The precursor of the outer membrane protein A (OmpA) was also purified recently (Crooke *et al.*, 1988b). It is difficult to delineate native and non-native conformations in this case since it is a membrane protein. It would also be problematic to clearly define folding rates except for the conversion to 'transport incompetent conformations'. A comparison of the precursors of the maltose binding protein and the ribose binding protein (Park *et al.*, 1988) with their mature forms also found that the signal sequence slows the folding rates. Therefore this 'folding retardation' may be a general phenomenon. It is tempting to speculate that a protein whose transport is post-translational, such as β-lactamase, must efficiently maintain a transport-competent conformation and needs a high kinetic barrier retarding precursor folding. The fact that a simple dilution of urea-solubilized pre-β-lactamase leads to only a low folding yield—in contrast to the other precursor proteins investigated (Park *et al.*, 1988)—may be a direct consequence of this high kinetic barrier. The presence of reduced cysteines probably does not bear on the question of cytoplasmic folding since it could be demonstrated that the reduced form of pre-β-lactamase can still be refolded to a native structure.

While the relationship between folding and transport into mitochondria has also been investigated for a synthetic hybrid protein (Eilers and Schatz, 1986, 1988; Eilers *et al.*, 1988; Endo and Schatz, 1988; Vestweber and Schatz, 1988), the influence of the signal on the folding rate has not yet been delineated in detail. It is still unknown how a transport competent conformation of the mitochondrial precursor is obtained. A distinct unfolding step (Chirico *et al.*, 1988; Deshaies *et al.*, 1988; Eilers *et al.*, 1988), protein factors stablilizing the unfolded state of newly synthesized proteins or an intrinsically slow folding rate might be important for obtaining such a conformation.

In conclusion, we could show that the signal sequence slows down the folding of pre-β-lactamase, but does not prevent its folding to a native-like structure. A non-native structure in which small subdomains can be transported through the membrane may be an essential requirement (Randall, 1983; Randall and Hardy, 1984). Possibly these subdomains should not be stabilized by interactions within the native globular domain so that they can be accessible to the export machinery. Further experiments are needed to clarify the precise role of soluble or membrane-bound factors (Müller and Blobel, 1984; Crooke and Wickner, 1987; Collier *et al.*, 1988) that may interact with the nascent polypeptide chain in bacteria. The importance of folding modulators may be dissectable by the use of this refolding assay which can be performed in the presence of other proteins.

Materials and methods

Enzymes and chemicals

Restriction endonucleases, *E.coli* DNA polymerase (Klenow fragment) and ligase were from New England Biolabs, Boehringer Mannheim, GIBCO BRL or Renner. ^{35}S-Labeled nucleotides were from Amersham. Nitrocefin was from BBL Microbiology Systems (Cockeysville, MD). Components of culture media were from Difco. Ultrapure bovine serum albumin (BSA) was from GIBCO BRL. Other biochemicals were from Sigma, Merck or Aldrich and were of the highest grades available.

Bacterial strains

E.coli K12 strains DH1 (F^-, *recA1, endA1, gyrA95, thi-1, hsdR17*(r_k^-,m_k^+), *sup E44, (relA1)*, λ^-) was from D.Hanahan, Harvard University. *E.coli* K12 strain POP2136 (F^-, *endA1, thi, hsdR, aroB, mal,* ▽ *cI857*) was from R.Kleene, Ludwigs-Maximilians-Universität, Munich and was used for construction with the λ P_L-promoter. *E.coli* K12 strain N4830 (F^-, su^-, *his, ilv, rpsL, Δ8* (λ *cI857, ΔBAM, ΔH1*)) was obtained from Pharmacia. *E.coli* K12 strain W3110 (F^-, λ^-, *IN (rrnD-rrnE)*) was obtained from W.Wetekam (Hoechst AG).

General methods

Bacterial growth was performed according to Miller (1972). Recombinant DNA techniques were based on Maniatis *et al.* (1982). Plasmid DNA was isolated as described by Ish-Horowicz and Burke (1981) or Birnboim and Doly (1979). Transformation of *E.coli* was carried out either by the method of Dagert and Ehrlich (1979) or Hanahan (1983). Polyacrylamide gel electrophoresis was as described by Fling and Gregerson (1986). Silver staining was carried out with a staining kit from Amersham. DNA sequencing was carried out by the dideoxy method (Sanger *et al.*, 1977) with 'quasi end labeling' (C.H.Duncan, *NEN Product News*, 4, Nr. 3, p. 6) modified for double strands (A.Skerra and A.Plückthun, unpublished data). Mutations in the β-lactamase gene were introduced using site-directed mutagenesis of the *Eco*RI−*Pst*I fragment of pTG2 subcloned in M13mp11 by the method of Zoller and Smith (1983).

Construction of overexpression vector

The source of the β-lactamase gene was the vector pTG2 (obtained from J.Knowles, Harvard University) (Kadonaga *et al.*, 1984). This gene contains two point mutations (Ser −22 to Arg and Ala −1 to Gly) in the signal sequence that were introduced to obtain unique restriction sites, but the gene product is identical to authentic RTEM β-lactamase in all respects investigated. Therefore, the pTG2 gene product is referred to as wild-type in this work. The numbering of the signal sequence is from −23 (Met) to −1, and that of the mature protein is from +1 to +263. Deletions are referred to by the first and last amino acids that are missing.

First, a plasmid was constructed in which β-lactamase was put under the control of the inducible *tac* promoter derived from pKK223-3 (Brosius and Holy, 1984). pKK223-3 was partially digested with *Bam*HI and treated with the Klenow fragment of *E.coli* DNA polymerase I to produce blunt ends. This DNA was then cut with *Pvu*I and this fragment containing the *tac* promoter and the N-terminal fragment of β-lactamase was isolated. pTG2 was linearized by partial *Xmn*I digestion, cut with *Pvu*I, and the largest fragment was isolated. After ligation of these two fragments, the resulting plasmid has both a functional ampicillin and tetracycline resistance, thus permitting manipulations of the *bla* gene. Next, the *Nde*I site near the origin of replication was removed by cutting with the enzyme, filling with the Klenow fragment and religating. An *Eco*RI fragment (filled in with Klenow polymerase) containing the *lacI*^{q} gene from the plasmid pJW271 (obtained from J.Wang, unpublished data) was then ligated into the *Pvu*II site. To place the β-lactamase under *tac*-promoter control, the region between the polylinker and the ATG of β-lactamase had to be removed. The unique *Eco*RI site of the polylinker was trimmed with nuclease S1 and the intervening sequence between this blunt end site and the *Pvu*I site of the *bla* gene was replaced by the small *Eco*RI−*Pvu*I fragment of pTG2 (coding for β-lactamase) and an oligonucleotide cassette connecting the blunt end of the promoter to the *Eco*RI site in codon 3 of the *bla* gene. The oligonucleotide also introduced a unique *Nde*I site preceding the start codon. Thus, in the resulting plasmid pAP5 (Figure 6), β-lactamase expression is under *tac*-promoter control and can be repressed by a *lac* repressor from a gene resident on the same plasmid. The placement of the *lacI*^{q} gene in the middle of the *rop* gene increases the copy number of pAP5.

The *Nde*Ic fragment of the plasmid pAS1 (Rosenberg *et al.*, 1983) was inserted into the single *Nde*I site of pAP5. This fragment contains the λ P_L-promoter and the nut_L and nut_R sites. The resulting plasmid pAL2 (Figure 6) provides ampicillin resistance to 50 μg/ml without previous heat induction.

Assay of β-lactamase activity

The β-lactamase activity was determined spectrophotometrically at 486 nm with the chromogenic substrate nitrocefin (O'Callaghan *et al.*, 1972) at 25°C. A molar extinction coefficient $\Delta\epsilon$ = 16 000 was used in the calculations (Sigal *et al.*, 1984). Because of the poor solubility of nitrocefin, stock solutions of this compound were prepared in dimethyl sulfoxide (DSMO), but the final concentration of DMSO in the assay never exceeded 0.5%. This concentration of DMSO was shown not to influence the enzymatic rate. The standardized assay was as follows: 20 μl of β-lactamase solution was mixed with 980 μl nitrocefin mix (0.2 mM nitrocefin, 0.25 mg/ml ultrapure BSA, 50 mM potassium phosphate, 0.5% DMSO, pH 7.0, preincubated at 25°C). The BSA in the assay mix was found to stabilize the wild-type, the Cys→Ala double mutant and the precursor alike.

For monitoring column fractions for β-lactamase activity, 200 μl nitrocefin assay mix without BSA were placed in wells of a microtiter plate and mixed with 10 μl of the column fractions.

For the determination of the K_M values, the nitrocefin concentration was varied but the DMSO concentration was always adjusted to 0.5%. The data were analyzed with Eadie−Hofstee plots. The protein concentrations used in the determination of k_{cat} were obtained from OD_{281} measurements in the case of the wild type RTEM enzyme and the Cys→Ala double mutant,

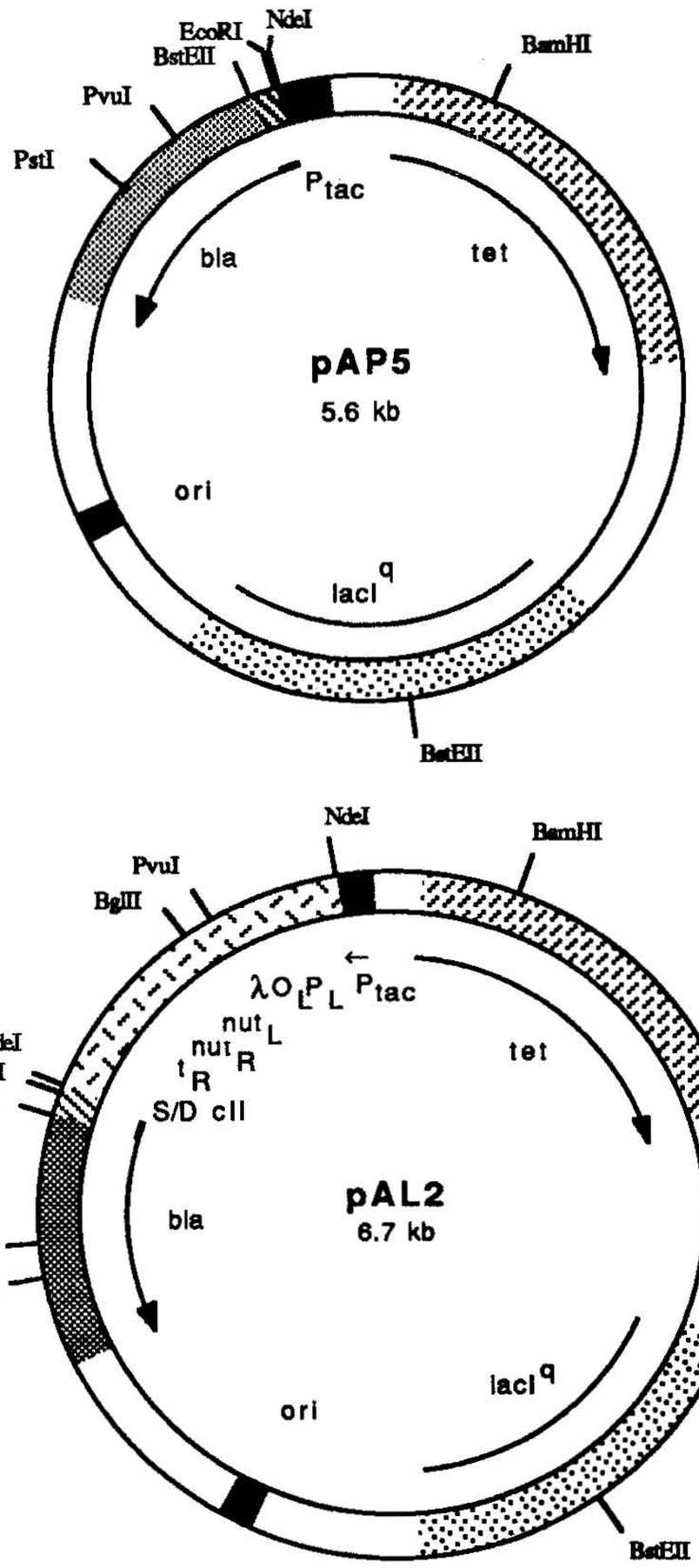

Fig. 6. Maps of the plasmids pAP5 and pAL2 used for the expression of β-lactamase and pre-β-lactamase, respectively. The construction is described in detail in the text.

using the known extinction coefficient of the enzyme (Fisher *et al.*, 1980). Since urea and β-mercaptoethanol in the precursor stock solution and several components of the refolding cocktail preclude most protein assays, quantitative gel scanning was carried out instead. Several dilutions of wild type β-lactamase and the precursor were prepared containing BSA or ovalbumin as an internal standard. From replicate experiments with several dilutions, the concentration of precursor was calculated using mature β-lactamase as the standard.

Fermentation

For the production of pre-β-lactamase, *E.coli* strain N4830 harboring plasmid pAL2 was grown in a 50-l fermenter at 30°C. This strain provides the thermolabile repressor λ cI_{857} the N-protein (Gottesman *et al.*, 1980). The 2AYCG medium used consisted of 2.75% K_2HPO_4, 0.9% KH_2PO_4, 0.2% $(NH_4)_2SO_4$, 0.05% Na-citrate·$2H_2O$, 1% casamino acids, 0.5% yeast extract and 1% glucose at pH 7.0. The fermentation culture was inoculated with 1 l of an overnight culture grown in the same medium at 30°C with 50 mg/l ampicillin. At an OD_{550} of 0.6, the temperature was shifted to 42°C for 30 min for inducing the λ P_L-promoter. The culture was then shifted to 37°C and the cells were grown for an additional 3 h. The formation of inclusion bodies was followed by phase contrast microscopy. The cells were centrifuged and stored at −20°C.

For the production of the Cys→Ala double mutant, the *E.coli* strain W3110 harboring a derivative of pTG2 (Kadonaga *et al.*, 1984) with the two point mutations was used. The fermentation was carried out in 2AYCG medium (see above). Growth at 37°C was continued to an OD_{550} of 5.7. The cells were harvested by centrifugation and frozen.

For the production of the wild-type enzyme, the *E.coli* strain W3110 harboring the plasmid pAP5, in which the β-lactamase is under control of the *tac* promoter, was used. When growth at 37°C had reached an OD_{550} of 1.0, isopropylthiogalactoside (IPTG) was added to a final concentration of 1 mM. Growth was allowed to continue for another 5 h. The cells were harvested by centrifugation and frozen.

Purification of pre-β-lactamase

Frozen cell paste was thawed in 4 ml/g TE50/2 (50 mM Tris−HCl, 2 mM EDTA, pH 8.0). Lysozyme and DNase I were added to final concentrations of 20 mg/ml and 0.1 mg/ml respectively, and the suspension was stirred for 1 h at room temperature. Triton X-100 was added to a final concentration of 1% and the stirring was continued for another hour. Unless noted otherwise, all subsequent steps were carried out at 4°C. The cell suspension was passed twice through a French Pressure Cell at 16 000 p.s.i. and centrifuged three times at 3200 *g* (1 h). The pellets were resuspended in TE50/2, combined, and washed twice with TE50/2 to remove residual Triton X-100.

This pellet was resuspended in TE50/2 (40 mg protein/ml) and diluted with urea to give a final concentration of 8 M urea by addition of a deionized urea solution [9 M, deionized with AG501-X8 mixed bed resin (Bio Rad)]. This suspension was stirred for 1 h at room temperature and centrifuged (45 000 *g*, 1 h, 4°C). To the cleared solution—the solubilized inclusion bodies—β-mercaptoethanol was added to a final concentration of 5%, and the solution was stirred for 1 h and finally cleared again with a 45 000 *g* centrifugation step (1 h). The latter two resulting pellets contained no pre-β-lactamase detectable by SDS−PAGE. The solubilized inclusion bodies were stirred with ammonium sulfate (25% saturation) overnight. The solution was centrifuged at 45 000 *g* (1 h, 4°C). The pellet was resuspended in 65 ml of a solution containing 8 M urea and 2% β-mercaptoethanol. Ampholines (5 ml of a 40% solution pH 5−7, LKB No. 1809−121) H_2O (30 ml) and Ultrodex (4 g, LKB No. 2117−510) were added to the suspension. A preparative isoelectric focussing step was carried out essentially as described elsewhere (see LKB application note 198, revised 1980). The focussing gel was run for 25 h at 10 W and 10°C. The pre-β-lactamase-containing gel section was detected by placing a dry filter paper on top of the gel slurry until wet and staining it with Coomassie brilliant blue R250. The gel section was transferred to a second gel slurry and run again under identical conditions. Pre-β-lactamase was eluted from the Ultrodex gel slurry with 8 M urea containing 1% β-mercaptoethanol in a small column. This fraction was completely homogeneous on SDS−PAGE detected by Coomassie stain, silver stain and antibody stain after Western blotting and was used for folding experiments.

Purification of wild-type and Cys→Ala β-lactamase

This procedure is a modification of the procedures of Fisher *et al.* (1980) and of Melling and Scott (1972). Unless noted otherwise, all subsequent steps were carried out at 4°C. Frozen cell paste (410 g) was thawed in 1 l of 0.2 M Na/K-acetate-buffer (pH 5.5). The cell suspension was passed twice through a French Pressure Cell with 16 000 p.s.i. The pH of the lysed cell suspension was adjusted to 4.9 with HCl and clarified by two 12 000 *g* centrifugation steps (30 min). The pH of the supernatant (670 ml) was adjusted to 5.5 with solid Na-acetate. Ammonium sulfate precipitations were carried out and the material precipitating between 25 and 70% saturation was collected.

The pellet was dissolved in 150 ml of 0.1 M potassium phosphate buffer (pH 7.0) and dialyzed against 25 mM 2-[*N*-morpholino]-ethanesulfonate (MES, pH 6.5) buffer. The dialyzed suspension was cleared by a 16 000 *g* centrifugation step for 90 min. The supernatant (200 ml) was then loaded onto a pre-equilibrated (25 mM MES, pH 6.5) DEAE−cellulose column (Whatman DE52, 3.0 × 26 cm), from which the protein was eluted with 100 mM MES (pH 6.5) buffer. Fractions with enzymatic activity were pooled and dialyzed against 25 mM Tris−HCl (pH 7.0) buffer. The dialyzed suspension (680 ml) was loaded onto a DEAE−cellulose column (Whatman DE52, 3.0 × 26 cm) pre-equilibrated with 25 mM Tris−HCl, pH 7.0. β-Lactamase was eluted with a 25−100 mM Tris−HCl (pH 7.0) buffer gradient. Fractions with enzymatic activity were pooled (435 ml). The protein was precipitated with ammonium sulfate of 80% saturation and centrifuged (16 000 *g*, 30 min). The pellet was resuspended in 50 ml of 10 mM Tris−HCl (pH 7.0) buffer and dialyzed against the same buffer. To the resulting protein solution, 5 ml of a 40% Ampholine solution (pH 4−6, LKB No. 1809−116) was added and the solution was adjusted to 100 ml with 10 mM Tris−HCl (pH 7.0) buffer. Then 4 g Ultrodex was added and the slurry was subjected to preparative isoelectric focussing for 20 h at 10 W and 4°C. β-Lactamase was focussed at pH 5.3−5.4. The protein was eluted from the gel matrix with 5 vol 25 mM Tris−HCl (pH 7.0) buffer. β-Lactamase was homogeneous by SDS−PAGE at this stage. To eliminate any denatured protein, an affinity chromatography step was carried out as the final step.

Affinity chromatography

Phenylboronate affinity columns with a hydrophobic spacer (B-type) on CH-Sepharose 4B or a hydrophilic spacer (L-type, Affigel 10, BioRad) were prepared according to Cartwright and Waley (1984). RTEM β-lactamase was found to bind only to the column with the hydrophobic spacer. The enzyme was dialyzed against loading buffer (20 mM triethlanolamine−HCl, 0.5 M NaCl, pH 7.0), and loaded onto a B-type column, which was washed to elute any inactive enzyme, and the active enzyme was eluted with 0.5 M borate, 0.5 M NaCl, pH 7.0.

Experiments with the refolded precursor were carried out with several buffers on both column types, but the active precursor enzyme could not be specifically eluted with borate. Therefore, we have so far not been able to selectively purify the refolded precursor protein.

Protein sequence determination

The β-lactamase precursor (0.3 mg/ml) was extensively dialyzed against 0.1% trifluoroacetic acid. From this solution, aliquots were withdrawn for gas phase sequencing on an Applied Biosystems 470 A gas phase sequenator and for quantitative amino acid analysis on a Beckman System 6 300 amino acid analyzer.

Protein folding assay

For carrying out folding experiments, all proteins first had to be in a reduced state in 8 M urea. The precursor was directly obtained in this state from purification and could be used without further steps. The wild-type enzyme was incubated in 8 M urea containing 10 mM DTT at 25°C for at least 1 h. SDS−PAGE stained with silver was used to confirm quantitative reduction. The Cys→Ala double mutant of β-lactamase was incubated in 8 M urea not containing reducing agent at 25°C.

Solutions of reduced proteins (wild type β-lactamase, Cys→Ala-β-lactamase or pre-β-lactamase) in 8 M urea were diluted 1:50 into refolding buffer. The basic refolding buffer was 50 mM potassium phosphate buffer pH 7.0 containing 10 mM DTT. By further experimentation (see Results) the following refolding cocktail was developed to increase the refolding yield: 100 mM urea, 100 mM ammonium sulfate, 0.01% Tween 20, 50 mM potassium phosphate, 10 mM DTT, pH 7.0. The final urea concentration is higher (tyically 260 mM) since urea is introduced from the enzyme stock solution as well. The folding reaction was followed by withdrawing samples (20 μl) and immediately assaying the enzymatic activity by the standardized nitrocefin assay (see above). The protein concentrations were such that these enzymatic activity assays could be carried out directly without further dilutions. In all cases (except for the first time point of the refolding assay of the mature enzymes) straight time courses were observed in the activity assays. This rules out significant further refolding taking place during the short time of the enzymatic activity assay (30 s).

For the determination of the refolding yield of wild type or Cys→Ala β-lactamase, aliquots from the same stock solution used for the denaturation were appropriately diluted and assayed for enzymatic activity.

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