The major coat protein (gene 8 product) of coliphage M13 is an integral protein of the host cell cytoplasmic membrane prior to virus assembly. It is synthesized as a precursor, termed procoat, with an extra 23 NH₂-terminal residues. We have studied the synthesis, assembly, and processing of procoat protein by amino acid pulse-labeling *E. coli* which are infected by either M13 or by M13 with amber mutations. Pulse-labeled procoat is found in the soluble fraction and little radioactivity is incorporated into membrane-bound coat protein by short pulse labeling.

For wild type M13-infected cells, the soluble procoat sediments at about 5 S in a sucrose gradient. Pulse-labeled procoat rapidly chases from the soluble fraction.

For amber 7 M13-infected cells, the pulse-labeled procoat is soluble and sediments at about 5 S, as in the wild type virus infections. During chase, procoat is lost from the soluble fraction and appears in the membrane fraction at short chase times as coat protein begins to appear. The appearance of coat protein during "chase" parallels the loss of procoat and is not affected by the addition of the protein synthesis inhibitor puromycin at the beginning of the chase.

These data for wild type and amber 7 M13 infections strongly suggest that soluble procoat is the precursor of membrane-bound procoat, which is then proteolytically cleaved to yield coat protein.

For amber 5 M13-infected cells, procoat is found in the soluble fraction with a sedimentation coefficient of 1.2 S, which is that expected for the monomeric protein. A portion of the procoat is found in the membrane; even after long chase times, only one-half of the membrane-bound procoat is converted to coat protein. The reasons for these alterations in procoat metabolism in M13 amber 5-infected cells are not known.

Proteins associated with biological membranes are of interest not only for their biological functions but also for their pathways of synthesis and assembly (1). Intrinsic membrane proteins have hydrophobic surfaces which anchor them to the bilayer. They require detergents for their solubilization and dispersal, yet presumably they are synthesized by the same cytoplasmic enzymic machinery which makes soluble proteins. How do they avoid aggregation during their synthesis? Proteins which span the bilayer have hydrophilic regions exposed on the outer surface of the membrane, separated from the site of protein synthesis by the hydrophobic fatty acyl membrane core. How do these polar protein surfaces cross this apolar barrier?

Transmembrane proteins and secreted, soluble proteins share this feature of crossing the bilayer (2). One of the most striking findings for both exported and membrane proteins is that the initial product of translation is usually a precursor molecule with an extra 15 to 30 amino acid residues (leader or signal sequence) at the NH₂ terminus (1). The "signal hypothesis" (3, 4) has proposed that this sequence guides the nascent protein to the membrane and is recognized by a peptide transport system. After a ribosome-membrane junction forms, the growing polypeptide chain is thought to extrude through (or into) the bilayer in an extended form via a proteinaceous pore. The "membrane-triggered folding hypothesis" (1) (membrane trigger hypothesis) suggests that the main function of the leader sequence is to modify the protein's folding pathway. Nascent membrane proteins initially fold in a manner which is compatible with their aqueous cytoplasmic environment. During, or shortly after, their synthesis, they encounter their target membrane, and this triggers their refolding into a conformation which penetrates the bilayer and exposes hydrophobic regions to the membrane's apolar center. These hypotheses differ in the timing of assembly events, in the function of the leader peptide, and in the proposal of a protein transport pore. It is important to note that each hypothesis has several parts and that many of the events involve topographic changes rather than the making and breaking of covalent bonds. At present, the actual role of the leader sequence and the mechanism of translocation of these proteins across the bilayer is not known.

One approach to the study of membrane assembly is to pulse-label precursor proteins in *vivo* with radioactive amino acids and to follow their intracellular localization and proteolytic processing during a "chase" with unlabeled amino acid. In the present paper, we report such studies of procoat, the precursor of the major capsid protein of the coliphage M13. Unlike lytic viruses which assemble in the cytoplasm of infected cells, M13 assembles during the extrusion of its DNA through the host cell surface (5). Prior to virus assembly, the M13 (gene 8) coat protein spans the cell's cytoplasmic membrane with its NH₂ terminus on the periplasmic surface (6, 7). This protein can account for one-third of the total membrane protein synthesis in infected cells (8). Our previous success in detecting procoat, the precursor of coat protein, in infected cells (9), showed that this system offers an excellent opportunity to follow the pathway of synthesis and assembly of an integral membrane protein. As presented below, pulse-labeled...
procoat is not integrally bound to the membrane, but it rapidly binds and is proteolytically processed to yield coat protein.

**MATERIALS AND METHODS**

**Chemicals**

[\textsuperscript{[\textsuperscript{3}H]}]Proline (111 Ci/mmole) was obtained from New England Nuclear.

**Growth and Infection of Bacteria**

M13 and its amber mutants in gene 5 (am5-H3) and gene 7 (am7-H2) were obtained from David Pratt, University of California, Davis. *Escherichia coli* strain HJM114 (F'lacpro/Vlacpro) (10) was grown at 37°C in medium 63 (11) with 0.5% glucose and 1 μg of thiamine/ml. At A₆₆₀ = 0.4, cells were infected at a multiplicity of 100. Pulse labeling was usually done 1 h after infection.

**Preparation of Soluble and Membrane Fractions**

All operations were performed at 0 to 4°C.

Sonic Lysis—Cells were converted to spheroplasts and disrupted by sonication as described previously (9, 12). After removal of unbroken cells by low speed centrifugation, the lysate was layer on 10 ml of 15% sucrose, 3 mM EDTA (pH 7.5) with a 0.5-m cushion of 70% sucrose, 3 mM EDTA (pH 7.5) and centrifuged (40,000 rpm, 38 min, in a Beckman SW 40 rotor).

**Lysis by Osmotic Shock and Freeze-Thaw—**Cells from 2 to 20 ml of cultures were harvested by centrifugation (10,000 rpm, 5 min, Beckman JA-21 rotor), washed with 2 ml of 0.03 M Tris/Cl (pH 8.1), 0.15 M NaCl, resuspended in 0.2 ml of 20% sucrose, 0.03 M Tris/Cl (pH 8.1), 0.15 M NaCl, and converted to spheroplasts by incubation for 30 min with 20 μl of a solution of 1 mg/ml of lysozyme dissolved in 0.1 M EDTA (pH 7.3). Many of the infected cells were lysed under these conditions; therefore, the supernatant obtained after centrifugation (15,000 rpm, 10 min) was saved for its cytoplasmic proteins. The pellet of spheroplasts was suspended in 0.2 ml of 0.01 M Tris/Cl (pH 8.1), 0.11 mM EDTA, 0.15 mM NaCl and subjected to three cycles of freezing and thawing using a dry ice/acetone bath. The lysate was incubated on ice with pancreatic DNase I (10 μg/ml), RNase A (50 μg/ml), and 10 mM MgCl₄ for 45 min. EDTA was then added to 10 mM. Unbroken cells and debris were precipitated by centrifugation (20,000 × g, 10 min) and this precipitate was washed once with 0.2 ml of 0.01 M Tris/Cl (pH 8.1), 0.1 mM EDTA, 0.15 mM NaCl. The three supernatant fractions were pooled (0.6 ml of crude lysate) and layered on a 3-ml gradient of 10 to 25% sucrose (in 0.01 M Tris/Cl, 0.1 mM EDTA, 0.15 μM NaCl) over a 0.2-ml shell of 70% sucrose (in the same buffer). "Soluble" and "membrane" fractions were obtained by collecting the top and bottom quarters of the gradient after centrifugation (54,000 rpm, 45 min, SW 56 rotor).

**SDS\textsuperscript{1} Gel Electrophoresis**

The method of Anderson et al. (13) was followed with modifications. A 1 ml stock solution of Tris/Cl (pH 8.4) was further titrated to pH 8.7 with concentrated NaOH. The separation gel contained 19.3% acrylamide, 0.074% N,N'-methylenebisacrylamide, 0.33% M Tris/Cl (above stock), 6 M urea, and 0.1% SDS. Urea, low cross-linking, and the inclusion of NaCl (generated by the titration of the Tris/Cl stock solution) were necessary for the resolution of procoat and coat proteins. The stacking gel contained 5% acrylamide, 0.13% N,N'-methylenebisacrylamide, 0.0625% M Tris/Cl (pH 6.8), 6 M urea, and 0.1% SDS.

Samples were mixed with an equal volume of ice-cold 10% trichloroacetic acid and centrifuged (2 min in a Brinkmann microfuge). The precipitates were washed with 1 ml of cold acetone, dissolved in 50 to 100 μl of SDS sample buffer (0.0625% M Tris/Cl (pH 6.8), 2% SDS, 5% β-mercaptoethanol, 10% glycerol) and boiled for 3 min. This precipitation was needed both for sample concentration and to eliminate salt which interferes with the separation of procoat and coat. Gels were fluorographed (14) to visualize isotopically labeled proteins.

**RESULTS**

**Detection of Procoat Protein in Crude Fractions**—The major virus-specific proteins synthesized after M13 infection of *E. coli* are the products of gene 5 (DNA-binding protein (15), Mₑ = 9,688) and gene 8 (major coat protein, Mₑ = 7,628 for procoat (16) and Mₑ = 5,280 for coat protein (17, 18)). The low molecular weight of these proteins allows their separation from the bulk of host proteins by SDS-polyacrylamide gel electrophoresis. The gene 5 protein, procoat, and coat protein of M13 amber 7-infected, [\textsuperscript{[\textsuperscript{3}H]}]proline-labeled cultures were resolved by SDS-gel electrophoresis in the presence of 6 mM urea (Fig. 1). M13 coat protein was predominantly found in the membrane fraction (Lane 1), whereas gene 5 protein was largely soluble (Lane 2). Procoat protein was found in both fractions, as expected for these specific labeling conditions (see below). As seen in Lanes 3 and 4 of Fig. 1, very little labeled protein from uninfected cells is seen in the coat, procoat, and gene 5 protein regions of the gel. The identity of these three M13-specific proteins is established by their mobilities relative to procoat and coat markers (Lane 5), by their absence from uninfected cells, by their reactivity with antibody to coat protein (not shown), and, in the case of procoat, by its disappearance during chase with unlabeled proline (see below).

[\textsuperscript{[\textsuperscript{3}H]}]Proline is employed in these studies because it is absent from *E. coli* prolioprotein and lipoprotein (20). When membrane fractions from [\textsuperscript{[\textsuperscript{3}S]}]methionine-labeled infected and uninfected cells were examined (Lanes 6 and 7), a major host protein band (prolioprotein or lipoprotein, or both) is seen to migrate at the same position as procoat. The resolution of procoat from coat is lost in the absence of urea from the gels (Lanes 10 and 11). It should be noted that faint bands with the same electrophoretic mobilities as procoat and coat can be seen in the uninfected sample (Lane 4). This becomes clearer when the gels are heavily exposed during fluorography.

**Subcellular Localization of Procoat and Coat**—Our previous analysis by trichloroacetic acid precipitation of total pulse-labeled cell protein (9) showed that procoat is rapidly (within 30 s) converted to coat protein in wild type or amber gene 3 M13 infection. In contrast, processing is delayed in phage mutants in genes 1, 5, and 7 which are defective in the formation of viral filaments (9). We also showed a considerable portion of pulse-labeled procoat was recovered in a soluble fraction prepared by sonication of amber 5-infected cells. To examine the kinetics of these processes in more detail, cells infected with either wild type, amber 5, or amber 7 M13 were pulse-labeled with [\textsuperscript{[\textsuperscript{3}H]}]proline for 30 s and chased with unlabeled proline for various times. Samples were quickly chilled by mixing with crushed ice, and soluble and membrane fractions were prepared from sonicated spheroplasts as described (9, 12).

Fig. 2A shows that in wild type infection, procoat can only be seen in the pulse-labeled soluble fraction (Lane 1). The amount of procoat is usually small compared to the total coat protein, indicating that assembly and processing are very rapid. Although assay of NADH oxidase, an inner membrane enzymatic activity, and analysis of protein profiles by SDS-gel electrophoresis showed that more than 90% of the membrane are recovered in the membrane fraction (9), some coat protein is still seen in the soluble fraction (Fig. 2A). Presumably, this is due to the formation of small membrane vesicles during sonication (see Figs. 6 to 8). In contrast, procoat is clearly enriched in the soluble fraction. This is not due to membrane fragments, since it is seen without sonication (Fig. 6); sucrose gradient centrifugation (Figs. 3 to 5) shows that this procoat has a low sedimentation velocity and does not co-sediment with the coat protein contaminant.

The tritium-labeled coat protein in the membrane (Fig. 2A, Lanes 7 to 12) decreases after approximately 5 min of chase. This is due to virus assembly; SDS-gel electrophoresis of the culture supernatant showed that labeled coat protein appears in the medium in parallel with the decrease of membrane-
bound coat protein label (Ref. 21 and Footnote 2).

In amber 7-infected cells, both the loss of procoat from the cytoplasmic fraction and the appearance of coat protein in the membrane are delayed. Pulse-labeled procoat only disappears from the cytoplasmic fraction after 1 min of chase (Fig. 2B, Lanes 1 to 4) with a concomitant increase in radioactivity in membrane-bound coat (Fig. 2B, Lanes 7 to 10). Small amounts of procoat protein are found in the membrane fraction at intermediate times. Proteolysis experiments with spheroplasts and with sonicated, inverted spheroplasts have shown that this coat protein spans the bilayer.

M13 amber 5-infected cells show a unique pattern of coat protein biosynthesis (Fig. 2C). Procoat protein is the major labeled soluble protein (Lane 1) and is chased with a half-life of approximately 5 min (Lane 5). The membrane fraction has comparable amounts of pulse label in procoat and coat (Lane 7) with little change during the chase. Thus, in M13 amber 5-infected cells, a portion of the procoat assembles rapidly into the membrane, and half of this membrane-bound portion is rapidly converted to coat protein. Neither the basis for this dramatic disruption of the normal assembly pathway nor the fate of this soluble procoat is clear at this time. However, this block in the processing of membrane-bound procoat has allowed us to purify procoat from this source.

Sedimentation Analysis of Soluble Procoat—We have further characterized the soluble, pulse-labeled procoat by sedimentation in sucrose gradients. Infected cells were treated with lysozyme and EDTA, followed by osmotic shock, freeze-thaw, and digestion by DNAs and RNAs as described under "Materials and Methods." Lysed cells were mixed with marker proteins, β-galactosidase (16 S), hemoglobin (4 S), and cytochrome c (1.7 S) and layered directly on a 10 to 25% sucrose gradient over a 70% sucrose shelf. Under the centrifugation conditions used, membranes were recovered on the 70% sucrose shelf near the bottom, whereas soluble proteins were displayed according to their sedimentation velocities along the gradient (Figs. 3 to 5).

In amber 5 M13 infections, the majority of the pulse-labeled procoat sediments near the top of the gradient (Fig. 3A). The procoat (M = 7,628) sediments more slowly than cytochrome c (M = 12,000) and is thus, presumably, monomeric. It disappears during chase with unlabeled amino acid (Fig. 3B). Upon more prolonged centrifugation (Fig. 3C), the procoat

1 P. Silver, M. Nokelainen, P. Hearne, and W. Wickner, manuscript in preparation.
Pulse-labeled procoat in amber 7 infection. Two 20-ml cultures were infected with amber 7 M13 and were pulse-labeled with \[^{3}H\]proline (A), chased for 1.5 min (B), and analyzed as described in the legend to Fig. 3. Procoat is marked by the vertical arrow: \(\beta\)-gal, \(\beta\)-galactosidase; \(Hb\), hemoglobin; \(cyt\), cytochrome c.

**Fig. 5.** Sedimentation of pulse-labeled proteins in amber 7 infection. Two 20-ml cultures were infected with amber 7 M13 and were pulse-labeled with \[^{3}H\]proline (A), chased for 1.5 min (B), and analyzed as described in the legend to Fig. 3. Procoat is marked by the vertical arrow: \(\beta\)-gal, \(\beta\)-galactosidase; \(Hb\), hemoglobin; \(cyt\), cytochrome c.
protein, or both. This procoat is not slow sedimenting because of its low buoyant density, since it sedimented to the bottom after additional centrifugation and its rate of sedimentation relative to other proteins was unaltered by higher sucrose concentrations (25 to 70%) in the gradient. These results show that newly synthesized procoat in wild type or amber 7 M13 infection is in a soluble complex sedimenting at 5 S.

**Effect of Cell Lysis Conditions on Localization of Procoat**—In order to test whether the presence of procoat in the soluble cell fraction was a function of cell lysis conditions, fractions from M13 amber 5-infected cells were assayed for procoat and coat by gel electrophoresis after lysis by different procedures (Fig. 6). Since infected cells are fragile (22), considerable amounts of cytoplasmic proteins are released by osmotic shock of EDTA-treated cells or by lysozyme treatment in hypertonic solution. Procoat was a major pulse-labeled protein in the osmotic shock fluid (Fig. 6, Lane 1), in the supernatant after lysozyme treatment in the presence of EDTA (Lane 7), or Mg²⁺ (Lane 10), in the soluble cytoplasmic fraction obtained by sonication in the presence of EDTA (Lane 2, no lysozyme treatment; Lane 8, after lysozyme treatment) or Mg²⁺ (Lane 5), and in the soluble fraction after osmotic lysis (Lane 11). Sonication in buffer containing Mg²⁺ and NH₄Cl, which had been used for detection of the precursor of the arabinose binding protein in the envelope fraction (25), yielded more than two-thirds of the protein in the precipitate fraction (Lanes 5 and 6). It should also be noted that some coat protein is found in the soluble fraction after sonication in the presence of EDTA (Lanes 2 and 8). Presumably, this is due to the generation of small membrane vesicles (see Figs. 2 to 5). In contrast, procoat protein is clearly found in the soluble fraction (i) irrespective of the presence or absence of Mg²⁺ or EDTA, (ii) with or without lysozyme treatment, and (iii) after sonication, osmotic lysis, or even simple lysozyme treatment at low temperature. Procoat in wild type and amber 7 M13 infection was also found in the soluble fraction after mild cell lysis by lysozyme-EDTA-freeze and thaw-osmotic shock treatment as shown in Figs. 4, 5, 7, and 8.

**Soluble Procoat as a Precursor of Membrane-bound Coat Protein**—To follow the early kinetics of synthesis and assembly in wild type and amber 7 M13 infections, cells were pulse-labeled for a shorter time (15 s) at a lower temperature (32°C) than in Fig. 2. In addition, cells were lysed by a lysozyme-EDTA-osmotic shock-freeze and thaw treatment (as under “Materials and Methods”), avoiding sonication, and the generation of small membrane pieces. With this technique, the membrane fraction contained more than 97% of the total lipid assayed by chloroform/methanol extraction of cells labeled with ['H]glycerol. The gel electrophoresis patterns (Figs. 7 and 8) also showed that membrane and soluble fractions prepared by this method have different sets of specific proteins with little cross-contamination.

The clearest data showing precursor-product relationships were obtained with amber 7 infection (Fig. 7). Procoat is initially seen only in the soluble fraction, with little labeled coat or procoat in the membrane fraction (compare Lanes 1 and 5). As in Fig. 2B, the loss of soluble procoat parallels the appearance of coat protein in the membrane fraction, and membrane-bound procoat can be seen at intermediate times. These data are consistent with the sequence of assembly: soluble procoat → membrane-bound procoat → membrane-bound coat.

In wild type M13 infection, the sequence of assembly and processing is similar to that of amber 7 infection, though the rate is faster (Fig. 8). Since, using this lysis technique, there is a soluble labeled host peptide which co-migrates with procoat on SDS gels (Fig. 4C), the soluble fractions were analyzed by sucrose gradient ultracentrifugation to distinguish this unrelated slow sedimenting protein from procoat. Gradient fractions were electrophoresed on SDS slab gels and fluorographed. The soluble fraction of the pulse-labeled sample contains appreciable labeled procoat protein (Fig. 8A) which sediments at 5 S and is identified by (i) its migration on SDS gels, (ii) its rapid loss during chase with nonradioactive amino acids (Fig. 8B), and (iii) its precipitation with antibody to coat protein (not shown). Immediately after the pulse, the membrane fraction has little labeled coat protein (Fig. 8C, Lane 1). Label appears in the coat protein fraction as it is lost from the soluble procoat (Lanes 2 to 4). These data indicate that soluble procoat is a precursor of membrane-bound coat in wild type infections.

Similar initial kinetics were obtained with amber 5 infection,
we can detect procoat protein in the soluble fraction at the earliest times of labeling (unless one assumes that the soluble and membrane-bound species are made by different mechanisms with different chain elongation rates and that soluble procoat is degraded at the same rate that membrane-bound coat protein is synthesized). These possibilities are tested by the following experiment with the protein synthesis inhibitor puromycin.

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**Fig. 8. Early kinetics of procoat assembly in wild type infection.** The experiment was the same as described in the legend to Fig. 7, except that wild type virus was used and that the times of sampling were 15, 30, 45, and 60 s after initiation of labeling. Soluble fractions were further fractionated by sucrose gradient centrifugation (3 ml of 10 to 25% sucrose in 0.01 M Tris-HCl (pH 8.1), 0.1 mM EDTA, 0.15 M NaCl over 0.2 ml of 70% sucrose in the same buffer) and centrifuged at 41,000 rpm for 13 h in an SW 56 rotor. The position of hemoglobin (Hb), which was added as a marker, is indicated. A, soluble fraction at 15 s (no chase); B, soluble fraction at 45 s (30 s chase); C, membrane fraction at 15 s (Lane 1), 30 s (Lane 2), 45 s (Lane 3), and 60 s (Lane 4). Procoat in part A is indicated by the vertical arrow.

that is the soluble fraction had pulse-labeled procoat and very little protein label was found in the membrane fraction (Fig. 9, compare Lanes 1 and 5). Therefore, at least a part of the soluble procoat in amber 5 infections is a precursor of membrane-bound coat and procoat. Unlike M13 or M13 amber 7 infections, the chase of part of the soluble procoat is quite slow in amber 5 infections and a significant part of the membrane-bound procoat remains unprocessed. Since detergent extracts of membranes from wild type, amber 7, or amber 5 M13-infected cells have comparable leader peptidase activity to uninfected cells, further studies will be necessary to determine the basis for this block.

It should be noted that all of the proteins of the membrane are poorly labeled by short pulses of radioactive amino acids (Figs. 7 to 9), but that substantial radioactivity appears in these proteins during chase. In these experiments, chemically similar amounts of protein were loaded on the gel irrespective of the chase time. This delay in the appearance of label is characteristic of membrane proteins, especially outer membrane proteins, as we reported previously (12). This delayed labeling could reflect the slow translocation process(es) of membrane proteins or, possibly, a slower rate of polypeptide chain elongation for membrane proteins than for soluble proteins. A low chain elongation rate is not responsible for the delayed appearance of coat protein in the membrane, since

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*C. Zwizinski and W. Wickner, unpublished data.*
Cells were pulse-labeled with [3H]proline for 0.5 min and supernatant fractions were prepared from spheroplasts broken by sonication (see "Materials and Methods"). These soluble fractions were diluted with an equal volume of buffer (0.02 M Tris/Cl, pH 8.1, 0.2 mM EDTA, 0.3 M NaCl, 4% Triton X-100) and incubated for 15 min at 37°C with 530 pg/ml of control γ-globulin. S. aureus ghosts (see below) were added, incubation was continued for 15 min, and the suspension was centrifuged, thereby removing ghost, adsorbed preimmune antibody, and proteins which nonspecifically bound to S. aureus ghosts or γ-globulin. The supernatant was then incubated for 15 min at 37°C with 530 pg/ml of either anticoat antibody (6, 7) or control γ-globulin, followed by addition of S. aureus ghosts and an additional 15-min incubation. The antigen-antibody-staphylococcus complexes were collected by centrifugation, washed twice with 2% Triton X-100, 0.01 M Tris/Cl, pH 8.1, 0.1 mM EDTA, 0.15 M NaCl, and then washed with this same buffer without detergent. They were then extracted by boiling 3 min with SDS sample buffer (see "Materials and Methods").

Effect of Puromycin—To test whether membrane-bound procoat protein does indeed come from the soluble procoat, M13 amber 7-infected cells were pulse-labeled for 30 s with [3H]proline. Chase with nonradioactive proline was done in the presence of the protein synthesis inhibitor puromycin and aliquots of cells were precipitated with trichloroacetic acid and analyzed by SDS-gel electrophoresis. Procoat, but very little coat, was labeled by the pulse (Fig. 10A, Lanes 1 and 5). Samples assayed after 1/2 min, 2 min, and 5 min of chase (Lanes 2, 3, and 4) showed a progressive loss of procoat and appearance of coat protein. The appearance of coat protein in the membrane was not complete after 30 s of chase, although the drug inhibited protein synthesis within 10 s after its addition (Fig. 10B). Furthermore, the appearance of coat protein was no slower in cells treated with 2 mg/ml of puromycin (Lane 1 to 4) than in parallel untreated controls (Lane 5 to 8). Thus, continued polypeptide chain elongation is not necessary for the appearance of coat protein during the chase of procoat. Pulse-labeled procoat, which is soluble in these cells (Figs. 2, 5, and 7), is, therefore, clearly the precursor of membrane-bound coat protein.

Immunoaassay of Procoat in the Soluble Fraction—As an independent assay of procoat found in the soluble fraction, we examined selective immunoprecipitates. Pulse-labeled soluble fractions were mixed with either affinity-purified antibody against coat protein (6, 7) or control γ-globulin. Antigen-antibody complexes were co-precipitated with *Staphylococcus aureus* (26). As shown in Table I, considerable radioactivity was specifically precipitated by anti-coat protein antibody from the soluble fraction of pulse-labeled infected cells. Less radioactivity is recovered in the immunoprecipitate after chase of the labeled cells, and immunoprecipitation of pulse-labeled cytoplasm of uninfected cells gave background radioactivity with both anti-coat and control antibodies. The radioactivity in anti-coat immunoprecipitates from pulse-labeled infected cells was found by SDS-gel electrophoresis to be almost exclusively in procoat (not shown).

The M13 coat protein offers an exceptional opportunity to follow the biosynthesis, assembly, and proteolysis of a membrane protein in *vitro*. Pulse-labeled procoat protein is apparently located in the cytoplasmic, soluble fraction of the infected cells. Pulse-chase experiments showed that this soluble procoat is integrated into the membrane and processed to membrane-bound coat protein. During M13 amber 7 infection of nonsuppressing hosts, the assembly and processing reactions are delayed, allowing the clearest demonstration of the assembly pathway. Pulse-labeled cells have soluble procoat and little or no membrane-bound procoat or coat (Figs. 2B and 7). Procoat chased from the cytoplasm, membrane-bound procoat is seen at early chase times, and coat protein appears in the membrane as procoat is lost. Addition of puromycin, immediately after completion of pulse labeling (Fig. 10), blocks polypeptide chain elongation but does not prevent the subsequent appearance of coat protein, showing that coat does indeed arise from the soluble procoat precursor.

The kinetics of procoat assembly and processing are more rapid in wild type infections, yet several lines of evidence suggest that the mechanism is the same in amber 7 infections. (i) In multiple experiments, where pulse-labeled infected cells were harvested by precipitation with trichloroacetic acid and analyzed by SDS-gel electrophoresis, from one-half to twice the amount of procoat as coat was found. (ii) Procoat is found in the soluble fraction (Figs. 2, 4, and 8). (iii) Soluble procoat has the same apparent sedimentation coefficient in wild type infections as in amber 7 infections (Figs. 4 and 5), and is found for the soluble procoat which is made in vitro (19) and which can post-translationally assemble into added membranes. (iv) Procoat synthesis is completed on polysomes which are not attached to the membrane (9), suggesting that the procoat is initially soluble. (v) The post-translational chase of procoat to coat is insensitive to arsenate and is sensitive to the same levels of azide, cyanide, dinitrophenol, and CCCP (carbonyl cyanide *p*-chlorophenylhydrazine) in amber 7 and in wild type M13 infections.

In amber 5 infection, where over twice the amount of coat protein is synthesized as in infection by wild type M13 (27) and viral single-stranded DNA is not produced, some of the procoat is assembled and processed very rapidly, other procoat is assembled rapidly but not processed, while some procoat remains in the soluble fraction in a monomeric state for several minutes (Figs. 2, 3, and 9). While we do not know the physiological basis of this behavior, it has been useful in three respects. (i) Procoat has been isolated in milligram quantities from the membranes of amber 5-infected cells. (ii) Procoat can be found in a soluble, monomeric state (Fig. 3) even though coat protein itself is quite water-insoluble (28) and the additional NH₂-terminal leader peptide is somewhat apolar. This is striking and novel evidence that the leader peptide can cause a protein to fold into a different conformation, in this case one which presumably buries hydrophobic regions in the protein interior. (iii) The persistent membrane-bound procoat in amber 5 infections provides additional evidence that cleavage of a leader sequence is not a prerequisite of membrane integration.
noted, or (iii) other factors. The only simple way to explain compared to larger membrane vesicles (as previously highly curved vesicles produced by French press lysis as possibly protein-protein interaction is transformed into a stable synthesis but not when they were added after the protein view of co-translational assembly may be to assume that the absence of procoat synthesis and integral membrane insertion; the presence of membranes during translation does not show that this assembly is not catalyzed by specific procoat assembly when membranes were added at the end of the small, highly curved vesicles produced by French press lysis as compared to larger membrane vesicles (as previously (19) noted), or (iii) other factors. The only simple way to explain the presence in vitro of a soluble precursor from the point of view of co-translational assembly may be to assume that the initial interaction of a nascent chain with the membrane is different from what is known of the stable protein-lipid interactions of integral membrane proteins. Before this weak (possibly protein-protein) interaction is transformed into a stable and integral state, the protein could be released from the membrane in vitro.

In contrast, post-translational assembly can explain the present findings without any particular assumption, as has already been discussed. In addition, many of the physiological properties of the assembly of procoat into membranes have been reproduced by our in vitro reconstruction experiments (19). Procoat synthesis can proceed without membranes. Procoat is initially water-soluble and then, post-translationally, assemblies integrally into the membrane. The rate of post-translational assembly was found to be proportional to the amount of added membrane. Finally, large unilamellar liposomes supported integral assembly of procoat. While this shows that procoat is capable of spontaneous assembly into lipid bilayers in vitro, it is important to emphasize that this does not prove that this assembly is not catalyzed by specific proteins in vivo.

The finding of soluble procoat is consistent with the idea that the leader peptide alters the folding pathway of newly made procoat protein to allow this otherwise water-insoluble protein to be compatible with the aqueous environment of the cytoplasm. This would answer the question of how hydrophobic membrane proteins avoid nonspecific aggregation during their synthesis. Whether conformational change as procoat encounters the lipid bilayer is enough for its subsequent integration into the membrane (19) or the process requires some catalysis (receptor, channel, pilot protein, or others) is an important question left for future studies.

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