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# Procoat, the precursor of M13 coat protein, requires an electrochemical potential for membrane insertion

(membrane assembly/uncouplers/membrane trigger hypothesis)

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ABSTRACT **The coat protein of coliphage M13 spans the host cell cytoplasmic membrane prior to its assembly into extruding virus. It is made as a soluble cytoplasmic precursor, termed "procoat," with 23 extra amino acid residues at the $NH_2$ terminus. Procoat binds to the cell membrane and is converted proteolytically to coat protein. When the electrochemical gradient of an infected cell is rapidly dissipated by uncouplers, procoat still binds to the plasma membrane but is not converted to coat. We report here that membrane-bound procoat is only detected at the inner face of the cytoplasmic membrane and that uncouplers prevent it from integrating into a transmembrane conformation.**

How are water-insoluble integral membrane proteins made by the water-soluble protein synthetic machinery of the cell? How is the synthesis of a membrane protein related to its insertion into the bilayer? To explore these problems, we have studied the biosynthesis of the major coat protein (gene 8 product) of coliphage M13. Its precursor form, termed "procoat," has an additional 23-residue leader peptide on its $NH_2$ terminus. It is synthesized by polysomes which are not membrane-bound (1). Procoat is initially found as a soluble, oligomeric protein (1, 2) which rapidly binds to the host cell plasma membrane and is proteolytically converted to coat protein by an enzyme termed "leader peptidase." The final product of this pathway, the mature coat protein, spans the plasma membrane with its $NH_2$ terminus exposed on the outer, periplasmic surface (3, 4), its hydrophobic central region exposed to the fatty acyl region of the membrane, and its COOH terminus exposed to the cytoplasm (5). Recently, we found that an electrochemical potential is required for the conversion of the membrane-bound procoat to coat protein, even though isolated leader peptidase has no such requirement (6). We now report the results of limited proteolysis studies which map the location of procoat and coat at each stage of this biosynthetic pathway.

## MATERIALS AND METHODS

**Materials.** Soybean trypsin inhibitor was purchased from Worthington. Trypsin, chymotrypsin, *o*-nitrophenyl galactoside, and *p*-nitrophenyl phosphate were from Sigma. Other chemicals were from sources as described (2, 6).

**Methods.** *Escherichia coli* HJM114, coliphage M13, and M13 amber 7 were from previously described sources (2). Cells were grown in minimal medium 9 (7), 0.5% glucose, and vitamin B-1 at 37°C to an $OD_{600} = 0.4$ and infected with virus at a multiplicity of 100. One hour after infection, cells were pulse-labeled for 30 sec with [$^3$H]proline (15–50 $\mu$Ci/ml; 110 Ci/mmol; 1 Ci $= 3.7 \times 10^{10}$ becquerels) and chased with unlabeled proline at 0.5 mg/ml and, where indicated, 60 $\mu$M carbonylcyanide *m*-chlorophenylhydrazone (CCCP). Subsequent procedures are described in the figure legends. Buffer A contained 20% (wt/vol) sucrose/30 mM Tris·HCl, pH 8.1/10 mM EDTA. Sample precipitation by trichloroacetic acid, washing with acetone, and analysis by $NaDodSO_4$/polyacrylamide slab gel electrophoresis and fluorography were as described (2, 6).

Alkaline phosphatase and $\beta$-galactosidase were assayed by hydrolysis of *p*-nitrophenyl phosphate and *o*-nitrophenyl galactoside, respectively; the increase in $A_{420}$ was measured.

## RESULTS AND DISCUSSION

Cells infected by M13 with an amber mutation in gene 7 (a virion assembly gene) show a delay in the conversion of procoat to coat, allowing easier analysis (1, 2). We therefore performed proteolytic mapping of the positions of procoat and coat in amber 7- as well as in wild-type M13-infected cells. These results provide an additional line of evidence that procoat metabolism follows the same pathway in each case, although at different rates. M13 amber 7-infected cells were pulse-labeled with [$^3$H]proline and chased with unlabeled proline in the presence of CCCP. This uncoupler of oxidative phosphorylation blocks the conversion of procoat to coat without affecting the binding of procoat to membranes (6). Analysis of these labeled cells by $NaDodSO_4$ gel electrophoresis and fluorography (Fig. 1*A*, lane 1) showed three prominent low molecular weight bands, identified (2) as the soluble product of gene 5, the procoat, and the coat protein.

*E. coli* is bounded by an inner (cytoplasmic) membrane and an outer membrane with an aqueous compartment, the periplasm, in between. Experiments were performed to determine the position, with respect to the plasma membrane, of the procoat that persists in the presence of CCCP. Proteolysis of intact cells by either trypsin or chymotrypsin had no effect (Fig. 1*A*, lanes 2–9), presumably due to the permeability barrier of the outer membrane. However, the outer membrane of M13-infected cells is known to be unusually fragile (8), and it was breached by suspending cells in a solution of sucrose/Tris/EDTA without "osmotic shock." Although such cells retained their rod-shape, added protease digested coat protein but did not digest either procoat, gene 5 protein, or various predominantly cytoplasmic proteins (Fig. 1*B*). This presumably reflects the exposure (3, 4) of the protease-sensitive (4, 9) $NH_2$-terminal region of coat on the outer surface of the cytoplasmic membrane. Marker enzyme analysis showed that exposure of infected cells to sucrose/Tris/EDTA released 46% of the periplasmic alkaline phosphatase but only 6% of cytoplasmic $\beta$-galactosidase. The effect of sucrose/Tris/EDTA did not depend on the exposure of the cells to protease. Because alkaline phosphatase ($M_r$, 80,000) is much larger than chymotrypsin ($M_r$, 24,300) or trypsin ($M_r$, 23,800), it is reasonable to conclude that the proteases had access to the outer surface of the inner membrane.

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Abbreviations: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

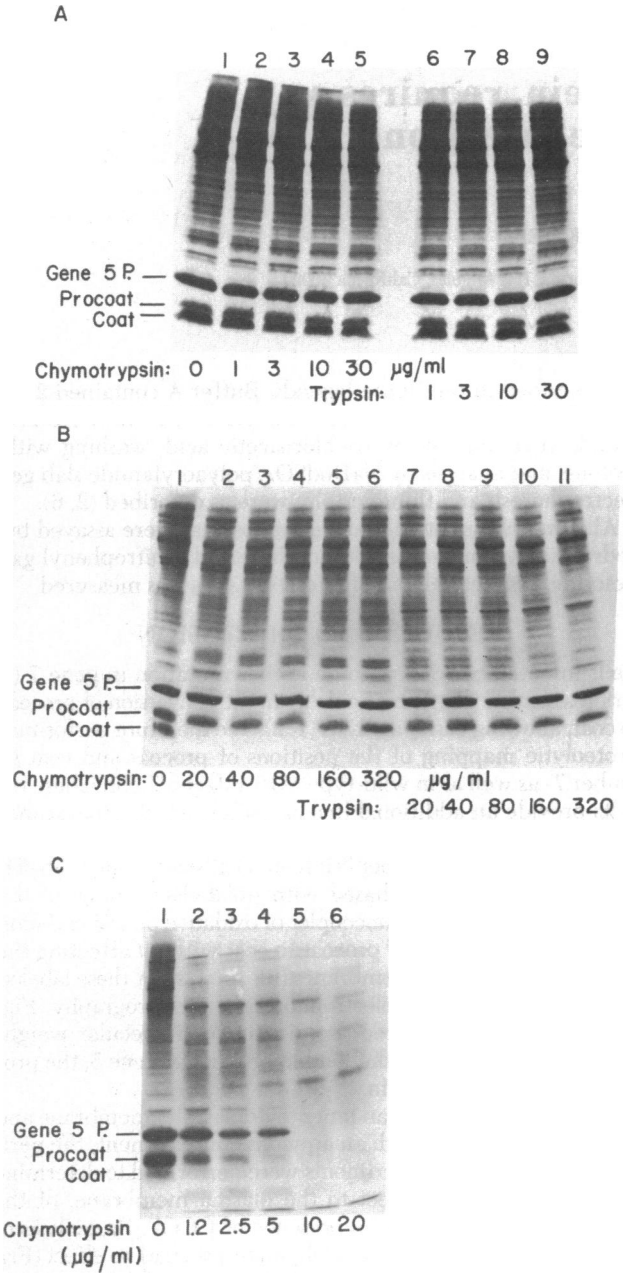


FIG. 1. Orientation of procoat in M13 amber 7-infected cells pulse-labeled and chased for 6 min in the presence of CCCP. (*A*) Cells (1.6 ml) were chilled on ice and mixed with EDTA (0.16 ml of 0.1 M). Aliquots (0.16 ml) were digested with the indicated concentration of trypsin or chymotrypsin for 30 min at 37°C and analyzed by Na-DodSO$_4$/polyacrylamide gel electrophoresis and fluorography. (*B*) Cells (2 ml) were chilled on ice and suspended in 2.4 ml of buffer A. Aliquots (0.2 ml) were digested with the indicated concentration of protease for 30 min at 23°C and analyzed as above. (*C*) Cells (20 ml) were collected by centrifugation, suspended in 2 ml of buffer A containing 1 mg of lysozyme, and incubated for 30 min at 23°C. The resulting spheroplasts were subjected to ultrasound (Heat Systems-Ultrasonics, Inc., model 350; output 1.5, 50% duty cycle, 3 min, 0°C). Aliquots (160 μl) of broken spheroplasts were mixed with an equal volume of buffer A and digested for 30 min at 37°C with the indicated concentration of chymotrypsin.

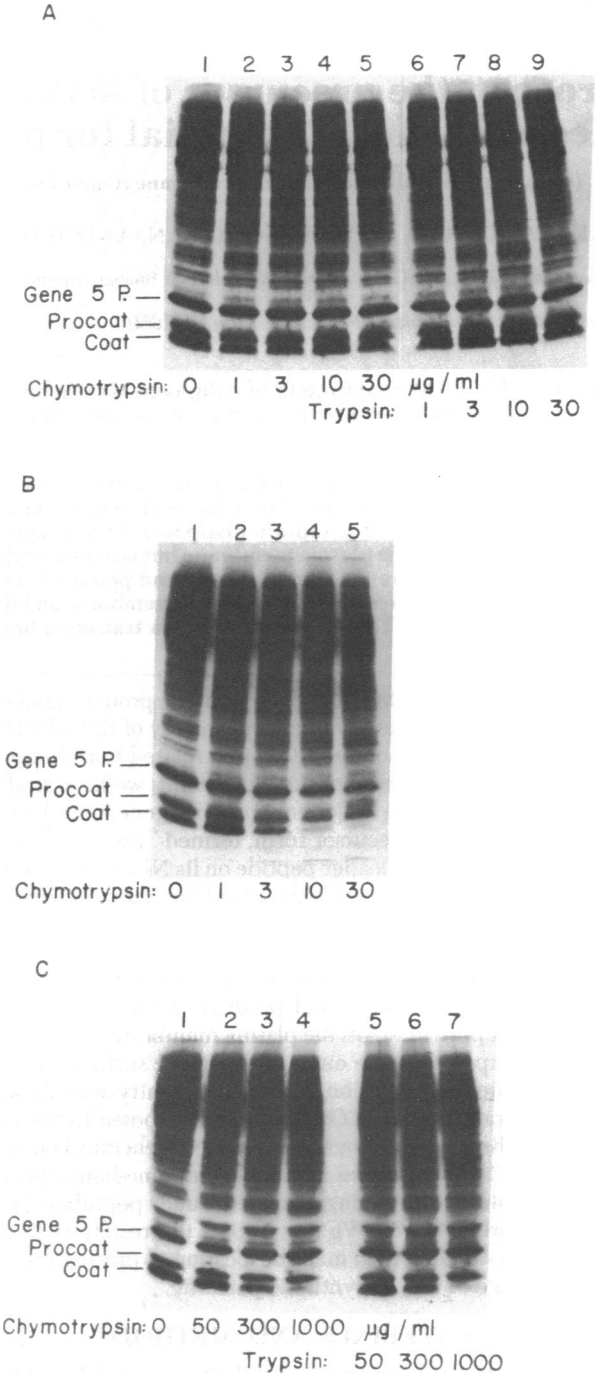


FIG. 2. Orientation of procoat in wild-type M13-infected cells pulse-labeled and chased for 2 min in the presence of CCCP. Cells were chilled on ice, collected by low-speed centrifugation, and suspended in their original volume of either M9 medium (*A*) or buffer A (*B*). Aliquots (0.16 ml) were digested with the indicated concentrations of chymotrypsin or trypsin for 30 min at 37°C. (*C*) Cells (7 ml) were collected by centrifugation, suspended in buffer A (0.7 ml) with 3.5 mg of lysozyme, and incubated for 1 hr at 0°C. Aliquots (50 μl) were mixed with buffer A (150 μl) and digested for 30 min at 0°C with the indicated concentration of protease.

Lysozyme converts sucrose/Tris/EDTA-treated cells to spheroplasts. Disruption of spheroplasts by sonication releases the cytoplasmic proteins and turns the plasma membrane fragments inside-out (10, 11), exposing their cytoplasmic surface. Under these conditions, as little as 2.5 μg of chymotrypsin (Fig. 1*C*, lane 3) or trypsin (data not shown) digested the procoat, showing that it is sensitive to protease when it is exposed. These data show that the procoat that persists in the presence of uncouplers is localized to the cell interior; earlier results (6) showed that it is bound to the plasma membrane. Similar data were obtained for wild-type M13-infected cells that were pulse-labeled and chased in the presence of CCCP. Neither

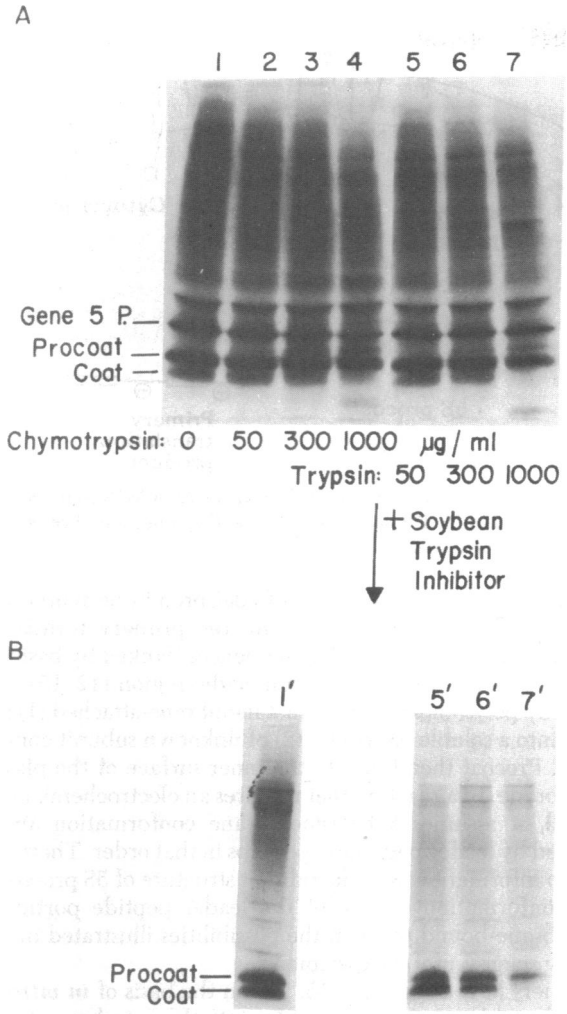


FIG. 3. Orientation of procoat in M13 amber 7-infected cells pulse-labeled but not chased. Cells (5 ml) were chilled by mixing with ice, harvested by low-speed centrifugation, and suspended in 0.5 ml of buffer A with 2.5 mg of lysozyme. After 1 hr at 0°C, aliquots (70 μl) were mixed with buffer A (130 μl) and digested for 30 min at 0°C with the indicated concentrations of protease. (*A*) Spheroplasts. A portion of each digestion (50 μl) was analyzed directly. (*B*) Membranes. The rest of each digestion (150 μl) was mixed with soybean trypsin inhibitor (40 μl of 5 mg/ml) and subjected to freezing and thawing (2) followed by centrifugation (30 min, 4°C, 14,000 × *g*). The precipitates were analyzed by gel electrophoresis. Lanes 1′,5′,6′, and 7′ correspond to the samples in lanes 1, 5, 6, and 7.

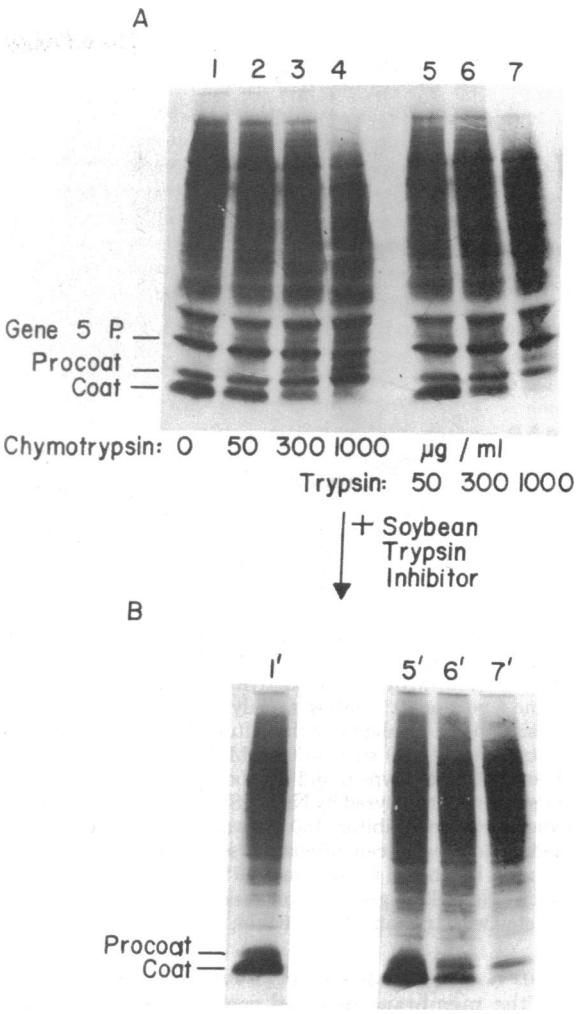


FIG. 4. Orientation of procoat in wild-type M13-infected cells pulse-labeled but not chased. The experiment was identical to that shown in Fig. 3, except that infection was by wild-type M13.

procoat nor coat was digested by protease added to intact cells (Fig. 2*A*), whereas coat protein but not procoat or gene 5 protein was digested by protease added to cells treated with sucrose/Tris/EDTA (Fig. 2*B*) or to spheroplasts (Fig. 2*C*). In the latter experiments, digestion was performed with high levels of protease at 0°C to avoid the problem of spheroplast lysis.

Similar experiments have been performed with pulse-labeled, M13-infected cells that were chased in the presence of cyanide, azide, or dinitrophenol. Each of these poisons prevents the generation of an electrochemical potential and inhibits the posttranslational conversion of procoat to coat (6). Procoat that accumulated in the presence of each of these inhibitors was not accessible to protease at the outer surface of the cytoplasmic membrane (data not shown).

Is procoat on the cytoplasmic surface of the inner membrane an intermediate in the normal biosynthetic pathway or is it only found in the presence of uncouplers? We have previously detected membrane-bound procoat as a transitory intermediate in pulse-labeled infected cells (2). In those studies, infected cells were pulse-labeled with [$^3$H]proline and chased with nonradioactive proline. At various times, cells were quickly chilled, converted to spheroplasts, lysed, and separated into soluble and membrane fractions. Each fraction was analyzed by Na$DodSO_4$/polyacrylamide gel electrophoresis and fluorography; procoat was found initially in the cytoplasm and then in the plasma membrane and was converted finally to coat protein. We therefore used the same protocol here to analyze whether the membrane-bound procoat is accessible to protease on the external face of the plasma membrane. M13 amber 7- and wild-type M13-infected cells were pulse-labeled, rapidly chilled to 0°C, and converted to spheroplasts. Digestion with chymotrypsin or trypsin (Figs. 3*A* and 4*A*) showed that only coat protein was accessible from the outer surface of the plasma membrane.

Because most of the pulse-labeled procoat is initially soluble, it was necessary to determine specifically whether the small fraction that had chased to the membrane was sensitive to external protease. Spheroplasts that were undigested or trypsin-digested were mixed with soybean trypsin inhibitor, lysed, and fractionated into membranes and soluble components. The membranes from another portion of the same spheroplasts analyzed in each lane of Figs. 3*A* and 4*A* were then analyzed by Na$DodSO_4$/polyacrylamide gels and fluorography (Figs. 3*B* and 4*B*; lane numbers indicate the corresponding sample

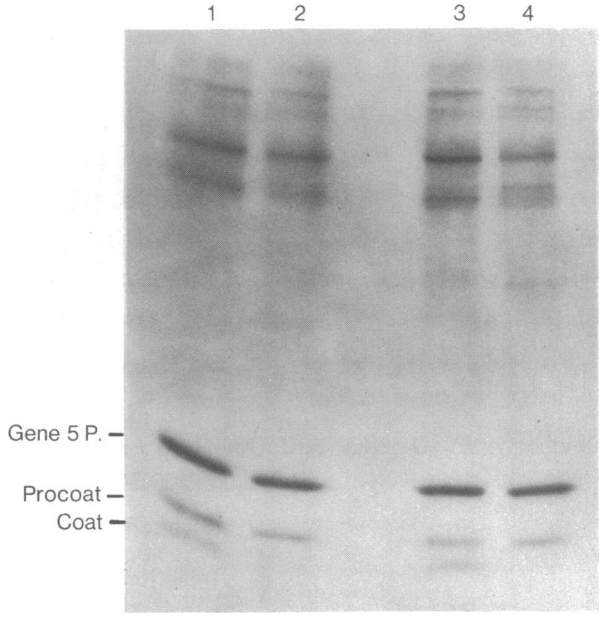


FIG. 5. Separate stages of membrane assembly. *E. coli* infected with amber 7 M13 were pulse-labeled for 15 sec with [$^3$H]proline and chased for 1.5 min with nonradioactive proline. Cells were rapidly chilled and a portion (0.18 ml) was analyzed by $NaDodSO_4$ gel electrophoresis (lane 1). An aliquot of the culture (0.62 ml) was mixed with 0.62 ml of 40% (wt/vol) sucrose/60 mM Tris·HCl, pH 8.1/20 mM EDTA and 26 $\mu$g of trypsin and incubated for 20 min at 23°C, a 0.36-ml portion was analyzed by $NaDodSO_4$ gel electrophoresis (lane 2). Soybean trypsin inhibitor (150 $\mu$g) was added and the cells were collected by low-speed centrifugation, suspended in 0.5 ml of M9 medium, and incubated for 20 min at 37°C. Half of these cells were analyzed by $NaDodSO_4$ gel electrophoresis (lane 3) and the other half was digested with trypsin (50 $\mu$g, 20 min, 23°C) before analysis (lane 4).

from part *A* of each experiment). Protease added to the outer face of the membrane digested coat protein but not membrane-bound procoat (Figs. 3*B* and 4*B*, lanes 5′–7′). Membrane-bound procoat, which was previously shown to be a biosynthetic intermediate, is now seen to be on the inner surface of the plasma membrane and to accumulate there when the transmembrane electrochemical potential is dissipated by CCCP. It is widely believed that cleavage of leader peptides occurs on the external (noncytoplasmic) membrane face, although there is little firm evidence on this question. If this belief is true, then the present data indicate that procoat that has assembled into a transmembrane conformation is quite rapidly cleaved to yield coat protein, because procoat is not sensitive to protease which is added to the periplasmic surface of the inner membrane.

The synthesis of procoat and its insertion into the bilayer and cleavage can be performed in two discrete stages (Fig. 5). M13 amber 7-infected cells were pulse-labeled with [$^3$H]proline and chased with nonradioactive proline and CCCP. $NaDodSO_4$ gel analysis of an aliquot of the cells (lane 1) showed gene 5 protein, procoat, and coat. These cells were rapidly chilled, exposed to Tris/sucrose/EDTA, and treated with trypsin; as expected (Fig. 1), only the label near the exposed $NH_2$ terminus of coat protein was removed (Fig. 5, lane 2). The spheroplasts were then washed at 0°C to remove protease and CCCP, mixed with soybean trypsin inhibitor to block any remaining protease, and warmed to 37°C for 20 min. A significant portion of the internal procoat was converted to coat (lane 3) and this coat protein was digested by addition of fresh trypsin (lane 4). This experiment provides another demonstration that internal procoat is competent to insert into the membrane posttranslationally and be cleaved to coat.

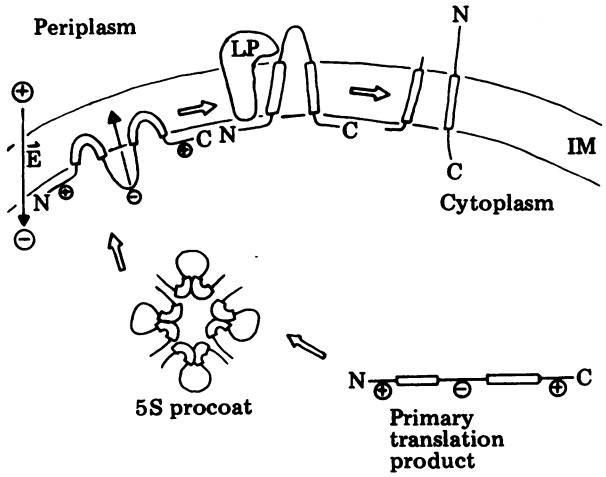


FIG. 6. Working model of the biosynthesis of M13 coat protein. See text for details. LP, leader peptidase; IM, inner membrane of *E. coli* (plasma membrane).

Our current working model of coat protein biosynthesis is shown in Fig. 6. Procoat protein, the primary translation product, has two hydrophobic sequences flanked by basic regions and separated by a central acidic region (12–15). It is made by polysomes which are not membrane-attached (1) and folds into a soluble 5S species (2) of unknown subunit composition. Procoat then binds to the inner surface of the plasma membrane. In a reaction that requires an electrochemical potential, it assumes a transmembrane conformation and is cleaved by leader peptidase, perhaps in that order. There is as yet no information as to the subunit structure of 5S procoat or the conformation or fate of the leader peptide portion of membrane-bound procoat; the possibilities illustrated in Fig. 6 may suggest useful experiments.

Others have suggested (15, 16) on the basis of *in vitro* experiments that procoat integrates into the membrane by cotranslational extrusion through a proteinaceous pore in a reaction driven by polypeptide chain elongation. However, it is clear from strictly *in vivo* studies of procoat biosynthesis (refs. 1, 2, and 6; this report) that procoat initially assumes a conformation that is consistent with its aqueous environment (17) and, upon binding to the membrane, exploits the electrochemical gradient for its integration. Our *in vitro* studies (18, 19) faithfully reflect this pathway of procoat metabolism. Further resolution of these processes will require the isolation of pure procoat, leader peptidase, and any other necessary protein factors for the faithful *in vitro* reconstitution of the assembly events.

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