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Biochemistry

Mechanisms of membrane assembly: Effects of energy poisons on the conversion of soluble M13 coliphage procoat to membrane-bound coat protein

(leader peptidase/membrane potential/integral membrane protein/uncoupler-resistant mutants/membrane trigger hypothesis)

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ABSTRACT **The coat protein (gene *8* product) of coliphage M13 spans the host cell plasma membrane prior to its assembly into extruding virions. It is made as a soluble precursor, termed procoat, with an extra 23 NH_2-terminal amino acid residues. We have examined the effect of metabolic poisons on the assembly of procoat into the plasma membrane and its proteolytic conversion to coat protein. Protein synthesis and proline uptake were measured to assess the effect of each poison on cellular high-energy phosphate and on the transmembrane protonmotive force, respectively. Arsenate, which abolished protein synthesis but did not affect proline uptake, had no measurable effect on the conversion of procoat to coat protein. In contrast, the uncoupler carbonylcyanide *m*-chlorophenylhydrazone (CCCP) blocked conversion of procoat to coat protein. Neither CCCP nor arsenate inhibited the ability of a detergent-solubilized and highly purified preparation of leader peptidase to convert procoat to coat protein in the presence of detergents. The procoat that accumulated in the presence of CCCP was membrane bound. A spontaneous mutant that grows in the presence of CCCP showed (*i*) CCCP-resistant proline uptake in whole cells, (*ii*) CCCP-resistant proline uptake in inner membrane vesicles, and (*iii*) CCCP-resistant conversion of procoat protein to coat protein. These data suggest that an electrochemical gradient is at least indirectly necessary for the proper assembly of procoat into the cellular membrane.**

M13 is a filamentous coliphage composed of a single-stranded circular DNA, 1–3 copies of an adsorption or "pilot" protein (1–4), and 2400 copies of coat protein, the product of virus gene *8* (1, 5–7). At each stage of virus infection, the coat protein is integrally bound to the host cell cytoplasmic membrane (8–11), with its NH_2 terminus on the outer surface and its COOH terminus exposed to the cytoplasm (12–14). The coat protein has 50 amino acid residues of known sequence (6, 7); residues 20–40 are hydrophobic and span the apolar center of the membrane. Both the coat protein from the infecting virus and the coat protein that is made *de novo* by the infected cell share this asymmetric orientation (12). Coat protein accounts for 7% of the cell's protein synthesis and a third of the inner membrane protein synthesis (9). Its abundance, known sequence, and easy isolation have made it a favorable object of studies of protein assembly into membranes (15–20).

As with many secreted and membrane proteins, coat protein is made as a precursor, termed "procoat." Procoat has 23 additional NH_2-terminal residues, termed its "leader" (17) or "signal" (16) peptide. This peptide's basic and hydrophobic sequence was deduced from the mRNA sequence (21) and confirmed by determining the nucleotide sequence of the DNA (22) and the amino acid sequence of procoat that was made with radioactive amino acids in a cell-free reaction (16). The leader peptide is rapidly removed after the protein assembles into the membrane (20); its subsequent fate is unknown.

Our recent studies (18) have shown that procoat is synthesized on polysomes that are not attached to the membrane. Pulse-labeled procoat is soluble and sediments in a sucrose gradient at 5 S (20). It is rapidly "chased" to the membrane fraction and is subsequently proteolytically cleaved to yield coat protein. These data indicate that the biosynthetic pathway is:

$$\text{Unattached polysomes} \xrightarrow{1} \text{Soluble procoat} \xrightarrow{2} \text{Membrane-bound procoat} \xrightarrow{3} \text{Membrane-bound coat}$$

Each of these three steps has been reproduced in crude or partially purified cell-free reaction mixtures. Procoat synthesized in a cell-free reaction (step 1) is initially soluble and sediments at 5 S (15) as it does *in vivo* (20). It assembles into membrane (step 2) after its synthesis is complete (18, 20). Not all membrane fractions serve equally well as "receptor" for procoat assembly (15); large membrane vesicles or liposomes appear optimal. Finally, the proteolytic processing of procoat (step 3), which had been reported to require detergent and to occur only during translation (16), has more recently been shown to occur posttranslationally in the absence of detergent (17). Leader peptidase, the membrane-bound enzyme that cleaves procoat, and procoat itself, have been purified to facilitate the study of these events (unpublished data).

The identification and isolation of the proper cell constituents that support the conversion of soluble procoat to membrane-bound coat may be aided by isolating mutants and identifying drugs that affect these steps. The identification of the soluble form of procoat and the demonstration that its processing, which occurs in the membrane, is independent of translation (20) have allowed us to study the effects of metabolic energy poisons on these events.

MATERIALS AND METHODS

Virus and bacterial strains, sources of isotopes and other biochemicals, and techniques of cell culture, pulse labeling, and analysis by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and fluorography were as previously described (18, 20). Carbonylcyanide *m*-chlorophenylhydrazone (CCCP) was purchased from Sigma.

A spontaneous mutant of *Escherichia coli* HJM114 was selected on rich agar plates (10-cm diameter) spread with 32 μmol of CCCP (dissolved in dimethyl sulfoxide). This mutant is named SWL14.

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Abbreviation: CCCP, carbonylcyanide *m*-chlorophenylhydrazone.

RESULTS

The assembly of soluble procoat into the membrane and its conversion to coat protein are delayed in cells infected with amber 7 M13 phage as compared to the wild-type virus infection (18). This allows these events (steps 2 and 3, above) to be studied with greater ease and clarity. To examine the effects of energy poisons, amber 7 and wild-type M13-infected cells were pulse-labeled with [^{3}H]proline. Cultures were chased with nonradioactive proline mixed with different poisons as indicated. Aliquots were removed after the pulse-labeling and after different times of chase. Each aliquot was precipitated with trichloroacetic acid, washed with acetone, boiled in sodium dodecyl sulfate, and electrophoresed on a sodium dodecyl sulfate/polyacrylamide slab gel. Fluorography of the dried gel was used to assay radioactivity in procoat and coat.

Arsenate. Procoat, but little coat, was labeled by a brief pulse of amber 7 M13-infected cells with [^{3}H]proline (Fig. 1*A*, lanes 7 and 10). During the succeeding 1½ min of chase, the procoat assembles into the membrane (18, 20) and is converted to coat protein (lanes 8 and 9). The addition of 0.1 M sodium arsenate with the chase of nonradioactive proline had no detectable effect on the rate or extent of conversion of procoat to coat (lanes 11 and 12). Qualitatively similar results were found in cells infected by wild-type M13 (lanes 1–3, no arsenate; lanes 4–6, with arsenate), though the conversion of procoat to coat was more rapid and therefore more coat protein was labeled during the pulse interval (18).

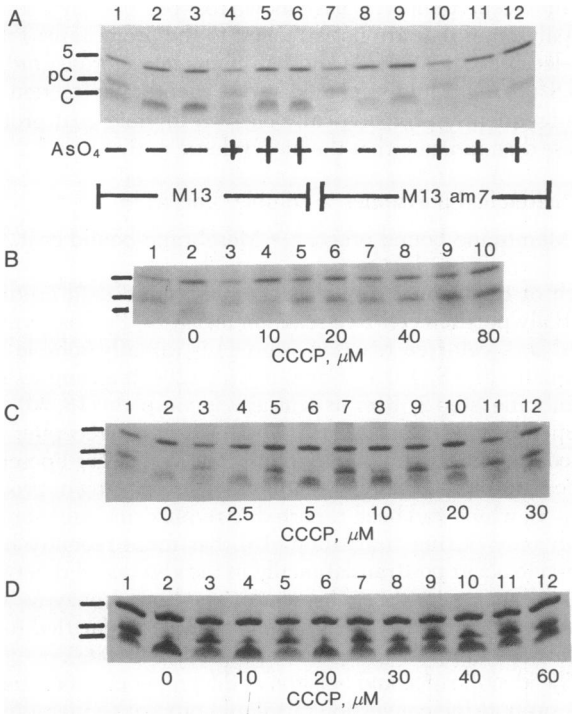

FIG. 1. Effects of poisons on the conversion of procoat to coat. (*A*) *E. coli* strain HJM114 was grown to OD_{600} = 0.4 at 37°C in GMA medium (23) and infected with either M13 or M13 amber 7 at a multiplicity of 100. After 1 hr of aeration at 37°C, two 0.6-ml portions of each culture were pulse-labeled for 15 sec with 30 μCi of [^{3}H]proline (1 mCi/ml, 100 Ci/mmol) (1 Ci = 3.7 × 10^{10} becquerels). One portion was chased with 0.5 mg of proline, the other with 0.5 mg of proline + $NaAsO_4$ (0.1 M final concentration). Lanes 1–6: wild-type M13-infected cells. Lanes 7–12: M13 amber 7-infected cells. Lanes 4–6 and 10–12: arsenate present during the chase. Samples were withdrawn, mixed with cold trichloroacetic acid, and analyzed by sodium dodecyl sulfate gel electrophoresis (18, 20). The positions of gene 5 protein (5), procoat (pC), and coat (C) are shown on the left. Each group of three lanes (1–3, 4–6, etc.) has cells harvested after only 5 sec of chase, followed by cells harvested after longer chase intervals. Chase times: lanes 1, 4, 7, and 10, 5 sec; lanes 2 and 5, 2 min; lanes 3 and 6, 6 min; lanes 8 and 11, 25 sec; lanes 9 and 12, 75 sec. (*B–D*) *E. coli* strain HJM114 (*B* and *C*) or SWL14 (*D*) was grown at 37°C in M9 + glucose medium (24) and infected at OD_{600} = 0.4 with either M13 (*C* and *D*) or M13 amber 7 (*B*) at a multiplicity of 100. After 1 hr, 0.4-ml portions of each were labeled for 15 sec with 20 μCi of [^{3}H]proline and chased with 0.3 mg of proline plus the indicated concentration of poison. Half of each portion was mixed with trichloroacetic acid after a 5-sec chase (odd lanes), the other half after 75 sec (even lanes).

Although arsenate had no measurable effect on the conversion of procoat to coat protein, it rapidly and completely stopped protein synthesis (Fig. 2*A*), presumably because of an abrupt drop in cellular pools of high-energy phosphates (25). Proline uptake was assayed as a measure of the protonmotive force (25). Arsenate had little effect on proline uptake in M13 amber 7-infected cells (Fig. 3*A*), as had been reported for uninfected cells (25). These data suggest that if there is any high-energy phosphate requirement for conversion of procoat to coat, it would have to have a low K_m.

CCCP. Posttranslational conversion of procoat to coat protein in M13 amber 7-infected cells was inhibited by 10 μM CCCP (Fig. 1*B*, lane 4) and was completely blocked by concentrations of 20 μM and above (lanes 6, 8, and 10). Similar inhibition was found in wild-type M13-infected cells (Fig. 1*C*). CCCP is thus a potent inhibitor of procoat processing; its mode of action was therefore studied in some detail. Marked inhibition of proline uptake was seen at 5 μM CCCP (Fig. 3*B*), presumably reflecting the dissipation of the protonmotive force. Higher levels of CCCP are necessary to block protein synthesis (Fig. 2*B*).

To confirm that CCCP inhibition of proline transport and of the conversion of procoat to coat protein was due to action at the same site, a spontaneous CCCP-resistant bacterial mutant, named SWL14, was isolated. This mutant showed CCCP-resistant growth in culture, CCCP-resistant proline uptake (Fig. 3*C*), and CCCP-resistant conversion of procoat to coat protein

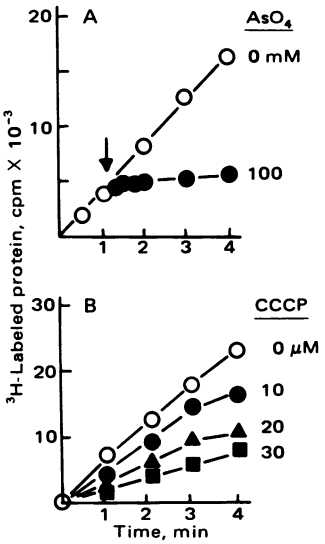

FIG. 2. Effects of poisons on protein synthesis. (*A*) HJM114 was grown to OD_{600} = 0.4 at 37°C in GMA medium and infected with M13 at a multiplicity of 100. After 1 hr, 1.2 ml of culture was mixed with [^{3}H]leucine (20 μCi, 3 μM, dissolved in 0.8 ml of GMA medium). After 1 min, 0.9 ml of the reaction mixture was mixed with 0.1 ml of 1 M sodium arsenate. Aliquots (0.1 ml) were withdrawn and assayed for acid-insoluble tritium. (*B*) One hour after infection of HJM114 (at OD_{600} = 0.4, 37°C, in M9 + glucose) with amber 7 M13, 0.36-ml portions of the culture were mixed with [^{3}H]leucine (6 μCi, 2 Ci/mol, in 0.24 ml of M9 medium) and the indicated concentration of CCCP. Aliquots (0.1 ml) were assayed for acid-insoluble tritium.

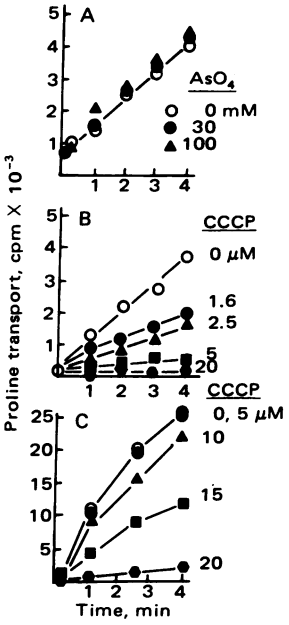

FIG. 3. Effects of poisons on proline transport (26). HJM114 (*A* and *B*) or SWL14 (*C*) was grown at 37°C in 21 ml of GMA (*A*) or M9 + glucose (*B* and *C*) to OD_{600} = 0.4 and infected with M13 amber 7 at a multiplicity of 100. After 1 hr, cells were collected by centrifugation and suspended in 1.4 ml of the respective minimal salt. Transport assays had 0.2% glucose, [^{3}H]proline (50 μM, 6 μCi), and the indicated poison in 3.0 ml of minimal salts solution. Reactions were initiated by the addition of 200 μl of cell suspension. Aliquots (0.55 ml) were removed at the indicated times and filtered through 0.45-μm-pore-diameter filters (Millipore). Filters were washed with 1 ml of proline-free salts solution, dried, and assayed for tritium.

(Fig. 1*D*) relative to HJM114, the parent strain. This mutant did not appear to have become CCCP-resistant by altering its drug permeability; proline transport by mutant strain plasma membrane vesicles, prepared according to Kaback (27), was CCCP resistant relative to vesicles from the parent strain, HJM114 (Fig. 4). These data suggest that: (*i*) an electrochemical gradient is required for the conversion of procoat protein to coat protein; (*ii*) a metabolite whose concentration is regulated by

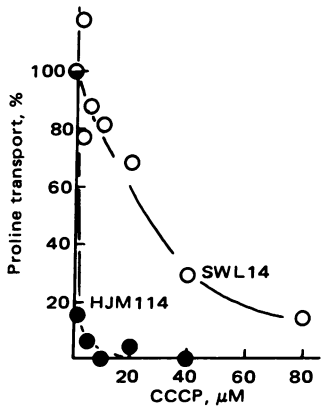

FIG. 4. Effects of CCCP on proline transport in membrane vesicles. Membranes were prepared as described by Kaback (27) from HJM114 and SWL14 grown at 37°C in medium 63 (28), 0.5% succinate, and vitamin B1 and were assayed as described (27) for D-lactate-driven proline transport. CCCP was present at the indicated levels; 100% transport was 3.7 nmol/mg·min for HJM114 and 5.0 nmol/mg·min for SWL14.

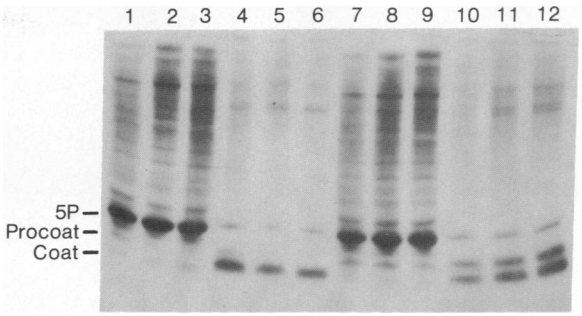

FIG. 5. Localization of procoat in the presence of CCCP. HJM114 was grown at 37°C in 12 ml of M9 + glucose to OD_{600} = 0.4 and infected with wild-type M13 at a multiplicity of 100. After 1 hr, the culture was divided in two and each portion was labeled with 200 μCi of [^{3}H]proline for 10 sec. One portion was chased with 1 mg of proline (lanes 1–6), the other with 1 mg of proline plus CCCP (30 μM final concentration, lanes 7–12). Aliquots (2 ml) were removed after 5 sec (lanes 3, 6, 9, and 12) of chase, converted to spheroplasts and disrupted by sonication, and separated into soluble (lanes 1–3, 7–9) and membrane (lanes 4–6, 10–12) fractions as described (18). Fractions were then concentrated by acid precipitation and analyzed by sodium dodecyl sulfate/polyacrylamide gel electrophoresis and fluorography.

the electrochemical gradient is needed for conversion of procoat to coat protein; or (*iii*) the protein whose mutation confers CCCP resistance is itself required for both processes.

To localize the procoat that persists in the CCCP-treated wild-type bacteria, HJM114 was infected with wild-type M13 and was pulse-labeled with [^{3}H]proline. Cells were then chased with an excess of nonradioactive proline and with either no poison or with CCCP. After 5, 20, and 50 sec of chase, portions of the culture were rapidly chilled, sonicated, separated into soluble and membrane fractions, and analyzed by sodium dodecyl sulfate gel electrophoresis and fluorography (Fig. 5). In the absence of drug (lanes 1–6), procoat was seen transiently in the soluble and membrane fractions (lanes 1 and 4) but rapidly disappeared from each. When CCCP was added during the chase (lanes 7–12), procoat persisted in both the soluble (lanes 7–9) and membrane (lanes 10–12) fractions, although it is clear that the bulk of the procoat was membrane bound (compare lanes 9 and 12).

Effects of Poisons on Leader Peptidase. The only step in the conversion of soluble M13 procoat to membrane-bound coat protein that has been shown to involve the making or breaking of covalent bonds is the proteolytic cleavage of membrane-bound procoat (step 3 as outlined above). We have solubilized and partially purified the responsible protease, termed leader peptidase, from uninfected *E. coli* (see Fig. 6 legend; unpublished). This enzyme was assayed by its ability to posttranslationally cleave procoat to coat (Fig. 6*A*). Neither 0.1 M arsenate (Fig. 6*B*, compare lanes 2 and 3) nor 300 μM CCCP (Fig. 6*C*, compare lanes 2 and 3) inhibited the leader peptidase. Because CCCP is clearly not an inhibitor of the isolated leader peptidase, its actions *in vivo* must be explained on other grounds. In contrast, as little as 0.1 mM dinitrophenol significantly inhibited this enzyme (Fig. 6*D*, lane 5), and proteolytic processing was completely blocked by 2 mM dinitrophenol (lane 3).

Effects of Other Metabolic Poisons. Cyanide, azide, and dinitrophenol each caused substantial inhibition of the post-translational conversion of procoat to coat protein (data not shown). The complexity of their metabolic effects, including the ability of each to inhibit isolated leader peptidase, precluded a detailed analysis of their effects on procoat metabolism.

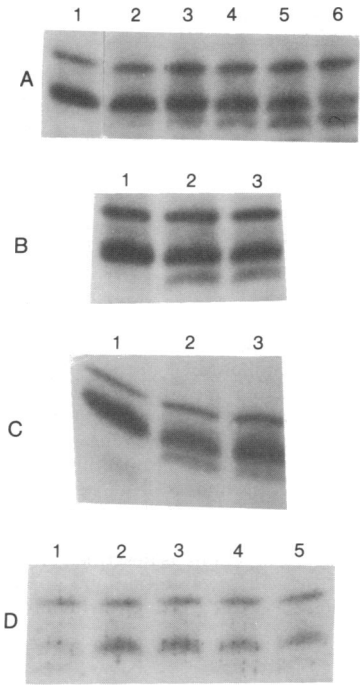

FIG. 6. Effects of poisons on soluble purified leader peptidase. Leader peptidase from *E. coli* Q13 (16, 17) was purified approximately 1000-fold by a procedure to be described in detail elsewhere. Briefly, a membrane fraction of Q13 was prepared by passage through a Mantin–Gaulin mill and differential centrifugation. Membranes were then extracted with Triton X-100 in a buffer containing Mg^{2+} (to inhibit solubilization of outer membrane proteins). This crude extract was dialyzed, adsorbed to DEAE-cellulose, and eluted with a gradient of KCl. Fractions containing leader peptidase activity were concentrated on a small column of DEAE-cellulose and subjected to gel filtration on Sephacryl S-300 (Pharmacia). Each assay contained (*i*) 10 μl of an *in vitro* M13 DNA-directed protein synthetic reaction mixture prepared in the presence of 1% Triton X-100 (17), (*ii*) 20 μl of reaction buffer (1% Triton X-100/50 mM triethanolamine·HCl, pH 7.5/5 mM $MgCl_2$) with sufficient inhibitor to give the indicated final concentrations, and (*iii*) 10 μl of leader peptidase in reaction buffer. After 60 min at 37°C, samples were subjected to sodium dodecyl sulfate gel electrophoresis and fluorography. (*A*) The assays in lanes 1–6 had 0, 13, 17, 55, 110, and 220 ng of leader peptidase. (*B*) Effect of arsenate. Lane 1, no enzyme; lane 2, 110 ng of enzyme; lane 3, 110 ng of enzyme + 100 mM arsenate. (*C*) Effect of CCCP. Lane 1, no enzyme; lane 2, 110 ng of enzyme; lane 3, 110 ng of enzyme + 300 μM CCCP. (*D*) Effect of 2,4-dinitrophenol. Lane 1, no enzyme; lanes 2–5, 55 ng of enzyme plus dinitrophenol at 4, 2, 1, and 0.1 mM, respectively.

DISCUSSION

The kinetics of procoat assembly into the membrane and of its proteolytic processing to coat protein have allowed these steps to be separated experimentally (18, 20) and, in the present study, to be examined for their energy requirements. Arsenate is thought to poison cells by substitution for phosphate in oxidative phosphorylation, forming a phosphate-arsenate anhydride bond with ADP, which quickly undergoes spontaneous hydrolysis (25). The primary effect of arsenate is therefore to deplete the cellular pool of high-energy phosphate bonds. This does not, however, affect the posttranslational metabolism of M13 procoat (Fig. 1*A*). In contrast, each of the uncouplers dinitrophenol, KCN, NaN_3, and CCCP inhibited the conversion of procoat to coat protein. At least part of the inhibitory effects of dinitrophenol, KCN, and NaN_3 might be due to their inhibition of leader peptidase. However, even 300 μM CCCP does not inhibit leader peptidase; this drug is therefore probably affecting procoat metabolism by its action as an uncoupler. The isolation of a mutant that simultaneously becomes CCCP resistant for energy metabolism and for the conversion of procoat to coat strongly supports the idea that an electrochemical gradient is needed for procoat metabolism. Although this could be an indirect requirement, such as the gradient being needed for maintenance of a proper intracellular ionic composition, the simplest interpretation is that the gradient itself is directly needed for procoat metabolism. CCCP does not dramatically affect the binding of procoat to the membrane, but it may affect the distribution of bound procoat across the plane of the bilayer. It is tempting to speculate that the transfer of the anionic region, residues 23–43, of procoat protein across the bilayer is driven by the electrical gradient, which is from negative inside the cell to positive outside. Whether or not this is the case, this pattern of inhibitor sensitivities will be a useful criterion of future cell-free reconstitutions of procoat assembly. Such cell-free studies will in turn be necessary to demonstrate the mechanisms of the inhibitors' actions.

The inhibition of procoat metabolism by uncouplers but not by arsenate also serves as an additional (18) demonstration that procoat enters the membrane and is proteolytically processed after its synthesis is complete rather than during translation. Protein synthesis requires nucleoside triphosphates, not a membrane potential. Procoat assembly and processing require approximately 60 sec in M13 amber 7-infected cells (18), yet they are insensitive to even high levels of arsenate (Fig. 1*A*), levels that shut off protein synthesis within 5 sec (Fig. 2*A*). In contrast, uncouplers inhibit procoat metabolism at levels that only partially inhibit protein synthesis. These findings are consistent with the membrane-triggered folding hypothesis (29) but are difficult to reconcile with cotranslational assembly, as suggested by the signal hypothesis (30, 31).

In addition to finding a requirement for an electrochemical gradient, these studies have shown that the conversion of procoat to coat shows the same response to each inhibitor in wild-type and amber 7 M13 infections, providing an additional line of evidence that the same mechanism is used in each case. As previously reported (18, 20), the conversion is slowed in amber 7 infections, yet in both cases the procoat is initially soluble and sediments at 5 S. Because of their delayed kinetics, amber 7-infected cells have provided a valuable physiological setting for these studies.

M13 procoat is not the only protein that requires energy for its posttranslational assembly into a membrane. The assembly of the ATP/ADP transport protein into the mitochondrial inner membrane has been shown to be CCCP sensitive *in vivo* and *in vitro* by Neupert and colleagues (32, 33). Recently, high-energy phosphate bonds have been shown to be necessary for the assembly of mitochondrial ATPase, cytochrome bc_1, and cytochrome c_1 peptides (34).

The ease of isolation of CCCP-resistant *E. coli* mutants is in agreement with reports of similar strains in yeast (35) and *Bacillus megaterium* (36). These strains may be useful to those studying energy metabolism *per se*, because conventional views of the mode of CCCP action suggest that resistant mutants should not occur.

The observation that leader peptidase is sensitive to dinitrophenol reinforces earlier observations (37) of the rather low specificity of uncouplers. This inhibitor may be useful in studying the mechanism of this unusual protease.

Note Added in Proof. Recent mapping studies have used proteases to show that both (*i*) the small proportion of pulse-labeled procoat that is membrane bound and (*ii*) the procoat that persists in the presence of CCCP are exposed only on the inner surface of the cytoplasmic membrane.

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