SECRETION AND MEMBRANE LOCALIZATION OF PROTEINS IN *ESCHERICHIA COLI* *

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I. INTRODUCTION

Probably no one would doubt that *Escherichia coli,* a prokaryote, has been used most widely for solving many fundamental questions in molecular biology. In this review, we attempt to introduce another aspect of *E. coli,* which provides important information for understanding how proteins are secreted across the membrane, and how the secreted proteins are transferred and assembled to their final destinations.

Taking advantage of the unique structure of the *E. coli* envelope, one can attempt to solve the questions mentioned above. *E. coli,* as well as other Gram-negative bacteria, are surrounded by an envelope consisting of two distinct membranes as observed by electron microscopy, the outer membrane and the inner or cytoplasmic membrane (Figure 1)[1,2] The cell wall or the peptidoglycan layer is located between the two membranes.[1] Both the outer and the cytoplasmic membranes show the typical features of a unit membrane having a thickness of about 75 A. Similar to the cytoplasmic membrane of the eukaryotic cells, the *E. coli* cytoplasmic membrane displays many important functions. In *E. coli,* energy metabolism, active transport, and synthesis of lipids, peptidoglycan, and lipopolysaccharides all occur in the cytoplasmic membrane. On the other hand, the functions of the outer membrane are still somewhat obscure. However, one of the roles of the outer membrane is to protect the cell from toxic materials such as bile salts and antibiotics by serving as a permeability barrier against them. The outer membrane is also involved in cell recognition processes as in mating.[4] Furthermore, it serves as a barrier to prevent many enzymes and proteins from leaking out from the space between the outer membrane and the cytoplasmic membrane. This space (see Figure 1), called the periplasmic space, includes binding proteins required for transport of nutrients such as sugars and amino acids inside the cell as well as hydrolytic enzymes such as ribonuclease, deoxyribonuclease, protease, peptidase, and alkaline phosphatase. It is important to keep these enzymes in the periplasmic space, since they would be quite harmful to the cell if they existed inside the cell, but they are important for the cell to digest the exogenous nutrients. In this regard, the periplasmic space is analogous to the lysozome in the eukaryotic cell.

Since there is no protein-synthesizing activity in the periplasmic space, both periplasmic and outer membrane proteins must be synthesized at the interior side of the cytoplasmic membrane and secreted through the cytoplasmic membrane. It is also im-

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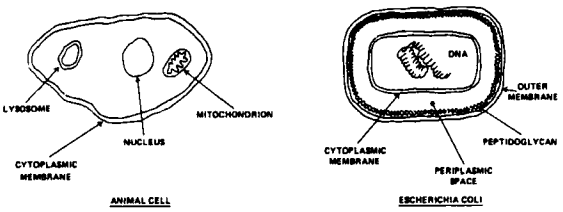

FIGURE 1. Schematic illustration of the membrane structures of the animal cell and the *E. coli* cell.

portant to point out that those cytoplasmic membrane proteins which exist at least in part at the exterior side of that membrane may also be considered as secreted proteins. In addition to the mode of secretion, another interesting problem which may be approached in the *E. coli* envelope system is the question of how proteins secreted across the cytoplasmic membrane reach their final locations in the envelope, the outer membrane, the periplasmic space, or the cytoplasmic membrane.

Since the molecular mechanisms of biosynthesis and assembly of the outer membrane proteins have been extensively investigated,[3] the proteins of the outer membrane provide a particularly suitable system for study of the molecular mechanism of secretion as well as membrane localization.

In this review, we will discuss the structures and properties of the precursors of the *E. coli* envelope proteins thus far identified, and their roles in translocation of the proteins across the cytoplasmic membrane and in determination of their final locations in the envelope.

II. PRECURSORS OF ENVELOPE PROTEINS

A. Outer Membrane Proteins

The outer membrane of *E. coli* contains a few major proteins (lipoprotein, tolG protein, and matrix protein) which exist in very large quantities.[3] Therefore, purification and characterization of these proteins are relatively easy. Thus, extensive studies have been done to elucidate the mechanism of biosynthesis and assembly of these proteins.[3] However, efforts to find precursors for these proteins by short pulse-label experiments have failed,[5,6] suggesting that processing and assembly of precursor molecules, if any, occur very rapidly. Therefore, another approach to examine the intermediate steps of outer membrane protein synthesis and assembly has been taken by making use of perturbants of the envelope structure. The first perturbant used was toluene treatment of cells[7,8] which is known to result in breakdown of the cellular permeability barrier with partial dissolution of the cytoplasmic membrane. Since the toluene-treated cells are permeable to molecules such as nucleotide triphosphates they have provided an important and easily obtainable system, partially in vitro, to study DNA synthesis,[9] RNA synthesis,[10] and peptideglycan synthesis.[11] Conditions were established in which cells may be treated with toluene and retain the ability for protein synthesis.[7,12] This system was found to be entirely dependent upon the addition of ATP, and to exclusively synthesize membrane proteins. When these membrane proteins were treated with antilipoprotein serum, two distinct peaks were observed during SDS-polyacrylamide gel electrophoresis of the immunoprecipitate: one comigrated with the in vivo lipoprotein, and the other appeared to be a new form of lipoprotein with an apparent molecular weight of 15,000.[8,12] This new form of lipoprotein had the same carboxyl-terminal structure as lipoprotein as determined by peptide mapping,[8] and amino-terminal analysis revealed methionine[8] instead of the glycerylcysteine in the case of lipoprotein. Assuming that the new protein contained an ami

tide extension on the lipoprotein in sequence, its amino acid composition was determined by double labeling experiments.[8] Thus the peptide extension was determined to contain at least 18 to 19 extra amino acid residues enriched in hydrophobic residues.[8] This new form of lipoprotein designated prolipoprotein, was believed to play a major role in the biosynthesis and assembly of lipoprotein. Thus, toluene treatment appeared to block processing of prolipoprotein to lipoprotein.

If the prolipoprotein accumulated in toluene-treated cells was a precursor of lipoprotein, one would expect that it would be seen as the primary translation product of the lipoprotein mRNA. In fact, the lipoprotein mRNA has been purified,[13-16] and the purified mRNA was active in cell-free protein synthesizing systems.[13,15,16] The product in the cell-free system was found to have a higher molecular weight than lipoprotein but the same carboxyl terminal structure as lipoprotein.[13] This protein was found to comigrate on SDS-gel electrophoresis with prolipoprotein from toluene-treated cells.[17] The amino acid sequence of the peptide extension at the amino terminus of lipoprotein was then determined by sequential Edman degradation of the cell-free products.[17] The complete amino acid sequence of the peptide extension of prolipoprotein consisting of 20 amino acid residues thus determined and shown in Table 1. Characteristics of this sequence will be discussed in the next section.

Treatment of cells with toluene for a shorter time, that is, 1.5 min instead of 10 min, enhanced the incorporation of (^{35}S)-methionine into new membrane proteins of higher molecular weights.[18] The production of two new membrane proteins was clearly evident, and based on results obtained by immunoprecipitation, SDS-polyacrylamide gel electrophoresis, and autoradiography it was demonstrated that these new proteins represented putative precursors of matrix and tolG proteins (promatrix, and protolG protein) with molecular weights about 2,000 higher than those of the two major outer membrane proteins. Since all three major outer membrane proteins may be formed from precursors that contain about 20 extra amino acid residues, a common mechanism for their biosynthesis seems to be involved. The amino terminus of both promatrix protein and prolipoprotein is methionine.[17,18] The distribution of leucine residues at the amino terminal region of the promatrix protein differs from that of the matrix protein, which suggests that the peptide extension is located at the amino terminal end of matrix protein, analogous to the case with prolipoprotein.

The use of phenethyl alcohol (PEA) instead of toluene as a membrane perturbant in order to accumulate precursors of secreted proteins proved to be a valuable tool in studying outer membrane protein synthesis and assembly.[19] Since PEA is water soluble up to 2%, the extent of PEA administration to the cells was quite easily controlled and unlike toluene the effects of PEA were reversible and less damaging in terms of macromolecular synthesis and membrane structure. At 0.3% PEA among the major outer membrane proteins, promatrix protein was specificially accumulated. In contrast to toluene-treated cells, the accumulation of promatrix protein was reversible. Upon removal of PEA and regrowth of the cells, promatrix protein was processed and normal assembly of the resulting matrix protein into the outer membrane was observed, indicating that there is a direct precursor-product relationship between the two proteins. The accumulation of protolG protein has also been observed when the fluidity of the membrane lipids was reduced.[135] The protolG protein thus accumulated was found to be chased into the mature tolG protein upon resuming the fluidity to normal.

In addition to the above systems, another system which allows study of outer membrane protein precursors is obtained by isolation of polysomes coding for these proteins from cells followed by cell-free synthesis. One such system has been established for λ phage receptor protein (*lam* B gene product) which was found to be made as a higher molecular weight form.[20]

TABLE 1

Amino Acid Sequences of the Peptide Extension of Precursor Proteins of *E. coli**

	23	22	21	20	19	18	17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1		Ref.
Prolipoprotein:				Met	Lys	Ala	Thr	Lys	Leu	Val	Leu	Gly	Ala	Val	Ile	Leu	Gly	Ser	Thr	Leu	Leu	Ala	Gly	Cys	17
Pro-β-lactamase:	Met	Ser	Ile	Gln	His	Phe	Arg	Val	Ala	Leu	Ile	Pro	Phe	Phe	Ala	Ala	Phe	Cys	Leu	Pro	Val	Phe	Ala	His	24
Pro-fd-major coat protein: (gene 8)	Met	Lys	Lys	Ser	Leu	Val	Leu	Lys	Ala	Ser	Val	Ala	Val	Ala	Thr	Leu	Val	Pro	Met	Leu	Ser	Phe	Ala	Ala	25a
Pro-fd-minor coat protein: (gene 3)						Met	Lys	Lys	Leu	Leu	Phe	Ala	Ile	Pro	Leu	Val	Val	Pro	Phe	Tyr	Ser	His	Ser	Ala	25b

* Data as of September 1978.

B. Periplasmic Proteins

Using DNA isolated from a transducing phage (ϕ80) containing the gene for alkaline phosphatase (*pho*A), cell-free synthesis of the enzyme was carried out in a coupled transcription-translation system.[21] The cell-free products from *pho*A$^+$ and *pho*A$^-$ transducing phage DNAs were examined with antiserum against purified alkaline phosphatase followed by SDS-gell electrophoresis. It was found that the in vitro product of the *pho*A gene has larger molecular weight than alkaline phosphatase by several thousand. It was believed that the in vitro product contained an extra amino acid sequence beside the alkaline phosphatase sequence and the authors were able to process the in vitro product to the correct size of alkaline phosphatase using an outer membrane preparation. The extra segment was found to be hydrophobic since the in vitro product bound irreversibly to a decyl-agarose column while alkaline phosphatase could be eluted from the column with 0.2*M* NaCl. Based on these results the in vitro product was presumed to be a precursor of alkaline phosphatase containing a hydrophobic peptide extension. The existence of alkaline phosphatase precursor has recently been confirmed using in vitro polysome systems.[22] However, in all systems above, the position of the extra peptide has not been determined.

Using the in vitro polysome system described earlier for the λ receptor, two additional periplasmic proteins (maltose binding protein and arabinose binding protein) were found to be synthesized as forms of about 3000 daltons larger than the mature proteins as determined by gel electrophoresis. In the case of the arabinose binding protein, the in vitro product was shown to contain a peptide extension located at or near the amino terminus of the mature protein by a pulse-chase experiment.[23]

Finally, the existence of a precursor for β-lactamase, a periplasmic enzyme which hydrolyzes penicillins, has recently been deduced based upon the nucleotide sequence of the plasmid gene coding for that protein.[24] It was found that the first 23 amino acids predicted by the DNA sequence did not appear in the mature, secreted protein (see Table 1). Therefore, although not directly shown, the β-lactamase gene would code for a precursor containing an amino terminal peptide extension of 23 residues long.

C. Cytoplasmic Membrane Proteins

Similar to the case of β-lactamase above, two cytoplasmic membrane proteins have been deduced to be synthesized from precursors based on the nucleotide sequences. From the nucleotide sequence of its mRNA, the bacteriophage fd major coat protein (gene 8 product) was found to be made from a precursor containing an amino terminal peptide extension of 23 residues (see Table 1).[25a] Furthermore, from the DNA sequence of gene 3 of bacteriophage fd, its minor coat protein was also found to be produced from a precursor with 18 extra amino acid residues at the amino terminus (see Table 1).[25b] In addition, bacteriophage M13 coat protein is also made from a precursor of 23 extra amino acid residues at the amino terminus, which can be assembled into the bilayer in an integral fashion upon exposure to *E. coli* membrane vesicles or to liposomes prepared from *E. coli* lipids.[26] Although these coat proteins are derived from bacteriophages, they are shown to be oriented asymetrically across the *E. coli* cytoplasmic membrane in phage-infected cells,[27] as will be discussed later in the section on localization of envelope proteins.

III. STRUCTURE OF THE PRECURSOR PEPTIDE EXTENSION

A. Amino Acid Sequence

From the amino acid sequence of the peptide extension of the prolipoprotein (Table 1), Inouye et al.[17] pointed out several unique features of the sequence which are possibly playing important roles for translocation of the protein across the cytoplasmic membrane.

1. The extended region is basic and positively charged at neutral pH because it contains two lysine but no acidic amino acid residues.
2. This region contains three glycine residues which are not present in lipoprotein.
3. Of the amino acid residues in the extended region, 60% are hydrophobic in contrast to 38% in lipoprotein.
4. The distribution of these hydrophobic amino acids along the peptide chain is completely different from their periodical distribution in lipoprotein.

Recently, the amino acid sequence of the precursor of β-lactamase (pro-β-lactamase) was determined from the nucleotide sequence of the gene for β-lactamase,[24] as discussed in Section II (Table 1). In contrast to the outer membrane lipoprotein, β-lactamase is a periplasmic enzyme. However, the amino acid sequence of the peptide extension of pro-β-lactamase is surprisingly similar to that of prolipoprotein. Furthermore, the amino acid sequences of the peptide extensions of precursors of two different coat proteins of bacteriophage fd[25] also show remarkable similarity to that of prolipoprotein (Table 1). These coat proteins are known to be produced from these precursors and their final location is in the cytoplasmic membrane as discussed in the previous section.

In view of the fact that the final locations of the processed products of these proteins are totally different from each other, the similarity seen in Table 1 must be derived from the common functions of extended peptides.

In this section, we would first like to discuss the general features of the peptide extension and then to discuss the possible function(s). In order to determine the general features of the peptide extension, the contents of certain amino acid residues were examined along the sequence of the extension. Figure 2A shows the distribution of basic amino acids, hydrophobic amino acids, and proline plus glycine residues. In this figure, the average content of certain amino acids at position N is calculated as the average content of the amino acids at position N−1, N, and N+1 in order to reduce fluctuations seen in the contents calculated for a single position. From this figure and the sequences in Table 1, the following general features can be deduced.

Rule 1 — The amino terminal section (Figure 2A, Section I) has a basic characteristic: the amino terminal section of the extension has the property of being very basic containing at least two basic residues. This basic section is separated by about 55 A from the cleavage site. It should be pointed out that regardless of the lengths of the peptide extensions, the first lysine or arginine residue appears at either the 16th or 17th position without fail, (See Table 1). In addition, no peptide extensions in Table 1 contain any acidic amino acid residues.

Rule 2 — There is a hydrophobic property of a section of about 50 A in length between the basic section and the cleavage site (Figure 2A, Section II). As clearly seen in Figure 2A, the content of hydrophobic amino acids sharply increases immediately after the basic Section I. Hydrophobicity is especially high between positions 6 and 15.

Rule 3 — There is an existence of a proline or a glycine residue at least 15 A to 20 A from the cleavage site in the hydrophobic Section II. As shown in Figure 2A, there

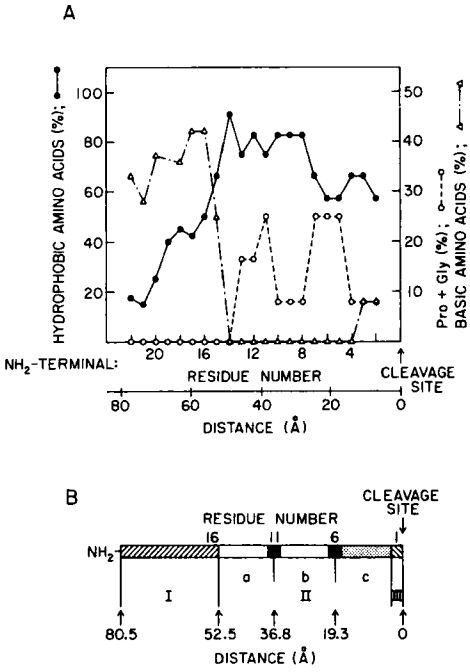

FIGURE 2. Amino acid distribution in the peptide extensions of precursor proteins in *E. coli*. (A) Using amino acid sequences listed in Table 1, the content (C) of amino acid residues of a particular class at each position (N) is calculated as follows; C = 100 X (total number of amino acid residues of a particular class at positions N − 1, N, and N + 1)/(total number of amino acid residues at positions N − 1, N, and N + 1). C is then plotted against residues numbers from the cleavage point or the distance (A) from the cleavage point: •———•, contents of hydrophobic amino acid residues (Ala, Cys, Val, Met, Ile, Leu, Tyr, Phe, and Trp except for Met at the amino terminal ends); Δ———Δ, contents of basic amino acid residues (Lys, His, and Arg); and O- - -O, contents of Pro and Gly. (B) The general structure of the peptide extension deduced from A. I, basic region; II, hydrophobic region; and III, recognition site for cleavage of the peptide extension. Section II is divided into three subsections, a, b, and c by Pro or Gly at positions 6 and 11. Distances are calculated using 3.5A for the distance between two adjacent amino acid residues.

are two peaks of proline plus glycine residues along the peptide extension. One peak lies between positions 11 and 13 (based on the average) and the other lies between positions 5 and 7. In fact, all four sequences in Table 1 contain a proline or a glycine residue between positions 4 and 7 (Gly-7 for 1, Pro-4 for 2, Pro-6 for 3, and Pro-6 for 4). As for the other peak, prolipoprotein contains a glycine residue at position 12, pro-β-lactamase contains a proline residue at position 12, and pro-fd-minor coat protein contains a proline residue at position 10, whereas pro-fd-major coat protein does not contain a proline or glycine residue at this region. These sites divide the hydrophobic Section II into three subsections a, b, and c (see Figure 2B). As can be seen from Figure 2A, Sections II-a, and II-b are extremely hydrophobic and Section II-c is somewhat less hydrophobic.

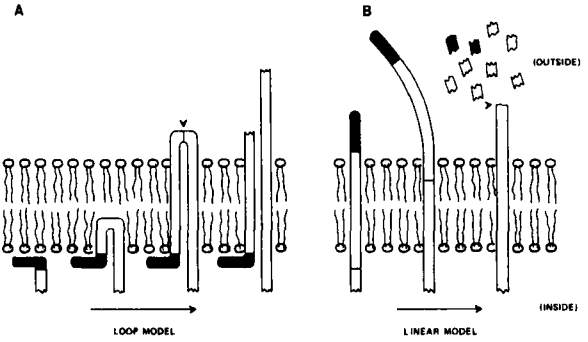

FIGURE 3. Loop model and linear model for the translocation of secretory proteins across the membrane. (A) Loop model, and (B) Linear model. Solid portions represent the basic regions of the first several amino acid residues of the peptide extension (section I in Figure 2). The following blank portions represent the hydrophobic regions of the peptide extension (section II) and the cleavage site (section III) followed by shaded portions of mature proteins. Small arrows show the cleavage sites of the precursor molecules.

Rule 4 — The amino acid residue at the cleavage site (Section III) consists of one of the amino acids whose side chain at the α-carbon contains none or only one carbon (i.e., Gly, Ala, Ser and Cys).

The general features of the peptide extensions, therefore, can be schematically drawn as shown in Figure 2B, where the peptide extension consists of (a) a basic section (I) which is 52.5 A away from the cleavage site, and whose length varies from 10 to 25 A, (b) a hycrophobic section (II) of about 50 A in length which is divided into three subsections a, b, and c by proline or glycine residues, and (c) an amino acid residue with a short side chain at the cleavage site (Section III).

B. Roles of the Peptide Extension

How are these general features functionally important for the peptide extensions? Since the proteins listed in Table 1 are drastically different in nature and incorporated into three different fractions of the *E. coli* envelope (outer membrane, periplasmic space, and cytoplasmic membrane), the general features discussed above are most likely to be essential to a common feature for all three different classes of secreted envelope proteins, but not to a specific function for the individual proteins. This common feature is translocation of a protein (in cases of the outer membrane proteins and periplasmic proteins) or a part of a protein (in case of the cytoplasmic membrane proteins) through the cytoplasmic membrane to the outside of that membrane.

Possible roles of the section of the peptide extension have been suggested.[3,17,28] The basic positively charged Section I allows the initial attachment of the precursor and consequently the polysomes to the negatively charged inner surface of the cytoplasmic membrane through ionic interactions (see Figure 3A). The *E. coli* membrane surface is charged negatively at neutral pH because of phosphatidyl glycerol. The next hydrophobic Section II is then progressively inserted into the cytoplasmic membrane by hydrophobic interaction with the lipid bilayer (Figure 3A). As the peptide further elongates, the loop formed by Section II is further extended into the lipid bilayer. Eventually the cleavage site of the peptide extension (Section III) becomes exposed to the outside surface of the cytoplasmic membrane, while the positively charged amino

terminal Section I remains on the inside surface of the cytoplasmic membrane (see Figure 3A). Since the length of Sections II + III is about 53 A, it is long enough to extend through the lipid bilayer to expose Section III to the outside surface of the membrane.

The role of proline or glycine residues in Section II may be important in bending the peptide at the position to form a loop. This proposed mechanism of protein translocation across a membrane is called the loop model.[28] An alternative mechanism is the linear model (signal hypothesis) proposed by Blobel and Dobberstein[29] for the transfer of eukaryotic secretory proteins across membranes. In this model it is proposed that the hydrophobic nature of the peptide extension of secretory protein precursors allows for initial interaction of the polysome with the membrane followed by interaction of the peptide extension with a specific receptor protein in the membrane. The result is activation of the receptor protein to mediate aggregation of proteins at the membrane-bound polysome site to form a transmembrane hydrophilic channel through which the peptide extension and the succeeding secretory protein may be linearly translocated across the membrane during its synthesis. This model does not take into account the specific characteristics of the peptide extensions as described above. However, no direct evidence has yet been provided to distinguish between the two models. It may not be difficult to distinguish between them experimentally since in the linear model the peptide extension is transported to the outside, while it is left in the cytoplasmic membrane in the loop model.

C. Comparison with Eukaryotic System

Existence of precursors for secretory proteins was first demonstrated in 1972 by Milstein et al.[30] They showed that immunoglobulin light chains are produced from their precursors of slightly larger molecular weights. They suggested that a short amino-acid sequence at the amino terminus of a precursor protein provides a signal which is required for the formation of membrane-bound polysomes as well as secretion of the protein. The signal hypothesis, proposed by Blobel and Dobberstein[29] offered a more precise description for translocation steps of secretory proteins. In the signal hypothesis, it is proposed that the peptide extension (signal peptide) at the amino terminus of the precursor is first recognized by receptor proteins in the membrane, which then form a transmembrane tunnel. Through this tunnel, the signal peptide as well as the remainder of the protein are linearly translocated from the inside to the outside of cells (the linear model: see the previous section). It is not known yet whether such specific receptors or tunnel exist in the eukaryotic membrane.

In view of the general features found in the peptide extensions of the *E. coli* secretory proteins (see the previous section), it is of great interest to examine whether they are also applicable to the peptide extensions of eukaryotic secretory proteins. Table 2 shows nine complete amino acid sequences and 10 partial sequences of the peptide extensions of eukaryotic secretory protein precursors so far determined. In this table, it should be noted that the secretory protein precursors in the eukaryotic system are termed preproteins in contrast to proproteins in *E. coli* system, since in some cases of eukaryotic secretory proteins there is another precursor form of the fully functional proteins which are called proproteins (i.e., preproinsulin and preproalbumin).

When the eukaryotic peptide extension sequences (only for the complete amino acid sequences one to nine in Table 2) are compared with those of the peptide extensions of the *E. coli* precursor proteins (Table 1), some eukaryotic precursor proteins show remarkable homology in their peptide extensions with those of *E. coli* protein precursors. Peptide extensions of prelysozyme, preconalbumin, and preproalbumin satisfy all four rules derived above from the structures of the peptide extensions of the *E. coli*

TABLE 2

Amino Acid Sequences of the Peptide Extensions of Precursors of Eukaryotic Secretory Proteins[a]

Complete Sequence	30	29	28	27	26	25	24	23	22	21	20	19	18
Prelysozyme (hen oviduct)													Met
Preconalbumin(hen oviduct)												Met	Lys
Preovomucoid (hen oviduct)							Met	Ala	Met	Ala	Gly	Val	Phe
Preproalbumin (rat liver)													Met
Preimmunoglobin κ-type light chain (mouse; MO8C-41)									Met	Asp	Met	Arg	Ala
Preimmunoglobin λI-type light chain (mouse)												Met	Ala
Preimmunoglobin λII-type light chain (mouse)												Met	Ala
Preparathyroid hormone (bovine)						Met	Met	Ser	Ala	Lys	Asp	Met	Val
Pregrowth hormone (rat)					Met	Ala	Ala	Asp	Ser	Gln	Thr	Pro	Trp
Partial Sequence													
Preproinsulin (rat)								X	Leu	Lys	Met	X	Phe
Prepromelittin (honey bee)										Met	Lys	Phe	Leu
Pretryprinogen 2 (dog)													
Preplacental lactogen (human)					Met	Pro	X	X	X	X	X	Leu	Leu
Preproinsulin (fish)								X	Ala	Leu	X	Leu	X
Preproinsulin (avian)						Met	Met	X	X	Leu	X	Leu	X
Preprolactin (rat)		Met	X	X	X	X	X	X	X	X	X	X	X
Preimmunoglobin κ-type light chain (mouse; MOPC321)											Met	X	Thr
Pregrowth hormone (bovine)				Met	Met	X	X	X	Pro	X	X	X	Leu
Pre-α-lactalbumin (rat)												Met	Met

[a] Data as of September 1978.

proteins: (a) a lysine or an arginine residue exists at position 17 or 18 (Rule 1), (b) there exists a long sequence of hydrophobic amino acid residues after the basic amino acid residue (about 55 A; Rule 2), (c) a proline or a glycine residue exists in the hydrophobic section (Pro-6 for 1, Gly-8 for 2, and Gly-5 for 4 in Table 2; Rule 3), and (d) there exists a glycine, alanine, or serine residue at the cleavage site (Rule 4). Furthermore, Rules 2 and 4 are applicable to all sequences. Rule 3 is also applicable to all sequences except for preparathyroid hormone. As for Rule 1, it is applicable to five out of nine sequences (sequences 1, 2, 4, 5, and 8 in Table 2). Generally speaking, it seems that the peptide extensions of precursors with longer sequences such as preovomucoid, preparathyroid hormone, and pregrowth hormone show markedly less similarity to the *E. coli* peptide extensions. Therefore it is possible that those secretory proteins which share general features with the *E. coli* secretory proteins are translocated through the membrane by a similar mechanism as in *E. coli*, while those which do not follow some of the *E. coli* rules may be secreted by more specialized mechanism(s). These more specialized systems are possibly diverged from a primitive *E. coli* system to accomodate physiological needs to secrete a protein through a specific intracellular membrane system (i.e., Golgi apparatus, mitochondrial membrane, nuclear membrane, etc.) and/or perhaps at different specific sites on the same membrane system. It should be noted that those peptide extensions of precursors with longer peptide extensions such as of preovomucoid and pregrowth hormone seem to share common features: (a) proline residue at position 6, (b) between this proline residue and the cleavage site there is an acidic amino acid (Asp-5 for 3, and Glu-4 for 9), (c) a cysteine residue at position 10, (d) a serine residue at position 13 or 14, (e) a glycine or a proline residue at position 20 or 19, and (f) Met-Ala at the amino terminus. These features may be associated with a specific receptor or a specific channel through the membrane.

TABLE 2 (cont'd.)

Amino Acid Sequences of the Peptide Extensions of Precursors of Eukaryotic Secretory Proteins[a]

17	16	15	14	13	12	11	10	9	8	7	6	5	4	3	2	1		Ref.
Arg	Ser	Leu	Leu	Ile	Leu	Val	Leu	Cys	Phe	Leu	Pro	Leu	Ala	Ala	Leu	Gly	Lys	31
Leu	Ile	Leu	Cys	Thr	Val	Leu	Ser	Leu	Gly	Ile	Ala	Ala	Val	Cys	Phe	Ala	Ala	32
Val	Leu	Phe	Ser	Phe	Val	Leu	Cys	Gly	Phe	Leu	Pro	Asp	Ala	Ala	Phe	Gly	Ala	33
Lys	Trp	Val	Thr	Phe	Leu	Leu	Leu	Leu	Phe	Ile	Ser	Gly	Ser	Ala	Phe	Ser	Arg	34
Pro	Ala	Gln	Ile	Phe	Gly	Phe	Leu	Leu	Leu	Leu	Phe	Pro	Gly	Thr	Arg	Cys	Asp	35
Trp	Ile	Ser	Leu	Ile	Leu	Ser	Leu	Leu	Ala	Leu	Ser	Ser	Gly	Ala	Ile	Ser	Gln	35
Trp	Thr	Ser	Leu	Ile	Leu	Ser	Leu	Leu	Ala	Leu	Cys	Ser	Gly	Ala	Ser	Ser	Gln	36
Lys	Val	Met	Ile	Val	Met	Leu	Ala	Ile	Cys	Phe	Leu	Ala	Arg	Ser	Asp	Gly	Lys	37
Leu	Leu	Thr	Phe	Ser	Leu	Leu	Cys	Leu	Leu	Trp	Pro	Gln	Glu	Ala	Gly	Ala	Leu	38
Leu	Phe	Leu	Leu	Ala	Leu	Leu	Val	Leu	Trp	Glu	Pro	Lys	Pro	Ala	Gln	Ala	Phe	39,40
Val	X	Val	Ala	Leu	Val	Phe	Met	Val	Val	Tyr	Ile	X	Tyr	Ile	Tyr	Ala	Ala	41
	Ala	X	X	Phe	Leu	Phe	Leu	Ala	X	Leu	Leu	Ala	Tyr	Val	Ala	Phe	Pro	42
Leu	X	X	X	Leu	Leu	X	Leu	X	Pro	X	X	X	X	X	X	X	Val	43
X	Phe	X	Leu	Leu	Val	Leu	Leu	Val	Val	X	X	X	X	X	X	Ala	Val	44
X	X	X	Leu	Leu	X	Leu	X	X	X	X	X	X	X	X	X	X		44
X	Leu	Leu	Leu	Leu	Met	Met	X	X	Leu	Leu	X	Cys	X	X	X	X	Leu	45
X	Thr	Leu	Leu	Leu	Trp	Val	Leu	Leu	Leu	Trp	Val	Pro	X	Ser	Thr	X	Asp	35
Leu	Leu	X	Phe	X	Leu	Leu	X	Leu	Pro	X	X	X	X	X	X	X	Phe	46
X	X	X	X	Leu	X	Leu	X	X	X	X	Leu	X	X	X	X	X	X	47

It is also noteworthy that prelysozyme, preconalbumin, and preovomucoid are synthesized in the same cell (hen oviduct) and secreted from it. However, the peptide extension of preovomucoid is much longer than the other two and its amino acid sequence is quite different from the other two which share common features with the *E. coli* secretory proteins. More dramatically, ovalbumin which is also synthesized and secreted from hen oviduct cells has been shown not to be made from a precursor containing a peptide extension at its amino terminus.[48,49] Furthermore, its amino terminal sequence does not demonstrate any common features with the peptide extensions of the three egg white precursor proteins discussed above.[31-33] Another unique feature of ovalbumin is that its amino terminal glycine residue is acetylated. These results indicate that ovalbumin, the major secretory protein of the hen oviduct (55% of the total protein synthesis is for ovalbumin in contrast to 10%, 10% and 2% for conalbumin, ovomucoid, and lysozyme, respectively),[50] is secreted by a distinctly different mechanism from that used for conalbumin, ovomucoid, and lysozyme.

In case of the *E. coli* system, it has been reported that the *traT* gene product, an outer membrane protein required for conjugation, is not produced from a precursor of higher molecular weight.[51] However, since the amino acid sequence of the amino terminal part of the protein has not been determined, it is premature at present to conclude that *E. coli* has also two distinct secretory mechanisms. This is because cleavage of the peptide extension is not an obligatory step for translocation of outer membrane proteins across the cytoplasmic membrane, as discussed later, or perhaps for assembly of *traT* protein.

It should be noted here that the membrane-bound penicillinase of *Bacillus lichemiformis* has an extra amino acid sequence at the amino terminus of penicillinase excreted into the culture medium.[52,53] These proteins have been well characterized and the membrane-bound enzyme is shown to be a precursor of the exoenzyme.[54] The extra amino

acid sequence consists of 24 amino acid residues (only 4 Asp, 4 Asn, 4 Glu, 3 Gln, 4 Ser, and 5 Gly) and a phosphatidylserine at the amino terminus.[52] This amino terminal peptide extension is sometimes believed to be a secretory-type of the peptide extension (for example, see References 37 and 55). However, in view of the amino acid sequence of this region it appears most likely not to play a secretory role for this enzyme but rather to maintain the enzyme membrane bound. Therefore, it is predicted that this protein is made from an additional precursor containing a secretory-like amino-terminal peptide extension.[53] In this regard, it has been found that the penicillinase can be formed as a hydrophobic precursor which can be processed.[136]

IV. MEMBRANE-BOUND POLYSOMES

A. Existence

Palade and coworkers have found that there is a correlation between the amount of ribosomes bound to the endoplasmic reticulum and the secretion of proteins.[56] From the fact that puromycin released incomplete chains to the interior of the microsomes it was suggested that the secreted protein passes through the membrane while the peptide chain is elongated.[57] From this and other results Blobel and Sabatini proposed a model in which membrane-bound ribosomes in animal cells are responsible for protein secretion across the membrane[58]. Later, Blobel and Dobberstein provided evidence for the involvement of membrane-bound ribosomes in protein secretion.[29]

By analogy, secreted proteins in prokaryotic cells might also be synthesized on membrane-bound polysomes and determination of the involvement of the amino terminal peptide extensions in membrane binding would be of great interest. In this regard, Cancedda and Schlessinger[59] examined the synthesis of alkaline phosphatase by isolated membrane-bound and free polysomes. They found that the periplasmic enzyme was preferentially made on the membrane-bound polysomes. This result was more recently confirmed and extended with the use of better new techniques for isolation of membrane-bound polysomes. Membrane-bound polysomes separated from free polysomes after sonic disruption of the cells were found to synthesize two classes of proteins, one which remained membrane bound after in vitro chain completion and another which became soluble upon release from the ribosomes.[60] The former were identified immunologically as outer membrane proteins, the latter as periplasmic proteins. The synthesis of alkaline phosphatase on membrane-bound polysomes has also recently been confirmed.[22,55,61]

If the nascent peptide is translocated across the membrane during protein synthesis, the nascent peptide should be exposed to the outside of the cell and should be accessible to chemical modification. This possibility was tested by using ^{35}S-acetylmethionyl methylphosphate sulfone which can label only amino groups exposed to the outside of the membrane surface since the drug cannot penetrate through the membrane.[55] When the membrane-bound polysomes were isolated from *E. coli* spheroplasts (only the outer membrane is permeabilized) labeled with the agent above, the radioactivity was recovered in peptidyl-tRNA. A portion of the labeled product after in vitro chain completion was identified as alkaline phosphatase. Hence, this result provides the first evidence that secreted proteins cross the membrane as growing chains.

B. Nature of Membrane Binding

In considering the role of membrane-bound polysomes in the mechanism by which the proteins traverse the membrane, two questions become apparent: (a) where does the energy for translocation arise from and (b) what is the role of the ribosome in these processes? It has been suggested[29] that the energy for movement of the growing chain through the membrane comes from protein synthesis. If this were the case, one

would expect that the ribosome should be anchored to the membrane apart from the growing chain so that as the peptide is further elongated, it would be "pushed" through the membrane. Such an attachment of the ribosome to the membrane has been reported in eukaryotic cells since the membrane-bound polysome was found to remain membrane bound even after release of the nascent chain by puromycin, and these ribosomes could then be released by high salt concentrations.[62,63] However in *E. coli*, evidence has recently been presented which suggests that there is no specific attachment of the membrane-bound ribosome to the membrane other than by way of the nascent peptide chain.[64] When isolated polysomes were treated with puromycin, it was observed that 75% of the polysomes were released from the membrane by the puromycin treatment regardless of the Mg^{++} and KCl concentrations. In addition, the mRNA could be ruled out as a mediator of membrane-bound ribosomes since the polysomes released from the membrane by puromycin were still intact (ribosome + mRNA) because the system had no ribosome release factor. From these results, it was concluded that since the ribosome itself has no firm attachment to the membrane other than by way of the nascent peptide chain, then the energy expended in the chain elongation of protein synthesis could not be used to push the growing peptide through the membrane barrier. However, it should be pointed out that in their study, 25% of the ribosomes in their membrane-bound polysome preparation was not released by puromycin. Among this 25% it was shown that 10 to 15% contained peptides that did not react with puromycin (even at 500μg/mℓ). Since secreted proteins in *E. coli* were shown to be more resistant to puromycin than cytoplasmic proteins[12,61,65] and since it was not determined what portion of the membrane-bound polysome fraction codes for secreted proteins, it is possible that the 25% of the polysomes which remained membrane bound after puromycin treatment engaged in the production of a class of secreted proteins. The fact that the puromycin-resistant membrane-bound polysome fraction (25%) could not be released by Mg^{++} or KCl as are puromycin treated eukaryotic membrane-bound ribosomes may be due to the nascent peptide attachment to the membrane. This polysome fraction may simply be due to an artifact or it may have an as yet undetermined functional attachment of ribosome to the membrane. At any rate, it would be of interest to determine which secreted proteins are produced in puromycin-sensitive and resistant membrane-bound polysomes. In this regard, it should be noted that no difference could be detected between outer membrane protein polysomes (rifampicin resistant polysomes) and other cellular polysomes when their protein components were analyzed.[66]

If protein synthesis does not provide the energy for the translocation of secreted proteins across the membrane in bacteria, alternative sources of energy for this process must be considered. One possibility is the stabilization of the protein transfer across the membrane by protein folding of the peptide as it crosses the membrane. Alternatively, the energy could somehow be derived from the membrane by an undetermined process. In this regard it is interesting to note that in a preliminary experiment no inhibition of outer membrane protein synthesis in glucose-grown cells was observed by addition of carboxylcyamide-*m*-chlorophenyl-hydrazene (CCCP) which is supposed to eliminate the membrane potential.[137]

An interesting aspect of outer membrane protein biosynthesis is the differential inhibition by certain antibiotics. In a pioneering study on envelope protein biosynthesis in vivo, Hirashima et al.[65] examined the effects of various and antibiotic inhibitors of protein synthesis on the biosynthesis of cytoplasmic and individual envelope proteins. These results established that there are major differences between biosynthesis of membrane and cytoplasmic proteins. In general, the biosynthesis of envelope proteins was strikingly more resistant to kasugamycin and puromycin than cytoplasmic proteins.

Conversely, envelope protein synthesis was more sensitive to tetracycline and sparsomycin. There were also differences among the envelope proteins. Tol G protein synthesis was more resistant to kasugamycin, sparsomycin, and chloramphenicol than the other envelope proteins, while lipoprotein was more sensitive to chloramphenicol. In the case of puromycin a much more severe differential effect was observed. Although the synthesis of envelope proteins was in general more puromycin resistant than that of cytoplasmic proteins, lipoprotein synthesis was strikingly more resistant to puromycin than the synthesis of the other envelope proteins. At 600 μg/mℓ puromycin, all other envelope protein synthesis was almost completely inhibited while lipoprotein was synthesized at 60% its normal rate. The reasons for puromycin resistance of envelope protein synthesis was believed to yield insights into their specific mode of biosynthesis. Since lipoprotein synthesis was extreme in this respect, the lipoprotein puromycin resistant biosynthesis was further studied.[12] Lipoprotein synthesis was examined in three systems in which the permeability barrier to puromycin was progressively disrupted by EDTA treatment, toluene treatment, and in an in vitro system using isolated polysomes. In each system lipoprotein synthesis remained puromycin resistant indicating that puromycin resistance was an intrinsic property of the lipoprotein biosynthetic machinery. Membrane-bound polysomes which are capable of synthesizing periplasmic and outer membrane proteins were found to be much more puromycin resistant than free polysomes.[60] Therefore, puromycin resistance may be a function of membrane binding of the polysomes, and therefore it may be a useful probe for determination of the extent of membrane binding of a particular polysome or class of polysomes.

A question which remains to be answered is on the nature of membrane binding of the polysome. As was discussed above, the nascent peptide may be the sole mode of attachment of the polysome to the membrane and the peptide extension of precursor proteins is generally believed to be responsible for such binding. However, the interaction of the peptide extension with the membrane is undetermined. In the previous section two models of such an attachment have been presented. In the loop model, the peptide extension itself has characteristics (as demonstrated in Figure 3A) such that it can interact with the membrane structure. In the linear model (signal hypothesis), the membrane is proposed to contain a specific receptor protein which recognized the peptide extension. If such a receptor protein would exist in *E. coli* it would be possible to locate a mutant of this protein in which the secreted protein could not transverse the membrane. No such mutant has yet been reported. In addition, if each ribosome of the secreted protein should have its own receptor protein, as implied in the signal hypothesis, and if secreted proteins have a common receptor, there must be as many as 10,000 receptor proteins per cell judging from the number of membrane-bound ribosomes per cell (about 10,000 to 40,000).[64] Therefore, it should be possible to isolate this protein from the cytoplasmic membrane.

In this regard, it should be noted that Miyoshi and Yamagata[67] have isolated very interesting mutants in which a single mutation appears to cause spectinomycin resistance in ribosomal function as well as hypersensitivity to antibiotics, dyes, and detergents. The spectinomycin resistance was sucrose dependent (resistant to spectinomycin only in the presence of 20% sucrose) and was found to be due to alterations in 30S ribosomal protein components S5, S4, or S3.[68] These alterations in ribosomal proteins appear to cause a specific change in the cytoplasmic membrane by losing a cytoplasmic membrane protein, I-19.[69,70] This protein I-19 seems to be related to ribosomes, since even in the wild type strain protein I-19 disappeared by the addition of spectinomycin.[70] The disappearance of protein I-19 was specific to the addition of spectinomycin and was not observed by other antibiotics such as neomycin, erythromycin, and chloramphenicol.[70] These results strongly suggest that there is an interaction between 30S

ribosome subunit and the cytoplasmic membrane. This interaction may be important for secretion of proteins across the cytoplasmic membrane.

Another approach toward elucidating the role of the peptide extension in membrane polysome binding would be to isolate mutants in the peptide extension which do not allow binding or translocation of the secretory protein across the membranes. A mutant has recently been isolated in which the hybrid protein of *lam*B-β-galactosidase, which will be discussed in the next section, can no longer be secreted across the cytoplasmic membrane.[71] The mutation is at the amino terminal portion of the hybrid protein and it was suggested to exist within the peptide extension. Characterization of this mutant as well as others in the peptide extension should yield insights into the precise functions of the peptide extension.

V. PROCESSING OF PRECURSOR PROTEINS

A. Enzymes

Enzymes required for processing of precursor proteins seem to have a very narrow range of specificity. As discussed earlier (Rule 4 in section III-B and also see Table 1 and 2) in almost all cases only Gly, Ala, Ser, or Cys is found at the cleavage site of the peptide extension (16 out of 17 cases; the one exception is Phe of pre-trypsinogen 2). This suggests that proteases with very similar specificities are responsible for the cleavage in both prokaryotic and eukaryotic systems. It should be pointed out that amino acid residues at either side of the position 1 residue of the peptide extension (position 2 and the amino terminus of the processed protein Tables 1 and 2) do not show any common feature. Therefore, the high specificity of the processing enzyme may depend on the conformation of the cleavage site rather than on the amino acid sequence of the region at the cleavage site. Since the amino acid residues found at the cleavage site of the peptide extension have short side chains, the position may easily be bent as shown in the loop model (Figure 3A) to make the cleavage site accessible to the processing enzyme.

Because of the difficulty of purification of the processing enzyme and of preparation of proper substrate for the enzyme, no report has yet been published for characterization of the enzyme in either prokaryotic or eukaryotic systems. However, there is a report which suggests that the processing enzyme in rat pituitary cells may be sensitive to L-1-tosylamide-2-phenylethylchloromethyl ketone (TPCK, a specific inhibitor for chymotrypsin).[72] However, treatment of *E. coli* cells with several different types of protease inhibitors (tosyl-lysine chloromethylketone (TLCK), antipain, leupetin, and diisopropylfluorophosphate) did not cause any accumulation of precursor molecules of envelope proteins.[73,74] One interesting observation in these works was that synthesis of matrix protein was severely inhibited by some of the inhibitors and that TLCK appeared to cause the formation of abnormal proteins. The reasons for these phenomena are not well understood at present.

It has not been determined whether the processing enzyme is an exo- or endopeptidase. However, a fragment of the peptide extension of pro-coat protein of bacteriophage f1 has been isolated which contains at least eight amino acid residues from the amino terminus of the pro-coat protein.[75] In the case of eukaryotic cells, fragments from the peptide extensions have been detected.[76,77] These results suggest that cleavage of the peptide extension may be caused by endoproteolytic action.

The location of the processing enzyme was shown to exist exclusively in the outer membrane fraction in the case of proalkaline phosphatase.[21] It has been shown that in eukaryotic[46,47] as well as in prokaryotic systems[75] the presence of membrane vesicles was absolutely required during protein synthesis in order for precursor proteins to be

processed. This implies that proper conformation of the precursor peptide extension and cleavage site in the membrane is required for processing to occur and proper insertion into the membrane occurs only when the synthesis takes place in the presence of membrane. However, in the case with proalkaline phosphatase it is not clear how the proper conformation of the peptide extension and cleavage site can occur with outer membrane fraction since, needless to say, proper insertion in vivo occurs in the cytoplasmic membrane. It should also be pointed out that the processing of proalkaline phosphatase to alkaline phosphatase in the presence of total envelope was very slow and even after 4 hr at 35° a substantial amount of unprocessed precursor was left in the reaction mixture.

The enzyme activity for processing appears to be latent in the eukaryotic system. It became active when rough microsomes were extracted by the detergent deoxycholate.[76] When the enzyme was extracted, it could cleave the peptide extension even after the precursor molecule was completed without the membrane fraction. It is also reported that processing of pro-f1 coat protein by the *E. coli* enzyme was strongly stimulated when the membrane fraction was present together with a nonionic detergent Nikkol.[75]

It should be noted that in eukaryotic systems, the processing enzyme shows a very wide range of species specificity; for example, microsomes from dog pancreas can cleave correctly precursors from many different animals,[44,46,47] and prepromelittin of honeybee can also be correctly processed in *Xenopus* oocytes.[41] This lack of species specificity indicates that the precise mechanism of processing (thus, translocation across the membrane) of secretory proteins is very well conserved during evolution.

Peptidase and proteases of *E. coli* have been described, [79] and recently two periplasmic enzymes aminoendopeptidase[79,80] and protease I[81] have been reported. The former enzyme was shown to be specific to L-alanine-p-nitroanilide, and to be inhibited by *N*-ethylmaleimide but not by EDTA and diisopropylphosphorylfluoride.[79] These enzymes are possibly required for digestion of exogenious nutrients but not for processing of secretory proteins.

B. Is Processing Required For Translocation?

As discussed earlier the amino terminal peptide extension or secreted protein precursors have a short lifetime with cleavage of the extended region occurring during or immediately after the membrane translocation process. From this point of view, it is an important question whether processing of the precursor protein (removal of the peptide extension) is coupled with and necessary for the translocation of the protein across the membrane or whether the two events occur independently. One way to answer this question is to accumulate the precursor form in the cell and examine its location as exterior or interior to the cytoplasmic membrane. This has been achieved by two independent means. First using phenethyl alcohol (PEA)-permeabilized cells, it is possible to specifically and reversibly accumulate promatrix protein.[19] Promatrix protein was subcellularly localized by a variety of methods. When trypsin was added to cells containing accumulated promatrix protein, promatrix protein was digested only in the absence of Mg^{++} under which condition trypsin penetrates the outer but not the cytoplasmic membrane. This result suggests that promatrix protein was located between the cytoplasmic and outer membranes. Promatrix protein was also solubilized from the envelope by sodium sarcosinate indicating it was not properly inserted into the outer membrane. This data raised the question of a cytoplasmic membrane location. However, when the cells were lysed with EDTA-lysozyme and the two membranes separated by sucrose density gradient centrifugation promatrix protein was only found in the outer membrane fraction. These results indicated that promatrix protein had a strong affinity to the outer membrane even if it were associated with the cytoplasmic

membrane and therefore remained with the outer membrane upon cytoplasmic-outer membrane separation. Unlike matrix protein, however, promatrix protein was released from this outer membrane fraction by 0.5*M* NaCl. The fact that promatrix protein was translocated across the cytoplasmic membrane in the absence of processing indicates that processing of the precursor is not required for translocation but rather is necessary for the proper assembly of the protein after the translocation step.

Secondly, using a mutant of prolipoprotein,[82,83] it was also shown that processing is not obligatory for translocation of the protein across the membrane. In this mutant, the glycine at the 14th position of proplipoprotein (position 7 in Table 1) was changed to an aspartic acid.[82] As a result, the prolipoprotein was not processed and was accumulated in these mutant cells. Upon separation of the outer and cytoplasmic membranes by sucrose density gradient centrifugation the mutant prolipoprotein was found to be distributed as 60% in the outer membrane fraction, 20% in the cytoplasmic membrane fraction, and 20% in the soluble cell fraction. This result suggests that prolipoprotein was translocated across the cytoplasmic membrane without being processed (see also Section VI.A).

In addition to the results above, processing of tolG protein was also inhibited by lowering lipid fluidity of the membrane. Under this condition pro tolG protein was accumulated and was localized similarly as promatrix protein as discussed above.[135] Based on results above, it is possible that there is an assembly intermediate of the outer membrane proteins which is still anchored on the outside surface of the cytoplasmic membrane by the peptide extension. At least in the case of promatrix protein, it probably also has some interaction with the outer membrane or the peptidoglycan. Hence, although processing of the precursor may occur during its synthesis under normal conditions, it is not an obligatory coupling to translocation across the membrane.

VI. DETERMINATION OF LOCATION IN THE ENVELOPE

A. Is the Peptide Extension Required for Localization?

As we have seen above, three types of proteins in *E. coli* are translocated at least in part across the cytoplasmic membrane: cytoplasmic membrane transmembrane proteins, periplasmic proteins, and outer membrane proteins. Although their final destinations are different, these proteins are produced from precursors containing hydrophobic amino-terminal peptide extensions (see Section III). What determines the final location of the secreted protein? It appears unlikely that the peptide extension is the determinant in localization of the final product since as discussed above the amino acid sequences of the peptide extensions for the three types of proteins do not vary significantly such that they can be classified into three distinct groups.

In order to investigate the question of secreted protein localization the PEA-permeabilized cell system was used.[19] As mentioned previously, PEA is soluble in water up to 2% and therefore the extent of PEA administration to cells is easily controlled. Therefore the effects of a range of PEA concentrations, from 0.3% to 1.0% PEA, on outer membrane protein synthesis was examined. At the higher PEA concentration processing of the membrane protein was inhibited. This inhibition occurred at different PEA concentrations for the different proteins, matrix protein being the most sensitive followed by tolG protein and lipoprotein being the most resistant. At lower concentrations of PEA a different effect on outer membrane protein assembly was observed. Although at 0.5% PEA processing of the precursor was not inhibited, properly assembled of the processed protein into the outer membrane did not occur (for tolG protein and lipoprotein). As a result, the processed protein was apparently accumulated in the periplasmic space as indicated by the fact that the processed tolG protein and lipoprotein were released from the cells by osmotic shock.

The inhibition of tolG protein assembly at 0.4% PEA was found to be reversible from the insertion of the processed protein into the outer membrane upon removal of PEA and regrowth of the cells in culture. This indicates that the periplasmic tolG protein is an intermediate form of the outer membrane protein which could be accumulated by PEA. This inhibition of assembly by PEA indicates that an additional step after processing of the precursor protein (removal of peptide extension) is required for proper assembly of the outer membrane protein into the outer membrane and that this step is PEA sensitive. In addition, this result implies that the peptide extension on the precursor is not required for assembly to the outer membrane but only for translocation across the cytoplasmic membrane. Last, ongoing synthesis of the protein is not required for its assembly into the outer membrane.

In the case of the mutant prolipoprotein[82,83] described in the previous section, the authors suggested that proteolytic cleavage of the peptide extension is not essential for the assembly of lipoprotein into the outer membrane, since 60% of the mutant prolipoprotein was found in the outer membrane fraction. However, the fact that the mutant cells have little or no lipoprotein bound to the peptidoglycan in contrast to the wild-type cells[84] suggests that the mutant prolipoprotein found in the outer membrane is not properly assembled. It is possible that the mutant prolipoprotein is simply inserted into the outer membrane by hydrophobic interaction between the lipid bilayer and the peptide extension, or by the lipoprotein interaction with the matrix protein.[107]

B. Genetic Approach

An elegant genetic experiment was designed to define the determinant(s) of localization of outer membrane proteins.[85] In this study, gene fusion between the *lacZ* gene for β-galactosidase, a cytoplasmic protein and the *lam*B gene for λ receptor, an outer membrane protein, was achieved which resulted in the formation of hybrid proteins comprised of an amino terminal sequence from λ receptor and the major portion of the carboxyl terminal sequence of β-galactosidase. The hybrid proteins were found to retain β-galactosidase activity. One (pop 3105) of such hybrid proteins which contained a very small portion of the amino terminal sequence of λ receptor was found in the cytoplasm as is normal β-galactosidase. On the other hand, in the case of a second hybrid protein (pop 3186) which contained a much larger portion of λ receptor, 40 to 70% of the β-galactosidase activity was found in the crude membrane fraction, and about 50% of the membrane-bound β-galactosidase activity was in the outer membrane fraction. These results are in contrast to a hybrid protein comprised of an amino terminal part of a maltose transport protein (*mal* F gene product; a cytoplasmic membrane protein) and a carboxyl terminal part of β-galactosidase.[86] In this case the β-galactosidase activity was found in the cytoplasmic membrane but not in the outer membrane fraction.

These results clearly indicate that an amino terminal segment of λ receptor is directing the hybrid protein into the outer membrane. The hybrid protein of strain pop 3186 was found to be larger by 10,000 to 15,000 daltons than β-galactosidase.[85] This indicates that the hybrid protein contains an amino terminal sequence of 90 to 140 amino acid residues out of a total of 500 amino acid residues of λ receptor. Therefore it is assumed that the information which is required for the determination of the final location in the outer membrane resides in this amino terminal fragment of λ receptor. It is possible that this amino terminal fragment has specific interaction with outer membrane components such as lipopolysaccharide, and such specific interaction might be required for protein localization in the outer membrane.

It should be pointed out that this gene fusion technique provides an excellent system to characterize not only the mechanism of protein localization in *E. coli* envelope but

also the molecular mechanism of translocation of secreted proteins across the cytoplasmic membrane. Since the growth of the hybrid strain pop 3186 is markedly inhibited by addition of inducer (maltose) to the culture, it is possible to isolate mutants which change the maltose sensitive to the maltose resistant phenotype.[72] Such mutants have been isolated and one of them has been shown to have a mutation in the region very close to the amino terminus, which resulted in failure to export the hybrid protein to the outer membrane and in retaining the hybrid protein precursor in the cytoplasm. In this mutant, it is most likely that the mutation caused the structural alteration in the peptide extension of the hybrid protein which did not allow the translocation of the protein across the cytoplasmic membrane. The determination of the exact change caused by the mutation will provide an important clue to the secretory mechanism.

C. Role of Processing in Assembly

An intriguing question is now, why is the peptide extension removed or is it necessary that the peptide extension be removed so that assembly may take place? It is suggested in the loop model (see Section III) that the peptide extension has specific interaction with the cytoplasmic membrane. If this were the case the outer membrane protein would have to be released from the cytoplasmic membrane by removal of the peptide extension in order that the protein proceed with the assembly process. However, if the processing of the precursor molecules is blocked by some means such as mutation (i.e., mutant prolipoprotein),[82-84] the precursor molecules may become outer-membrane bound without being processed. This may be caused if the specific interactions of the precursor molecules with an outer membrane component(s) are stronger than that of the peptide extension with the cytoplasmic membrane. However, since it has not been established whether the mutant prolipoprotein in the outer membrane is assembled in the normal fashion or simply bound to the outer membrane with use of the peptide extension, it is premature to conclude that the processing of the precursor molecules is not required for assembly of the outer membrane protein in the final location.

In the case of the periplasmic proteins, it is predicted that the precursor molecules would remain bound to the cytoplasmic membrane if the processing of the precursors is inhibited by some means. Suggestive evidence for accumulation of periplasmic proteins in the cytoplasmic membrane has also recently been presented.[87] In spheroplasts of *E. coli*, a smaller amount of protein is secreted concomitant with a larger proportion becoming membrane bound. By using antibodies directed against alkaline phosphatase, only 40% of the alkaline phosphatase was secreted by spheroplasts as opposed to 99.6% secretion of intact cells. Based on these results it was suggested that partial depletion of outer membrane-bound protease(s) involved in processing of precursors of secreted proteins was caused during spheroplast formation, resulting in the significant defect of processing of precursors which then remain membrane bound.

In the case of cytoplasmic transmembrane proteins, cleavage of the peptide extension would not necessarily be a prerequisite for assembly. As discussed in Section II, bacteriophage M13 coat protein could be synthesized as procoat protein in vitro in a coupled transcription-translocation system.[26] This procoat protein synthesis did not require membrane, and in a membrane-depleted extract the procoat protein initially folded into a water-soluble complex in contrast to the mature coat protein.[26] However, upon exposure to *E. coli* membrane vesicle or to liposomes prepared from *E. coli* lipids, the procoat protein was found to be inserted into the bilayer in an intergral fashion. Since the mature coat protein could not interact with the lipid vesicles, it was suggested that refolding of the procoat protein occurred as it interacted with lipids, and was inserted into the bilayer without protein receptors or channels. Although this result is very suggestive, it has not been shown that the procoat protein assembled in

the membrane vesicles is in the same proper conformation as the mature coat protein. Therefore, it is premature to conclude that the preformed precursor of the cytoplasmic transmembrane protein can be assembled in membrane vesicles. In this regard, it should be noted that in many cases the presence of membrane vesicles is absolutely required during protein synthesis in order for precursor proteins to be processed (see Section V).

D. Excreted Proteins

In addition to the envelope proteins discussed above, there exist a class of proteins in *E. coli* which have been thought to be excreted out of the cell into the external medium, such as collicins and toxins. However, it has been recently shown that more than 90% of the colicin E3 synthesized after induction of colicinogenic bacteria was cell bound, about half of it being salt-extractable state at the cell surface.[88] Their results suggest that secretion of colicin E3 into the culture medium had never occurred. It is interesting to note that they have isolated mutants which were defective in translocation of the colicin to the cell surface.[88] The biosynthesis and assembly of these proteins must share common characteristics with those of the envelope proteins since they must also be translocated across the cytoplasmic membrane. However, their assembly process may involve an additional translocation across the outer membrane to the cell exterior or cell surface. It has not as yet been determined whether these proteins are made from precursors similar to those of the other envelope proteins discussed above, and there is at present no information on the mode of excretion across the outer membrane. However, a system has recently been reported[89] in which mutants in production of plasmid coded *E. coli* enterotoxins can be isolated. It is hoped that this approach would be viable in determination of the modes of translocation and excretion of these proteins.

E. Involvement of Bayer's Junctions

Bayer has reported that in the *E. coli* envelope there exist sites of apparent adhesion or junctions between the cytoplasmic and the outer membranes as seen under the electron microscope.[90] It has been shown that capsular polysaccharides[91] and lipopolysaccharide[92] are inserted at these junctions to the outer membrane. It has recently been reported that the newly synthesized matrix proteins in *Salmonella typhimurium* can be found preferentially at these junctions.[93] Therefore it was suggested that matrix proteins are transferred from the cytoplasmic membrane to the outer membrane through these junctions. However, it has not been determined if the Bayer's junctions play a role in the assembly of other envelope proteins besides matrix protein.

F. Effects of Lipid Fluidity

An important consideration in the translocation and assembly mechanisms or processes is the fluid state of the cytoplasmic and outer membrane. By using an unsaturated fatty acid auxotroph the fatty acid compositions of the membrane lipids can be dictated. By growing the cells on specific unsaturated fatty acids the fluid state of the membrane can be controlled by altering the growth temperature. It has been shown that when the membrane of *E. coli* is in the crystalline state the induction of alkaline phosphatase is arrested.[94] When the membrane is allowed to return to the noncrystalline, or fluid state, induction of the enzyme can proceed. There are two possible explanations for these results: (a) alkaline phosphatase monomers are synthesized but their translocation or assembly is blocked, or (b) the actual synthesis of the monomers is arrested. In a recent study the latter possibility[6] was eliminated when it was found that at the crystalline state secretion of alkaline phosphatase was drastically decreased or

abolished depending upon the temperature, but that cross-reactive material with antialkaline phosphatase serum was found associated to the membrane fraction.[87] However, it was not determined if the membrane-bound form was unprocessed precursor or its precise location in the envelope.

Similar experiments were performed to examine the effects of membrane fluidity on the assembly of the cytoplasmic membrane and outer membrane proteins.[95] The results suggested that a fluid membrane is required for the normal assembly of the membrane proteins and that this requirement is more stringent for the outer membrane proteins than for the cytoplasmic membrane proteins. When the effects of the fluid state of the membrane on the assembly of the individual major outer membrane proteins were examined more carefully, there were remarkable differences among them.[135] Under conditions of a crystalline membrane state the assembly of the lipoprotein is hardly affected; however, the assembly of the matrix protein is completely inhibited. The tolG protein is synthesized, apparently, but not assembled when the membrane is in a crystalline state; it can be assembled upon subsequent shifting to conditions necessary to form a fluid membrane. The tolG protein synthesized under conditions of a crystalline membrane state, was found to be in the form of protolG protein. The tolG protein accumulated under these conditions was found to be loosely bound to the membrane fraction, and appears to be translocated across the cytoplasmic membrane. Therefore the change in the fluid state of the membrane seems to allow translocation of the protolG protein but not processing and assembly. The reasons for the differences in the effects of fluidity changes on the outer membrane proteins are not understood at the present time. It is important to find out at which step the assembly of the matrix protein is inhibited; at translation of the mRNA, at translocation across the cytoplasmic membrane, at processing of the precursor protein, or at the insertion of the matrix protein into the outer membrane.

It is quite interesting to note that the differential effects on outer membrane protein assembly by PEA[19] as described previously are remarkably similar to the effects of lipid fluidity above, with synthesis and assembly of matrix protein being the most easily affected followed by tolG protein and lipoprotein the least affected. In this regard it is interesting that PEA is structurally a type II local anesthetic. Since these compounds are known to increase the fluid state of the membrane, the effects of PEA on fluidity of *E. coli* envelope was examined by using 5-doxylstearate as an ESR probe.[19] It was found that PEA drastically increased the fluid state of the envelope membranes over the range at which it exerted its effects on outer membrane protein assembly. Therefore it appears that not only low but also high fluid states of the membrane cause similar inhibitory effects on the assembly of the outer membrane proteins. This indicates that a proper fluidity of the membrane is required to maintain the machinery for processing of precursor proteins and for transferring the outer membrane proteins from the cytoplasmic membrane to the outer membrane. It is possible that the change in lipid fluidity might cause severe effects on the structure and function of Bayer's junction, which then result in accumulation of precursors of the outer membrane proteins in the cytoplasmic membrane or the processed proteins in the periplasm.

G. Interaction with Other Components

The factors which would determine the localization of the outer membrane proteins should clearly involve the specific properties of the outer membrane proteins and their interactions with envelope components. Probably, the interactions which maintain the outer membrane protein assembly may be playing an important role in the localization of the protein to the outer membrane. Especially the interaction with the lipopolysaccharides is considered to be most important. If outer membrane proteins have specific

affinity to the lipopolysaccharides which are exclusively located in the outer leaflet of the outer membrane, newly formed outer membrane proteins can be easily self-assembled in the outer membrane. In fact, the self-assembly of the outer membrane protein with the lipopolysaccharides[96,97] and the specific interaction of outer membrane proteins with the lipopolysaccharides have been demonstrated.[97-101] Recently, Schweizer et al.[102] showed that the amino terminal moiety of tolG protein are active as phage receptors and protected from protease digestion in the presence of the lipopolysaccharides. These results indicate that the amino terminal portion of tolG protein has specific affinity to the lipid A part of the lipopolysaccharides. Beside the interaction between the lipopolysaccharides and the outer membrane proteins, a specific interaction between matrix protein and the peptidoglycan[103,104] and λ receptor and the peptidoglycan[105] have also been reported. Matrix protein appears to form a highly periodic array which exhibits threefold symmetry[105] and also has shown to interact with lipoprotein in the outer membrane.[107] However, the interaction between matrix protein and lipoprotein may not be essential for the determination of the final location of these proteins in the outer membrane, since mutants which lack one of these proteins have been isolated.[3]

H. Posttranslational Modification

Posttranslational modification may also play an important role in determination of the final localization of proteins. Among the *E. coli* membrane proteins, a most complex posttranslational modification can be seen in the lipoprotein of the outer membrane.[108] The posttranslational modification of lipoprotein may conceptually be divided into stages including five independent enzymatic reactions. The first stage, similar to the other secretory proteins, is the translocation and processing of the precursor. In the second stage the newly formed amino terminal cysteine residue is extensively modified. At least three independent reactions appear to be involved in modification of the cysteine residue. In one reaction, acylation of the free amino group occurs to yield an amide-linked fatty acid at this position. The other modification results in the attachment of a diglyceride group to the SH group of the amino terminal cysteine residue. The last stage of lipoprotein assembly involves the covalent attachment of one third of the lipoprotein molecules to the peptidoglycan. In this reaction the ξ-amino group of the carboxyl terminal lysine residue on lipoprotein is bound in peptide linkage to the carboxyl group of the diaminopimelic acid in the peptidoglycan.

In view of the extensive posttranslational modification of lipoprotein described above, it is of great interest which stages, if any, are involved in the assembly of lipoprotein into the outer membrane. As described above, the second stage of lipoprotein modification involves the modification by fatty acids. The fatty acids impart to lipoprotein a very high hydrophobic nature suggesting a role of this modification in the assembly of lipoprotein into the outer membrane. Clearly, the amide-linked fatty acid can only be attached after processing of the prolipoprotein (removal of the peptide extension). However, it is not known if the attachment of diglyceride occurs before or after processing. In the case of the prolipoprotein mutant[82] the accumulated mutant prolipoprotein lacks the covalent diglyceride. One line of data which suggests the involvement of the lipoprotein fatty acid modification may be essential for the proper lipoprotein assembly in the outer membrane comes from the PEA-permeabilized cell systems.[19] Assembly intermediates of tolG protein and lipoprotein may be accumulated by PEA as processed proteins in the periplasmic space. In the case of the periplasmic lipoprotein, it was found that the protein was sensitive to trypsin treatment.[19] As opposed to the case of the outer membrane lipoprotein. Since the trypsin resistance of lipoprotein is considered to be due to the presence of the covalently linked fatty acids,

the periplasmic lipoprotein, although the peptide extension was removed, must be lacking in one or more of these fatty acids. Complete analysis of the amino terminal structure of the periplasmic lipoprotein is believed to provide insights into the precise requirements of the fatty acid modifications for assembly of the lipoprotein.

The conversion to bound form is apparently not essential to the insertion of lipoprotein into the outer membrane, since free form is first inserted into the outer membrane and a part of free form is converted into bound form.

It has been reported that both matrix protein and tolG protein contain sugar. Matrix protein possibly contains 1 mol of glucosamine/mole,[109] whereas tolG protein contains 2 mol of reducing sugar/mole.[110] Besides this posttranslational modification by sugar, the existence of α-aminoadipic acid δ-semialdehyde (allysine) in matrix protein has been reported.[111] The positions of lysince residues converted to allysine was found to be nonspecific. The function of allysine residues in matrix protein is not understood at present, although it has been suggested that it may be involved in crosslinking between matrix protein with other outer membrane components.[111]

VII. REGULATION

A. Flexibility in Protein Composition

It is an intriguing question how the biosynthesis and assembly of membrane proteins are regulated. It is rather surprising that in the case of some outer membrane proteins only a few hundred molecules exist in a cell, whereas in the case of each of the major outer membrane proteins such as lipoprotein matrix protein, and tolG protein there are more than 10^5 molecules per cell.[3] It is also interesting to note that the protein composition of the outer membrane is flexible; when one or several proteins are lost or drastically reduced due to mutation there is a compensatory increase in one or several of the other proteins.[3] For instance, when matrix protein Ia is absent, the level of matrix protein Ib or both Ib and tolG proteins increases so that the total amount of outer membrane protein remained constant.[112] In a mutant strain lacking matrix protein, the amount of tolG proteins was approximately equal to the total amount of matrix and tolG proteins in the wild type strain.[113] Conversely, mutants lacking tolG protein partially compensated for this loss by producing increased amounts of matrix protein.[114] The reason for this flexibility in composition is unknown at present.

Another interesting aspect of the biosynthesis and assembly of the outer membrane proteins is that in a merodiploid strain for the structural gene of lipoprotein there is twice as much lipoprotein in the outer membrane as in a haploid strain,[115] whereas in a merodiploid strain for the tolG protein gene there is no apparent gene dosage effect when the amount of tolG protein in the outer membrane was examined.[116] The reason for the difference is also unknown at present; however, it may be due to the difference in the regulatory mechanisms of gene expression between the two proteins. Alternatively, the difference may be simply because the number of assembly sites for tolG protein is limited in contrast to lipoprotein. It should be noted that in a merodiploid strain of lipoprotein, the amount of the free form was doubled, whereas the amount of bound-form remained constant.[115] This suggests that the assembly sites for the bound-form lipoprotein are saturated in a haploid strain in contrast to the sites for the free-form lipoprotein.

The flexibility of the membrane-protein composition is also observed in those proteins which are inducible. For example, λ receptor protein is a minor protein in cells grown in the absence of maltose, whereas it becomes a major protein in the outer membrane upon induction of maltose.[117]

Another interesting fact to note is the control of gene expression of matrix protein. Recently this protein is shown to be produced from two independent genes which are located at 21 min (for matrix protein Ia) and 48 min (for matrix protein Ib), respectively, on the circular *Escherichia coli* chromosome map.[118] The relative amount of matrix proteins Ia and Ib vary greatly depending on growing conditions, especially when the composition of the growth medium is altered.[3] For instance, when *E. coli* K-12 cells were grown in the presence of glucose and a high concentration of sodium chloride, only matrix protein Ib was produced, whereas in nutrient broth with no fermentable carbon source, matrix protein Ia was almost completely missing from the outer membrane.[119]

Furthermore, a mutation on a third gene at 74 min (*omp* B) resulted in a complete elimination of both matrix proteins Ia and Ib.[3,120] Both matrix proteins Ia and Ib can also be replaced in strains lysogenic for the lambdoid phage PA-2 by a large amount of a new protein coded by the phage.[121] It is also interesting to note that in mutant strains which lack both matrix proteins Ia and Ib new proteins with similar properties to matrix proteins are produced.[122,123] The mechanism of this induction of new proteins is not known at present.

B. Cell Cycle Dependent Synthesis and Assembly

In addition to the regulatory aspects described in the preceding section, another type of control over the synthesis of envelope proteins has been reported. It has been found that certain envelope proteins are produced only at specific times during the cell cycle. In one instance it was reported that phosphatidylglycerol was metabolized in a stepwise manner at a specific stage in the division cycle approximately 20 to 30 min prior to cell division.[124] Coordinated with this metabolic cycle was the synthesis of certain cytoplasmic membrane proteins such as cytochrome b1. A temperature-sensitive mutant was subsequently isolated which could not produce these cell cycle specific envelope components at nonpermissive termperatures.[125,126] Thus, it was suggested that membrane proteins essential for cell division are under the strict control of a protein which is synthesized or activated 20 to 30 min before cell division.[125,126]

It has also been reported that many envelope proteins are synthesized in a stepwise manner early in the cell cycle, while phospholipid synthesis appears to be continuous.[127] A similar coordination of membrane protein synthesis with the cell cycle has been reported for lipoprotein,[128] λ receptor,[129] maltose binding protein,[130] and the β-methylgalactoside transport system.[131] It is also very interesting to note that some proteins such as λ receptor[129] and maltose binding protein[130] appear to be inserted in discrete sites in the cell envelope. These results may have important implications for the regulation of membrane biogenesis, including the secretion and assembly of envelope proteins. It is not known what is the role of the metabolic coordination of certain envelope components during the cell cycle. It is possible that at least some of these apparent regulatory events might be artifactually produced by the method of cell division synchronization. In addition, some envelope proteins such as lipoprotein demonstrate gene dosage effects and would thereby result in an increase in synthesis at the time the particular gene is duplicated during DNA replication.

C. Analysis of Base Sequences of Genes and mRNAs

A promising approach toward understanding some aspects of the regulation of envelope protein secretion and assembly is the analysis of the nucleotide sequences of the genes and the mRNAs for these proteins. For example, the lipoprotein mRNA has been purified[132] and the partial sequence has been determined.[15,133] It was found that the lipoprotein mRNA and the mRNA for the eukaryotic BMV coat protein share a

common 12 base sequence at the 5′ terminus, including the ribosomal binding site.[134] Another sequence at the 3′ end of the lipoprotein mRNA revealed a possible loop structure which may be involved in the high stability of the lipoprotein mRNA.[138] Further analysis of not only the lipoprotein mRNA sequence but also the sequence of mRNAs for other secreted proteins may shed additional light on the role(s) of possibly unique structures of the mRNAs for secreted proteins. In addition to mRNA sequences, the sequencing of the genes for secreted proteins, such as β-lactamase,[24] may reveal unique aspects important in the regulation of protein production.

VIII. CONCLUSION

We have seen that proteins which must traverse the cytoplasmic membrane (secreted proteins) seem to have at least one common feature. That is, they are made from precursors which contain an amino terminal peptide extension of about 18 to 23 residues in length. This was found to be true for outer membrane proteins, periplasmic proteins, and cytoplasmic membrane proteins which, at least in part, traverse the cytoplasmic membrane.

In addition, as pointed out in Section III, there would appear to be four unique features in the amino acid sequences of the peptide extensions regardless of the final location of the secreted proteins. The four rules for the structure of the peptide extension (Section III) were deduced from the four known amino acid sequences of the peptide extensions so far determined in the *E. coli* system. The rules were also applicable to the structure of many of the sequences of the peptide extensions of eukaryotic secretory proteins. The striking homology among the peptide extensions, regardless of the final location of the secreted proteins implies that all the peptide extensions share a common function in the assembly of the proteins, that is, translocation of the secretory protein across the cytoplasmic membrane. Thus, the loop model for translocation of precursor molecules across the membrane has been proposed to accommodate all four common features (see Figure 3A).

The purpose of the peptide extension is most likely only for translocation of the precursor molecules across the membrane but not for determination of the final location of the proteins in the *E. coli* envelope. As discussed in Section VI, at least for outer membrane proteins, the amino terminal section of the processed protein appears to recognize the final location. Figure 4 illustrates a possible mechanism of secretion and localization of the final location of secreted proteins in *E. coli*. In the cases of the three different envelope proteins, the initial step of secretion is assumed to proceed according to the loop model (see Figure 3A). The peptide extension is cleaved off and as the peptide elongates, the newly formed amino terminal region of the processed protein begins to form a secondary structure, thus making the translocation process irreversible.[3,28] If this newly formed amino terminal portion has a specific affinity to a component(s) of the outer membrane, this portion begins to be inserted and assembled into the outer membrane. In view of the fact[93] that the matrix protein appears to be inserted into the outer membrane through the sites of adhesion between the outer and cytoplasmic membrane (Bayer's junction), the newly formed amino terminal region would bind newly synthesized outer membrane components at Bayer's junction. This interaction then leads the protein into the outer membrane as the outer membrane component flows through Bayer's junction as shown in Figure 4A. The lipopolysaccharide, which is a specific component of the outer membrane, is the most likely component which is involved in this interaction as described in Section VI.G.

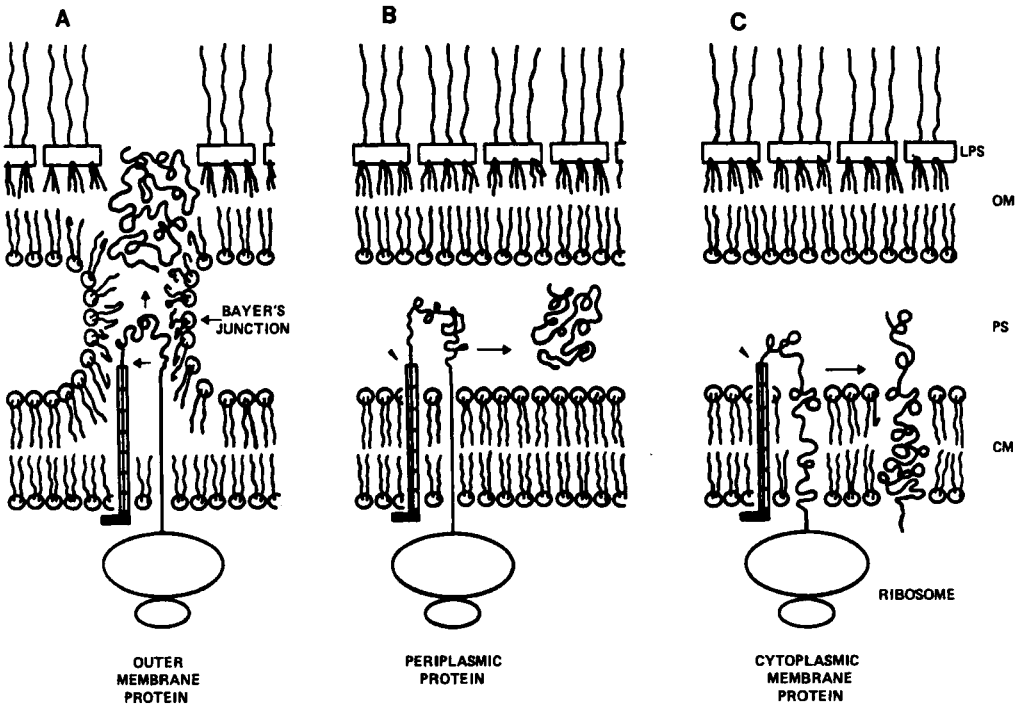

FIGURE 4. Mechanism of secretion and final localization of envelope proteins in *E. coli*. (A) Outer membrane proteins. When the cleavage site is exposed to the outside of the cytoplasmic membrane, the peptide extension is cleaved off before the completion of the chain. The resultant amino terminal part of the nascent peptide of the outer membrane protein begins folding, and then interacts specifically with an outer membrane component(s) such as the lipopolysaccharides in a junction between the cytoplasmic and outer membranes (Bayer's junction). (This specific interaction is not shown in the figure.) The interaction between the lipopolysaccharides and the amino terminal part of the outer membrane protein leads the outer membrane protein leads the outer membrane protein to the outer membrane structure through the Baye's junction. (B) Periplasmic proteins. When the cleavage site is exposed to the outside surface of the cytoplasmic membrane, the peptide extension is cleaved off and the resultant nascent peptide begins to fold. Because of hydrophilic property of the proteins, the part which extrudes to the outside of the cytoplasmic membrane remains soluble in the periplasmic space. (C) Cytoplasmic membrane proteins. After the peptide extension is cleaved off as in the case of the outer membrane proteins and periplasmic proteins. The new amino terminal part of the protein stays in the periplasmic space, while the other region is started to assemble in the lipid bilayer of the cytoplasmic membrane as the peptide is further synthesized because of the hydrophobic property of this portion of the protein. In this illustration a part of the carboxyl terminal portion of the protein as a transmembrane protein is exposed in the cytoplasm. Solid portions and the following thick portions indicate basic sections I (see Figure 2), and hydrophobic sections II plus section III, respectively. Large arrows show the sequence of assembly. LPS, lipopolysaccharides; OM, outer membrane; PS, periplasmic space; and CM, cytoplasmic membrane. The peptidoglycan layer is not illustrated between the outer membrane and the cytoplasmic membrane for simplicity.

In addition to the lipopolysaccharides, the peptidoglycan may also be an important component that is required for the assembly of the outer membrane proteins, since its specific interactions with matrix protein[103] and λ receptor[105] have been shown. Self-assembly of the matrix protein with the peptidoglycan has also been demonstrated.[104] Other possible components required for the assembly of the outer membrane proteins are the outer membrane proteins themselves.

It is also possible some proteins are translocated to the outer membrane through the Bayer's junctions, while other proteins can assemble into the outer membrane independent from the junctions. For example, the assembly of the lipoprotein has been shown to be very insensitive to PEA[19] as well as the low membrane fluidity.[135] Thus the lipoprotein may assemble into the outer membrane quite differently from the other outer membrane proteins. The extensive posttranslational modification of the lipoprotein (see Section VI.H.) may be playing an important role for the assembly.

As shown in Figure 4B and 4C, the final location for the periplasmic and cytoplasmic membrane proteins is possibly determined by the nature of the peptide which emerges through the cytoplasmic membrane. If the peptide is hydrophilic as a whole, the final product stays in the periplasmic space, whereas if the hydrophilic amino terminal region is followed by a very hydrophobic segment, this segment interacts strongly with the lipid bilayer of the cytoplasmic membrane and is forced to stay in the cytoplasmic membrane (Figure 4). If a carboxyl terminal part of this protein is hydrophilic, this portion remains in the cytoplasm. Thus this protein becomes a transmembrane protein. A typical example of transmembrane protein is glycophorin, a protein found in the human erythrocyte membrane.[134] The complete amino sequence of this protein shows that it consists of (a) a hydrophilic amino terminal section of 64 amino acid residues which is exposed to the outside of the cytoplasmic membrane, (b) a very hydrophobic section of 32 amino acid residues which is buried in the membrane, and (c) a hydrophilic section of 35 amino acid residues which remains in the cytoplasm. However, it has not yet been shown whether glycophorin is produced from a precursor having a peptide extension. It should be noted that not all of the cytoplasmic membrane proteins are necessarily produced from precursors since a protein without the peptide extension could be inserted into the cytoplasmic membrane because of its hydrophobicity.

On the basis of what we have seen in this review, we now can ask many more specific and important questions concerning the molecular mechanism of translocation of secreted proteins across the membrane and of the determination of the final location of the secreted proteins in the *E. coli* envelope.

1. Are there specific receptors or channels for the peptide extension?
2. Where and how is the peptide extension cleaved off?
3. What is the energy source for translocation of secreted proteins across the membrane?
4. What is the minimal requirement for the amino acid sequence of the peptide extension?
5. How is the secondary structure of the nascent peptide of secreted proteins kept on the ribosome until the peptide extension reaches the cytoplasmic membrane?
6. Is Bayer's junction actually playing an active role for transferring the outer membrane proteins from the cytoplasmic membrane to the outer membrane?
7. What are the specific interactions required for the membrane localizations of envelope proteins?

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