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Secretion of Beta-Lactamase Requires the Carboxy End of the Protein

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Summary

Synthesis and secretion of β-lactamase were studied in Salmonella typhimurium infected with P22 phage carrying the structural gene for β-lactamase (the *bla* gene) in mutant or wild-type form. The wild-type gene was shown to specify two forms of β-lactamase which differ in molecular weight by about 2500 daltons. This difference is consistent with removal, predicted on other grounds, of 23 amino-terminal residues (the "signal" sequence). All *bla*⁻ mutants, including chain-terminating mutants lacking as much as 50% or as little as 10% of the protein, were apparently unaffected in this processing step. Pulse-chase experiments showed that more than 85% of the wild-type (as well as mutant) proteins are synthesized as complete overlength precursors before being processed to their mature forms. Virtually all the mature wild-type protein appears in the periplasmic space whereas a large fraction of the precursor appears in the cytoplasm. In contrast, both the precursor and processed forms of β-lactamase proteins synthesized by chain-terminating mutants (including one which lacks only 10% of its residues from the carboxy end) are not secreted and apparently remain soluble in the cytoplasm. These results show that the carboxy-terminal amino acid sequence (at least) of β-lactamase is essential to successful transport across the cytoplasmic membrane, and suggest that the presence (and probably also the act of removal) of the signal sequence does not suffice to ensure secretion.

Introduction

Virtually all organisms can transport specific proteins across a membrane without destroying the functional integrity of that membrane. Many proteins in both eucaryotes and procaryotes which are specifically transported across membranes have in common the unusual feature that they are synthesized with 15–30 extra amino acids at their amino termini (the "signal" sequence or peptide) which are not present in the mature protein. This observation stimulated the idea that the extra amino acids (and/or the process of their removal) were implicated in (possibly sufficient for) the transport of these proteins into or across membranes (Milstein et al., 1972; Blobel and Dobberstein, 1975).

Such ideas were strongly supported by work in E. coli, where genetic manipulations were performed to fuse genes such that fusion proteins between membrane proteins and the soluble cytoplasmic enzyme β-galactosidase were synthesized by the cell (Silhavy et al., 1977; Bassford, Silhavy and Beckwith, 1979). In many cases (although not all) the fusion proteins (which had portions of the amino terminus of one or another membrane protein attached to the beginning of the β-galactosidase protein) were found associated with a membrane. Moreover, mutations which cause changes of an amino acid in the signal sequence eliminated transport of these proteins (Bedouelle et al., 1980; Emr et al., 1980).

The notion that the signal region of a secreted protein might suffice to cause its secretion found some support in experiments in which sequences specifying eucaryotic proteins were fused to the genes of a secreted protein. Specifically, Villa-Komaroff et al. (1978) fused the gene for rat insulin to the first two thirds of the periplasmic β-lactamase in E. coli, and found evidence for insulin protein sequences in the periplasm.

We have investigated the secretion of the TEM β-lactamase through the cytoplasmic membrane of S. typhimurium, a member of a bacterial species closely related to E. coli. The β-lactamase (*bla*) gene was originally found on the translocatable ampicillin resistance element Tn*1* and can therefore be introduced into many gram-negative species of bacteria. The enzyme is a monomer (Richmond, 1975) which is secreted into the periplasmic space (that is, the space between the inner and outer membrane; J. Knowles, personal communication; P. Model, personal communication) of any gram-negative species of bacteria in which it has been tested. The nucleotide sequence of the gene as well as the protein sequence of the mature enzyme have been determined (Sutcliffe, 1978; Ambler and Scott, 1978); comparison between them shows the presence of a signal sequence coding for 23 amino acid residues (Sutcliffe, 1978).

Using a derivative of the temperate Salmonella phage P22 containing an insertion of Tn*1*, we isolated and mapped more than 300 mutations in the structural gene of β-lactamase. Using a series of deletions constructed in vitro we were able to determine quite accurately the positions of the chain-terminating mutations in the nucleotide sequence of the gene.

The fact that the wild-type and mutant β-lactamase genes are carried on the genome of phage P22 allowed us to label specifically the β-lactamase protein along with other phage proteins after infection of cells irradiated with ultraviolet light, and to follow the secretion of the labeled β-lactamase into the periplasm. In this paper we describe experiments examining the synthesis and transport of wild-type and mutant β-lactamases. The results indicate that the β-lactamase is synthesized initially as a complete precursor whose

size is consistent with the presence of the signal peptide, and only after the completion of its synthesis is the protein processed to its final mature length. We present evidence that processing occurs efficiently on prematurely terminated fragments of the β-lactamase protein only about half the length of the wild-type protein, although we have not yet proved that this processing is identical to that which occurs for wild-type. Finally, we show that the processed mutant fragments as well as the unprocessed wild-type precursor protein are not secreted into the periplasmic space, and that they probably remain soluble in the cytoplasm. Since the removal of as few as 21 amino acid residues from the carboxy terminus results in failure to transport the protein across the cytoplasmic membrane, we conclude that the carboxy terminus is essential to normal secretion and that the synthesis and removal of the signal sequence are not sufficient for secretion.

Results

Phage-specified proteins are easily identified using SDS-polyacrylamide gel electrophoresis because phage-specified proteins can be labeled specifically after infection of a host which has been irradiated with ultraviolet light (Studier, 1973). We have used this system to investigate the synthesis and maturation of the β-lactamase protein in S. typhimurium by incorporating the structural gene for β-lactamase into the genome of the temperate phage P22, whose normal host is S. typhimurium. β-lactamase could be readily visualized under conditions essentially identical to those used to identify most of the P22 phage proteins (Botstein, Waddell and King, 1973; Poteete and King, 1977).

The P22 derivative used began as an insertion mutant (called P22 Ap31) in which the translocatable ampicillin resistance element Tn*1* was inserted into the anti-repressor gene of phage P22 (Weinstock, Susskind and Botstein, 1979). A spontaneous deletion which removes some Tn*1* and some P22 DNA but which retains the ampicillin resistance determinant was selected. This phage (P22 Ap31 *pfr*1) was extensively characterized and has normal P22 early and late functions, lysogenizes and integrates its prophage normally, and synthesizes β-lactamase both after infection and as prophage (Winston, 1980). β-lactamase synthesis is not under phage control. DNA heteroduplex analysis in the electron microscope (not shown) indicates that P22 Ap31 *pfr*1 amounts to a simple substitution of the β-lactamase structural gene for the dispensable secondary immunity region (*imm*I) of P22.

A large number of mutations in the β-lactamase structural gene (the *bla* gene) carried in P22 Ap31 *pfr*1 were isolated and mapped relative to a set of deletion mutations derived from a P22 Ap30 prophage (Weinstock et al., 1979) as well as to a set of deletions with endpoints of known nucleotide sequence made in vitro and provided by K. Talmadge and and W. Gilbert. The approximate map positions of about 150 of these mutations are shown in Figure 1. The central part of the figure shows the positions of a set of protein chain-terminating mutations (that is, suppressible nonsense mutations or suppressible frameshift mutations) whose map position on the physical map (the nucleotide sequence published by Sutcliffe, 1978) could be deduced from mutagen specificity, the in vitro deletions and the known specificity of nonsense and frameshift suppressors. Details of the genetic analysis are to be published elsewhere (D. Koshland, K. Talmadge and D. Botstein, manuscript in preparation).

Analysis of the Protein Products of Wild-type and Mutant β-Lactamase Genes by SDS-Polyacrylamide Gel Electrophoresis

To identify the products of the *bla* gene carried on P22 Ap31 *pfr*1 we examined autoradiograms of SDS-polyacrylamide gels on which were displayed the labeled proteins synthesized in irradiated cells after infection with *bla*$^+$ or *bla*$^-$ phages (see Experimental Procedures). It was possible to identify three bands as products of the *bla* gene because all were absent or shifted to positions corresponding to lower molecular weights when the infection was with phage carrying chain-terminating *bla*$^-$ mutations. All these bands were missing when a phage carrying a deletion of the entire *bla* gene was analyzed. The existence of three *bla*$^+$ bands (a single band and a faster-migrating doublet; see Figure 4, lane i) is interpreted as follows: the doublet co-migrates with purified mature β-lactamase, which migrates as a doublet due to incomplete denaturation under the standard conditions for preparation of samples for SDS-polyacrylamide gel electrophoresis (J. Knowles, personal communication and our unpublished results); often only a single band is visible. The slower-migrating band corresponds in molecular weight to a protein about 2500 daltons larger than the mature β-lactamase. Its size is what might be expected of a precursor in which the 23-residue signal peptide predicted by comparison of the β-lactamase protein sequence and the β-lactamase structural gene DNA sequence (Sutcliffe, 1978) has not been removed.

Analysis of the bands produced after infection with phages carrying *bla*$^-$ mutations allowed us to distinguish three different phenotypic classes, all of which had in common a pair of bands differing in apparent molecular weight by about 2500 daltons. As summarized in Table 1, class I mutants showed a set of bands indistinguishable from those found with wild-type; class II were missing the bands at the wild-type positions, but had two new bands which migrate more rapidly although they are still separated by an appar-

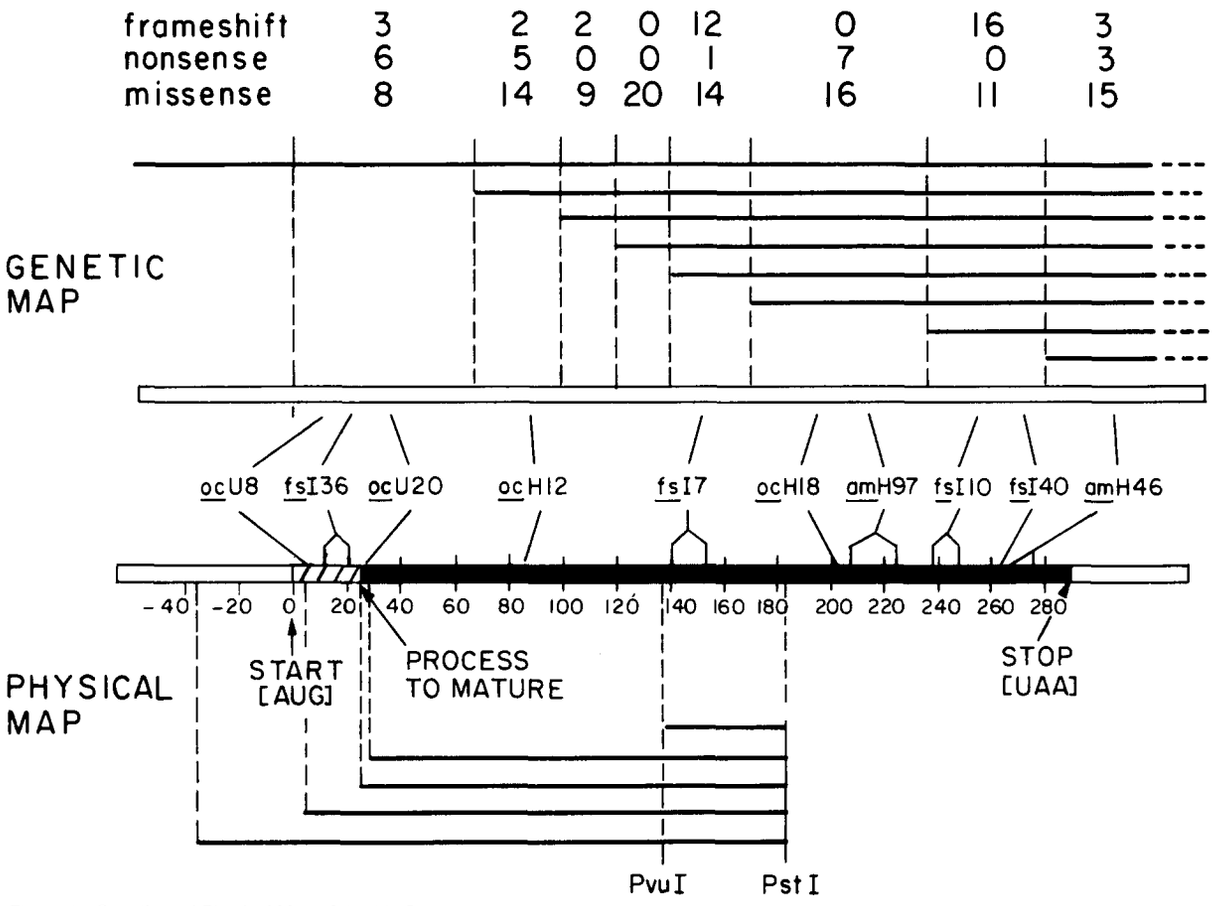

Figure 1. Genetic and Physical Map of the *bla* Gene

The phage P22 Ap31 *pfr*1 (*bla*$^+$) was mutagenized with hydroxylamine (H), ultraviolet light (U) or ICR-191 (I). Phages which could not transduce wild-type S. typhimurium to ampicillin resistance were defined as having a Bla$^-$ phenotype. These mutations were classified as amber (*am*), ochre (*oc*) or frameshift (*fs*) if a phage carrying a *bla*$^-$ allele could stably transduce to ampicillin resistance Salmonella strains carrying the appropriate suppressors (Winston, Botstein and Miller, 1979). The frameshift suppressors (Roth, 1974) can only redirect the reading frame from the +1 frame to the proper reading frame. Therefore all suppressible frameshift mutations must alter the reading frame to the +1 frame.

To generate deletions of the *bla* gene in vivo, we constructed a phage carrying both a Tn*1* insertion and a Tn*10* insertion at separate sites in the P22 phage genome. Taking advantage of properties of P22 and the transposable element Tn*10*, we recovered deletions which start at the Tn*10* element and extend into the *bla* gene (Chan et al., 1972). The in vitro deletion which removes the DNA between the Pst and Pvu restriction sites was constructed in our laboratory. All other in vitro deletions were given to us by K. Talmadge, who constructed them and determined their DNA sequence in W. Gilbert's laboratory at Harvard University.

ent molecular weight difference of about 2500; finally, class III mutants were like those of class II but also contained a third, much more rapidly migrating band.

We interpret class I mutations as being missense mutations. This interpretation is supported by their revertibility, their failure to be suppressed by nonsense or frameshift suppressors, and their production of normal amounts of (presumably inactive) protein. Class II mutations are interpreted as chain-terminating mutations, which is directly proved by the observation that every one of them is suppressible by host nonsense or frameshift suppressors. In one case (*am*H97) the intensities of both the faster-migrating bands characteristic of the mutant were diminished, and bands which co-migrated with the products of the wild-type gene reappeared, when a host carrying an amber suppressor was used (data not shown). The correct identifications of bands corresponding to all the amber, ochre and frameshift peptides found are supported by the correlation between observed apparent molecular weights and the molecular weights predicted from the positions of the mutations in the *bla* gene (Table 1). Class III mutations are also suppressible and thus appear to be chain-terminating mutations. Both members of this class are close together in map position, and we suppose that the fragment produced by these mutants is extraordinarily sensitive to proteolytic degradation, producing the third and fastest-migrating band.

The observation that all the class II and class III (chain-terminating) mutants produce bands differing in apparent molecular weight by about 2500 daltons suggests that the removal of the 23 amino acid signal sequence from the amino terminus occurs in all of them. If β-lactamase is indeed synthesized as a precursor which is subsequently processed from the

Table 1. Molecular Weights of β-Lactamase-Associated Proteins in Chain-Terminating *bla*⁻ Mutants

Allele	Predicted Molecular Weight	Observed Molecular Weight[a]	Difference in Molecular Weight; Precursor-Mature[a]
bla⁺	32. 29.5	30.5 27.5	3.0
Class I mutants: missense			
H3	32. 29.5	30.5 27.5	3.0
Class II mutants: chain-terminating (two β-lactamase bands)			
*am*H46	29.1 or 30.3 26.6 or 28.8	29.5 27.5	2.0
*fs*I40	26.0 or 29.0 23.5 or 26.5	28.0 26.0	2.0
*fs*I10	26.0 or 29.0 23.5 or 26.5	27.0 24.5	2.5
*fs*17	17.6 15.1	16.5 14.0	2.5
Class III mutants: chain-terminating (three β-lactamase bands)			
*oc*H18	22.0 19.5	21.5 19.0 (m1) 15.0 (m2)	2.5 (p-m1)
*am*H97	23.0 or 25.0 20.5 or 22.5	22.5 19.5 (m1) 16.0 (m2)	3.0 (p-m1)

[a] Figures in these columns are given to the nearest 0.5 kilodalton. The predicted molecular weights (in kilodaltons) are derived from genetic mapping of the mutations as summarized in Figure 1, using the assumption that an average amino acid has a molecular weight of 110. In the case of frameshift mutations we know the number of amino acids because the suppressibility of the mutations requires that the frame be shifted into the +1 frame (Roth, 1974). The next termination codon was found in this frame on the nucleotide sequence (Sutcliffe, 1978) and the length so determined was used to calculate the molecular weight. The observed molecular weights were calculated from autoradiograms of SDS-polyacrylamide gels using as molecular weight standards: BSA, 64,000; tomato bushy stunt virus protein, 42,000; and lambda repressor, 26,500. (p), (m1) and (m2) indicate the putative precursor, the highest molecular weight mature species and the lower molecular weight mature species, respectively.

amino end, then the presence of the carboxy end of the protein is apparently not essential for the processing.

Precursor-Product Relationships

The preceding results prompted a direct test of the possibility that the two bands differing by about 2500 daltons in wild-type and all the mutants exhibit a precursor-product relationship. If infected cells are exposed to a short pulses of radioactive amino acids, then the precursor form (by hypothesis, the slower-migrating species) should be labeled preferentially. After increasing time of incubation with unlabeled amino acids (chase), stoichiometrically related amounts of radioactivity should disappear from the putative precursor and appear in the product [by hypothesis, the faster-migrating band(s)].

We performed an experiment in which irradiated host cells were infected with P22 Ap31 *pfr*1 (*bla*⁺) and subjected to a 30 sec exposure to ^{35}S-methionine followed immediately by the addition of excess unlabeled methionine. Samples were added directly to sample buffer at 90°C immediately after addition of the unlabeled methionine and every 30 sec thereafter. Figure 2 shows an autoradiogram of an SDS-polyacrylamide gel analysis of these samples, and Figure 3 shows a plot of the fraction of radioactivity found in the putative precursor and product bands on that gel as measured with a scanning densitometer (see Experimental Procedures). From these figures it is clear that at least 80% of the radioactivity is present in the putative precursor band at the beginning of the chase, while more than 90% of the radioactivity is in the putative product after 270 sec. The radioactivity in the sum of all the β-lactamase-related bands remained constant during the chase, supporting the conclusion that incorporation of ^{35}S-methionine was effectively halted by addition of the unlabeled methionine. This conclusion was also supported by analysis of radioactivity in the band corresponding to the P22 coat protein, which also remained constant during the chase.

The results of this experiment satisfy the conditions for a meaningful pulse-chase analysis, and we conclude that most of the β-lactamase synthesized in the cell after infection with P22 *bla*⁺ is made first as a precursor about 2500 daltons larger than the mature enzyme. The conclusion does not depend upon the irradiation of the host with ultraviolet light, since a similar experiment using unirradiated cells (not shown) also gave results consistent with a precursor-product relationship. Another experiment (also not shown) using uniformly labeled ^{14}C amino acids instead of ^{35}S-methionine gave similar results, eliminating the possibility that the result represents an artifact of the use of ^{35}S-methionine.

When similar experiments were carried out using P22 phages carrying various mutant forms of the *bla* gene, the results again showed a precursor-product relationship. In every case the slowest-migrating β-lactamase-related protein contains most of the radioactivity after the pulse, and the faster-migrating bands contain most of the radioactivity after 5 min of chase.

Cellular Location of β-Lactamase

The ability to identify the protein products of the several alleles of the *bla* gene allowed us to examine

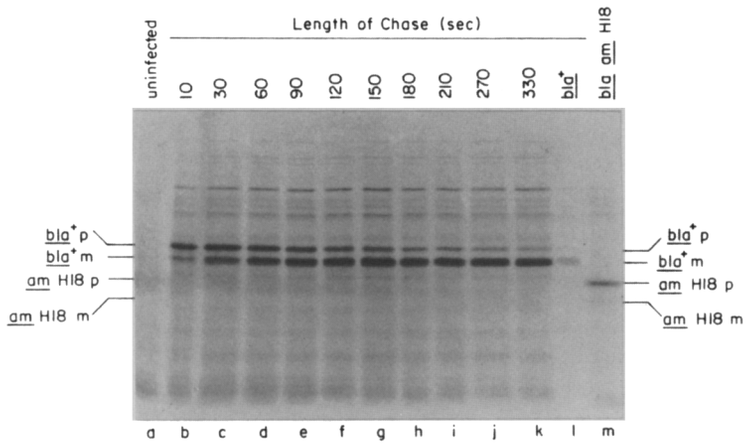

Figure 2. Autoradiogram of ^{35}S-Methionine-Labeled Extracts from Pulse-Chase Analysis of *bla*$^+$ Gene Products

The details of the pulse-chase experiment are described in Experimental Procedures. Lanes (b–k) are samples taken from cells infected with *bla*$^+$ phage, pulse-labeled for 30 sec with ^{35}S–methionine and chased with excess cold methionine. The number above each slot indicates the time (in seconds) after the addition of the chase. The multiplicity of infection was 10. A constant volume of 20 μl was loaded in each slot. Electrophoresis was on a 12.5% gel and the autoradiogram was exposed for 22 hr. Lane (a) contains an extract of uninfected cells pulsed with ^{35}S–methionine for 30 sec. Lane (l) contains an extract of irradiated cells infected with *bla*$^+$ phage and pulsed with ^{14}C amino acids followed by a short chase. Lane (m) contains an extract of *bla-am*H18-infected cells pulsed with ^{35}S–methionine for 30 sec.

the effect of changes in the structure of the β–lactamase on its cellular location. Since β–lactamase is normally secreted from the cell into the periplasm, we adapted the osmotic shock procedure of Neu and Heppel (1965) to distinguish proteins located in the periplasm from proteins present in the cytoplasm or attached firmly to the cytoplasmic membrane. Irradiated cells were infected with P22 *bla*$^+$ or the various P22 *bla*$^-$ phages and labeled after infection with either ^{35}S–methionine or uniformly labeled ^{14}C amino acids. The cells were transferred from hypertonic to hypotonic medium by centrifugation and resuspension as described in Experimental Procedures. In the end, three samples were produced: the periplasmic fraction (supernatant), the cytoplasmic/membrane-bound fraction (pellet) and an unfractionated control.

The results of such an experiment with P22 *bla*$^+$ (wild-type β–lactamase) are shown in Figure 4. Among the labeled proteins (which include many phage proteins as well as the β–lactamase-related ones) only the mature form of β–lactamase is found in the periplasmic fraction. This result verifies the periplasmic location of β–lactamase in the case of the P22-infected S. typhimurium. The remaining proteins, notably the β–lactamase precursor form, remain in the cytoplasmic/membrane-bound fraction. Quantitation of these results by measurement of the intensity of the bands in the autoradiograms (Table 2) showed that about 90% of the mature β–lactamase is in the periplasmic fraction, whereas about 90% of the precursor is in the cytoplasmic/membrane-bound fraction.

To verify that the larger molecular weight species in the preceding experiment behaves like authentic chasable precursor, a short pulse-label experiment was performed and the samples were immediately fractionated. The results showed that when most of the total β–lactamase-associated radioactivity was in the precursor form, 80% of that material was either cytoplasmic or membrane-bound; when a sample was

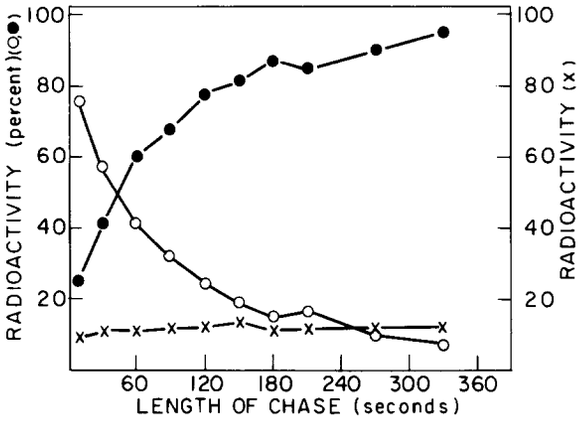

Figure 3. Quantitation of Pulse-Chase Analysis of *bla*$^+$ Protein Products

The radioactivity in specific bands of the autoradiogram of the gel presented in Figure 2 was measured by quantitating the intensity of those bands with a Zeineh Soft Laser Scanning Densitometer. To ensure that the intensities of the bands in this autoradiogram were linear with time of exposure, three separate exposures of 5.5, 11 and 22 hr were made on preflashed film. On the right abscissa is plotted the percentage of the total radioactivity in *bla*$^+$ bands present in either precursor or mature forms. On the left abscissa is the total radioactivity present in a specific band corresponding to the P22 phage head protein p5. Plots of radioactivity in the precursor and mature bands versus time are virtually superimposible with the plot of the percentage of total radioactivity in *bla*$^+$ bands in the precursor and mature bands versus time shown above. (○——○) Percentage of total radioactivity in *bla*$^+$ bands present in the precursor form; (●——●) percentage of total radioactivity in *bla*$^+$ bands present in the mature form; (×——×) total radioactivity in p5.

taken after a few minutes of chase, most of the radioactivity was shifted into mature protein located in the periplasmic fraction (data not shown).

Using the various *bla*$^-$ mutants, we examined the cellular locations of the precursor and product forms of the β–lactamase in experiments similar to the one described above for the wild-type. For mutants which cause premature termination of the polypeptide chain

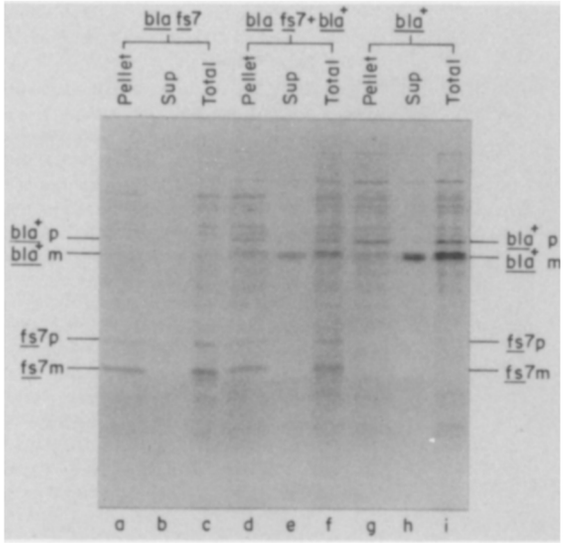

Figure 4. Osmotic Shock Analysis of ^{14}C-Labeled Cells Infected with *bla-fs*I7 and *bla*$^+$ Phages

A culture of DB4381 was irradiated and infected with *bla-fs*I7, *bla*$^+$ or a mixture of the two phages. The multiplicity of infection was held constant at 20; for the mixed infection each phage was used at a moi of 10. The infected cells were labeled with ^{14}C amino acids and osmotically shocked (see Experimental Procedures). 20 μl aliquots were loaded on a slot. Electrophoresis was on a 15% gel and the autoradiogram was exposed for 24 hr. (Pellet) represents the membrane-bound and cytoplasmic fraction. The supernatant (Sup) represents periplasmic proteins. (Total) is an equivalent aliquot of the same labeled cells which were not shocked.

(that is, mutants *am*H46, *fs*I7, *fs*I40, *oc*H18, *am*H97; see Figure 1), the two bands which differ by 2500 daltons were both found in the cytoplasmic/membrane-bound fraction after fractionation. This unexpected result suggests that deletion of even a small part of the carboxy terminus of β-lactamase results in failure of secretion of the polypeptide into the periplasm despite the apparently normal processing of the precursor into a "mature" form.

A typical experiment using P22 *bla-fs*I7 is shown in Figure 4. As in the case of wild-type, 90% of the precursor-sized protein was found in the cytoplasmic/membrane-bound fraction (pellet); however, in contrast to wild-type, 90% of the mature *fs*I7 protein is also found in this fraction (Figure 4, lanes a and b). To eliminate possible artifacts attributable to the necessity of carrying out the osmotic shock procedure on different samples, we carried out a control infection in which the same cells were infected with both P22 *bla-fs*I7 and P22 *bla*$^+$ phages simultaneously. Since the prematurely terminated β-lactamase proteins synthesized by the mutant migrate to a position in the SDS-polyacrylamide gel electrophoresis distinct from the position of the wild-type β-lactamase proteins, the fractionation of both mutant and wild-type proteins can be observed in the same samples. The results (Figure 4, lanes d and e) clearly show that in the mixed infection the mature wild-type β-lactamase appears as expected in the periplasmic fraction while most of the mutant protein stays in the cytoplasmic/membrane-bound fraction. Quantitation of these results is given in Table 2.

These results were unchanged when the cellular location experiments were performed as above, with the exception that the periplasmic proteins were released by conversion of infected labeled cells to spheroplasts (Witholt et al., 1976) instead of by osmotic shock (not shown).

Figure 4 also provides evidence that the synthesis of wild-type β-lactamase is proportional to the multiplicity of infection (lanes d–f represent moi = 10; lanes g–i represent moi = 20). However, the same relative amount of the total β-lactamase protein is found as the mature species in the periplasm, indicating that neither the secretion nor protein processing mechanisms is saturated.

Table 2 also summarizes the results of osmotic shock experiments similar to the one in Figure 4 with other chain-terminating mutations in the *bla* gene. In every case (including one mutation, *am*H46, which terminates either 11 or 21 amino acid residues from the normal carboxy terminus of the protein) the precursor and the mature protein (that is, the two forms that differ by about 2500 daltons) are not recovered in the periplasmic fraction (less than 20% of the total), but instead remain in the cytoplasmic/membrane-bound fraction. It should be noted that some of the mutants tested (*fs*I40 and *am*H46) produce protein fragments whose molecular weight is close enough to that of the wild-type β-lactamase to preclude resolution of mutant and wild-type bands on the SDS-polyacrylamide gel of the mixed infections. In these cases it was observed that the radioactivity migrating at the position of mature wild-type protein was divided about equally between the periplasmic and cytoplasmic/membrane fractions, as expected if the mutant form is not transported while the wild-type is.

Further examination of the data in Table 2 reveals that the chain-terminating mutants which produce a third band in addition to the precursor and mature forms (class III) show a slightly different behavior: about half of the extra band appears in the periplasmic fraction, although no precursor or mature protein does. This further processing (or degradation) of the protein appears to facilitate secretion, since the third band is recovered in the periplasmic fraction much more efficiently than either of the larger β-lactamase-related peptides made by these mutants.

The preceding experiments suggest that the carboxy terminus of the β-lactamase protein is essential for successful secretion of the protein into the periplasm, even though the processing of the precursor occurs normally. Since we examined a mutant in which the chain is terminated at residue 265 (or 275) of the protein sequence, the last 21 (or 11) amino acid residues are essential.

Table 2. Fractionation of β-Lactamase-Related Peptides by the Cold Osmotic Shock Procedure

Infection	Band[a]	Recovery (%)[b]	Periplasmic (Supernatant) (%)	Nonperiplasmic (Pellet) (%)
Mutant: *bla-fs*I7 (terminates after 167 residues)				
Wild-type	p(*bla*$^+$)	100	10	90
	m(*bla*$^+$)	113	90	10
Mutant	p(*bla*$^-$)	111	10	90
	m(*bla*$^-$)	100	18	82
Mixed	p(*bla*+)	100	12	88
	m(*bla*+)	108	73	27
	p(*bla*$^-$)	72	<10	>90
	m(*bla*$^-$)	99	15	85
Mutant: *bla-am*H97 (terminates after 206 or 226 residues)				
Wild-type	p(*bla*+)	86	4	96
	m(*bla*+)	106	87	13
Mutant	p(*bla*$^-$)	140	<10	>90
	m1(*bla*$^-$)	118	23	77
	m2(*bla*$^-$)	84	50	50
Mixed	p(*bla*+)	110	<10	>90
	m(*bla*+)	100	78	22
	p(*bla*$^-$)	75	<10	>90
	m1(*bla*$^-$)	110	9	91
	m2(*bla*$^-$)	69	44	56
Mutant: *bla-fs*I40 (terminates after 251 residues)				
Wild-type	p(*bla*+)	86	<10	>90
	m(*bla*+)	91	83	17
Mutant	p(*bla*$^-$)	175	<10	>90
	m(*bla*$^-$)	116	12	88
Mixed	p(both)	ND	ND	ND
	m(both)	100	60	40
Mutant: *bla-am*H46 (terminates after 264 or 274 residues)				
Wild-type	p(*bla*+)	67	<10	>90
	m(*bla*+)	94	83	17
Mutant	p(*bla*$^-$)	100	<10	>90
	m(*bla*$^-$)	98	13	87
Mixed	p(both)	112	<10	>90
	m(both)	96	35	65

[a] (p) means precursor band, (m) means mature band; where there are two mature forms (class III mutants), (m1) is the larger and (m2) is the smaller as in Table 1, which gives the actual molecular weights.

[b] Recovery was determined by dividing the sum of the radioactivity in particular precursor or mature bands in the pellet and supernatant fractions by the radioactivity in the same band(s) in the unfractionated sample. Equivalent amounts of each fraction (see Figure 4) were applied to each lane of the SDS-polyacrylamide gel.

All the experiments were performed as described in the legend to Figure 4 (from which the relevant data here are derived). The autoradiograms were exposed for 20–40 hr on preflashed film and scanned as described in Experimental Procedures. The results for another class III mutant (not shown) are very similar to the ones given here for *am*H97. (ND) Not determined.

Solubility of Processed Mutant β-Lactamase Proteins

The preceding experiments show that all the β-lactamase precursor proteins and all the matured prematurely terminated mutant proteins (with the partial exception of the additional fragments in class III mutants) are either cytoplasmic or membrane-bound. This ambiguity is in the nature of the osmotic shock procedure, which separates periplasmic proteins from the others. To resolve this ambiguity, we fractionated proteins bound to membranes from soluble proteins using the method of Silhavy et al. (1976). Irradiated cells were infected with P22 *bla*$^+$ or P22 bla$^-$ phages and then labeled with ^{14}C amino acids. The labeled cells were collected by centrifugation and lysed by sonication or lysozyme-osmotic shock. Intact cells

(comprising less than 10% of the radioactivity) were removed and the membranes were collected by centrifugation at 100,000 × g for 2 hr. The membrane pellet and supernatant fluid were separated and assayed for β-lactamase proteins by SDS-polyacrylamide gel electrophoresis as before. To monitor the success of the fractionation procedure, the SDS-polyacrylamide gel was stained with Coomassie blue. The membrane fractions contained only a few staining protein bands, with molecular weights corresponding to the major membrane proteins of S. typhimurium (Osborn et al., 1972), while the cytoplasmic fractions contained a large number of staining protein bands, as expected. Examination of the intensities of Coomassie blue stain in several isolated membrane bands showed that the percentage of total membrane protein which remained in the soluble fraction was less than 20%.

The results of a membrane isolation experiment in which the cells were lysed by lysozyme-osmotic shock are shown in Figure 5 and Table 3. In every case we found that most of the processed β-lactamase-associated proteins are soluble. The majority of the precursor forms of these proteins are also found in the soluble fraction, although in the case of infections with *am*H46 and wild-type a substantial fraction of the total precursor protein is membrane-bound (see Table 3). The same results were obtained if the cells were lysed by sonication (data not shown). These observations support the idea that most of the nonsecreted mature mutant forms and at least some fraction of all precursor forms are both cytoplasmic and soluble.

Discussion

Analysis of the synthesis and secretion of β-lactamase after infection of S. typhimurium with P22 phage carrying the *bla* gene in mutant or wild-type form can be summarized as follows:

—All alleles of the *bla* gene have in common that they produce two forms of the β-lactamase protein which differ in molecular weight by about 2500 daltons. In the case of the wild-type, the smaller form co-migrates with authentic purified β-lactamase.

—The larger form of the protein, both in chain-terminating mutants and in wild-type, is a kinetic precursor of the smaller form(s). In a pulse-chase experiment, more than 85% of the radioactivity which ultimately appears in the mature form can be observed first in the precursor form.

—Although mature β-lactamase is located in the periplasmic space, as expected, the kinetic precursor form is not secreted.

—Analysis of β-lactamase synthesis and secretion by chain-terminating mutants shows that removal of as few as 21 (possibly 11) amino acid residues from the carboxy terminus of the protein eliminates secretion of the protein (despite apparently normal processing of the amino terminus). The "matured" mutant peptides apparently remain soluble in the cytoplasm.

Role of the Carboxy Terminus of β-Lactamase in Secretion

The strongest conclusion which can be drawn from the experimental results described above is that the carboxy terminus of the β-lactamase contains information essential to the normal secretion of the protein from the cytoplasm into the periplasmic space. The failure of the product specified by the *bla-am*H46 mutant to be secreted implies that at least a portion of the essential information must reside in the last 21 amino acid residues.

This conclusion suggests either that the total protein conformation is somehow involved in the secretion process, perhaps as proposed in the "membrane trigger" hypothesis (Ito, Mandel and Wickner, 1979), or that the carboxy-terminal amino acids provide part or all of a site which interacts directly with the transport machinery. Further characterization of the unusual degradation products (m2) of the class III mutants, which are secreted more efficiently than any

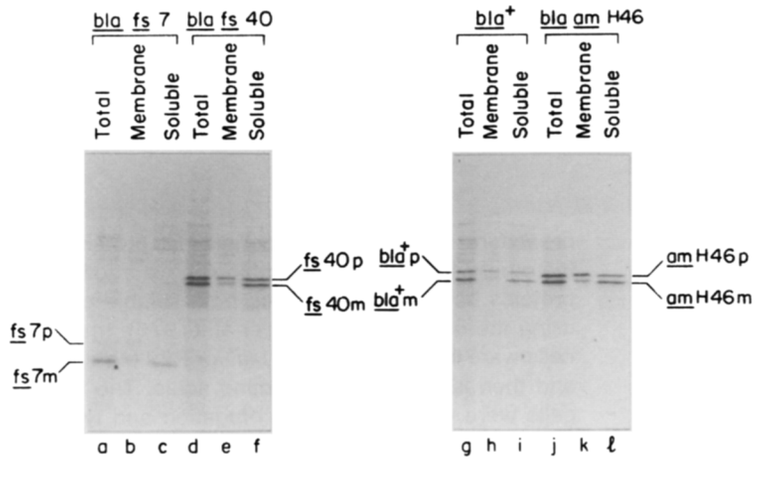

Figure 5. Membrane Isolation of Cells Infected with *bla*$^+$ and *bla*$^-$ Phages

A culture of DB4381 was irradiated and infected with *bla-fs*I7, *bla-fs*I40, *bla-am*H46 or *bla*$^+$ phages at a moi of 20. The infected cells were labeled with ^{14}C amino acids. These cells were lysed by lysozyme osmotic shock and their membranes were isolated by centrifugation as described in Experimental Procedures. (Total) represents the labeled cellular proteins after removal of the periplasmic fraction; the mature wild-type β-lactamase present represents the small fraction of this protein which was not released into the periplasmic fraction. 20 μl of each fraction were loaded on a slot; care was taken so that each sample represents an equivalent amount of culture. Electrophoresis was on a 15% gel and the autoradiogram was exposed for 8 days.

Table 3. Fraction of β-Lactamase-Related Peptides by Membrane Isolation

Infection	Band	Recovery (%)	Soluble (%)	Membrane (%)
*fs*I7	p	ND	ND	ND
	m	66	78	22
*fs*40	p	100	78	22
	m	88	89	11
*am*H46	p	91	54	46
	m	95	77	23
bla$^+$	p	117	62	38
	m	106	91	9

The experiment was performed as described in the legend to Figure 5. The autoradiograms were exposed for 8–16 days on preflashed film and scanned as described in Experimental Procedures. (p), (m) and (Recovery) are defined as in the legend to Table 2. Apparently due to the differences in total protein loaded, the width of lanes containing unfractionated proteins or soluble proteins was substantially greater than that of those lanes containing only membrane proteins. Since the scanning densitometer measures the radioactivity in a band over a fixed width, these differences in lane width between fractions were taken into account in calculations of the amount of radioactivity present in a band of a given fraction. (ND) Not determined.

other products of chain-terminating mutants [including the larger, more normal "mature" products (m1) produced by the same class III mutants] may help to distinguish between these alternatives.

The presence of information essential to protein secretion in the carboxy terminus of β-lactamase does not preclude the possibility that essential information for secretion also resides in other regions of the protein, for example, the signal sequence. Clearly, however, the presence of the signal sequence (and probably also its processing) is not sufficient to ensure the secretion of β-lactamase. The finding that all the fusion proteins combining the amino terminus of the E. coli maltose-binding protein with the bulk of β-galactosidase fail to be secreted even though most of them include an intact signal sequence is another case in which the signal sequence may not contain all the information required to ensure protein secretion (Bassford et al., 1979).

It should also be noted that some of the chain-terminating mutations are frameshifts that encode a run of incorrect amino acids preceding the out-of-frame termination codon. Formally, the failure of secretion of such mutant proteins could be attributed to the incorrect amino acids instead of to the absence of the normal carboxy terminus. However, from the fact that these frameshift mutations are suppressible we know their reading frame, and from the nucleotide sequence of the gene we have determined that the number of incorrect amino acid residues in each case is small (fewer than 20). It thus seems more likely that the failure of these proteins to be secreted is due, as in the case of the nonsense mutations, to the absence of the crucial carboxy-terminal residues.

The observation that all the processed proteins produced by chain-terminating mutants (whether they result from nonsense or frameshift mutations) not only fail to be recovered in the periplasmic fraction after the osmotic shock procedure but are also absent from the membrane fraction after the sonication or lysozyme-osmotic shock procedures suggests that the untransported mutant proteins remain soluble in the cytoplasm. However, the possibility of some weak association with either surface of the inner membrane is not absolutely ruled out. One might imagine that these mutant proteins stick to the membrane either nonspecifically because they are improperly folded, or specifically because they lack a sequence required to release them from the "transport machinery" (a terminal step in secretion). We regard these interpretations as improbable for the following reasons. The labeled cells are treated identically in the osmotic shock procedure and the lysozyme-osmotic lysis procedure (that is, suspended in sucrose-EDTA and then rapidly resuspended in water), except in the latter case the cell wall had previously been digested by the lysozyme. It therefore seems reasonable to assume that any membrane association the mutant proteins may have had in osmotically shocked cells should have been maintained in osmotically lysed cells. Further amino-terminal fragments missing as few as 10% and as many as 50% of the carboxy-terminal residues of β-lactamase all show the same general behavior, and would all have to show the same special degree of stickiness to the membrane were our results to be interpreted in terms of a weak membrane association. We therefore prefer the idea that the inability to release the mutant proteins by osmotic shock or spheroplasting combined with their apparent lack of association with isolated membranes is a true indication of their cytoplasmic nature.

Essentially all the precursor forms produced by chain-terminating mutants or wild-type fail to be recovered in the periplasmic fraction after osmotic shock procedure, yet a large fraction of these precursor molecules are not found associated with the membrane. Applying the same arguments that we used for the mature mutant proteins above, we suggest that this fraction of the precursor molecules may also be in the cytoplasm. However, in some cases (*am*H46 and wild-type) a sizable fraction of the precursor forms was also found in the membrane fraction. The work of Emr et al. (1980) and Bedouelle et al. (1980) suggests that a functional signal sequence is required to initiate transport. Since we suggest that these precursor molecules have an intact signal sequence, their association with the membrane may indicate an initial weak binding which is an intermediate in transport.

If the mutant proteins are truly cytoplasmic, then we are obliged to consider the possibility that the information in the carboxy-terminal residues of the protein is used to initiate the secretion process or is involved

in some step in the process soon after initiation. If this were not the case, it would be hard to see a simple way in which the aborted secretion could result in a soluble cytoplasmic protein. It should be emphasized that the localization of some essential information for secretion to the extreme carboxy end may be a specific feature of the β-lactamase; other secreted proteins might have sequences essential to transport elsewhere in their nonsignal regions. Alternatively, one might suppose that the total conformation of a protein must meet some specific requirements of the secretion mechanism in order to be transported successfully.

Relationship of Protein Processing to Secretion

Examination of the β-lactamase-related proteins made by a series of chain-terminating mutants showed a good correlation between position of the mutations on a genetic/physical map and the length of polypeptide fragments synthesized. Wild-type β-lactamase appeared as two bands, a kinetic precursor and the mature enzyme, which differed in mobility on an SDS-polyacrylamide gel by an amount equivalent to 2500 daltons. The mutants also had two bands, differing in mobility by a comparable amount, which bear the same precursor-product relationship. The value of 2500 daltons is in good agreement with the expected weight of the signal sequence (23 amino acid residues) predicted by the difference between the mature protein sequence and the sequence of the β-lactamase gene. It thus seems reasonable to assume that the protein processing in both wild-type and mutant represents the cleavage of the signal sequence from the beginning of the protein.

It should be emphasized, however, that we have not shown directly in any of these cases that the two protein species in our gels which differ by about 2500 daltons have the primary structures dictated by this assumption. In view of the fact that the cleavage is associated with transport through the membrane only in the case of wild-type, the possibility that the cleavage in the mutants represents the adventitious removal of a similar length of protein by some cytoplasmic protease unrelated to protein secretion remains to be ruled out.

If, on the other hand, it turns out (as we are inclined to believe) that the removal of 2500 daltons of protein from both wild-type and mutant β-lactamases represents the normal cleavage of the signal sequence from the precursor protein, our results have dissociated the protein processing function from the secretion of the protein. If one takes at face value all the data relating to the locations of the precursors and cleaved forms of the mutant and wild-type proteins, then our results imply that protein processing can occur without transport through the membrane and that the protease(s) responsible for the processing are accessible to the cytoplasm.

The relationship between processing of the signal sequence and transport of proteins through or into membranes has not been clear in the past. In some cases (Bedouelle et al., 1980; Emr et al., 1980) it is clear that mutations in signal sequences simultaneously abolish cleavage and transport. In other cases (Lin et al., 1978) transport in the absence of processing has been observed. Our results suggest the converse for β-lactamase: cleavage in the absence of transport. The possibility must therefore be raised that the actual cleavage of the sequence is not related mechanistically to secretion.

Relationship of Secretion to Synthesis and Processing of the β-Lactamase

The labeling kinetic experiments described above show that most of the β-lactamase protein is completed before it is processed. Furthermore, we have provided evidence that the processed form of the wild-type protein is located in the periplasmic space while the kinetic precursor is not. The evidence suggests that a large fraction of the precursor can be found in the cytoplasm, although the difficulties of fractionating the cell quickly have made it difficult to establish unambiguously the location of the true precursor (as opposed to a possibly unprocessable and/or untransportable product). Evidence that proteins which are transported across a membrane can be initially synthesized as soluble precursors in the cytoplasm comes from studies of certain mitochondrial proteins (Chua and Schmidt, 1979).

These observations fit nicely with the evidence that the secretion of β-lactamase requires its carboxy-terminal amino acid residues in some capacity. The notion that the carboxy end of a protein might be involved in the earliest steps of its transort through the membrane requires that the synthesis of the protein by virtually complete prior to its secretion. Clearly all these observations are very difficult to reconcile with the idea of co-translational "vectorial" transport of the protein through a membrane (Blobel and Dobberstein, 1975).

Conclusions

Our analysis of the synthesis and secretion of β-lactamase through the cytoplasmic membrane of S. typhimurium suggests that the simple presence (and possibly also the removal) of the amino-terminal signal sequence is not sufficient for secretion. This result, along with the observation that synthesis of the wild-type protein precedes protein processing and secretion, militates against simple models of co-translational vectorial transport. A more complicated recognition system which must somehow use information from the carboxy-terminal amino acid residues therefore has to be invoked in the secretion of this particular protein. Since the β-lactamase is widely distributed in nature among the gram-negative bacteria, this more

complicated secretion mechanism must be equally widespread and general among this large class of bacteria.

Experimental Procedures

Strains and Materials

Construction of the P22 specialized transducing derivative (P22 Ap31 *pfr*1) carrying the β-lactamase gene is described by Winston (1980). Isolation, characterization and mapping of mutants of this phage defective in the *bla* gene and the correlation of the genetic map with the nucleotide sequence will be described in detail elsehwere (D. Koshland, K. Talmadge and D. Botstein, manuscript in preparation). Mutants derived using the mutagens hydroxylamine, ICR191 (a frameshift mutagen) and ultraviolet light are designated by the letters H, I and U, respectively, preceding the isolation number. Mutants known to be amber, ochre or +1 frameshifts on the basis of response to host tRNA suppressors are marker *am*, *oc* and *fs*, respectively.

All infections were with S. typhimurium strain DB4381 [*his*G46 Δ(*bio-uvrB*)], whose UvrB phenotype allows suppression of host-specified protein synthesis at a relatively low dose (Botstein et al., 1973). The medium used was M9 minimal (Miller, 1972) supplemented with 20 μg/ml histidine and 1 μg/ml biotin.

Labeling of Phage-Encoded Proteins after Infection

DB4381 cells growing exponentially at a density of 2×10^8/ml in supplemented M9 minimal medium were concentrated 2.5 fold by centrifugation and resuspension in growth medium. The cells were then irradiated with 2700 ergs/mm^2 of 254 nm light. 2 ml aliquots of irradiated culture were added to a prewarmed (37°C) flask containing 3 ml of growth medium containing phage at the desired multiplicity of infection (usually 10 phages per cell). After incubation for 40 min, 1 ml aliquots were removed to Eppendorf centrifuge tubes containing 50 μCi uniformly labeled ^{14}C amino acids (New England Nuclear). After a 5 min incubation at 37°C, excess amino acid mixture (enzymatic casamino acids; Nutritional Biochemicals) was added and the tubes were centrifuged for 2 min. The cell pellets were frozen in liquid nitrogen and stored frozen until analysis, when they were resuspended in sample buffer (Laemmli, 1970) and processed as described below.

SDS-Polyacrylamide Gel Electrophoresis and Autoradiography

Slab gels (Studier, 1973) were prepared using the stacking system and buffers of Laemmli (1970). Gels were fixed in 10% acetic acid (sometimes containing Coomassie blue) before drying in a heated apparatus (Hoefer) under vacuum. Dried gels were exposed to preflashed Kodak XR5 film at room temperature (Laskey and Mills, 1975). Autoradiograms were traced and the areas under the peaks were integrated using a Zeineh Soft Laser Scanning Densitometer (Biomed Instruments, Chicago). For quantitations, several exposures of a gel were scanned to verify linearity of response.

Pulse-Chase Experiments

Strain DB4381 cells were grown and irradiated as before. However, infected cells were labeled 40 min after infection by adding 0.5 ml to a centrifuge tube containing 250 μCi of ^{35}S-methionine (Amersham/Searle, Arlington Heights, Illinois). After 30 sec, 50 μl of chase mixture (10% casamino acids, 10 mg/ml methionine) were added. Samples were taken immediately and every 30 sec thereafter by transferring an aliquot to Eppendorf tubes containing an equal volume of 2 fold concentrated sample buffer preheated to 90°C.

Fractionation Procedure for Periplasmic Proteins

Osmotic Shock

The method used was adapted from Neu and Heppel (1965). Labeled infected cells were centrifuged in an Eppendorf centrifuge for 2 min and the pellet was put on ice. All subsequent steps were carried out at 4°C. Pellets were resuspended in 0.15 ml cold solution containing 20% sucrose, 10 mM Tris-HCl (pH 7.5). Then 5 μl of 0.5 M EDTA (pH 8) were added and incubation on ice was continued for 10 min. One third of the sample was removed and saved as the untreated control. The remaining cells were centrifuged in an Eppendorf centrifuge for 5 min in the cold. The supernatant fluid was quickly removed and the pellet was rapidly resuspended by vigorous agitation in 0.1 ml cold distilled water. The mixture was incubated for 10 min on ice and then centrifuged again for 5 min. The supernatant was removed and saved as the periplasmic fraction; residual fluid was carefully drained off and the remaining pellet was resuspended in 0.1 ml water and saved as the cytoplasmic/membrane-bound fraction. 2× sample buffer was added to each tube, and samples were heated to 90°C for 2.5 min and applied to an SDS-polyacrylamide gel as above.

Conversion of Labeled Infected Cells to Spheroplasts

The method used was adapted from Witholt et al. (1976). Labeled infected cells were centrifuged in an Eppendorf centrifuge for 2 min and the pellet was put on ice. All subsequent steps were carried out at 4°C. Pellets were resuspended in 75 μl of 0.1 M Tris-HCl (pH 8), 0.5 mM EDTA, 0.5 M sucrose. 7.5 μl of 2 mg/ml lysozyme were added, followed by 75 μl of cold H_2O. The sample was incubated for 25 min on ice. The success of spheroplasting was monitered by the roundness of cells and the extent of lysis after dilution as judged by phase-contrast microscopy. One third of the sample was saved as untreated control. To stabilize spheroplasts, 3 μl of 1 M $MgCl_2$ were added; cells were centrifuged in an Eppendorf centrifuge for 5 min. The supernatant was saved as the periplasmic fraction; the residual fluid was carefully drained off and the remaining pellet was resuspended in 0.1 ml water and saved as the cytoplasmic membrane fraction. 2× sample buffer was added to each tube, and samples were heated to 90°C for 5 min and applied to an SDS-polyacrylamide gel as above.

Membrane Isolation

The method used was adapted from Silhavy et al. (1976). Infected and labeled cells were spheroplasted as described above, except the $MgCl_2$ was omitted. After the spheroplasts had been pelleted and the supernatant removed, they were rapidly resuspended in 100 μl H_2O. 250 μl of a saturated culture of DB4381 cells lysed by the identical method were added to this mixture to serve as carrier. The procedure disrupted more than 90% of the cells as judged by examination in the microscope. The remaining intact cells were removed by centrifugation of the extracts at 1000 × g for 10 min, and the lysates were fractionated by centrifugation at 100,000 × g for 2 hr in a Beckman ultracentrifuge. The membrane pellet was purposely not washed before resuspension in sample buffer to avoid disturbing any weak protein membrane interaction.

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