Assembly of Proteins into Membranes

William Wickner

Summary. Two pathways for protein assembly into biological membranes have been proposed. The "signal hypothesis" emphasizes the role of specific membrane proteins in binding the growing polypeptide and conducting it into the bilayer during its synthesis. The "membrane-triggered folding" hypothesis emphasizes self-assembly and the role of changing protein conformation during transfer from an aqueous compartment into a membrane. These ideas provide a framework for reviewing recent data on the biogenesis of membrane proteins.

The basic structural feature of a membrane protein is a hydrophobic surface which anchors it to the bilayer. This surface may be a long sequence of apolar amino acids, an apolar surface of an α-helix, or noncontiguous portions of the polypeptide chain which are brought together by the folding pattern of the protein. Membrane proteins also have polar surfaces that are exposed to water. Once a protein reaches its final conformation with respect to the membrane, its rate of spontaneous inside to outside rotation across the bilayer's hydrocarbon core is unmeasurably slow.

Each of these facts suggests specific questions about the assembly of proteins into membranes. These questions are listed in Table 1 along with the answers suggested by models or experiments (or both). Membrane proteins differ in their detail of assembly. However, specific models are useful guides to the design and interpretation of experiments. Two models have dominated thinking in this field. Many scientists have considered the insertion of proteins into membranes from the perspective of protein secretion by the rough endoplasmic reticulum (RER); in contrast, enzymologists who purify membrane proteins have thought about assembly as being guided by the structure and solubilities of each protein. The development of each of these schools of thought is discussed in this article.

Protein secretion. Ultrastructural studies by Palade (*1*) and others showed that secretory tissues such as pancreas and liver have an abundant RER (*2*). Secreted proteins first appear within the RER. They pass to the Golgi, secretion vesicles, and cell exterior by closed vesicles that bud from one organelle and fuse with the next. This pathway has gained widespread, although not complete (*3*), acceptance and has been well described (*1*). It fulfills a second function of conducting many integral membrane proteins from the RER to other organelles, such as the plasma membrane (*4*). This has led to the suggestion (*5*) that the same mechanism is used for inserting membrane proteins into the lipid phase of the RER as for transferring secreted proteins across it and into the lumen.

In support of this suggestion is the fact that the processes of protein secretion and protein assembly into membranes share several properties. Both involve transfer of at least part of a polypeptide chain across a bilayer. Both types of proteins frequently are made with an NH_2-terminal leader or signal sequence which is removed during, or very shortly before or after, transit across the bilayer. Both types of proteins often have unusual sensitivity to ribosomally directed antibiotics (*6*), suggesting that they are made at an unusual site within the cell.

There are, however, significant a priori differences between secretion and membrane assembly. Secreted proteins all go to only one place (outside the cell) and can therefore be made by a pathway without spatial branches, whereas hydrophobic proteins go to the many different membranes of a cell and require a spatially branching pathway. All secreted proteins cross the bilayer completely, whereas membrane proteins may assume many different topographies with respect to their membrane. Finally, many proteins are made in the cytoplasm and undergo "intracellular secretion" into a specific organelle such as the mitochondrion, where they are found in either a soluble compartment (matrix) or in a membrane within the organelle.

Despite these differences between secretion and membrane assembly, the development of data and ideas about secretion has influenced current thinking about membrane biogenesis. The isolation of RER as well as polysomes that were not attached to membranes allowed assay, in an in vitro translation reaction, of the proteins synthesized by each class of polysomes. Several groups of investigators (*7*) found that hepatocyte-secreted proteins, such as albumin or transferrin, were predominantly made on RER while cytoplasmic proteins such as ferritin were completed by unattached polysomes. Characterization of liver RER showed that release of the bound polysomes required both puromycin (which causes polypeptide chain release) and a high concentration of salt, presumably reflecting direct attachment by both the nascent polypeptide chains and by ribosome-membrane ionic interactions (*8*). The nascent polypeptide chains themselves were not susceptible to proteolysis, indicating a tight junction between ribosome and membrane with immediate extrusion of the new protein across the bilayer (*9*). Two polypeptides that might participate in such junctions have been identified in rough endoplasmic reticulum and are termed ribophorins (*10*). They span the RER membrane and are close to the bound ribosomes. Further studies may indicate whether they are part of a ribosome-binding site or even of a nascent polypeptide transport pore. Recent success in reconstituting RER from messenger RNA (mRNA), ribosomes, and ribosome-depleted membranes may aid in such studies (*11*). Recently, smooth endoplasmic reticulum, which has no ribophorin, has been reported to efficiently sequester and proteolytically process nascent secretory proteins (*12*).

The RER may synthesize cytoplasmic as well as secretory and membrane-bound proteins (*13*). In bacteria, several secreted and outer membrane proteins are made on membrane-attached polysomes, while unattached polysomes make some cytoplasmic proteins such as elongation factor Tu and plasma membrane proteins such as M13 virus coat protein (*14, 15*). Davis and colleagues (*16*) have shown that membrane-bound polysomes bear nascent polypeptides that cross the bilayer in 21 residues, which is too short for tertiary structure.

The author is an associate professor of biological chemistry and molecular biology at the University of California, Los Angeles 90024.

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Puromycin alone is sufficient to release the polysomes, an indication that there is no stable binding of ribosome to membrane and that the polypeptide chain is not being pushed through the bilayer by the force of chain elongation. These data suggest that new proteins often cross the bilayer during their synthesis, and that there is variation in the association between ribosome and membrane in different tissues and organisms.

The signal hypothesis. In 1972, Milstein *et al*. (*17*) detected a novel precursor form of immunoglobulin light chain in a reticulocyte lysate which was incubated with mouse myeloma mRNA. This precursor was approximately 1500 daltons larger than authentic light chain and was efficiently labeled by [^{35}S]-formylmethionine transfer RNA, indicating that it was the primary product of translation. When mouse myeloma RER was used instead of the mRNA, authentic-sized light chain was made, although polysomes derived from this RER made precursor. Peptide mapping showed that the precursor had an altered NH_2-terminus. Milstein *et al*. concluded that ". . . secretory proteins are generally thought to be synthesized on microsomes. The signaling device whereby this segregation is achieved is unknown. It seems to us that a short amino acid sequence at the N-terminus of a precursor protein would be a simple way to provide such a signal." Blobel and Dobberstein (*18*) confirmed and significantly extended these results. They showed that, when ribosome-free RER (stripped RER) was added to the protein synthesis reaction, authentic-sized light chain was produced. This light chain was sequestered inside these RER membranes where it was not accessible to added protease. When the stripped RER was added after protein synthesis was complete, neither proteolytic processing nor sequestration of the precursor occurred. These results were the basis of the signal hypothesis, which envisioned several discrete steps in protein secretion (see Fig. 1A) as follows. (i) Protein synthesis begins on unattached polysomes. (ii) Proteins destined for secretion would have an NH_2-terminal extra piece, the "signal sequence." This would of course be the first part of the protein to emerge from the ribosome. (iii) The signal sequence would bind to the RER by virtue of its hydrophobic sequence or by its

Table 1. Answers to the questions of membrane assembly.

Question	Answers	
	Signal hypothesis	Self-assembly models
What is the primary translation product?	The protein with an NH_2-terminal signal sequence	Either the same as the mature protein or the mature protein plus an NH_2-terminal leader sequence
What are the functional units of the protein and of the membrane which specify the insertion of the protein and its asymmetry?	Separate regions of the protein sequence, each acting at a different time and each recognized by a specific receptor: (i) signal sequence to initiate linear insertion; (ii) start and stop sequences for bilayer transit	Domains of the partially or fully folded protein, which may include several regions of the polypeptide which are not contiguous in the 1° sequence
How do hydrophobic proteins being made in an aqueous environment avoid aggregation?	Insertion during synthesis (membrane-bound polysomes) by means of topographic catalysis (a polypeptide transport pore)	Spontaneous insertion during synthesis (membrane-bound polysomes) Alternative conformation of initially water-soluble species made on unattached polysomes (i) 4° (multimer versus monomer); (ii) 2° or 3°; (iii) carrier protein
How is membrane choice made?	No clear data or hypothesis	No clear data or hypothesis
What is the mechanism of transit of polar regions of a protein across the lipid bilayer?	By proteinaceous pore	Spontaneous by the protein shielding its own polar groups as they cross; charge pairs, charge delocalization, nonionized forms of side chains Protonation or deprotonation of charged groups
What is the form of the integrating protein species?	A polypeptide chain, starting with an end, "threaded" through a pore in an extended form	Transfer of an α-helical polypeptide chain, starting with an end Loops or more complex 3° structures, with interactions between noncontiguous portions of the 1° sequence
What is the source of energy for driving assembly?	Polypeptide chain elongation	Protein/lipid interaction and added water/water interaction; "spontaneous" ±altered conformation ATP (but not for protein synthesis) or ~ P Electrochemical gradient Proteolysis, glycosylation, or other covalent modification of the part of the protein which has finished crossing the bilayer
What factors specify a protein's asymmetry across the plane of the bilayer?	Synthesis on only one side of the membrane	Synthesis on only one side of the membrane Preexisting asymmetries of the membrane: (i) lipid polar head group or fatty acyl chain; (ii) proteins to which the new protein might bind; (iii) electrochemical potential; (iv) physical properties of lipids including fluidity and lateral compressibility, in each half of the bilayer; (v) enzymes of covalent modification

recognition of an integral membrane receptor protein (or both). (iv) The receptor protein, possibly in conjunction with other membrane proteins, would form a protein "pore" around the leader sequence. (v) Noncovalent bonds would form directly between the pore and the ribosome. (vi) As polypeptide chain elongation continued, the protein would be extruded through the pore in an extended conformation. (vii) A protease on the noncytoplasmic face of the RER would remove the signal peptide. This hypothesis addresses each of the major questions in the field of protein secretion and membrane assembly and has been a great stimulus to research in this area. It is useful to realize that the signal hypothesis, as outlined above, is really a number of separable hypotheses, of which some (but not others) may apply to a particular protein. This model places great emphasis on topographic catalysis (that is, catalysis of reactions where substrates and products are distinguished by spatial coordinates instead of by covalent structure) rather than self-assembly.

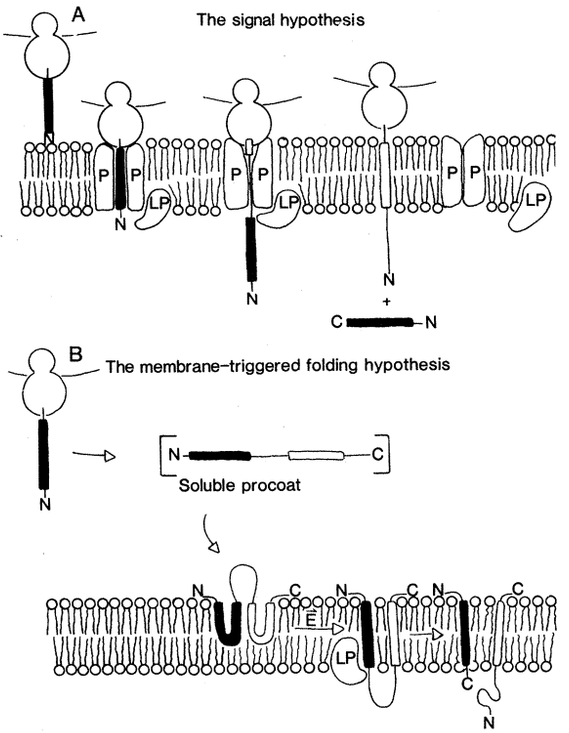

Fig. 1. Mechanisms proposed by (A) the signal hypothesis and (B) the membrane trigger hypothesis for the assembly of M13 procoat into the plasma membrane of *E. coli* and its proteolytic cleavage to coat protein. Procoat protein is 73 residues long; thick lines refer to hydrophobic sequences, thin lines to polar ones. The shaded thick line is the hydrophobic part of the leader sequence. See Fig. 2 for details of the coat and procoat structures. (A) *P*, pore protein; *LP*, leader peptidase. (B) Brackets indicate the unknown conformation of soluble procoat. An electrochemical gradient, *E*, is necessary for the transit of the acidic portion of procoat across the bilayer but may not be needed for the assembly of other membrane proteins. It is not known whether procoat first inserts into the bilayer and is then cleaved (as illustrated) or whether it is first cleaved and the coat protein then inserts.

During the last 5 years, extra NH_2-terminal sequences (leader peptides) of 15 to 30 residues have been found on a large number of secreted proteins synthesized in vitro (*19*), and their existence has also been inferred from DNA and RNA sequences (*20*). Leader peptides have a pattern of polar and often basic residues near the initiator methionine and near the site of cleavage with largely apolar residues in between, but they do not show true sequence conservation such as is seen when comparing the sequences of a specific enzyme isolated from several species (*21*). Even though secreted proteins from a very wide range of tissues and organisms have been examined, the addition of ribosome-free RER (stripped) from dog pancreas at the start of the protein synthetic reaction causes correct proteolytic cleavage, sequestration of the newly made protein within the vesicle lumen, and correct core glycosylation for many of these proteins. None of these reactions are seen when membranes are added after the protein synthesis is completed.

Despite this large body of evidence, no specific receptor or proteinaceous secretory pore has been identified (*22*). Furthermore, in most of the studies the microsomes used were prepared by one technique from dog pancreas (*19*); the absence of posttranslational activity might be due to the method of membrane isolation.

Recent studies have revealed unexpected requirements for secretion which possibly reflect a diversity of secretion pathways. Secretion of several bacterial proteins appears to require ongoing phospholipid synthesis (*23*). One of these proteins, alkaline phosphatase, also requires a fluid membrane phase for secretion (*24*). Ito *et al.* (*25*) studied bacterial secretion by brief labeling of cells with radioactive amino acids and then following the appearance of newly made protein in the cytoplasm, plasma membrane, periplasm, and outer membrane. They found that the labeling of the periplasm was very delayed. Several secreted bacterial proteins, protein S of *Myxococcus xanthus* (*26*), the maltose-binding protein, β-lactamase, and cloacin DF13 of *Escherichia coli* (*27*), are initially synthesized as soluble cytoplasmic proteins and only later secreted across the cell membrane. A growing number of studies of "intracellular secretion" across the limiting membranes of organelles and into their matrix or internal membranes have established a clearly posttranslational mechanism. The small subunit of ribulosebisphosphate carboxylase is made on unattached polysomes and is initially a soluble cytoplasmic preprotein; it then enters the chloroplast, is proteolytically processed, and combines with the large subunit (*28*). A similar pathway has been found for many mitochondrial proteins, including carbamyl phosphate synthetase (*29*), ornithine transcarbamylase (*30*), cytochrome c peroxidase (*31*), and several proteins of the inner membrane (discussed below). Neupert and colleagues (*32*) have taken a very general approach through brief labeling of *Neurospora* mitochondria with radioactive amino acids. They found that the matrix proteins which are of cytoplasmic origin enter the mitochondrion posttranslationally. The peroxisomal enzymes catalase and uricase are also made on unattached polysomes and posttranslationally enter the organelle (*33*).

Ovalbumin, a major secretory product of the chicken oviduct, is made without a transient NH_2-terminal leader peptide (*34*). Ovalbumin fused to the polar and acidic NH_2-terminus of *E. coli* β-galactosidase has been shown to be efficiently secreted by this organism (*35*). Thus, a leader (signal) sequence appears to not be essential to the secretion of this protein. It has been reported that ovalbumin has a central region which, when isolated as a tryptic peptide, fulfills the signal function (*36*). However, several aspects of this work (*37*) raise doubts about whether the conclusions that were drawn are justified by the data presented.

Secretion has now been studied by genetic means. The gene for *E. coli* β-galactosidase, a soluble enzyme, has recently been fused (*38*) with genes of the maltose operon that code for outer membrane, periplasmic, or inner membrane proteins. It was found that (i) an altered leader sequence can alter protein localization, (ii) the addition of a leader sequence alone is not sufficient to change the localization of β-galactosidase (*39*), and (iii) the fusion of β-galactosidase to a substantial portion of an inner membrane or periplasmic protein causes the protein to be bound to the inner membrane,

while the fusion product between β-galactosidase and an outer membrane protein is found in both membranes of the cell and in the cytoplasm. Thus, the leader sequence is not the only part of the protein that influences localization. This same conclusion is supported by studies of the periplasmic protein β-lactamase. Koshland and Botstein (*40*) have found that amber mutations near the COOH-terminus of this protein caused localization of the enzyme to the cytoplasm rather than the periplasm. The leader peptide was accurately removed in these strains.

These examples illustrate the diversity of pathways by which proteins entirely cross a membrane. A unifying hypothesis is nevertheless useful to suggest experiments and stimulate investigation; the signal hypothesis has clearly succeeded admirably in this regard. The proposed protein transport pore is the most difficult part of the signal hypothesis to evaluate; nevertheless, the signal hypothesis has stimulated the search for such a protein.

Signal hypothesis of membrane assembly. The signal hypothesis has been adapted to integral membrane proteins. Rothman and Lenard (*5*) have proposed that such proteins undergo cotranslational extrusion through a protein pore in the same fashion as that proposed for secreted proteins. Steiner and colleagues (*41*) have suggested that the nascent chain has a specific loop conformation as it passes through this protein pore. Blobel (*42*) has proposed that a long sequence of hydrophobic residues, such as spans the bilayer in several membrane proteins, would function as a "stop transfer" sequence. This would release the newly made protein into the membrane with the COOH-terminus exposed to the cytoplasm and the NH_2-terminus on the opposite membrane face. This model is supported by in vitro studies of histocompatability antigen (*43*) and of the capsid proteins of lipid-coated vesicular stomatitis virus (*44*) and Semliki forest virus (*45*). In each case, the preprotein was synthesized in a cell-free reaction. When ribosome-free RER was present throughout the reaction, each protein was properly inserted into the membrane, proteolytically processed, and glycosylated. These reactions were not seen when stripped RER was added after the protein synthesis reaction was complete, suggesting that assembly into the membrane was cotranslational in this in vitro reaction and, presumably, in vivo.

Membrane enzymology. A second, almost entirely separate perspective on membrane assembly has developed during the last 10 years. This perspective, termed the hypothesis of membrane-triggered folding (*46*), emphasizes self-assembly and the folding properties of proteins as they pass from cytoplasm to membrane. Enzymologists have now isolated a variety of membrane proteins and have found that their salient common feature is insolubility in water. Many membrane proteins can only be extracted by detergents and require detergents for their dispersal during isolation. As summarized by Helenius and Simons (*47*), detergents provide membrane proteins with a microenvironment that mimics the lipid bilayer in having both polar and apolar regions.

The inherent water insolubility of integral membrane proteins derives more from their conformations than from their amino acid compositions or primary sequences. Capaldi and Vanderkooi (*48*) cataloged the amino acid compositions of 224 soluble and membrane proteins and found a broad overlap of their polarities. Sequences of approximately 20 hydrophobic amino acid residues are present in several integral membrane proteins. However, such hydrophobic sequences are also present in soluble proteins (*49*), where the central regions of the folded protein are quite apolar. The main distinction between soluble and membrane proteins appears to be less a question of hydrophobic sequences than one of hydrophobic surfaces of the fully folded protein. Helenius and Simons (*50*) and Clarke (*51*) showed that detergents such as Triton X100 or deoxycholate bind to integral membrane proteins but not to soluble proteins. When membrane proteins are freed of both detergent and lipids, their exposed hydrophobic surfaces cause aggregation and often denaturation. This appears to be the sole common trait of membrane proteins and therefore one that is likely to carry at least part of the information for their localization to cellular membranes.

The earliest studies of hydrophobic membrane segments focused on small, abundant proteins that could be readily isolated. The major coat protein of coliphage M13 spans the host cell cytoplasmic membrane prior to its assembly onto extruding virus (*52*). Its amino acid sequence, determined in 1969 (*53*), before it was known to be a membrane protein (*54*), has a hydrophobic central region of 20 residues, which spans the bilayer. Similarly, glycophorin spans the human erythrocyte membrane with 23 apolar residues (*55*). This type of segment would be consistent with a "stop transfer" sequence as envisioned in the signal hypothesis. However, there are far more complex intramembranous segments. Every third to fourth residue of the *E. coli* outer membrane lipoprotein is hydrophobic, suggesting an α-helical structure with an apolar face (*56*). Self-association of lipoprotein would then lead to an oligomeric structure with a hydrophobic exterior in contact with the membrane lipids. The most complex membrane protein for which detailed structural information is available is bacteriorhodopsin, a light-harvesting protein of *Halobacterium halobium*. Bacteriorhodopsin is a 27,000-dalton polypeptide that crosses the membrane seven times as a folded α-helical rod (*57*), and amino acid sequence (*58*) and topographic mapping studies have led to a three-dimensional model (*59*). While the protein is hydrophobic in overall composition, charged and polar residues are found in the intramembrane regions. The model of bacteriorhodopsin suggests that polar residues are oriented toward the center of the protein and that the exterior is entirely apolar. The common theme is therefore not a long sequence of apolar amino acids but rather an apolar "face" that can be turned to the lipid fatty acyl chains. This idea is supported by the amino acid sequences of protein II* (*60*) and protein I of the *E. coli* outer membrane (*61*). Protein I is a 37,200-dalton polypeptide that forms pores in natural or synthetic membranes (*62*). This function strongly suggests that it spans the bilayer, yet its amino acid sequence shows no runs of apolar amino acids longer than four. Protein I is also found in the periplasm, and in this form can spontaneously integrate into membranes and form pores (*63*). This observation is especially important for our concept of assembly, in that it indicates that the same protein can either be soluble or expose its apolar residues and integrate into a bilayer.

Many membrane polypeptides cross the bilayer several times. This pattern is difficult to reconcile with a threading-through-a-pore model. Bacteriorhodopsin and protein I, discussed above, are two examples of such complex folding. Band III protein, the anion transport channel of human erythrocytes, also crosses the bilayer at least three times (*64*). All transport proteins may either be oligomeric or span the bilayer several times in order to shield a polar transport region from the fatty acyl phase.

Most membrane proteins cannot rapidly tumble across the plane of the bilayer once they have achieved their mature conformation. Furthermore, all the copies of each particular protein share a common asymmetric distribution across the membrane. Mapping studies have shown that human erythrocyte gly-

cophorin (*55*), coliphage M13 coat protein (*52*), murine histocompatibility (H-2) antigen (*65*), and vesicular stomatitis virus G protein (*44*) have their COOH-terminus on the cytoplasmic membrane surface and their NH_2-terminus on the opposite side, as predicted by the signal hypothesis model of cotranslational extrusion. However, band III protein (*64*), isomaltase (*66*), and aminopeptidase (*67*) have their NH_2-termini on the cytoplasmic membrane face and their COOH-termini exposed on the opposite side. Although there is not a large number of proteins with known sidedness of their NH_2- and COOH-termini, there does not yet seem to be a uniform rule. One protein, 5′-nucleotidase, appears to even reorient after its insertion into the membrane is completed (*68*).

An early observation that led to the signal hypothesis was the presence of transient NH_2-terminal leader peptides on secreted proteins. Many integral membrane proteins also have transient leader sequences: virus capsid proteins—such as M13 coat protein (*69*), vesicular stomatitis virus (VSV) G protein (*70*), and Semliki forest virus (SFV) p62 and E1 proteins (*45*)—proteins of the *E. coli* outer membrane [lipoprotein (*71*), λ receptor (*72*), matrix protein (*73*), tolG protein (*73*), membrane protein a (*74*)], chloroplast thylakoid membrane proteins (*75*), and mitochondrial proteins—*Neurospora* proteolipid (*76*), subunit 2 of cytochrome oxidase (*77*), and subunits α, β, and γ of yeast ATPase (*2, 78*). However, there are even more examples of membrane proteins that are not made with leader sequences, including cytochrome P-450 (*79*), cytobrome b_5, and NADH cytochrome b_5 reductase (*80*), opsin (*81*), Sindbis virus protein PE2 (*82*), several membrane proteins of *E. coli* [lactose permease (*83*), proteins coded by *che*M and *mot*B (*84*), D-lactate dehydrogenase (*85*), and ten membrane proteins coded by the F-factor (*86*)], mitochondrial membrane proteins [cytochrome c (*87*), ATP/ADP transport protein (*88*), and subunits 1 and 3 of cytochrome oxidase (*89*)], lens membrane protein MP26 (*90*), and proteolipids (*91*) (of yeast, *E. coli*, spinach chloroplasts, and bacterium PS-3).

Do proteins enter the membrane during their synthesis (cotranslational assembly) or afterward (posttranslational assembly)? There is strong evidence from cell-free studies that VSV G protein (*44*), SFV capsid proteins (*45*), and histocompatibility antigen (*43*) must begin membrane assembly well before their synthesis is completed. While this probably faithfully reflects the timing of events in vivo, there is also a possibility that the dog pancreas microsomes used in each of these experiments had lost their natural capacity to support posttranslational assembly. Many proteins assemble into membranes posttranslationally. Ito *et al.* (*25*) showed that there is a dramatic delay in newly made *E. coli* outer membrane or periplasmic proteins reaching their final compartment. The degree of delay varied with the specific outer membrane protein (being far greater for protein I than for protein II*) and was affected by membrane fluidity. Several groups of investigators, including Neupert and colleagues (*32*), have shown that most mitochondrial proteins are initially made as soluble cytoplasmic precursors. This has been specifically shown for cytochrome c_1 (*92*), cytochrome c oxidase (*32, 93*), the ATP/ADP transport protein—atractyloside binding protein (*32*)—and several subunits of the F_1 ATPase (*78*). High-energy phosphate is needed for the assembly of ATPase, cytochrome bc_1, and cytochrome c_1 polypeptides (*94*), while an uncoupler-sensitive electrochemical potential is needed for the assembly of mitochondrial ATP/ADP transporter (*32*) and M13 coat protein (*95*) into their respective membranes. Chloroplast thylakoid membrane proteins assemble posttransla-

Table 2. Classification of membrane proteins according to their relation to assembly questions. See the text for discussion and literature references for each protein.

Membrane proteins with leader sequences
- M13 coat protein
- VSV G protein
- *E. coli*: lipoprotein, matrix protein, λ receptor, tolG protein, protein A
- Mitochondria
  - Cytochrome oxidase subunit 1
  - *Neurospora* proteolipid
  - ATPase subunits (α, β, and γ)
  - Cytochrome c, subunit 5
- Chloroplast proteins 15 and 16

Membrane proteins without leader sequences
- Cytochrome P-450
- Opsin
- Sindbis virus protein PE2 and Semliki forest virus pE2 and E1
- *E. coli* membrane proteins
  - Lactose permease (M protein)
  - *che*M and *mot*B protein
  - Ten F-factor coded proteins
  - D-Lactate dehydrogenase
- Mitochondrial proteins
  - Cytochrome c
  - ATP/ADP transport protein
  - Cytochrome oxidase subunits 1 and 3
- Proteolipids of yeast, spinach chloroplast, *E. coli*, and bacterium PS-3
- Lens protein MP26
- NADH cytochrome b_5 reductase
- Cytochrome b_5

Considerations of structural complexity
- Band III protein (erythrocyte)
- Rhodopsin
- Bacteriorhodopsin
- *E. coli*
  - Porin
  - Lipoprotein

Orientation of termini
- N in, C out
  - Band III
  - Sucrase
  - Aminopeptidase
- N out, C in
  - Glycophorin
  - M13 coat protein
  - VSV G protein
  - Histocompatibility antigens
  - Sindbis virus glycoproteins
  - Influenza HA glycoproteins

Orientation independent of side of synthesis
- Proteolipid (*N. crassa* versus yeast)
- Small subunit of ribulose bis-P carboxylase (chloroplasts versus blue-green algae)

Timing of insertion
- Cotranslational
  - VSV G protein
  - SFV capsid proteins
  - Sarcoplasmic reticulum ATPase (glycosylated subunit)
  - Band III
  - Cytochrome P
- α-Glucosidase
- λ-Receptor
  - Cytochrome P-450
  - NADH cytochrome P-450 reductase
- Posttranslational
  - M13 procoat
  - *E. coli* porin
  - Sarcoplasmic reticulum ATPase (catalytic subunit)
  - Cytochrome b_5
  - Cytochrome b_5 reductase
  - Mitochondrial proteins
    - Cytochrome c
    - Cytochrome c peroxidase
    - ATP/ADP transporter
    - Subunits IV–VII of cytochrome oxidase
    - Nitrate reductase
    - F_1 ATPase subunits
    - Cytochrome bc_1
    - Succinate dehydrogenase
  - Sucrase
  - Lens protein MP 26
  - Glyoxosomal malate synthase
  - Cytochrome b_5
  - NADH cytochrome b_5 reductase
  - Chloroplast proteins 15 and 16

Spontaneous insertion without topographic catalysis
- Toxic or lytic proteins
  - Melittin
  - Complement
  - α-Toxin
  - Alamethicin
  - Gramicidin
  - Cholera toxin
  - Diphtheria toxin
  - Colicins Ia, E1, and K
- Purified membrane proteins
  - Cytochrome oxidase
  - Dicarboxylic acid transport proteins
  - Pyruvate oxidase
  - Malate oxidase
  - Protein II*
  - D-β-Hydroxybutyrate apodehydrogenase
  - Glycerol 3-P dehydrogenase
  - Cytochrome b_5 reductase
  - D-Lactate dehydrogenase

tionally (*75*). Posttranslational insertion into cytoplasmic membranes has been documented for a variety of proteins ranging from prokaryotic M13 coat protein (*95*), nitrate reductase (*96*), and succinate dehydrogenase (*97*) to intestinal epithelial sucrase (*98*) and lens protein MP26 (*90*). Malate synthase assembles posttranslationally into glyoxysomal membranes (*99*). Cytochrome b_5 and NADH cytochrome b_5 reductase assemble posttranslationally into microsomal membranes (*80*).

It is certainly difficult to prove that any assembly event is not catalyzed in vivo. However, a wide variety of proteins and peptides will assemble into biomembranes or liposomes without topographic catalysis. Many of these proteins are toxic or lytic agents, such as bee venom melittin (*100*), serum complement (*101*), the oligopeptide antibiotics alamethicin (*102*), valinomycin (*103*), and gramicidin (*103*), and the bacterial toxins such as α-toxin (*104*), cholera toxin (*105*), diphtheria toxin (*106*), and colicins Ia, El, and L (*107*). Many purified membrane proteins, such as cytochrome oxidase (*108*), D-β-hydroxybutyrate dehydrogenase (*109*), glycerol-3-phosphate dehydrogenase (*110*), cytochrome b_5 and its reductase (*111*), galactosyl transferase (*112*), D-lactate dehydrogenase (*113*), and protein II* (*114*) assume a water-soluble conformation when they are freed of detergent and will then spontaneously insert into bilayers. Both *E. coli* pyruvate oxidase and malate oxidase are integral membrane proteins in the presence of their substrates and cofactors but are fully soluble in water in their absence (*115*). The *E. coli* dicarboxylic acid transport proteins have been purified and shown to spontaneously reconstitute into liposomes, *E. coli* membranes, or even animal cell plasma membrane (*116*). The precursor form of M13 coat protein, termed procoat, will assemble into protein-free liposomes (*117*). Each of these integration events may be accompanied by a change in conformation or in the protein's multimeric state. Such changes have been documented for several of these proteins (*118*) as well as for model peptides with limited solubility in both fatty acyl phases and water (*119*). These studies of membrane proteins are summarized in Table 2.

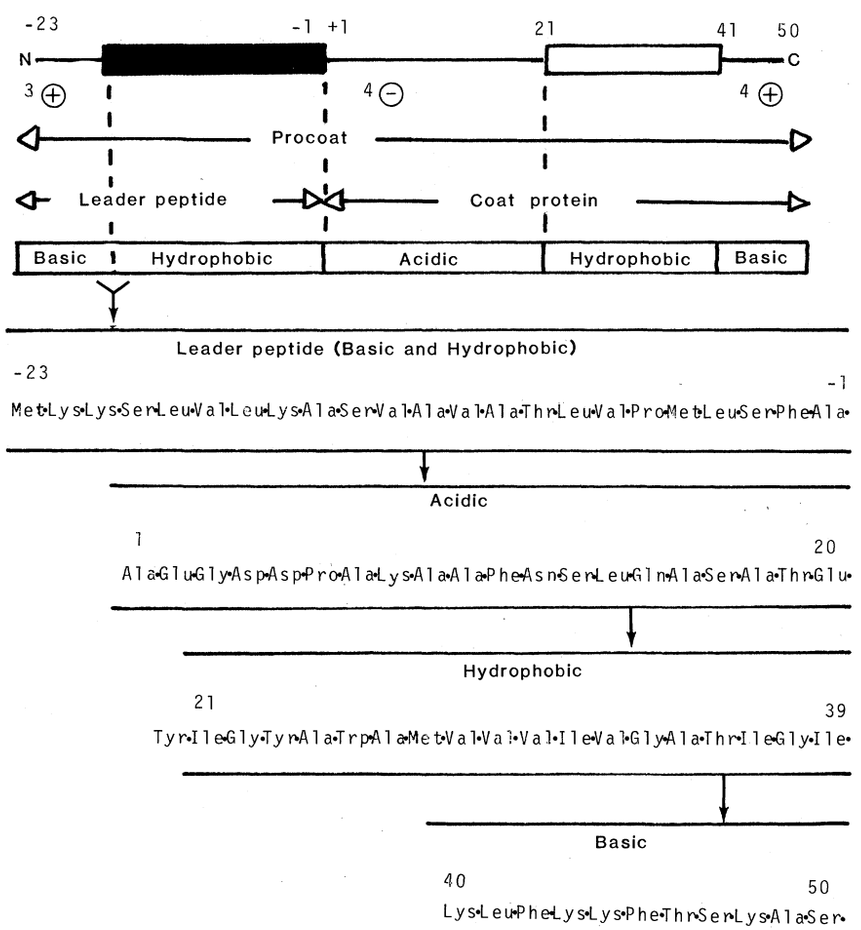

Fig. 2. Structure and sequence of M13 procoat. Abbrevations for the amino acid residues are: Ala, alanine; Arg, arginine; Asn, asparagine; Asp, aspartic acid; Gly, glycine; Glu, glutamic acid; Ile, isoleucine; Leu, leucine; Lys, lysine; Met, methionine; Phe, phenylalanine; Pro, proline; Ser, serine; Thr, threonine; Tyr, tyrosine; Val, valine.

Self-assembly models. Membrane proteins have diverse answers to the questions of assembly: some have simple intramembrane segments of entirely apolar amino acids, others have intramembrane segments with apolar surfaces of noncontiguous portions of the polypeptide chain. Some have their NH_2-terminus "out" and their COOH-terminus "in," others have the opposite orientation. Some span the bilayer once, others span the bilayer several times or are only anchored to the membrane by a short hydrophobic region. Some are synthesized with an NH_2-terminal transient leader segment, others without one. Finally, some proteins assemble into the membrane during their synthesis while others do so afterward.

One property which is characteristic of all integral membrane proteins is an apolar surface that is in contact with the fatty acyl chains of membrane lipid. Tanford has explored the hydrophobic effect in biological systems and has suggested that it is a major driving force in the folding of proteins, in the assembly of organelles ranging from ribosomes to membranes, and in the assembly of proteins into membranes (*120*). Although these concepts of the stability of hydrophobic proteins in membranes are fundamental, nevertheless, it is important to seek specific pathways for assembly that are kinetically sufficient for rapidly growing systems. Bretscher (*121*) in 1973 suggested that membrane proteins are synthesized in the cytoplasm and "whether a cytoplasmically synthesized protein remains as a soluble component, or partially dissolves in the inner surface of the bilayer, or dissolves in the membrane so that it traverses the bilayer is determined by the nature of the protein."

I have recently proposed (*46*) that membrane assembly is an integral part of the folding pathway of certain proteins as they encounter an amphipathic surface. Assembly can be initiated during or shortly after polypeptide synthesis and does not require a pore (topographic catalysis). Leader peptides, when present, might suitably alter the folding pathway and, upon their proteolytic removal, render it irreversible. Some proteins (especially larger ones that take longer to synthesize) may begin assembly during synthesis and others after their synthesis is complete. The latter may initially be led to a conformation without hydrophobic faces by their entirely aqueous environment. Upon encountering a membrane, these proteins may be triggered to refold (in secondary, tertiary, or quaternary structure, or a combination thereof) by the availability of the hydrocarbon core of the bilayer. The transit of

basic and acidic residues across the membrane may occur as charge-neutral pairs or after their deprotonation or protonation to the uncharged species (*122*). During the assembly event, polar portions of the protein may be shielded from the fatty acyl chains by more apolar residues. This proposal is illustrated in Fig. 1B and is termed the "hypothesis of membrane-triggered folding" or, for short, membrane trigger hypothesis.

Two models that suggest specific protein conformations during self-assembly into the membrane have been described. The "direct transfer model" of von Heijne and Blomberg (*123*) suggests that the assembly of proteins into a membrane or the transfer of secreted proteins across a bilayer is explained by the energetics of partitioning of the amino acid side chains between water and lipid, assuming a common α-helical conformation during these processes. Their quantitative treatment of the energetics of assembly has the virtues of predictive value and testability. Inouye (*124*) has proposed that the first few residues of the leader sequence, which are often basic, bind to the acidic phospholipid surface. As polypeptide chain longation continues, the chain forms a loop that extrudes through the bilayer without the aid of topographic catalysis. He has called this idea the loop model. While each of these suggestions of conformation during assembly may apply to specific proteins, it seems likely that the folding pathways of membrane proteins will prove as diverse as the proteins themselves.

Membrane Assembly of M13 Coat Protein

The major coat protein (gene 8 product) of coliphage M13 has been studied by proponents of several hypotheses and thus offers a useful object for comparison (*125*). Coat protein spans the plasma membrane of infected cells prior to its association with the extruding virus DNA (*52, 54*). It is made as a precursor, termed procoat, with an extra 23 amino acid residues on the NH_2-terminus (*69*). The sequence of procoat (Fig. 2) has been independently determined by DNA, RNA, and protein sequencing techniques.

Procoat synthesis and metabolism has been studied in intact M13-infected cells (*95*). Procoat protein is only made by polysomes that are not attached to membranes and procoat in short-term labeling experiments is initially found in the soluble fraction. It rapidly moves to the inner surface of the plasma membrane. Procoat then integrates into the membrane in a reaction which requires the transmembrane electrochemical potential. Finally, it is cleaned by a protease, becoming coat protein plus leader peptide. These in vivo studies (*95*) have established that posttranslational assembly is the physiological pathway.

Procoat protein is also a major synthetic product of cell-free protein synthesis reactions directed by M13 DNA or polysomes (*117, 126, 127*). One analysis of cell-free synthesis revealed that newly made procoat is a soluble 5*S* (presumably oligomeric) species that will posttranslationally assemble into large *E. coli* membrane vesicles or even large protein-free liposomes (*117*). Another series of experiments employed small, inverted *E. coli* membrane vesicles in this same M13 DNA-directed protein synthesis system (*126*). Procoat was processed to coat when these membranes were present during protein synthesis, but not when they were added 1 hour later. This lack of posttranslational processing is only seen after prolonged incubation; procoat assembles into added membranes for several minutes after its synthesis is complete (*128*). Thus, failure to obtain posttranslational processing in a particular set of experiments does not prove that posttranslational processing cannot occur at all.

Figure 1 shows the assembly pathways envisioned by each model, illustrated for the M13 coat protein. The signal hypothesis (Fig. 1A) suggests that proteins assemble into membranes by cotranslational extrusion through a proteinaceous pore, while the membrane trigger hypothesis (Fig. 1B) suggests that the specific conformations of different proteins during assembly will be as varied as their conformations after assembly.

Conclusion

Proteins which initially insert into the membrane of the RER fulfill many predictions of the signal hypothesis, whereas the membrane proteins of other organelles do not. It is now clear that there is no single answer to each question of how proteins assemble into membranes. Some of these questions and the suggested answers are outlined in Table 1. If the success or failure of a hypothesis can be judged by the interesting experiments it has suggested, then each school of thought has achieved a great measure of success. The central question in membrane assembly and protein secretion is whether there is topographic catalysis; that is, whether proteins are conducted into the bilayer or across it by a porelike transport system.

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