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Genetics

Substitution of tyrosine for either cysteine in β-lactamase prevents release from the membrane during secretion

(periplasmic proteins/protein folding/*Salmonella typhimurium*/hydroxylamine mutagenesis)

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ABSTRACT **Six independent secretion-defective mutations were found that result in failure to release protein from the membrane into the periplasmic space of *Salmonella typhimurium* after removal of the signal peptide. The mutant protein is found in a membrane-bound form accessible to trypsin added to intact spheroplasts. The phenotype of these mutations supports the existence in general of an intermediate in bacterial secretion. All six mutations changed one or the other of the two cysteine residues in the mature protein to tyrosine, suggesting that these residues are involved in the release of protein into the periplasmic space, most likely by affecting protein folding.**

Secretion of a soluble protein can be thought of as involving several events. First, the protein or its precursor must be recognized as destined for secretion by the cellular export apparatus; second, it must traverse one or more membranes; third, it must be released from the membrane into the cellular compartment for which it is destined in its mature, water-soluble, fully folded form. For many soluble eukaryotic and prokaryotic proteins that are synthesized with an amino-terminal "signal sequence" that is later removed, it is clear that the presence of the signal peptide is necessary to achieve recognition of the precursor by the export apparatus (for reviews, see refs. 1–4). The removal of the signal peptide is generally observed to occur after the precursor has at least partly traversed the membrane, and the bacterial signal peptidase has been shown to be a membrane protein (4). Thus the primary structure of secreted polypeptides with cleaved signal sequences has a role in the secretion process at two points: at the very beginning (recognition) and at some point nearer the end (maturation by signal peptidase).

The role of secondary and tertiary protein structure and, indeed, the points at which precursors fold (and possibly also refold) in the secretion process is less clear. The classical form of the signal hypothesis (5–8) envisions the essentially linear passage of the polypeptide chain through the membrane once the signal sequence has been recognized by the secretion machinery. Folding of the protein is not an integral aspect of the translocation process in these models. Subsequent modifications of this theory have included the possibility of internal "stop transfer" sequences, an idea that assumes such a linear procession of the polypeptides through membranes (4, 7). Where translocation through a membrane and translation itself are thought to be necessarily tightly coupled, this view is particularly attractive, even inevitable. Folding of the protein in such a tightly coupled system first becomes possible after translocation through the membrane and/or removal of the signal peptide.

Alternative views suppose that substantial domains of protein are synthesized before translocation across membrane actually commences. For most bacterial proteins, this condition is known to apply *in vivo* (9–15). An example of this kind of model is the "membrane trigger" hypothesis, under which the signal sequence and/or the translocation machinery bound to it constrain the folded state until interaction with the membrane "triggers" a conformational change that directs traversal through the membrane (9, 10). After translocation, the precursor is thought to be released from the membrane by removal of the signal peptide. After or during the cleavage by signal peptidase, the protein is supposed to fold into its mature, stable, water-soluble form.

A number of investigations have implicated experimentally a role for changes in folding during secretion (4, 12, 13, 16, 18–21). Changes in tertiary structure are often inferred from differential sensitivity to proteases, since the mature forms of many secreted soluble proteins are characteristically resistant to proteases. Randall and Hardy, in a recent example (17), have shown that the *Escherichia coli* maltose-binding protein can be translocated only if it has not folded into its mature, protease-resistant conformation, implicating a structural constraint before traversal of the membrane. Others have found evidence for structural changes during the final release of proteins into the target compartment (11, 13, 21).

The TEM β-lactamase is a soluble, protease-resistant enzyme that is secreted into the periplasmic space of Gram-negative bacteria. It is synthesized with a 23-residue amino-terminal signal peptide that is cleaved after translocation through the membrane and before release as a soluble form into the periplasm. There are three cysteine residues in β-lactamase: one is in the signal sequence (residue 18), whereas the other two (residues 75 and 121) have been shown to form a disulfide bond in the mature protein (20). After demonstration that this enzyme is secreted posttranslationally in *Salmonella typhimurium* (11, 12), Koshland (ref. 21, see also ref. 13) proposed the division of the secretion process into three stages. Each stage is experimentally defined on the basis of properties of putative intermediates accumulated with different β-lactamase mutations blocking secretion. The first stage (recognition and translocation through the membrane) is blocked by some mutations in the signal sequence, leaving a soluble precursor in the cytoplasm; the second stage (cleavage of the signal peptide) is blocked by different mutations in the signal sequence, leaving a membrane-bound precursor at least partly exposed on the outer surface of the inner membrane; the third stage is blocked by carboxyl-terminal nonsense mutations that result in a truncated, protease-sensitive protein that is membrane-bound but nevertheless has its signal peptide removed with normal kinetics (11, 13). Further support for this last intermediate comes from a recent finding that such a form can be detected kinetically during secretion of the wild-type β-lactamase at low temperatures (19). Both groups propose that the release of the cleaved protein from the membrane involves a change in folding.

We report here strong evidence that release into the periplasm of a cleaved, membrane-bound intermediate involves a specific change in folding of the protein. Six of six

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independent mutations that were found to fail in secretion with concomitant accumulation of a cleaved membrane-bound intermediate substitute tyrosine for one or the other of the two cysteine residues in the mature protein.

MATERIALS AND METHODS

The experiments described here were done with bacteriophage P22 strains carrying intact or mutant β-lactamase (*bla*) genes; these phage strains were used to infect appropriate marked *S. typhimurium* hosts. The origin and methods of propagation of these phage and bacterial strains have been described (11–13, 21). Specifically, the origin of the six hydroxylamine-induced mutants was described by Koshland (21). General bacteriological and recombinant DNA methods, including the methods for mutagenesis and screening for *bla* mutants were carried out as described before (22). Amersham was the source of vectors, enzymes, and radioactive substrates for DNA sequence analysis; after subcloning into the sequencing vectors, dideoxy sequence analysis was carried out according to the directions of the manufacturer.

Pulse–chase labeling and analysis of wild-type and mutant β-lactamase were also done as described before (11–13, 21). Each P22-borne *bla* allele was used to infect *S. typhimurium* strain DB4381 (*uvrB-bio his leu*) host cells irradiated with ultraviolet light (270 joules/m^2, 254-nm light). Proteins made 30–45 min after infection were labeled with a 2-min pulse of [^{35}S]methionine followed by a "chase" with excess nonradioactive methionine for 2 or 10 min. Samples were withdrawn from the infected cultures at various times. Sometimes, cell fractionation and determination of the trypsin-accessibility of β-lactamase in spheroplasts were done as previously described (12, 13) before analysis by $NaDodSO_4$/polyacrylamide gel electrophoresis (11, 13). As shown previously, when the β-lactamase sample is heated at 95°C for 2 min in the presence of 2-mercaptoethanol, the mature wild-type protein forms a doublet (13, 21).

RESULTS

A large collection of independent β-lactamase mutations carried on a *bla*-transducing variant of the *Salmonella* phage P22 had previously been made (21) by isolating, after mutagenesis *in vitro*, phages no longer able to confer resistance to ampicillin. This collection was later screened for the appearance in lysates of material capable of crossreacting with polyclonal antibodies directed against wild-type enzyme (J. Jackson and D.B., unpublished results). Surprisingly, many apparent missense mutations resulted in production of no crossreacting material. Under the conditions used, β-lactamase bound tightly to membranes would not be expected to compete effectively in the radioimmunoassay employed.

We investigated the nature of the protein products produced by 25 such mutants by carrying out pulse–chase labeling with [^{35}S]methionine after infection of UV-irradiated *Salmonella*, following the production of labeled β-lactamase by $NaDodSO_4$/polyacrylamide gel electrophoresis. We found in this way six mutants with a particular secretion-defective phenotype: the precursor is translocated through the membrane and the signal peptide is apparently cleaved, but the mature form is not released into the periplasm, as shown in detail below.

The six mutants were all originally isolated after heavy hydroxylamine mutagenesis of phage particles *in vitro* (21). To ensure that each induced mutation was an independent event, the treated phage particles were plated directly and the resulting plaques were screened for their content of β-lactamase (21, 22). The six mutants were mapped into two different deletion intervals by a marker-rescue technique (H. Kimsey and D.B., unpublished) using deletions made *in vitro* (J.M., H. Ma, and D.B., unpublished). DNA sequence analysis showed that two of the six mutations (*blaH19* and *blaH62*) altered codon 75, changing TGT (Cys) to TAT (Tyr); the independence of the two mutations is attested to by the fact that in *blaH62* the adjacent codon 76 is also changed, from GGC (Gly) to AGC (Ser). The other four mutations (*blaH10*, *blaH11*, *blaH17*, and *blaH38*) all change codon 121 from TGT (Cys) to TAT (Tyr). All the changes are G→A transitions consistent with their having been induced by the hydroxylamine mutagenesis; they affect the only two cysteine residues in the mature β-lactamase (24).

The result of a simple pulse–chase experiment for one of the mutant alleles, *blaH19*, is shown in Fig. 1; the behavior of this mutant is typical of the six with this phenotype. The precursor–product relationship is clear in both the wild-type and mutant infections, although the rate of processing (i.e., cleavage of the signal peptide) is noticeably slower in the mutants. The most striking difference is that the wild-type mature form appears as a doublet, whereas the mutant displays only a single band. Previous investigation of this phenomenon (13, 21) showed that the appearance of doublets depends experimentally upon the vigorousness with which samples are treated with reducing agents before electrophoresis. Direct sequence analysis has shown that doublets are not due to heterogeneity in the amino terminus. Doublets are seen only in precursors and mature forms that have traversed, at least in part, the membrane (13, 20, 21). Doublets are apparently caused by incomplete reduction of a disulfide bond. The result that all six mutants are missing one or the other cysteine that would be required for the disulfide bond confirms this view of the doublet phenomenon.

To determine the cellular location of the mutant proteins, irradiated bacteria were infected with phage carrying either *blaH19* (Cys-75→Tyr) or *blaH10* (Cys-121→Tyr), labeled, and subjected to cell fractionation before electrophoresis. As seen in Fig. 2, lanes b and c, both mutants release at most a trace of β-lactamase protein into the periplasm. Most of the protein remains with the spheroplasts, meaning that secretion, in the sense of delivery of the mature, soluble protein to the target compartment, is incomplete.

To determine where in the secretion process the protein is stuck, we examined the accessibility of the mutant proteins associated with spheroplasts to added trypsin. Lanes d and e in each of the panels of Fig. 2 show that the protein is extensively digested by the trypsin while the spheroplasts are still largely intact [excess trypsin eventually lyses the spheroplasts (lane f)]. This result shows two things: first, the protein is accessible and therefore must have traversed, at least

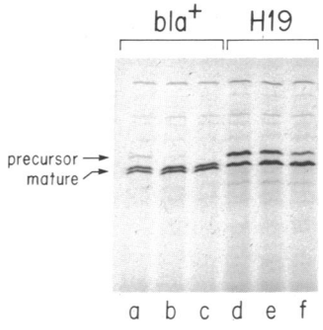

FIG. 1. Pulse–chase labeling of wild-type and mutant β-lactamase. Proteins made 30–45 min after infection with P22 *bla* phage were labeled with a 2-min pulse of [^{35}S]methionine (lanes a and d), followed by a "chase" with excess nonradioactive methionine for 2 min (lanes b and e) or 10 min (lanes c and f). Samples were withdrawn from the infected cultures at the times indicated. The positions of the precursor and the mature form of the protein are marked.

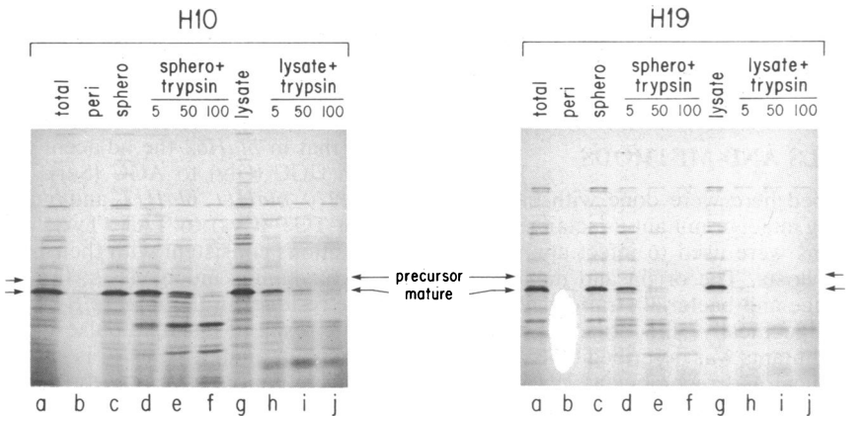

FIG. 2. Cellular location of β-lactamase. Cells were infected with P22 phages carrying either the *blaH10* (Cys-121→Tyr) or the *blaH19* (Cys-75→Tyr) allele and then were labeled for 2 min and chased for 5 min. Lanes a: unfractionated aliquot of infected culture. Lanes b: periplasmic fraction. Lane c: spheroplast fraction. Lanes d, e, and f: spheroplasts were treated with 5, 50, or 100 μg of trypsin, respectively. Lanes g, h, i, and j: spheroplasts lysed with water before treatment with trypsin.

partially, the membrane. Second, the mutant proteins are susceptible to trypsin (see also lysate control, lanes h, i, and j) and therefore cannot have acquired the final, highly protease-resistant conformation characteristic of the mature wild-type β-lactamase.

In summary, we obtained cell fractionation data showing that the substitution of tyrosine for either of the cysteines in the mature β-lactamase protein results in the production of a mutant protein that has lost its signal peptide but has become stuck in the membrane. The trypsin-accessibility data indicate that with both mutants the protein is exposed, at least partly, on the periplasmic face of the membrane.

DISCUSSION

Two quite different issues can be addressed by the results described above. The first involves the further definition, by examination of the phenotype displayed by these simple point mutations in the coding sequence for the mature protein, of an intermediate in the secretion pathway. The second involves the significance of the observation that the six independent mutations with this same phenotype involve changes to tyrosine of the only two cysteine residues in the protein, cysteines known to form a disulfide bond.

With respect to the first issue, our results can easily be interpreted according to the model shown in Fig. 3, a model (13, 21) that incorporates previous results concerning secretion of β-lactamase (11, 12). The protein is first synthesized in the cytoplasm, appearing as a trypsin-sensitive form called PI. Association of the precursor with the secretion apparatus (a step blocked by some signal-sequence mutations and some drug treatments) results in a membrane-bound precursor form, PII, that is accessible to trypsin added to intact spheroplasts. When PII is acted upon by signal peptidase (a step blocked by mutations in the latter part of the signal sequence), a new membrane-bound form, MI, also digestible by trypsin added to intact spheroplasts, results. The final step in the model is the release of the protein (possibly concomitant with final folding into the soluble, protease-resistant conformation) into the periplasm. The experiments presented here strongly buttress the evidence for the MI form as an intermediate in secretion of β-lactamase. The first evidence

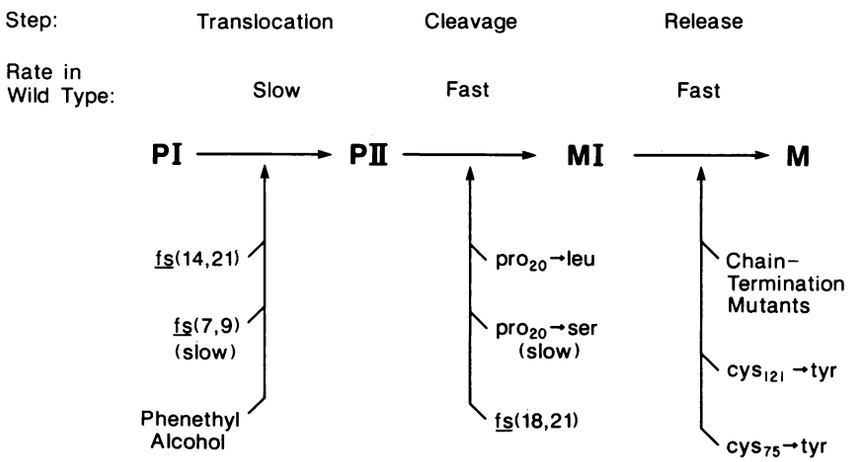

FIG. 3. Proposed pathway for secretion of β-lactamase into the periplasmic space. The first recognizable intermediate (PI) is a full-length precursor, containing the signal peptide, that is soluble in the cytoplasm and does not form doublets in $NaDodSO_4$/polyacrylamide gels (13). It can be seen in pulse–chase experiments with wild type (Fig. 1), and hence transition to the next form is inferred to be slow; PI also accumulates in certain signal-sequence mutants and in the presence of phenethyl alcohol (13, 21). PII has traversed the membrane but still retains the signal peptide, remains membrane-bound but accessible to trypsin added to spheroplasts, and forms doublets (12, 13, 21). It is observed with some signal-sequence mutants (13). MI is PII with the signal peptide removed; it is the form accumulated in the mutants studied here. MI had been observed previously with carboxyl-terminal mutants (11–13, 21) and in wild-type at low temperature (19). Secretion is complete when MI is released from the membrane and folds properly into mature β-lactamase. *fs*, pairs of compensating frameshift mutations affecting the codons indicated in the parentheses (see refs. 13 and 21).

for such an intermediate derived from analysis of carboxyl-terminal nonsense mutants (11); kinetic studies at low temperature have recently detected this intermediate during secretion of the wild-type protein (19).

The fact that all six independent mutants we found with this MI-blocked phenotype have one or the other cysteine in the mature protein changed to tyrosine is more difficult to interpret. The simplest view would be that the disulfide bond these two cysteines are known normally to make is directly involved in release of the protein from the membrane. This view, however, must be modified to take into account the recent finding of Schultz *et al.* (23) that substitution of serine for cysteine at codon 75 results in a fully active, secreted, but somewhat less stable enzyme. Thus the disulfide bond itself cannot be essential for either activity or secretion. Instead we propose that achievement of the three-dimensional spatial conformation consistent with the disulfide bond is required for release of the protein from the membrane into the periplasm.

There is considerable precedent for the idea that loss of cysteine residues involved in disulfide bonds need not cause failure to fold (25). Thus the fact that the tyrosine substitution has properties that serine substitution does not have can be accounted for by the fact that tyrosine has a very large side chain, whereas serine has a small one. The observation (23) that the serine at position 75 causes folding difficulties supports the notion that tyrosine at that position might cause more extreme difficulties.

The remarkable fact that all the mutants of this phenotype had Cys→Tyr substitutions at positions 75 and 121 remains to be explained. The finding of repeated independent changes at the same site raises the possibility that we might even have saturated the sites mutable with hydroxylamine that produce the full MI-blocked phenotype. The reason the substitution is always tyrosine at codons 75 and 121 is due to the specificity of the mutagen: the only way the codon TGT can be altered by hydroxylamine is to TAT. The reason other mutations at other positions were not found in abundance is more puzzling: one might well imagine that there are many possible changes elsewhere in the structure that should cause equally severe folding failures.

The recently published crystal structure (26) of a β-lactamase from *Staphylococcus aureus* that is related to the TEM β-lactamase protein we studied suggests a possible answer. Although there are cysteines at neither of the positions occupied by the two cysteines in TEM β-lactamase, one of the positions lies in an α-helix near the active site and makes its closest approach to a distant part of the molecule such that the α carbon of the other position is consistent with a disulfide bond were the two residues cysteines. It may be that the conformation required for secretion requires this close proximity of the helix to the other chain, a proximity clearly obstructed by tyrosine at either position, consistent with serine, and likely to be stabilized by a disulfide bond.

Although the requirement for a conformation consistent with the normal disulfide bond is the most natural explanation for our results, alternatives must be considered. It might still be that the substitution of tyrosine for cysteine simply leaves the protein in a lipid-soluble form: MI would not really be an intermediate. If this were true, the observation of an MI-like form as an intermediate during secretion of the wild-type protein at low temperature (19) would have to be dismissed as coincidence or artifact.

In conclusion, we believe that the observation that six out of six MI-accumulating mutants of β-lactamase alter one of the two cysteines in the mature protein strongly supports the idea that a conformation consistent with formation of a disulfide bond is involved in the release of the protein from the membrane after the signal peptide has been removed. It suggests that it may generally be the case that achievement of the proper folding of a secreted soluble protein is required for successful delivery of the protein to its intended subcellular compartment.

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