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Evidence for Posttranslational Translocation of β-Lactamase across the Bacterial Inner Membrane

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Summary

Secretion of β-lactamase was studied in Salmonella typhimurium infected with P22 phage carrying wild-type and mutant alleles of the structural gene. Cellular location of precursor and mature products of wild-type and temperature-sensitive and chain-terminating mutants was analyzed by cell fractionation and by trypsin accessibility in intact and lysed spheroplasts. The precursors of wild-type and all these mutants (none of which alter the signal peptide) are found sequestered within the cell, while all the mature forms have at least partially been translocated across the inner membrane. Thus most β-lactamase molecules traverse the membrane after completion of their translation. It seems that the carboxyl terminus of β-lactamase is not required for translocation across the inner membrane but is required for the protein to appear in the periplasm as a soluble species.

Introduction

The periplasmic proteins of gram-negative bacteria are soluble proteins that reside on the opposite side of the plasma membrane from the cytoplasm; for this reason they are referred to as secreted proteins. The secretion of specific proteins from the cytoplasm to the periplasm of gram-negative bacteria can be divided formally into three steps: the interaction of these proteins with the cytoplasmic side of the inner membrane, their translocation across the inner membrane and their release from the periplasmic side of the inner membrane into the periplasm. We have characterized the translocation step of secretion for one periplasmic protein, TEM β-lactamase.

The question of how periplasmic proteins translocate across the inner membrane has been addressed previously. Smith et al. (1977) were able to label nascent chains of alkaline phosphatase, a periplasmic protein, by exposing spheroplasts of Escherichia coli to membrane-impermeable labeling reagents. Smith (1980) was also able to show that alkaline phosphatase is sequestered into inverted vesicles of the inner membrane only if these vesicles are present during its in vitro synthesis. These experiments suggest that this protein is translocated across the inner membrane cotranslationally. Mutants of E. coli have been isolated that inhibit or enhance the translocation of more than one periplasmic protein (Emr et al., 1981; Oliver and Beckwith, 1981; Ito et al., 1981) These mutants have been interpreted as lesions that alter the function of a machinery required for the translocation step.

We have investigated the secretion of TEM β-lactamase in Salmonella typhimurium, a bacterial species closely related to E. coli (Koshland and Botstein, 1980). Our results supported the hypothesis that β-lactamase is initially synthesized with a signal peptide at its amino terminus (Sutcliffe, 1978) and showed that the signal peptide of β-lactamase can be cleaved posttranslationally from the amino terminus. Josefsson and Randall (1981) extended this analysis in E. coli, and their work confirms that the signal peptide of β-lactamase is usually processed from the amino terminus only after the synthesis of β-lactamase is completed. The role of the signal peptide in the secretion of β-lactamase is discussed by Koshland et al. (1982).

Using chain-terminating mutations in the β-lactamase structural gene (*bla*), we also examined the synthesis and secretion of prematurely terminated peptides of β-lactamase (Koshland and Botstein, 1980). Results of these experiments suggested that the carboxyl terminus of β-lactamase is not required for faithful processing of the signal peptide from the precursor forms of these peptides. In addition, when periplasmic proteins are fractionated from other cellular proteins, the processed forms of the chain-terminating alleles of *bla* are not recovered as soluble periplasmic proteins. When the membrane and cytoplasmic proteins are separated from each other, these peptides fractionate as though they were cytoplasmic. These results raised the possibility that the carboxyl terminus of β-lactamase is required for the successful translocation of β-lactamase across the inner membrane; in any case, they indicated that successful secretion (that is, deposition of the protein as a soluble species in the periplasm) somehow fails in the absence of the carboxy-terminal residues of β-lactamase.

We describe here experiments that examine specifically the translocation of β-lactamase across the inner membrane. We used cell-fractionation and trypsin-protection experiments to determine the cellular location of the precursor and mature forms of β-lactamase produced by the wild-type, chain-terminating and temperature-sensitive alleles of *bla*. Though we cannot resolve whether some initial interaction between β-lactamase and the inner membrane occurs during or after its synthesis, the results of these experiments indicate that the majority of β-lactamase molecules translocate across the inner membrane posttranslationally. We also present evidence that at least a substantial portion of the mature peptides of the chain-terminating alleles of *bla* are exposed to the periplasm. These results are not compatible with the localization of the mature forms of the chain-terminating alleles of *bla* to the cytoplasm, and suggest instead that the processed forms of these alleles are bound by some weak interaction to the surface of a membrane in the periplasm. Thus the presence of the carboxy-terminal residues of β-lactamase appears to be important (either directly or by their effect on

protein conformation) not for translocation per se but on some later step required for the protein to appear as a soluble species in the periplasm.

Results

Phage-specified proteins are easily identified with SDS-polyacrylamide gel electrophoresis because phage-specified proteins can be labeled specifically after infection of a host that has been irradiated with ultraviolet light (Studier, 1973). We previously established (Koshland and Botstein, 1980) the usefulness of this system for analysis of the synthesis and maturation of the β-lactamase protein encoded by a derivative of bacteriophage P22 carrying the *bla* gene (P22*bla*) in S. tymphimurium; in all the experiments described below this labeling procedure was used.

Pulse-Chase Cell Fractionation of Wild-Type Precursor

We investigated the cellular location, by cell fractionation, of the products of the wild-type *bla* allele as a function of time. Irradiated cells that were infected with P22*bla*$^+$ phage were labeled with ^{35}S-methionine for 30 sec, and then nonradioactive methionine was added. Samples of the labeled cells were removed at various times after the addition of the chase and immediately chilled to 0°C. The cells in each sample were converted to spheroplasts, and then the periplasmic proteins were separated from the spheroplasts by centrifugation. The spheroplasts were then lysed osmotically, and the membrane and other macromolecular structures were separated from the soluble proteins of the cytoplasm by ultracentrifugation. The proteins in each fraction were separated by SDS-polyacrylamide gel electrophoresis. The total protein in each fraction was visualized by Coomassie blue stain, while labeled proteins were visualized by autoradiography. The intensities of the labeled bands on the autoradiographs were quantitated with a scanning densitometer (see Experimental Procedures).

To obtain meaningful results from these pulse-chase cell-fractionation experiments, we had to satisfy several conditions. The most important of these concerned the effectiveness of chilling in stopping conversion of any intermediates in secretion and the reliability of the cell-fractionation system. These were checked as described in the Experimental Procedures, with the result that for *bla*$^+$ alleles chilling is adequate and the cell-fractionation system correctly separates known cytoplasmic, membrane-bound and periplasmic proteins.

A typical autoradiograph from a pulse-chase cell-fractionation experiment with irradiated cells infected with P22*bla* phage is presented in Figure 1. The precursor species and mature form fractionate very differently. The majority of label in newly synthesized precursor fractionates as soluble in the cytoplasm (Figure 1, lanes b, e and h). The mature species accumulates as the precursor disappears, and is found almost exclusively in the periplasmic fraction (Figure 1, lanes a, d, g and j). The percentage of newly synthesized precursor that fractionates as periplasmic is so small that the appearance of precursor in this fraction can be accounted for entirely by cross-contamination of one fraction with another (see legend to Figure 1).

During the chase most of the labeled precursor is apparently processed to give the mature species, indicating that the precursor detected in these experiments is converted to the mature β-lactamase. The cell fractionation suggests that the chaseable precursor is either soluble in the cytoplasm or weakly bound to the surface of a membrane.

Trypsin-Accessibility Experiments

Cell-fractionation experiments are, regrettably, subject to ambiguities and artifacts. Proteins that appear membrane-bound by cell-fractionation experiments could be either on the cytoplasmic side or on the periplasmic side of the inner membrane. In addition, proteins that are weakly bound to the membrane might disassociate from the membrane when the membrane is disrupted. Proteins with this property would appear soluble in the cytoplasm by cell-fractionation procedures. To resolve whether the precursor or mature species of particular *bla* alleles are on the cytoplasmic or periplasmic side of the inner membrane, we probed the localization of these proteins by trypsin-accessibility experiments (Halegoua and Inouye, 1979).

The rationale for these experiments is simple. If a protein is localized in the periplasm or on the periplasmic side of the inner membrane, then it should be digested by proteases when cells are converted to spheroplasts, as well as when the spheroplasts are lysed. However, if this protein is localized in the cytoplasm, then it should be protected from the degradative action of proteases when the cells are converted to spheroplasts but not when the spheroplasts are lysed.

The actual design of these protease experiments is very similar to the pulse-chase cell-fractionation experiments. Irradiated and infected cells were pulse-labeled with ^{35}S-methionine for 30 sec, followed by the addition of unlabeled methionine (and in some cases chloramphenicol was added as well). Samples were removed approximately 20 sec and several minutes after the addition of the chase and chilled immediately to 0°C. Each sample was converted to spheroplasts and then split into two aliquots. The spheroplasts in one aliquot were lysed osmotically. Then the aliquot containing the intact spheroplasts and the aliquot containing the lysed spheroplasts were each subdivided and treated with increasing amounts of trypsin. The trypsin digests were ended after 10 min by transfer of the digests to sample buffer preheated

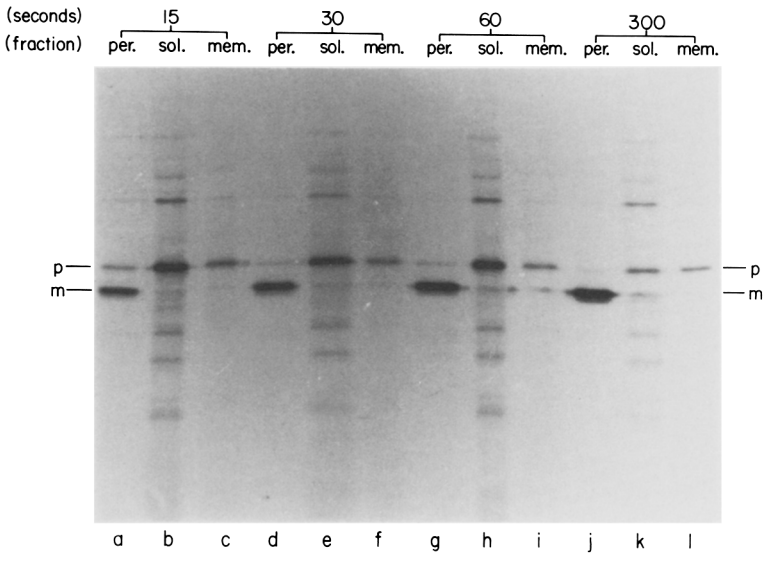

Figure 1. Pulse-Chase Cell Fractionation of Cells Infected with *bla* $^{+}$ Phage

A culture of DB4381 was irradiated and infected with P22*bla* $^{+}$ phage at a multiplicity of infection (moi) of 20. The infected cells were pulse-labeled with ^{35}S-methionine and chased with nonradioactive methionine. Samples were removed at various times during the chase and chilled to 0°C. The cellular proteins in each sample were partitioned by cell fractionation (see Experimental Procedures) into three fractions, periplasmic (lanes per.), soluble cytoplasmic (lanes sol.) and membrane-bound (lanes mem.). Twenty microliters of each fraction was loaded in each lane. Electrophoresis was carried out on a 12.5% gel, and the autoradiograph was exposed for 2 days. The length of the chase (in seconds) and the cellular fractions are listed above each lane. p: precursor form. m: mature form.

to 95°C. Then these samples were subjected to electrophoresis through SDS-polyacrylamide gels. Autoradiographs of the gels were made, and the intensities of the relevant bands were measured with a densitometer. The trypsin accessibility of several non-*bla*-encoded proteins was always followed, and was found to be consistent with the known cellular location of these proteins (see Experimental Procedures).

The results of the trypsin-accessibility experiments with cells infected with *bla* $^{+}$ phage are summarized in Figure 4 (panels 3a and 3b). After 20 sec of chase, 65% of the label in *bla*-encoded proteins is in the precursor form. This protein is digested by trypsin when the spheroplasts are lysed, but is not digested when the spheroplasts are intact. Five minutes after the addition of the chase, the small amount of precursor that remained (20% of the total label in *bla*-encoded proteins) remains protected from trypsin in intact spheroplasts. The mature β-lactamase is not digestible by trypsin in either lysed cells or spheroplasts (see below).

The trypsin insensitivity of wild-type precursor in spheroplasts, but not in lysed cells, seemed due to its location on the cytoplasmic side of the inner membrane. However, an alternative interpretation of these data is possible. Since the wild-type mature species apparently exists in a trypsin-resistant conformation, it is possible that the wild-type precursor also exists in a trypsin-resistant conformation on the periplasmic side of the inner membrane. The precursor form, unlike the mature form, would be converted to a trypsin-sensitive conformation upon lysis of the spheroplasts.

To distinguish between these interpretations, we performed trypsin-accessibility experiments with mutant alleles of *bla* whose precursor and mature forms are both trypsin-sensitive in crude lysates. If the precursor form of one of these alleles were trypsin-resistant in intact spheroplasts, then we could be reasonably confident that the apparent trypsin resistance of the precursor form was due to its protection by the inner membrane and not because the precursor form assumes an intrinsically trypsin-resistant conformation.

Four alleles whose precursor and mature forms are trypsin-sensitive in crude lysates are: two temperature-sensitive alleles of *bla* (*tsH1* and *tsH41*) and two chain-terminating alleles (*fsI7* and *amH46*). Except for trypsin sensitivity, the precursor and mature forms of the *tsH41* allele behave identically to the precursor and mature forms of the wild-type allele in cell-fractionation experiments and in pulse-chase experiments (Koshland and Botstein, 1980; see also below).

Figure 2 shows a typical autoradiograph from a trypsin-accessibility experiment for the *bla-tsH41* allele. The intensities of *bla*-encoded proteins were quantitated, and the results are summarized in Figure 4. An examination of lanes a and f of Figure 2 (samples in which no trypsin was added) show that the majority of precursor labeled at 20 sec is eventually processed to the mature species. Lanes b and c of Figure 2 show that this chasable precursor is not digested by trypsin in intact spheroplasts, while the mature species is. When the spherolasts are lysed, then the precursor is digested (Figure 2, lane e). After 5 min of chase, the small amount of precursor that remains is not digested by trypsin when spheroplasts are intact. In contrast, the majority of the newly processed mature protein is digested by trypsin under these conditions (Figure 2, lanes f–i). Apparently trypsin digests the mature species in intact spheroplasts to small peptides, because exposure of intact spheroplasts to trypsin causes the disappearance of the band representing the mature species but does not produce any new discrete bands that migrate faster than the mature species. The precursors and mature species of *amH46* (Figure 3;

Figure 4, panels 2a–2d), *fsl7* and *tsH1* (data not shown) all behave similarly to *tsH41* in trypsin-accessibility experiments.

These results strongly suggest that the precursors of these four alleles of *bla* are located on the cytoplasmic side of the inner membrane, while the mature species to which they give rise are located on the periplasmic side of the inner membrane. They also carry the implication that the apparent trypsin insensitivity of the wild-type precursor in spheroplasts is also due to its sequestration on the cytoplasmic side of the inner membrane, and not to some intrinsic trypsin resistance of the precursor form. Finally, they suggest that a substantial portion of the mature pep-

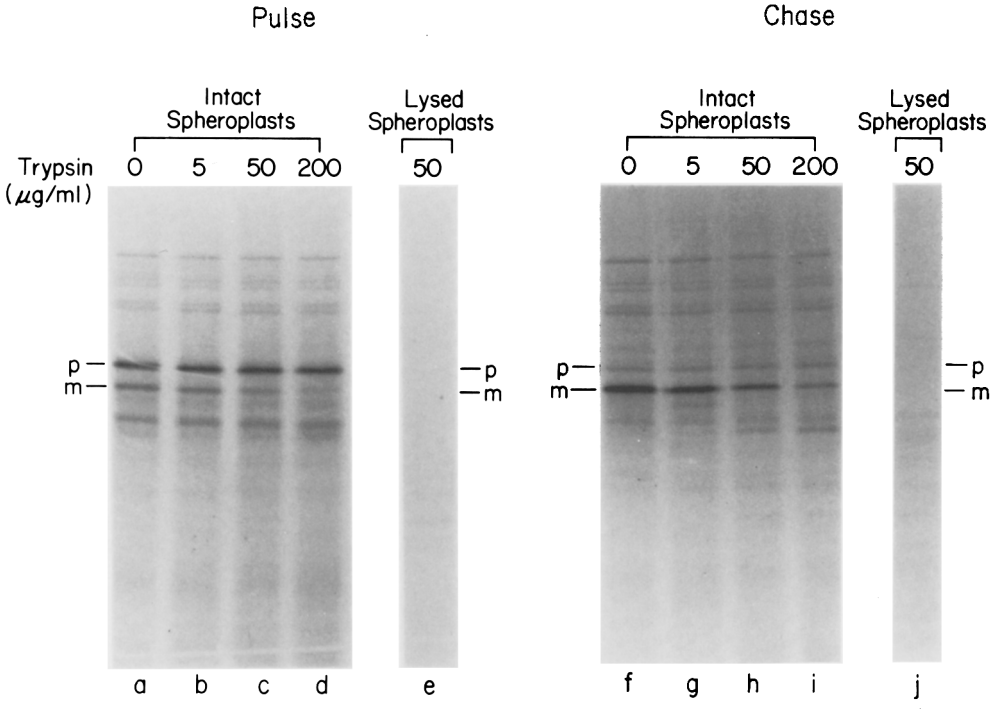

Figure 2. Pulse-Chase Trypsin-Accessibility Experiment with Cells Infected with P22*bla-tsH41*

A culture of DB4381 was irradiated and infected with P22*bla-ts*H41 phage at an moi of 10. The infected cells were labeled with ^{35}S-methionine and chased in the presence of nonradioactive methionine and chloramphenicol. Samples were removed immediately after the addition of the chase and 5–10 min later, and chilled to 0°C. The cells in each sample were converted to spheroplasts and then split in two. Half of the spheroplasts were pelleted and lysed osmotically (after the supernatant containing the periplasmic fraction was removed). Aliquots of the intact and lysed spheroplasts were exposed to increasing amounts of trypsin for 10 min. Twenty microliters of each digestion was loaded in each lane. Electrophoresis was carried out on a 12.5% gel, and the autoradiograph was exposed for 2 days. p: precursor form. m: mature form.

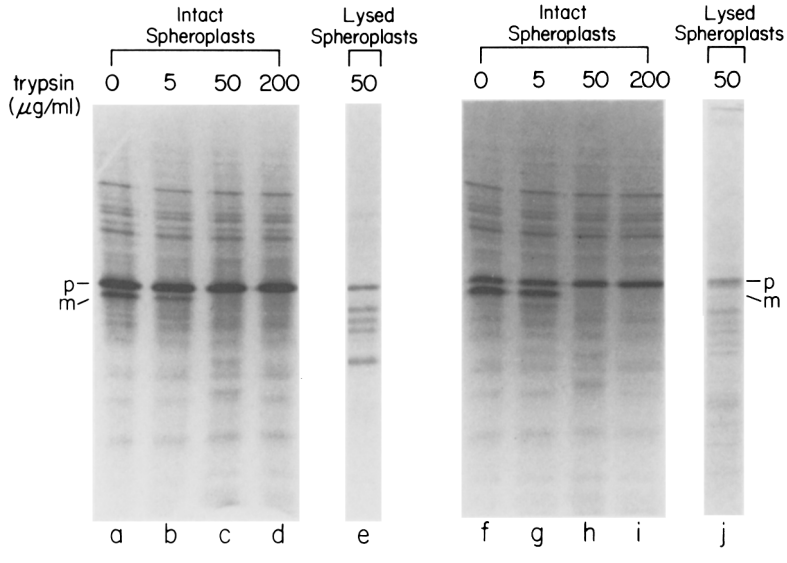

Figure 3. Pulse-Chase Trypsin-Accessibility Experiment with Cells Infected with P22-*amH46*

A culture of DB4381 was irradiated and infected with P22-*amH46* phage at an moi of 10. The experiment was performed exactly as described in the legend to Figure 2, except chloramphenicol was omitted.

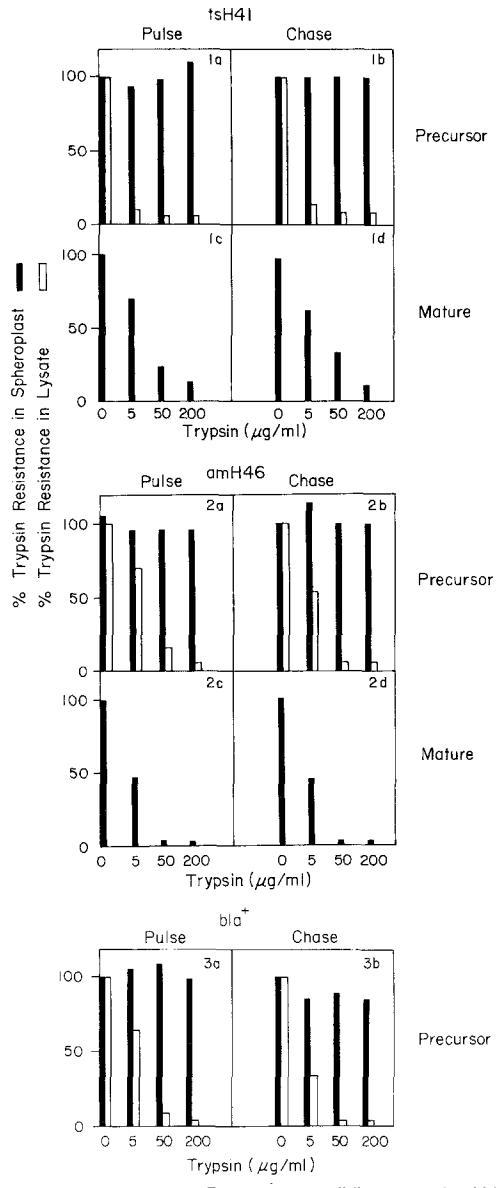

Figure 4. Pulse-Chase Trypsin-Accessibility Analysis of *bla*-encoded Proteins

Trypsin-accessibility experiments were performed with cells labeled with ^{35}S-methionine and infected with P22*bla* phage carrying the allele *tsH41*, *amH46* or wild-type as described in the legend to Figure 2. The autoradiographs were exposed for 20–40 hr. Then, for each of the alleles the intensities of the bands due to *bla*-encoded proteins were quantitated as described by Koshland and Botstein (1980). Solid bars: percentage of total label in precursor or mature form that remains after intact spheroplasts are treated with trypsin. Open bars: percentage of total label in precursor or mature form that remains after lysed spheroplasts are treated with trypsin.

tides encoded by the chain-terminating alleles of *bla* are exposed on the periplasmic side of the inner membrane.

The Amino-Terminal Amino Acid Sequence of Mature Forms of β-Lactamase

If the translocation of β-lactamase across the inner membrane in irradiated and infected cells is identical to its translocation under normal growth conditions, then one would expect that the translocated form of the mature species should have the same amino terminus in irradiated and infected cells as do normal cells. To test this expectation, we determined the partial amino acid sequence of several *bla*-encoded mature proteins from irradiated and infected cells. Cells infected with the appropriate *bla* phage were labeled with either ^{3}H-alanine and ^{3}H-valine for wild-type and *fsl7*, or just with ^{3}H-valine (*amH46*), and then the labeled proteins were subjected to SDS-polyacrylamide gel electrophoresis. The labeled *bla*-encoded proteins were purified from the gel and subjected to automated Edman degradations (for experimental details see Koshland et al., 1982). In some cases, the steps of the degradation procedure that contained peak amounts of label were checked for their amino acid composition (data not shown).

The data from these experiments is summarized in Figure 5. The peaks of radioactivity for the mature forms, *amH46*, *fsl7* and wild-type, occur in steps that correlate with the peaks one would expect given the published amino acid sequence of the *bla*$^{+}$ mature form (Ambler and Scott, 1978; Sutcliffe, 1978) and the labeled amino acids used in these experiments. These results show that the processing of the signal peptide from the wild-type and chain-terminating (*amH46* and *fsl7*) precursors in irradiated and infected cells is the same as in normal cells.

Intrinsic Protease Sensitivities of the *bla*$^{+}$ Precursor and Mature Forms

To investigate further the apparent protease insensitivity of the mature β-lactamase, we modified a standard trypsin-accessibility experiment. After 20 sec of chase, labeled cells infected with *bla*$^{+}$ phage were chilled and subsequently lysed. Aliquots of this crude lysate were treated with increasing amounts of different proteases for 30 min.

The results from this experiment are shown in Figure 6. A comparison of lane a of Figure 6 with the other lanes shows that while all other proteins including the wild-type precursor are digested completely by trypsin (Figure 6, lanes h and i) and pronase XIV (Figure 6, lanes e–g), the wild-type mature form appears to be virtually undigested by these enzymes. We conclude that the wild-type precursor and mature species must differ significantly in their conformation to exhibit such different sensitivities to these proteases in lysates.

Discussion

Analysis of the cellular location of *bla*-encoded proteins after infection of S. typhimurium with P22*bla* and its derivatives can be summarized as follows. First, the precursor form of wild-type β-lactamase, which apparently chases efficiently to the mature form, fractionates mostly as a soluble cytoplasmic protein. Second, the precursor forms of the wild-type, *tsH41*,

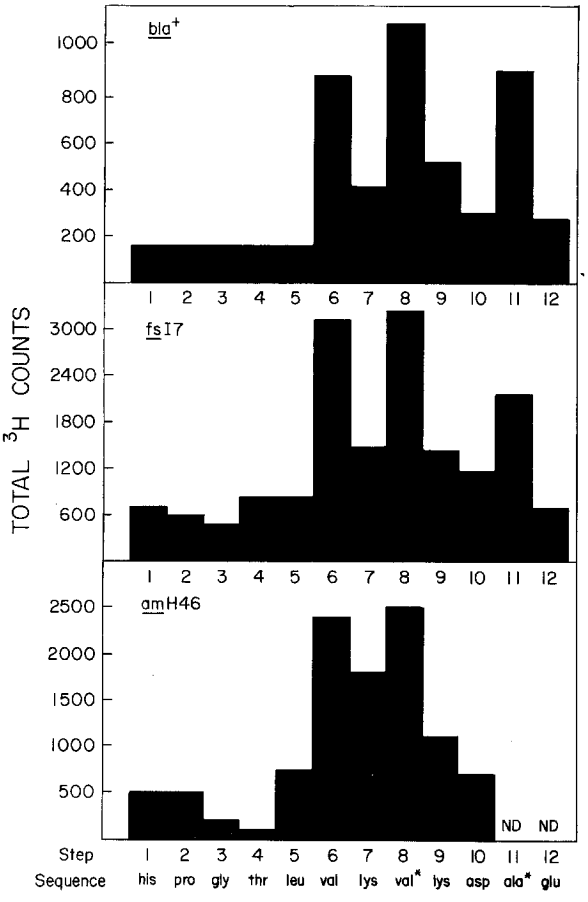

Figure 5. Partial Amino-Terminal Sequence of Mature Forms of β-Lactamase

A culture of DB4381 was irradiated and infected with P22*bla* phage carrying the wild-type, *fsI7* or *amH46* allele at an moi of 10. The irradiated and infected cells were labeled with ^{3}H-valine and ^{3}H-alanine (for infections with the wild-type and *fsI7* alleles) or just ^{3}H-valine (for infections with *amH46*). The labeled *bla*-encoded proteins were purified and subjected to automated Edman degradation as described by Koshland et al. (1982). The total ^{3}H counts detected in each step of Edman degration are given on the ordinate. Asterisks: steps in which label is predicted to appear. ND: not determined.

tsH1, fsI7 and *amH46* alleles, which all chase to the corresponding mature forms, are trypsin-accessible in lysed but not intact spheroplasts. These results suggest that the precursor forms of these alleles are sequestered by the inner membrane. Third, the mature forms of the chain-terminating alleles, *amH46* and *fsI7,* and temperature-sensitive alleles, *tsH41* and *tsH1,* of *bla* are trypsin-accessible in intact spheroplasts, suggesting that these proteins reside on the periplasmic side of the inner membrane. Finally, the precursor form and the mature form of wild-type β-lactamase exhibit radically different sensitivities to proteases. Therefore, these forms apparently exist in different conformations.

Posttranslational Translocation across the Inner Membrane

For all the alleles of *bla* we have examined the precursor forms apparently are sequestered by the inner membrane yet can be chased to mature forms that are either periplasmic (*bla*$^{+}$, *tsH41*) or bound to the surface of a membrane in the periplasm (*fsI7, amH46*). This result suggests that β-lactamase can translocate across the inner membrane after its synthesis is complete.

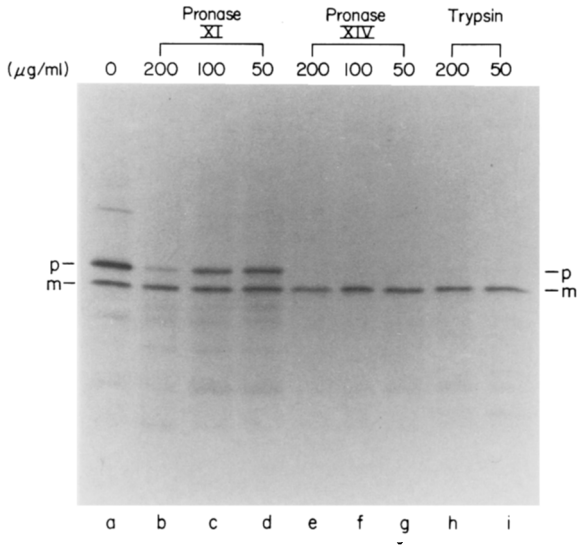

Figure 6. Sensitivity of *bla* $^{+}$-Encoded Proteins to Proteases in Crude Lysates

A culture of DB4381 was irradiated and infected with P22*bla* $^{+}$ phage at an moi of 10. The infected cells were labeled with ^{35}S-methionine for 30 sec and then chilled to 0°C. The cells were converted to spheroplasts and lysed as described for trypsin-accessibility experiments (see Experimental Procedures). The periplasmic fraction was added to the lysed spheroplasts to reconstitute a crude lysate of total cellular proteins. Samples of this crude lysate were exposed to varying concentrations of different proteases for 30 min. Twenty microliter aliquots were loaded in each lane. Electrophoresis was carried out on a 12.5% gel, and the autoradiograph was exposed for 15 hr. p: precursor form. m: mature form.

Several precedents exist that support the notion that proteins can translocate posttranslationally across a membrane. First, the processes that localize specific proteins encoded by nuclear DNA to the matrix of the mitochondria are topologically analogous to the secretion of periplasmic proteins. Proteins destined for the mitochondria can be synthesized in the cytoplasm and then translocated posttranslationally across mitochondrial membranes (Chua and Schmidt, 1978; Highfield and Ellis, 1978; Maccecchini et al., 1979). Second, evidence in procaryotes has been presented that suggests that an integral membrane protein, the M13 coat protein, can insert posttranslationally into the inner membrane (Wickner et al., 1978; Ito et al., 1979). The portion of this integral membrane protein that resides on the periplasmic side of the inner membrane has in fact translocated posttranslationally across the inner membrane.

The observation that β-lactamase can translocate across the inner membrane posttranslationally places a constraint upon the mechanism by which this protein is translocated across the inner membrane. The uncoupling of translocation and protein synthesis elim-

inates the hydrolysis of GTP during protein elongation as the energy source that drives the protein through the inner membrane. Recently, other investigators have demonstrated that membrane potential is required for the processing of several periplasmic proteins (Daniels et al., 1981; Enquist et al., 1981). It is possible that the membrane potential is required as an energy source to translocate the precursor forms of these proteins across the inner membrane and to make them accessible to the processing enzyme.

It is important to emphasize that our data address only certain aspects of one step in the secretion of β-lactamase: its translocation across the inner membrane. From these data we cannot tell whether prior to the translocation step itself some interaction between the signal peptide and the inner membrane must occur during translation. Though the pulse-chase cell-fractionation experiments suggest that the precursor forms are soluble and cytoplasmic, it is possible that these forms are weakly membrane-bound by an interaction, for example, between the inner membrane and the signal peptide. This interaction might be disturbed during our cell-fractionation procedures (see below). Second, our data do not indicate whether the mechanism for translocation of β-lactamase across the inner membrane does or does not involve some membrane-embedded machinery. Finally, we can not eliminate the possibility that a minor fraction of β-lactamase molecules translocate across the inner membrane cotranslationally. What our data do indicate is that the translocation of β-lactamase across the inner membrane is not usually or necessarily coincident in time with translation.

Role of the Carboxyl Terminus of β-Lactamase in Secretion

Previously we reported that the processed forms of β-lactamase that are missing the carboxyl end of the protein as the result of chain-terminating mutations in *bla* are not free periplasmic proteins. We preferred at that time the hypothesis that these proteins were cytoplasmic as opposed to weakly bound to a surface of a membrane because it was unclear why a protein that is weakly membrane-bound should be disassociated from the membrane by osmotic lysis of spheroplasts but not by osmotic shock of whole cells.

Our trypsin-accessibility experiments indicate that these peptides are nevertheless, in reality, exposed to the periplasm. The trypsin-accessibility data are not compatible with the hypothesis that the mature forms of the chain-terminating alleles of *bla* reside in the cytoplasm. To reconcile the still reproducible cell-fractionation experiments and the trypsin-accessibility experiments, we suggest now that the mature forms of the chain-terminating alleles are weakly bound to the surface of a membrane in intact spheroplasts. This weak interaction is disrupted when the cell undergoes osmotic lysis, making these proteins appear cytoplasmic. The absence of any large protected fragment of these peptides indicated that the bulk of the mature species is most likely on the periplasmic side of the membrane. These data suggest, contrary to our previous implication, that the carboxyl terminus of β-lactamase apparently is not required for the translocation of β-lactamase across the inner membrane. Similar experiments with the maltose-binding protein and the arginine-binding protein were reported that suggest that the carboxyl termini of these proteins are not required for their translocation across the inner membrane (Ito and Beckwith, 1981; Celis, 1981).

The weak interaction between the membrane and the peptides encoded by chain-terminating mutants has several possible explanations. A trivial explanation supposes that the protein, as a result of its missing carboxyl terminus, may assume a denatured conformation that is naturally sticky to a membrane. A more interesting alternative supposes that the wild-type protein must pass through and eventually be released from the inner membrane, and that removal of the carboxyl terminus may greatly reduce the efficiency of this terminal release step in secretion. A eucaryotic precedent is the immunoglobulin IgM, which is found in two forms, a truly secreted form and a membrane-bound form in which almost the entire molecule has traversed the plasma membrane. These two molecules differ only by a peptide at their carboxyl termini (Alt et al., 1980; Rogers et al., 1980). At this time we cannot tell which, if either, of these two explanations is correct.

Many investigators that study in vivo the secretion process in procaryotes have relied on the use of cell-fractionation experiments to localize proteins to particular cellular compartments. Our experience with the prematurely truncated mature peptides of β-lactamase suggests that these methods may not have sufficient resolution to conclude that a protein (a precursor to a secreted protein, for example) that is found in the cytoplasmic fraction is not weakly bound to either side of the inner membrane and released by the particular fractionation procedure. Recently other inadequacies in this method of localization were reported. Ito et al. (1981) showed that the apparent membrane association of the hybrid protein between the lambda receptor protein and β-galactosidase (previously assumed to be a membrane protein) depended on the salt concentration. Our results, coupled with these other reports, clearly indicate that the localization of a protein by cell fractionation must be treated as only a first approximation of the true localization of a protein, and that it by no means provides a definitive answer to this question.

Conformation and the Translocation Step

The different sensitivities of the wild-type precursor and mature form to trypsin and other proteases in crude lysates suggest that these two forms of β-lactamase are in different conformations. Since we have shown that the trypsin-sensitive mature form of *tsH41*

translocates across the inner membrane, the conformational difference between the wild-type precursor and mature form as measured by their differential trypsin sensitivities apparently is not essential to the execution of the translocation step. Other conformational differences may exist between the precursor and the mature form that are essential to translocation but that are not reflected by trypsin sensitivity. If a conformational change is essential to translocation of β-lactamase across the inner membrane, then it must occur in a domain of the amino-terminal portion of the protein, because the prematurely truncated products of the chain-terminating alleles apparently translocate across the inner membrane. Alternatively, β-lactamase may have evolved to assume a conformation (the trypsin-sensitive precursor form) that is compatible—that is, is, does not interfere—with posttranslational translocation. Once β-lactamase translocates across the inner membrane it may then assume its final conformation, a conformation that is not compatible with translocation but that is the active form of the enzyme.

Conclusions

Our analysis of the secretion of β-lactamase through the inner membrane of S. typhimurium suggests that the translocation of β-lactamase across the inner membrane can occur posttranslationally; previously we (Koshland and Botstein, 1980) and others (Josefsson and Randall, 1981) had shown only that processing can occur posttranslationally. The translocation step apparently does not require the presence of the carboxyl terminus of β-lactamase.

These results are inconsistent with an obligatory cotranslational model of secretion, as proposed by Blobel and Dobberstein (1975). Our results are also poorly reconcilable with models that require the presence of the entire protein (as would be expected if the conformation of the entire protein were important in translocation; Ito et al., 1979). Our results are more easily reconciled with the model proposed by Blobel (1980) for mitochondria or with the models proposed by DiRienzo et al. (1978) and by Engelman and Steitz (1981).

Experimental Procedures

Strains and Materials

Construction of the P22 specialized transducing derivative, P22Ap31pfr1, which carries the structural gene for β-lactamase (*bla*) is described by Winston and Botstein (1981). We refer to it as P22*bla*. Isolation and characterization of the *bla* alleles *amH46, tsI7, tsH1* and *tsH41*, and the genetic and physical mapping of all *bla* alleles, are described elsewhere (D. Koshland, D. Shortle, P. Grisafi, K. Talmadge and D. Botstein, manuscript in preparation). Mutants derived with the use of the mutagens hydroxylamine, ICR-191 or ultraviolet light are designated by the letters H, I or U. Mutations known to be amber, ochre or frameshift on the basis of their response to host tRNA suppressors are marked *am, oc* or *fs*.

Labeling of Phage-Encoded Proteins after Infection

Phage-encoded proteins were preferentially labeled as described by Koshland and Botstein (1980), with the following modification. Infected and irradiated cells were labeled 30 min after infection with ^{35}S-methionine. Usually 100–200 μCi label was used per milliliter of culture. The pulse was ended by addition of unlabeled methionine and/or chloramphenicol.

Gel Electrophoresis

SDS-polyacrylamide gel electrophoresis, autoradiography and the quantitation of the intensities of bands in autoradiographs have been described previously (Koshland and Botstein, 1980).

Cell Fractionation

The conversion of irradiated and infected cells to spheroplasts and the separation of the membrane and cytoplasmic fractions from lysed spheroplasts have been described previously (Koshland and Botstein, 1980).

Pulse-Chase Cell Fractionation

The purpose of this procedure is to examine the localization of *bla*-encoded proteins by cell fractionation as a function of time. A culture of DB4381 was grown and irradiated as described above. The culture was infected with P22*bla* $^+$ or a *bla* derivative at an moi of 20. Thirty minutes after the infection began, 2 ml culture was labeled with 400 μCi ^{35}S-methionine for 30 sec, whereupon 200 μl of 10 mg/ml unlabeled methionine was added. At intervals, 500 μl labeled culture was transferred to microfuge tubes that were prechilled to 0°C in an ice-water bath. All subsequent steps were performed at 4°C.

Each 500 μl aliquot was separated into three fractions (membrane, periplasmic and cytoplasmic) by the following procedure. The labeled cells were converted to spheroplasts as described above. The spheroplasts were pelleted in the microfuge, and the supernatant was saved as the periplasmic fraction. The spheroplasts were lysed, and the membrane and cytoplasmic fractions were separated by centrifugation as described above.

We assumed that by placing the samples at 0°C we effectively ended the chase; if any intermediates in the secretion process existed, they would be trapped by chilling the samples to 0°C. For alleles of *bla* that produced mature β-lactamase, this assumption was checked as described below. We reported previously (Koshland and Botstein, 1980) a pulse-chase experiment in which the chase was ended by mixing the aliquot of labeled culture with sample buffer heated to 95°C. It is safe to assume that under these conditions all cellular processes, including secretion, stop almost instantly. Therefore, if chilling also stops secretion, then the ratio of label in precursor to that in mature species at specific times during the chase in which the chase was ended by chilling (Figure 1) should be equal to the ratio of label in precursor to that in mature species at the same times during the chase in which the chase was ended by transferring an aliquot to sample buffer at 95°C (Koshland and Botstein, 1980). Such comparisons (data not shown) indicate that these conditions are met in experiments with the *bla* $^+$ allele.

To be able to compare the behavior of the products of *bla*-encoded proteins in separate fractionations, we examined the cell fractionation of unlabeled and labeled proteins not encoded by *bla*. These proteins partition to the same cellular fraction independent of whether they are from two separate fractionations within the same experiment (Figure 1) or two fractionations from different experiments (data not shown). In addition, the pattern of proteins of each cellular fraction gave the expected result. The cytoplasmic fraction includes the bulk of the Coomassie-blue-stained and labeled proteins (Figure 1). The membrane fraction has a smaller number of protein bands than the cytoplasmic fraction, and among these is included the majority of the outer-membrane proteins (data not shown). The periplasmic fraction has very few proteins as detected by Coomassie blue, but greater than 90% of the labeled mature β-lactamase present at a particular time point is recovered in the periplasm (Figure 1).

Pulse-Chase Trypsin-Accessibility Experiments

This procedure allows one to follow, as a function of time, the appearance of *bla*-encoded proteins on the periplasmic side of the inner membrane, whether they remain associated with the inner membrane or become soluble periplasmic proteins. A culture of DB4381 was grown and irradiated as described above. The culture

was infected with P22*bla* or a *bla* derivative at an moi of 10. Thirty minutes after infection, 2 ml culture was labeled with 400 μCi ^{35}S-methionine for 30 sec. Two hundred microliters of 10 mg/ml unlabeled methionine or 20 μl of 20 mg/ml chloramphenicol was added. Approximately 20 sec after the addition of the chase, 1.0 ml labeled culture was removed to a microfuge tube that was prechilled to 0°C in an ice-water bath. After 5 min, the remaining 1.0 ml labeled culture was transferred to a second tube prechilled to 0°C.

The cells in each aliquot were converted to spheroplasts as described above. The final volume was 200 μl. Half (100 μl) was divided into 25 μl aliquots that were transferred to four tubes. To the first tube was added 2.5 μl of 0.1 M Tris (pH 8); to the second, 2.5 μl of 2 mg/ml trypsin in 0.1 M Tris (pH 8); to the third, 2.5 μl of 500 μg/ml trypsin in 0.2 M Tris (pH 8); and to the fourth, 2.5 μl of 50 μg/ml trypsin in 0.1 M Tris (pH 8). After 10 min of incubation on ice, the 27.5 μl of digest were transferred to a tube containing 50 μl of 1.5-fold-concentrated sample buffer preheated to 95°C. These samples represented the test of trypsin accessibility of labeled proteins in intact spheroplasts.

The remaining 100 μl spheroplasts were centrifuged; the supernatant was removed and saved as the periplasmic fraction. The spheroplasts in the pellet were then lysed by resuspending them in 100 μl of distilled water. The 100 μl of lysed spheroplasts (minus the periplasmic fraction) were divided into 25 μl aliquots and subjected to trypsin digestion exactly as described above. Note that the trypsin digestions for lysed spheroplasts and intact spheroplasts were done in slightly different solutions (that is, the intact spheroplast samples contained sucrose and buffer). When these experiments were repeated such that the lysed spheroplasts were treated with trypsin in the identical solutions, the results were not affected.

The validity of the conclusions obtained from these experiments depends on the success of two procedures: the conversion of the labeled cells to intact spheroplasts and the lysis of these spheroplasts. In all the experiments reported, the conversion of cells to spheroplasts (greater than 90%), the intactness of those spheroplasts (80%–95%) and their subsequent lysis (80%–90%) were judged by examination of the cells by phase-contrast microscopy. Consistent with these observations was the correlation between the cell fractionation of non-*bla*-encoded species and the extent of their digestion with trypsin in these experiments. Essentially all labeled proteins not encoded by *bla* fractionated as soluble in the cytoplasm. When labeled cells were converted to spheroplasts and treated with trypsin, 80% or more of each labeled non-*bla*-encoded protein was recovered intact as measured by comparing the intensity of the labeled bands from spheroplasts treated with no trypsin and increasing amounts of trypsin (Figure 2, lanes a, b, c, d, f, g, h and k; also see Figure 3). The majority of cellular proteins that were stained by Coomassie blue were not digested by trypsin when cells were converted to spheroplasts. The exception, a major staining band, may be the product of the Salmonella *ompA* gene, because the product of this gene in E. coli was shown to be sensitive to trypsin under these conditions (Inouye and Yee, 1972). In contrast, when the spheroplasts were lysed and then treated with trypsin, less than 10% of the label in non-*bla*-encoded proteins was recovered (Figure 2, lanes e and f). In addition, many of the Coomassie-blue-stained proteins were absent.

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