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Translational and post-translational cleavage of M13 procoat protein: Extracts of both the cytoplasmic and outer membranes of *Escherichia coli* contain leader peptidase activity

(membrane assembly/M13 coat protein/signal hypothesis/membrane trigger hypothesis)

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ABSTRACT **The coat protein of coliphage M13 is an integral protein of the host cytoplasmic membrane at all stages of the infectious cycle. Both in *in vivo* and DNA-directed *in vitro* synthesis, it is initially made with an NH_2-terminal "leader peptide" of 23 amino acids and is termed procoat. We now report that leader peptidase, an activity which removes the leader peptide and converts procoat to coat, is found in both the inner (cytoplasmic) and outer membrane of *Escherichia coli*. However, only cytoplasmic membranes will catalyze cleavage of procoat in the absence of detergent. Leader peptidase will cleave procoat either during translation or after protein synthesis is complete.**

The assembly of proteins into cellular membranes poses several intriguing questions (1): How are proteins that have hydrophobic surfaces in their final conformation made by the same protein synthesis machinery that makes soluble proteins? How do proteins "choose" the proper membrane? Finally, how do proteins assume their final conformation in the bilayer? Answers to this last question must account for the fact that some proteins span the bilayer with polar domains exposed on either face and that some membrane proteins traverse the bilayer several times in a unique pattern. The sole chemical clue to these puzzles has come from the observation that secreted proteins (2–4) and membrane proteins (5–9) are synthesized with a hydrophobic NH_2-terminal "leader peptide" of 15–30 residues which is removed during, or shortly after, the export across or assembly into the bilayer. The function of these leader sequences is still unclear.

To study these problems, we have exploited the fact that the coat protein of the filamentous coliphage M13 (10) is integrally bound to the cytoplasmic membrane of the infected cell (11–14). The coat protein is a 5260-dalton peptide of known sequence (15, 16) with a central hydrophobic region. It spans the membrane *in vivo* with its NH_2-terminus exposed on the cell surface (17) and its COOH-terminus exposed to the cytoplasm (18). Similar asymmetric orientation has been achieved in *in vitro* reconstitution experiments (17, 18). Coat protein is synthesized and inserted into the cytoplasmic membrane of infected cells at a prodigious rate, reaching 26% of the membrane protein synthesis (12). Studies of the coat protein gene (19), of its messenger RNA (8), of its DNA-directed *in vitro* synthesis (7, 9), and of its synthesis in intact infected cells (K. Ito and W. Wickner, unpublished results) have shown that it is made as a precursor, termed procoat, with an additional 23 NH_2-terminal basic and hydrophobic amino acids. *In vivo*, procoat rapidly associates with the cell membrane and is cleaved to coat protein plus leader peptide. Chang *et al.* (9) have recently reported the detection of detergent-dependent leader peptidase activity in cell extracts and have convincingly shown that it cleaves procoat precisely after the leader sequence.

This communication reports the localization of leader peptidase activity to both the inner and outer membranes of *Escherichia coli*. The requirements for detergents and membranes for the proteolytic conversion of procoat to coat protein are examined. Detergent-free conditions are described for procoat cleavage by inner, but not outer, membranes. The ability to process procoat post-translationally is found to be a function of the microenvironment in which the procoat is synthesized, and of whether the synthesis is programmed by M13 duplex DNA or by a polysome fraction.

MATERIALS AND METHODS

Chemicals. [^{35}S]Methionine (200 Ci/mmol) (1 Ci = 3.7×10^{10} becquerels) was purchased from New England Nuclear. The detergent Nikkol was from the Nikko Chemical Co., Tokyo, Japan.

Protein Synthesis. Procoat was synthesized in a DNA-directed coupled transcription–translation system as described (20, 21). Products were boiled for 3 min in 1% sodium dodecyl sulfate/30% glycerol/0.1 M dithiothreitol and analyzed by electrophoresis as described by Laemmli (22) on 1-mm-thick slab gels containing 6 M urea, 15% acrylamide, and 1.6% bisacrylamide. Gels were either stained with Coomassie blue or fluorographed (23). It should be noted that procoat has three methionine residues and coat protein has one. Procoat and coat bands of equal intensity would therefore correspond to a 1 to 3 molar ratio of procoat/coat.

Membrane Preparations. Membranes were isolated from uninfected *E. coli* strain HJM114 (F'lacpro/∇lacproXIII) either by the method of Osborn *et al.* (24) or as described by Chang *et al.* (9) with an additional 66% sucrose shelf to isolate outer membranes in the sedimentation equilibrium step. Membranes were resuspended in solution A (0.05 M triethanolamine·HCl, pH 7.5/5 mM magnesium acetate/1 mM dithiothreitol) and stored at −90°C.

Detergent Extracts. Membrane suspensions (0.7 mg of protein per ml) were incubated for 30 min at 37°C with 1% Nikkol or Triton X-100 in solution A and then centrifuged at 23°C and 14,000 × *g* for 15 min. The supernatant (0.5 mg of protein per ml) was stored at −20°C for up to 1 month without loss of activity.

RESULTS

Membrane Separations. To localize leader peptidase, we purified membranes in the presence of Mg^{2+} from cells disrupted by French press treatment, as reported by Chang *et al.* (9), and in the presence of EDTA from spheroplasts disrupted by ultrasound, as described by Osborn *et al.* (24). Both tech-

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niques provided well-resolved preparations of cytoplasmic and outer membranes (Fig. 1 A and B) with 40- to 90-fold higher NADH oxidase specific activities (25) in the cytoplasmic membrane fractions. The resolution of the membrane fractions prepared by the Osborn technique is further documented by sodium dodecyl sulfate/acrylamide gel electrophoresis (Fig. 1C). The membrane fractions prepared by both techniques were used interchangeably throughout the experiments.

Localization of Leader Peptidase. M13 DNA-directed protein synthesis by crude extracts, which were free from cellular DNA and membranes, produced procoat but no coat protein (Fig. 2, lane a). When 1% Triton X-100 and equivalent amounts of either cytoplasmic or outer membranes were added to the synthesis reaction, significant coat protein was produced (lanes b and c). Addition of Triton X-100 alone did not cause procoat proteolysis (lane h). Processing was also achieved when a detergent extract of either inner or outer membrane was added to the synthesis reaction (lanes e, f, and g). These results confirm and extend those of Chang *et al.* (9); as they suggested, the ability of detergent alone to activate procoat cleavage in their system may have been due to endogenous membranes in their protein synthetic reaction.

Membrane Specific Cleavage. Protein synthesis was done in the absence of detergent and in the presence of various amounts of either cytoplasmic or outer membrane. Addition of cytoplasmic membrane to the protein synthetic reaction caused conversion to mature coat protein (Fig. 3, lanes b, c, and d). No coat protein was observed in synthesis reactions containing equivalent or higher concentrations of outer membrane (lanes e–g). The presence of Triton X-100 enhanced the processing activity of low levels of cytoplasmic membrane and permitted significant processing to occur by outer membrane (compare lanes c and h and lanes f and i).

Post-Translational Processing. As reported by Chang *et al.* (9), little conversion of procoat to coat was found when membranes were added after the completion of M13 DNA-directed polypeptide synthesis (Fig. 4, lanes c and h). When, however, detergent was present during synthesis, post-translational leader peptidase activity was observed (Fig. 4). Detergent, therefore, appeared to affect the conformation of the procoat, allowing it to be a substrate for leader peptidase. To refine this hypothesis, we performed a 60-min synthesis in the presence of concentrations of Triton X-100 that were either below or above the critical micelle concentration of 0.025% (26). A constant amount of cytoplasmic membrane extract and enough detergent to raise the final Triton X-100 concentration to 0.1% were added after synthesis was complete. The reaction was then continued for an additional 60 min. Although each reaction contained an identical amount of detergent-solublized leader peptidase, the enzyme was only able to cleave its procoat substrate when synthesis had occurred at detergent concentrations above the critical micelle concentration (Fig. 4). The extent of processing was the same whether the membrane extract was present during the synthesis of procoat or whether the same concentration of extract was added post-translationally (compare lanes f and g with lane b).

Polysome-Directed Synthesis. Upon resolution of membrane-bound and unattached polysomes (27, 28) from either lysozyme or sonic lysates of M13-infected cells, it was found that only the unattached polysomes synthesized procoat (W. Wickner, unpublished results). Cytoplasmic membranes will post-translationally convert procoat to coat in this system even

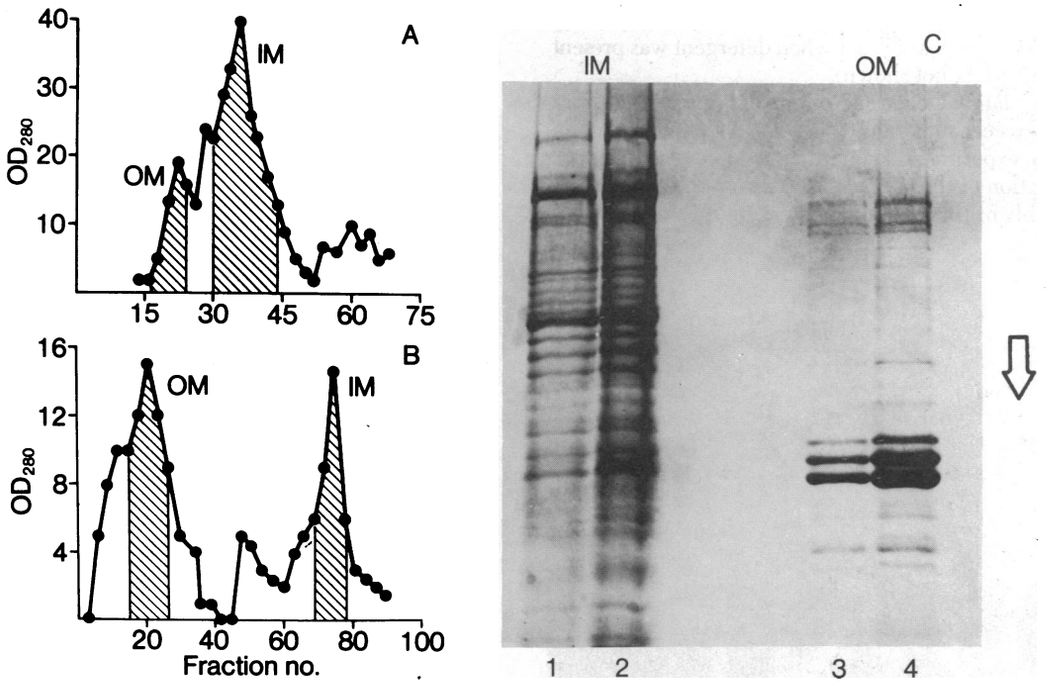

FIG. 1. Isopycnic sucrose gradient centrifugation of total membrane fractions from *E. coli* K-12 strain HJM114. (*A*) Membranes were prepared according to Chang *et al.* (9) with 66% sucrose in solution A in the bottom quarter of the gradient. NADH oxidase specific activity (25) in the pooled inner membrane fraction was 43 times that in the outer membranes. (*B*) Membranes were fractionated according to Osborn *et al.* (24). NADH oxidase specific activity in the inner membrane was approximately 92-fold higher than that in the outer membrane. In both *A* and *B*, crosshatching indicates the fractions pooled for the inner membrane (IM) and outer membrane (OM) preparations used throughout this study. (*C*) Polyacrylamide gel electrophoresis of inner and outer membrane proteins fractionated by the method of Osborn *et al.* (24). The membranes were prepared for electrophoresis and were analyzed on slab gels containing 10% acrylamide, 0.53% bisacrylamide, and 0.1% sodium dodecyl sulfate and stained with 0.1% Coomassie blue. Lanes 1 and 2, 6.3 and 12.6 μg, respectively, of inner membrane protein; lanes 3 and 4, 6.3 and 12.6 μg, respectively, of outer membrane protein.

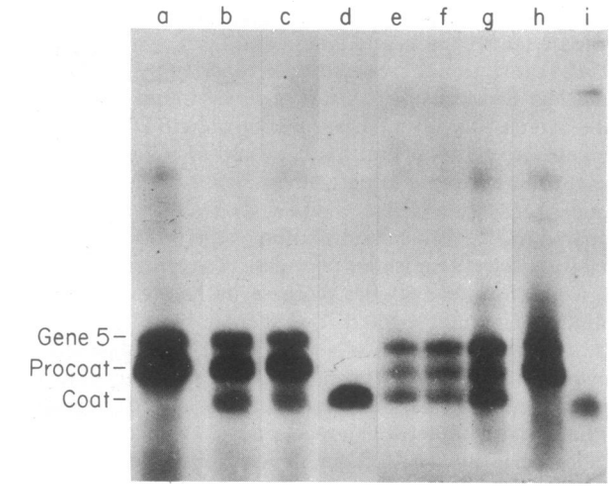

FIG. 2. Processing of M13 procoat is dependent upon a membrane fraction. Protein synthesis by crude extracts was done in the presence of the following. Lanes: a, no additions; b, 0.15 μg of cytoplasmic membrane protein and 1% Triton X-100; c, 0.15 μg of outer membrane protein and 1% Triton X-100; e and f, 11 μl (1.8 μg) of a diluted Triton X-100 extract of inner or outer membrane (respectively), prepared as described in methods; g, 4 μl of a 1% Nikkol extract of inner membrane; h, 1% Triton X-100 (no membrane). Lanes d and i contained ^{35}S-labeled coat protein from the virus. In this and subsequent figures, migration was from top to bottom.

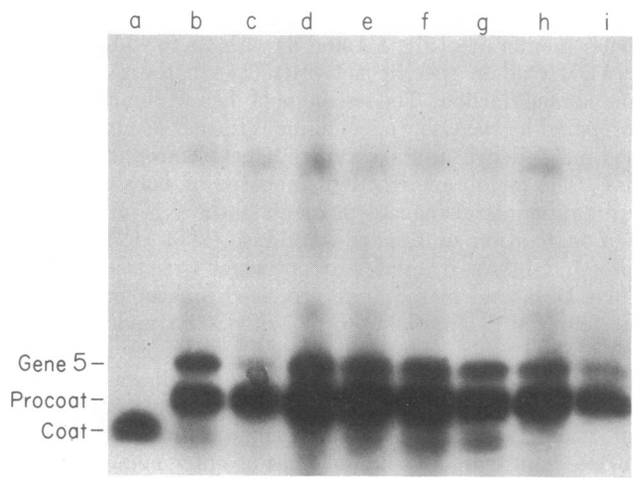

FIG. 4. Procoat that is synthesized in the presence of detergent can later be cleaved to coat protein. Synthesis and proteolytic processing reactions were performed sequentially. M13 DNA-directed protein synthesis was done with different concentrations of Triton X-100 near this detergent's critical micelle concentration of 0.025% (26). Lanes: c and h, no detergent; d, 0.01%; e, 0.02%; f, 0.03%; g, 0.04%. Each reaction then received Triton X-100 (to a final concentration of 0.1%), diluted 1% Triton X-100 extract of cytoplasmic membranes (0.08 μg of protein), and an excess of nonradioactive methionine (5 mM). Samples were then incubated for 60 min at 37°C and analyzed by sodium dodecyl sulfate/gel electrophoresis and fluorography. Lanes: a, ^{35}S-labeled coat protein from M13 virus; b, membrane extract and 0.1% Triton X-100 were present during protein synthesis; i, no detergent was present during protein synthesis and 0.1% Triton X-100, but no membrane extract, was present in the second incubation.

when detergent is added after polypeptide synthesis (Fig. 5, lane c). Some conversion is seen post-translationally in the absence of added detergent (lanes a and b) at membrane concentrations that give substantial cleavage when added during synthesis. Outer membranes, added post-translationally, were also able to process procoat to coat when detergent was present during synthesis (data not shown).

The post-translational cleavage of procoat synthesized from polysomes far exceeds that found in the DNA-directed reaction under the same experimental conditions (Fig. 5, lanes c and f). One interpretation of these results is based on the assumption that the assembly of procoat into a detergent micelle or membrane is critical for its cleavage. Coat protein is a DNA-binding protein when it is in the virus and a membrane protein when it is in the cell. In the DNA-directed reaction, the DNA may bind procoat and thereby inhibit its efficient processing.

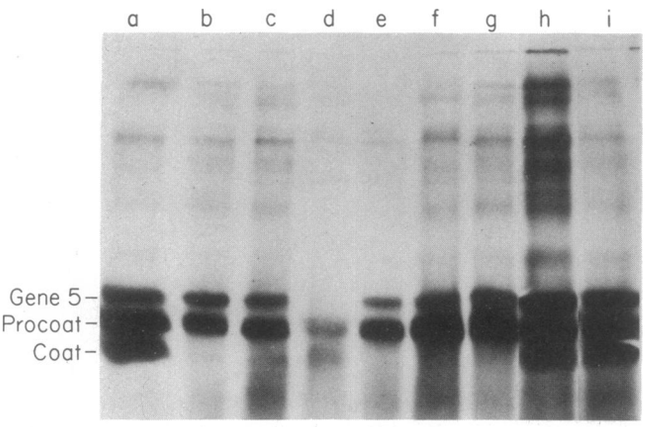

FIG. 3. Membrane-specific cleavage. Lane a, [^{35}S]-labeled coat protein, procoat, and gene 5 protein. M13 DNA-directed protein synthesis was carried out in the presence of the following. Lanes: b–d, 0.35, 0.70, and 1.4 μg of cytoplasmic membrane protein, respectively; e–g, 0.80, 1.6, and 3.2 μg of outer membrane protein, respectively; h, 0.70 μg of cytoplasmic membrane protein and 1% Triton X-100; i, 1.6 μg of outer membrane protein and 1% Triton X-100.

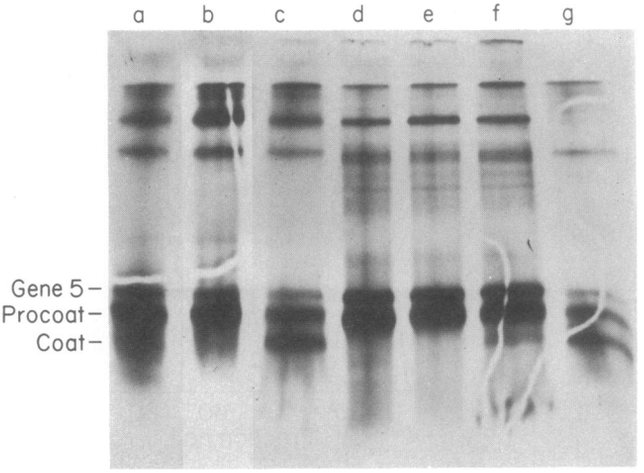

FIG. 5. Post-translational processing of procoat synthesized from soluble polysomes (28). The two-stage reaction was as described in Fig. 4. In lanes a–c, soluble polysomes were used in place of ribosomes and M13 DNA to program procoat synthesis in stage 1. Additions to stage 2: Lanes a and d, 2.3 μg of cytoplasmic membrane protein; lanes b and e, 6.9 μg of cytoplasmic membrane protein; lanes c and f, 2.3 μg of cytoplasmic membrane protein and 1% Triton X-100. Lane g contained procoat and coat protein markers.

DISCUSSION

Leader peptidase is the only known enzyme activity that is equally distributed between the inner and outer membranes of *E. coli*. The composition of the two envelopes is otherwise quite distinct, as revealed by sodium dodecyl sulfate/acrylamide gel analysis (ref. 24; Fig. 1*C*) and by the assay of individual enzyme activities (ref. 29; Fig. 1). We do not yet know whether or not each membrane has a physically distinct leader peptidase with overlapping substrate specificities. If the enzymes of the two membranes were identical, this raises the important question of how this enzyme of membrane assembly is itself segregated between the two membranes and integrated into them. The presence of leader peptidase in both the inner and outer membranes presumably reflects the fact that proteins of each membrane have leader peptides (5–9, 30). The presence of leader peptidase in uninfected cells strongly suggests that this activity functions in processing nascent membrane proteins of the host. The cleavage of each particular membrane protein by leader peptidase will presumably occur in only one membrane and will reflect the assembly properties of the nascent peptide, the substrate specificity of leader peptidase, and the topography of leader peptidase and nascent proteins. Understanding these problems will undoubtedly require the isolation of leader peptidases. Solubilization of the enzyme with nonionic detergents (Figs. 2 and 4) has allowed us to begin its fractionation (C. Zwizinski and W. Wickner, unpublished results).

While many of the steps of membrane assembly are topographic, the two known chemical features of this process are the presence of hydrophobic NH_2-terminal leader peptides and their cleavage from the new proteins during or shortly after their integration into the bilayer. Others have observed that leader peptidase activity is usually seen exclusively *during* synthesis of nascent polypeptides (2–4, 9), although post-translational cleavage has been reported (31). These studies and those reported here suggest that leader peptidase may provide a sensitive assay for the assembly of new proteins into the correct conformation in cell membranes or in detergent micelles. Leader peptidase processing of M13 procoat is now shown to occur post-translationally. Membrane-selective processing has also been demonstrated during procoat synthesis in the absence of detergent.

It may be useful to summarize here our current picture of the synthesis of M13 coat protein and its integration into the host cell plasma membrane. Coat protein enters the membrane by two distinct processes—i.e., from the infecting virus and from *de novo* synthesis within the infected cell. Both parental and newly synthesized coat protein span the cytoplasmic membrane with their NH_2-terminus on the cell surface (17, 18) and their COOH-terminus on the cytoplasmic side (19). Both species of coat protein are used in virus assembly (14). The similar orientation of coat protein that assembles by these two means, as well as model studies of the assembly of coat protein into liposomes (17, 18), suggests that orientation is determined by the hydrophobic interactions of coat protein and lipid near the fatty acyl phase-transition temperature. Procoat is made *in vivo* on free polysomes in the cytoplasm and, within a minute, assembles into the membrane and is converted to coat (K. Ito and W. Wickner, unpublished results). When procoat is made in a cell-free reaction, it is found in a water-soluble form, which will assemble into protein-free liposomes (21). We now show that leader peptidase can act on procoat that is either in a detergent micelle or in its physiological (inner) membrane.

These data are not consistent with certain aspects of the signal hypothesis (2–4). This model proposes that the leader peptide guides the ribosome–peptidyl-tRNA complex to the membrane and that the protein is conducted across the bilayer through a specific pore as synthesis proceeds. In the case of procoat, (*i*) synthesis is completed on polysomes that are not attached to the membrane, (*ii*) procoat assembles integrally into protein-free liposomes without any protein pore (21), and (*iii*) procoat can be post-translationally processed by the correct (cytoplasmic) membrane.

We propose an alternate model (1), termed the membrane-triggered folding hypothesis (membrane trigger hypothesis) to explain these data. Upon binding to an appropriate membrane, a newly synthesized protein is proposed to fold into a conformation that exposes hydrophobic residues to the bilayer's fatty acyl chains. This protein–membrane interaction may begin before synthesis of the protein is complete or, as in the case of procoat, may occur after completion of the protein. The NH_2-terminal leader oligopeptide's role is to influence the folding pathway of the growing peptide chain. This leader peptide thereby activates the protein for assembly and, in many cases, will finally be proteolytically removed, driving the assembly. The central distinctions between this model and the signal hypothesis are the absence of a peptide transport system and the proposed role of the NH_2-terminal leader peptide in facilitating the proper protein folding as it encounters the bilayer. This model does not call for specific ribosome–membrane interactions. Additional work is clearly necessary to distinguish which aspects of the signal and membrane trigger hypotheses are valid for the assembly of coat protein and other integral proteins into cellular membranes.

Note Added in Proof. Prolipoprotein is cleaved to lipoprotein (a major outer membrane protein) by detergent extracts of either outer or inner membrane, though only outer membrane will act in the absence of detergent (G. Mandel, M. Inouye, and W. Wickner, unpublished results). This suggests that the membrane-selective *in vitro* processing of procoat and prolipoprotein occurs by a physiological path.

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