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Secretion of β-lactamase into the periplasm of *Escherichia coli*: Evidence for a distinct release step associated with a conformational change

ABRAHAM MINSKY, RICHARD G. SUMMERS, AND JEREMY R. KNOWLES*

Department of Chemistry, Harvard University, Cambridge, MA 02138

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ABSTRACT **The secretion of β-lactamase (EC 3.5.2.6) into the periplasm of *Escherichia coli* has been followed by pulse–chase labeling at 15°C. Though the periplasmic fraction contains only the mature form of the enzyme, the spheroplast fraction contains the completed precursor *and* a hitherto undocumented processed form. When whole spheroplasts are treated with trypsin, the processed form in this fraction is completely digested. This is in contrast to the native mature enzyme localized in the periplasm, which is trypsin resistant. The β-lactamase is evidently processed after translocation to a trypsin-sensitive form that is transiently bound to the periplasmic face of the inner membrane. The release of this processed form into the periplasm occurs concomitantly with a conformational change that results in the soluble, catalytically active, trypsin-resistant structure.**

One of the clearest differences between the two most favored models for the secretion of proteins across membranes, the signal hypothesis and the membrane-trigger hypothesis, concerns the role of conformational changes that occur during the secretion process. The signal hypothesis was first developed to account for the behavior of secreted proteins in eukaryotic systems (1–5). According to this hypothesis, as soon as the signal peptide emerges from the ribosome, it interacts with a signal recognition particle and further elongation is blocked. Subsequent binding of this complex with a membrane-associated docking protein releases the translation blockade, and the resumed polypeptide elongation allows the cotranslational translocation of the molecule through a protein channel in the membrane. The protein is extruded linearly: folding and processing into its mature form occur either during or after translocation across the membrane. The signal hypothesis emphasizes the function of particular polypeptide sequences [including those required for insertion and stop-transfer (6)] in the translocation pathway, while postulating the process to be essentially independent of tertiary structure or folding patterns.

In contrast, the essence of the membrane-trigger hypothesis is that the thermodynamics of protein folding govern the secretory process (7, 8). This model was originally formulated to account for the assembly of the M13 phage coat protein into bacterial membranes but has been extended to accommodate the phenomena associated with the transport of secreted proteins. The signal peptide is postulated to allow the nascent polypeptide to fold in a manner compatible with the aqueous cytoplasmic environment and, at the same time, to prevent the chain from adopting the mature, water-soluble tertiary structure that could not be translocated. A conformational change, triggered by interaction with the membrane, allows the protein to become embedded in the apolar membrane. Upon removal of the signal peptide by a peptidase located on the outer face of the membrane, the protein refolds into its mature, water-soluble form and is released into the periplasm. Implicit in the belief of a conformational change at the outer face of the inner membrane is the existence of a distinct step in the secretory process that involves the release of the protein in its water-soluble conformation (9). Indeed, the aborted secretion of a C-terminally truncated β-lactamase (EC 3.5.2.6) (10) has been interpreted in terms of the inhibition of such a release step.

To detect functional intermediates in the secretion pathway, we have conducted pulse–chase and cell fractionation experiments at low temperature (15°C) that show the existence of a distinct release step in the secretion of the wild-type β-lactamase linked to a change in the conformation of the protein.

MATERIALS AND METHODS

Culture media were prepared using the products of Difco. [^{35}S]Methionine was purchased from Amersham. Trypsin (from bovine pancreas), trypsin inhibitor (from soybean), and protein A-Sepharose were from Sigma.

Pulse–Chase Cell Fractionation. Spheroplasts were prepared from stationary-phase bacteria [*Escherichia coli* K-12 strain DH1, transformed with plasmid pTG2 that encodes the RTEM β-lactamase (11)] by slight modification of the method of Witholt *et al.* (12). An overnight culture (5 ml) grown on M9 medium supplemented with lactose (4 mg/ml), $CaCl_2$ (20 μg/ml), $MgSO_4 \cdot 7H_2O$ (200 μg/ml), Casamino acids (2.5 mg/ml), thiamine (2 μg/ml), and tetracycline (2 μg/ml) was centrifuged at 5000 × *g* for 5 min. The cells were resuspended and incubated for 30 min at 37°C followed by 10 min at 15°C in a medium similar to that described above in which the Casamino acids were replaced by a solution containing 18 amino acids at 2.5 mg/ml but lacking methionine and cysteine. $MgSO_4 \cdot 7H_2O$ was replaced by $MgCl_2$. [^{35}S]Methionine (400 μCi; 1 Ci = 37 GBq) was then added to the culture, and, after 30 s at 15°C, unlabeled methionine (to 2.5 mg/ml) and unlabeled cysteine (to 2.5 mg/ml) were added. At intervals, portions (2 ml) of the culture were transferred to prechilled tubes containing chloramphenicol [20 μl of a solution (50 μg/ml) in EtOH]. All subsequent operations were performed at 4°C. After 10 min, the samples were centrifuged at 5000 × *g* for 5 min and the cells were resuspended in spheroplast buffer [0.2 ml, containing 100 mM Tris·HCl (pH 8.0), sucrose (0.5 M), and EDTA (0.5 mM)]. A freshly prepared solution (10 μl) of lysozyme (2 mg/ml in H_2O) was then added, followed by addition of spheroplast buffer (0.4 ml) in H_2O (0.4 ml). The cells were gently mixed and then allowed to stand for 20 min. The spheroplasts were then pelleted at 12,800 × *g* for 30 s and the supernatant was saved (this is the periplasmic fraction). The pellet was

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*To whom correspondence should be addressed.

vigorously suspended in 1 ml of 10 mM Tris·HCl buffer (pH 8.0) containing EDTA (1 mM) to lyse the spheroplasts. The suspension was allowed to stand for 15 min and was then centrifuged at 50,000 × *g* for 1 hr. The supernatant was saved (this is the cytoplasmic fraction), and the pellet was washed with 1 ml of 10 mM Tris·HCl buffer and then resuspended in 1 ml of 10 mM Tris·HCl buffer (pH 8.0) containing EDTA (1 mM) and Triton X-100 (2.5%, vol/vol). This suspension was allowed to stand for 15 min and then centrifuged at 50,000 × *g* for 1 hr. The supernatant was saved (this is the membrane fraction).

Trypsin Accessibility Experiments. Cells were grown, labeled, and converted to spheroplasts as described above. The spheroplasts were then gently resuspended in 100 mM Tris·HCl buffer (pH 8.0) containing sucrose (0.3 M) and $MgCl_2$ (10 mM). The spheroplast suspension and the periplasmic fraction were each divided into two equal portions. Trypsin (0.1 mg/ml) was added to one sample of each fraction, the second portion serving as a control. All samples were then incubated at 15°C for 1 hr, whereupon trypsin inhibitor (0.2 mg/ml) was added. The spheroplasts were then collected and lysed as described above.

Immunoprecipitation. Anti-β-lactamase antiserum (1 μl) was added to the various fractions and the samples were allowed to stand overnight at room temperature. Protein A-Sepharose (100 μl) preswollen in 100 mM phosphate buffer (pH 7.5) was then added. The mixture was incubated at room temperature for 30 min with frequent shaking and then centrifuged at 12,800 × *g* for 5 min. The supernatant was discarded and the pellet was washed twice with 50 mM Tris·HCl buffer (pH 7.6) containing NaCl (500 mM) and Triton X-100 [0.8 ml of a 2.5% (vol/vol) solution] and then twice with 50 mM Tris·HCl buffer containing NaCl (100 mM) and Triton X-100 (2.5%, vol/vol). The pellet was resuspended in gel electrophoresis buffer [50 μl of 62.5 mM Tris·HCl buffer (pH 6.8) containing sodium dodecyl sulfate (3%, vol/vol), 2-mercaptoethanol (5%, vol/vol), and glycerol (10%, vol/vol)]. The resulting mixture was then heated to 95°C for 5 min, cooled, and loaded onto denaturating polyacrylamide gel.

Enzyme Assays. The efficiency of the cell fractionation procedure was confirmed by assaying the catalytic activities of β-galactosidase, triosephosphate isomerase, and β-lactamase, in each of the different fractions. β-Galactosidase activity was assayed using *o*-nitrophenyl β-D-galactoside as substrate and monitoring the rate of increase in the absorbance at 373 nm (13). Triosephosphate isomerase activity was measured by the conversion of D-glyceraldehyde 3-phosphate to dihydroxyacetone phosphate in a coupled assay containing α-glycerophosphate dehydrogenase and NADH (14). Assays of β-lactamase activity were performed in 100 mM potassium phosphate buffer (pH 7.0) by using Nitrocefin as substrate and monitoring the rate of increase in the absorbance at 482 nm (15).

RESULTS

Cellular Location of β-Lactamase Species. *E. coli* cells, transformed with the plasmid pTG2 that encodes a β-lactamase (11), were treated in stationary phase with [^{35}S]methionine for 30 s at 15°C, followed by a chase with unlabeled methionine. Portions of cells were treated with chloramphenicol, followed by lysozyme/EDTA, and then separated into a periplasmic fraction and a fraction containing intact washed spheroplasts. The β-lactamase species in each fraction were immunoprecipitated and subjected to electrophoresis in denaturing polyacrylamide gels. Fig. 1 presents the results from a pulse–chase experiment at 15°C in which a 30-s pulse of [^{35}S]methionine was followed by a chase of unlabeled methionine. First, the periplasmic fraction contains only the mature form of the enzyme, **m** (of M_r ≈29,000), the amounts in this fraction increasing with the length of the chase, as expected. Second, the spheroplast fraction contains, at the start of the chase, some completed precursor, **p** (of M_r ≈31,500), some mature form (**m**), and a variety of bands of lower molecular weight (M_r ≈19,000–29,000) that appear to be incompletely translated enzyme (**i**). [The existence of explicit bands in this region (e.g., at M_r ≈23,500 and 24,500) rather than an even smear of radioactivity suggests that there may be "pauses" in the translational process. The possibility, however, that these bands represent the appearance of particularly strongly antigenic epitopes cannot be ruled out. Determination of the nature of these shorter polypeptides as well as their chasing patterns requires scrutiny.] As the chase proceeds, these smaller fragments disappear, and the precursor band at M_r 31,500 intensifies. Presumably the smaller fragments are completed during the chase period, and the radioactivity is chased into completed

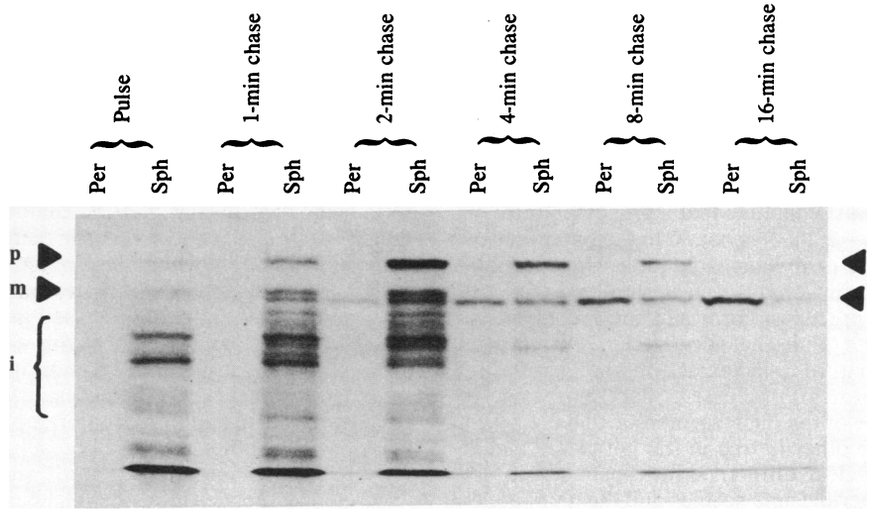

FIG. 1. Pulse–chase cell fractionation of cells transformed with pTG2. Cells were pulse-labeled with [^{35}S]methionine (15°C, 30 s) and chased with unlabeled methionine. Samples were removed at various times during the chase into prechilled tubes and chloramphenicol was added. The cellular proteins in each sample were partitioned by cell fractionation into periplasmic (Per) and spheroplast (Sph) fractions. (The spheroplast fraction contains soluble cytoplasmic as well as membrane-bound proteins.) The length of the chase (in minutes) and the cellular fraction are listed above each lane. **p**, Precursor; **m**, mature; **i**, incompletely synthesized β-lactamase.

precursor, **p**. At even longer times, the precursor and the mature forms in the spheroplast fraction are both chased into the periplasm.

Although most of the bands in Fig. 1 are what would be expected from a simple slowing down of the normal sequence of translation of precursor, translocation, processing, and release of the mature form into the periplasm, the existence of mature β-lactamase in the spheroplast fraction was a surprise. In principle, this band could derive from (*i*) contamination of the spheroplast fraction by periplasmic material, (*ii*) "improper" processing of cytoplasmic precursor by intracellular proteases, or (*iii*) properly processed precursor (i.e., precursor that has been processed on the outer face of the inner membrane by the signal peptidase) that has for some reason not yet been released from the spheroplast into the periplasm. The first possibility has been eliminated by a series of control experiments that confirm the validity of our cell fractionation procedures. These controls have shown that (*a*) >97% of the catalytic activities of the intracellular enzymes β-galactosidase, triosephosphate isomerase, and plasmid-encoded β-lactamase that completely lacks a signal sequence are found in the lysed spheroplast fraction and (*b*) >97% of the wild-type β-lactamase catalytic activity is found in the periplasmic fraction.

The second and third possibilities were explored as follows. A protein species that appears in the spheroplast fraction in principle could be either cytoplasmic or weakly bound to one or the other face of the inner membrane of the cell. If washed spheroplasts are lysed and further separated into soluble and membrane fractions, only those species that are strongly associated with membrane will be distinguished from soluble cytoplasmic proteins, whereas the location of weakly bound proteins may be misassigned. Therefore, to determine the location of the mature β-lactamase band seen in the washed spheroplast fraction, intact washed spheroplasts were treated with trypsin. Cytoplasmic proteins will be protected from protease digestion, but species on the outer face of the inner membrane that are accessible to and digestible by trypsin will be degraded. This experiment was performed with intact washed spheroplasts that had been pulsed with [^{35}S]methionine for 30 s and then chased with unlabeled amino acid for 90 s (all at 15°C); Fig. 2 shows that, whereas the mature, soluble periplasmic β-lactamase was resistant to trypsin, the mature β-lactamase associated with the spheroplasts was completely digested. It is also evident that neither the precursor (**p**) nor the incompletely synthesized β-lactamases (**i**) were significantly digested, consistent with the expectation that these forms are inside the spheroplast (either free or attached to the inner face of the cytoplasmic membrane). These two species (**p** and **i**) *can* be digested if the spheroplast membrane is disrupted by osmotic lysis before trypsin treatment, demonstrating that these species are not intrinsically resistant to tryptic digestion. From these data, we must conclude that a processed form of the β-lactamase (of $M_r \approx 29{,}000$) is bound to the outer face of the inner membrane and is, in intact spheroplasts, accessible to and sensitive to trypsin. This is to be contrasted with the *inaccessibility* of the precursor form and the incompletely translated proteins and with the accessibility but trypsin *insensitivity* of the mature β-lactamase in the periplasm. There are thus two forms of processed β-lactamase: one is bound to the outer face of the inner membrane and is trypsin sensitive, whereas the other is free in the periplasm and is trypsin resistant. The clear difference in trypsin sensitivity between the mature, periplasmic species and the membrane-bound form further confirms the validity of the cell fractionation procedure and eliminates the possibility of contamination of the spheroplast fraction by periplasmic β-lactamase.

Is the Mature Membrane-Bound Form an Intermediate in Secretion? Though the results in Figs. 1 and 2 demonstrate the existence of a mature membrane-bound form of the β-lactamase, the chase patterns of Fig. 1 do not define its fate. Is this membrane-bound form a true intermediate in the secretion pathway or is it a "dead-end" product? To answer this question, the fate of the membrane-bound form at higher temperatures has been examined in spheroplasts and in growing cells. In the first case, intact washed spheroplasts containing the processed membrane-bound form of the β-lactamase were suspended in an isotonic wash solution at either 15°C or 37°C and the fate of the membrane-bound intermediate was followed. The results are shown in Fig. 3*A*: the membrane-bound form of the β-lactamase is released into the wash solution slowly at 15°C and relatively rapidly at 37°C. The released β-lactamase is catalytically active and is resistant to tryptic digestion (data not shown). Other experiments in which the temperature shift (from 15°C to 37°C) was made 5 min or 30 min after "spheroplasting" showed that there is only one species the release of which is temperature dependent.

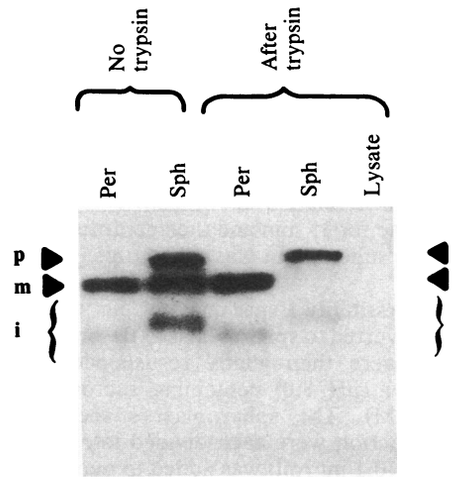

FIG. 2. Trypsin accessibility experiment with cells transformed with pTG2. Cells were pulse-labeled with [^{35}S]methionine for 30 s at 15°C and chased with unlabeled methionine for 90 s at 15°C. The cells were fractionated into periplasmic (Per) and intact spheroplast (Sph) fractions and incubated with or without trypsin. In the extreme right-hand lane (lysate), trypsin-treated *lysed* spheroplasts were loaded. **p**, Precursor; **m**, mature; **i**, incomplete forms of β-lactamase.

The fate of the membrane-bound intermediate in growing cells was also investigated. Cells were labeled at 15°C for 30 s with [^{35}S]methionine and then chased for 2 min at 15°C, for 1 min at 15°C and then 1 min at 37°C, or for 2 min at 37°C. Temperature-dependent chase patterns of the processed membrane-bound form into the periplasmic species are clearly observed (Fig. 3*B*). The results shown in Figs. 1 and 3 indicate that during the first minutes of chase at 15°C, a steady-state concentration of the membrane-bound polypeptide is obtained, in which the amount of β-lactamase synthesized, translocated, and processed is comparable to the amount of material being released into the periplasm. (This interpretation explains the roughly constant intensity of the membrane-bound band. Depletion in the level of precursor due to translocation, on the one hand, and blockage of protein synthesis due to addition of chloramphenicol at the end of the chase, on the other, eventually result in the disappearance of the membrane-bound polypeptide and the completion of the secreted sequence for all radiolabeled molecules.)

DISCUSSION

In this paper we report the existence of a species that appears to be an intermediate in the secretion pathway of the

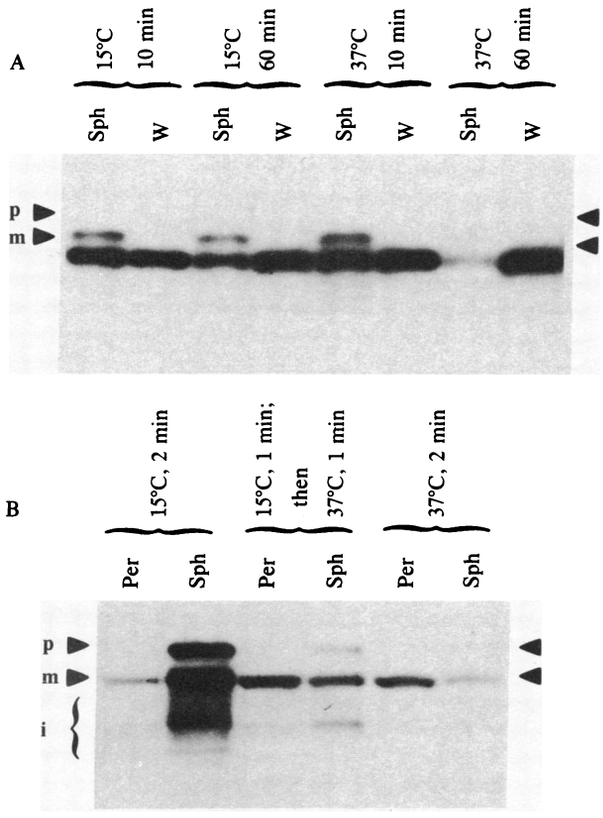

FIG. 3. Fate of the membrane-bound β-lactamase. (*A*) [^{35}S]Methionine-pulsed cells were converted to spheroplasts. The spheroplasts were resuspended in an isotonic solution and incubated at either 15°C or 37°C for various times, as shown. The spheroplasts were then lysed, and the lysed spheroplasts (Sph) and the isotonic wash solution (W) were loaded on the gel. **p**, Precursor form; **m**, mature form. (*B*) Cells were pulsed for 30 s at 15°C and split into three portions. Sample 1 was chased for 2 min at 15°C; sample 2 was chased for 1 min at 15°C and then for 1 min at 37°C; sample 3 was chased for 2 min at 37°C. Each sample was then partitioned into periplasmic (Per) and spheroplast (Sph) fractions. **p**, Precursor; **m**, mature; **i**, incomplete forms of β-lactamase.

wild-type TEM β-lactamase. This intermediate fractionates with the spheroplasts yet has a molecular weight identical to that of the mature periplasmic molecule. Trypsin treatment of the intact spheroplasts results in the complete digestion of the new species, showing not only that it is bound to the outer face of the inner membrane but also that its conformation is different from that of the released periplasmic enzyme (which is completely resistant to tryptic digestion). The release of the membrane-bound species into the periplasm therefore occurs *after* processing and is accompanied by a conformational change that produces the native, catalytically active, β-lactamase. It seems likely that the conformational change involves some "inside-out" process, during which apolar residues at the surface (which allow the protein to be translocated through the hydrophobic core of the membrane and cause the observed membrane association) are turned inside. The ultimate fate of the mature membrane-bound species indicates that it is an intermediate in the secretion pathway and not a dead-end product deriving from the low temperature at which the pulse–chase was conducted.

The membrane-trigger hypothesis for protein secretion is based upon the central principle that integral membrane proteins and fully secreted proteins can undergo reversible, relatively facile conformational changes between at least two conformations. The rationale behind this requirement is obvious: a protein synthesized in the cytoplasm and subsequently inserted into the membrane must switch from a water-soluble conformation to one that is more stable in the membrane. Indeed, the many examples of protease-sensitivity differences between precursor and mature secreted proteins (10, 16) attest to the existence of more than one protein conformational state. Even if the membrane assembly or secretion processes are not entirely posttranslational events but occur by the sequential incorporation or translocation of domains of the protein (9, 17–19), the requirement for local conformational flexibility still holds. Extension of the model for insertion of integral membrane proteins to include fully secreted proteins implies a second conformational change that occurs after translocation and that results in the water-soluble periplasmic form. Our results indicate that such an event can indeed be observed when the secretion process is followed at reduced temperatures.

Examples of protein conformational switching that is triggered by detergents or lipids are well documented (7). For instance, the diphtheria toxin protein binds the detergent Triton X-100 only after having been dialyzed against sodium dodecyl sulfate (20). The dialysis step is assumed to cause a rearrangement in which a hydrophobic segment, which in the native form is masked by polar residues, becomes exposed. This kind of conformational change may explain the transport of the toxin A-chain through the membrane. As far as β-lactamase is concerned, there is evidence that the enzyme can be triggered to undergo conformational isomerization, and it has been shown that different classes of β-lactamase substrates and substrate analogues can produce reversible changes in the susceptibility of the enzyme to proteases and to chemical modification (21, 22).

Though supporting the tenets of the membrane-trigger hypothesis, these examples do not provide proof that conformational changes occur during membrane insertion or secretion. Moreover, even if protein folding changes occur in the course of export, there are no indications whether these processes are necessary for, or are merely linked to, the translocation sequence. No data have hitherto been available on the temporal relationships between protein folding and the various steps of the secretion pathway. What we have observed here, however, is a conformational change concomitant with a membrane release step. Though our results are not inconsistent with other models of the secretory process (1–5, 9), such a linkage between a conformational change of the protein and its release from the membrane is clearly required by the membrane-trigger hypothesis. A conformational change that occurs prior to the actual translocation step and renders the protein competent for secretion through the apolar membrane necessitates a second structural isomerization that allows the translocated protein to be released into the aqueous periplasm. The behavior we have seen for the complete β-lactamase may also provide an explanation for the results observed with several periplasmic proteins [maltose- and ribose-binding proteins (23, 24), glycerophosphate phosphodiesterase (25), as well as β-lactamase (10)], the genes of which have been modified to produce C-terminally truncated proteins. In each of these cases, the shortened proteins are translocated across the membrane but are not released into the periplasm. It was not clear, however, whether the C-terminal truncation blocks a step in the normal sequence of events or whether the observed behavior of the mutant is an aberrant diversion from the normal course of protein secretion. Our results using the wild-type β-lactamase allow a distinction between these possibilities and show that the *normal* pathway for the secretion of proteins from prokaryotic cells requires a conformational change at the point of release from the membrane.

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