Proc. Natl. Acad. Sci. USA
Vol. 76, No. 3, pp. 1251–1255, March 1979
Cell Biology

Membrane biogenesis: Cotranslational integration of the bacteriophage f1 coat protein into an *Escherichia coli* membrane fraction

(bacteriophage f1 DNA/coupled transcription–translation system/post-translational proteolysis/sequence determination/topology of coat protein)

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Communicated by Norton D. Zinder, November 30, 1978

ABSTRACT **The coat protein (CP) of bacteriophage f1 is integrated into an *Escherichia coli* plasma membrane fraction consisting of inverted vesicles when it is synthesized in a cell-free, coupled transcription–translation system supplemented with the inverted vesicles. By using proteolytic enzymes as probes, we found by subsequent peptide mapping and determination of the sequence of the proteolytic products that CP was inserted into the inverted vesicles in an orientation indistinguishable from that in inverted vesicles prepared from infected *E. coli*: only a COOH-terminal portion of ≈10 residues was accessible to proteolysis, whereas the remainder of CP (CP′) was entirely protected. Protection of CP′ was dependent on the integrity of the vesicle membrane, because it was abolished when proteolysis was done in the presence of nonionic detergents. Insertion was observed when the inverted vesicles were present during translation in the cell-free system, not when they were added after translation. Thus, the asymmetric insertion of this type of integral membrane protein is strictly coupled to translation. These findings are discussed with respect to prokaryotic membrane biogenesis and are related to bacteriophage f1 assembly and infection.**

As part of the assembly process of bacteriophage f1, its coat protein (CP) is integrated into the plasma membrane of infected *Escherichia coli* (1). CP is a protein of 50 amino acid residues (2), which spans the membrane with its NH_2-terminal portion of ≈20 residues protruding into the periplasmic space (3). Its COOH-terminal segment of ≈10 residues has been proposed to extend into the cytoplasm (4). In an *E. coli* cell-free system that is largely devoid of membranes, CP is synthesized as a larger form (preCP) containing an additional "signal" sequence of 23 residues (5, 6).

Recently we have demonstrated (5) that this signal sequence is cleaved, yielding CP and signal peptide, when the cell-free system is supplemented with "inverted" vesicles derived from the plasma membrane of *E. coli*. Because cleavage was observed only when the vesicles were present during translation, we proposed, by analogy to eukaryotic secretory proteins (7, 8), that cleavage resulted from a translation-coupled penetration of nascent preCP, NH_2 terminus first, into the vesicle membrane. Our conjecture, therefore, was that this penetration would not only provide access to signal peptidase associated with the luminal aspects of the inverted vesicles (equivalent to periplasmic aspects of the plasma membrane) but would ultimately yield an asymmetric integration into the vesicle membrane of the processed and completed CP in an orientation that is indistinguishable from that achieved *in vivo*. Our present data provide direct evidence for these proposals. An account of this work was presented at the 17th annual meeting of the American Society for Cell Biology (9).

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METHODS AND MATERIALS

Protein synthesis was carried out in the coupled transcription–translation system as described (10, 11). A typical 50-μl reaction mixture contained 25 μCi of [^{35}S]Met (1 Ci = 3.7 × 10^{10} becquerels) and 0.28 A_{260} units of closed, circular replicating form I f1 DNA (11). Incubation was for 60 min at 37°C after which there was no additional incorporation of radioactivity into hot trichloroacetic acid-insoluble material. The various posttranslational assays that were carried out after the 60-min incubation for protein synthesis are detailed in the figure legends.

An inverted vesicle fraction from uninfected *E. coli* C90 was prepared as described (5), except that an additional EDTA treatment was carried out to remove remaining ribosomes. The described sucrose gradient fraction (35%–45%) containing inverted vesicles was sedimented (5) and resuspended in solution A (50 mM triethanolamine·HCl, pH 7.5/10 mM EDTA/1 mM dithiothreitol) at a concentration of 10 A_{280} units/ml [the absorbance was determined from a portion that was solubilized in 3% sodium dodecyl sulfate ($NaDodSO_4$) at 25°C]. Portions (8 ml) were layered over 2 ml of 0.58 M sucrose in solution B [same as solution A, except that it contains 5 mM $Mg(OAc)_2$ instead of 10 mM EDTA]. The rationale for including Mg^{2+} ions in solution B was to bind remaining EDTA. Centrifugation in a Spinco 40 rotor at 100,000 × *g* for 2 hr yielded a pellet that was resuspended in solution B at a concentration of 75 A_{280} units/ml (measured as above) and stored at −80°C.

The methods we have used to prepare membranes lead to the formation of "inverted" vesicles (12, 13). This seemed useful because they will present the same aspect to the protein synthesizing machinery as is found *in vivo*. We have not studied "right side out" vesicles prepared as discussed by Kaback (14).

E. coli MRE600 (15), *E. coli* K38, and bacteriophage f1 (16) were from our collection. *E. coli* C90 (17) was obtained from Worthington. An immunoselected IgG fraction prepared from a rabbit antiserum against f1 CP (3) was a generous gift of W. Wickner.

[^{35}S]Met at 1050 Ci/mmol was purchased from Amersham/Searle. [^{3}H]Asp at 25 Ci/mmol, [^{3}H]Lys at 72 Ci/mmol, [^{14}C]Pro at 248 mCi/mmol, [^{14}C]Glu at 260 mCi/mmol, and Triton X-100 were obtained from New England Nuclear. Bovine pancreatic DNase I, bovine pancreatic RNase A, and *N*-tosylphenylalanine chloromethyl ketone-treated trypsin were from Worthington. Pronase was from Boehringer Mannheim. Protein A-Sepharose CL-4B was from Pharmacia. Trasylol (aprotinin) was from Mobay Chemical Co., New York, and phenylmethylsulfonyl fluoride was from Calbiochem.

Abbreviations: CP, coat protein; $NaDodSO_4$, sodium dodecyl sulfate.

RESULTS

f1 DNA-directed protein synthesis was carried out as before, and the translation products were analyzed by $NaDodSO_4$/urea/polyacrylamide gel electrophoresis. Under the conditions used, there are several abundant products synthesized (Fig. 1 *left*, lane 4) (5, 11). Among these, CP is the most prominent product and is synthesized as a preCP, which has an additional 23 amino acid residues (signal sequence) at the NH_2 terminus of mature CP (lanes 3 and 4) (5, 6). As previously shown (5), nascent preCP can be processed cotranslationally to mature CP by a membrane fraction prepared from uninfected *E. coli* (lane 3). However, preCP cannot be processed to CP by a 1-hr post-translational incubation with the membrane fraction (lane 4). With an earlier preparation of membranes, only ≈20% of preCP was converted to CP in the cotranslational incubation (5). We have improved the extent of conversion of nascent preCP to CP with the use of an EDTA-treated membrane fraction. By titrating the amount of membranes required for optimal processing, we find a plateau at 9.4 A_{280} units/ml (data not shown).

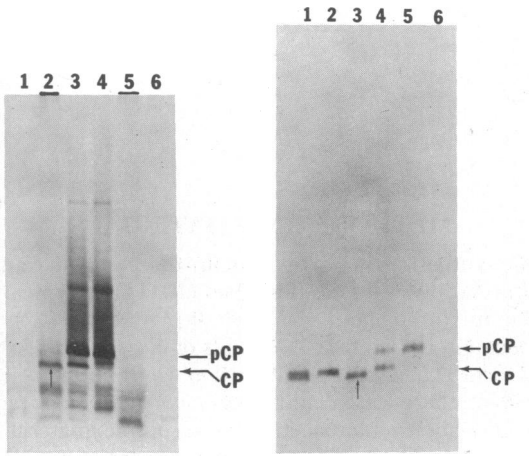

FIG. 1. (*Left*) Cotranslational cleavage and segregation of nascent preCP by inverted membranes. Bacteriophage f1 DNA was incubated in the coupled transcription–translation system either in the absence (lanes 4–6) or in the presence (lanes 1–3) of membranes (9.4 A_{280} units/ml). Following completion of translation, an equivalent amount of membranes was added to the reaction mixture from which they were absent during translation (lanes 4–6). Both reaction mixtures were subsequently incubated with DNase I (50 μg/ml) for 30 min at 37°C and with RNase (25 μg/ml) for 30 min at 4°C, were layered over 2 ml of 0.58 M sucrose in solution C (same as solution A except that solution C also contained 500 mM NaCl), and were centrifuged in a Spinco 40 rotor at 4°C for 2 hr. The pellets, containing the membrane fraction, were resuspended in solution D (150 mM NaCl/10 mM $CaCl_2$/50 mM Tris·HCl, pH 8.0) in a volume 4 times that of the original translation mixture. Equal-sized portions were then incubated in an ice bath for 24 hr either with no additions (lanes 3 and 4), with Pronase at 100 μg/ml (lanes 2 and 5), or with Pronase at 100 μg/ml and Triton X-100 at 1% final concentration (lanes 1 and 6). Proteolysis was terminated by the addition of 5 mM phenylmethylsulfonyl fluoride and incubation in a boiling water bath for 2 min. Analysis was by $NaDodSO_4$/urea/polyacrylamide gel electrophoresis and autoradiography of the dried slab gel. Horizontal arrows point to preCP (pCP) present in lanes 3 and 4, and to CP present only in lane 3. Vertical arrow points to CP′, present only in lane 2. There is a band in lane 4 with a mobility intermediate between preCP and CP. This intermediate is probably not related to CP because it cannot be immunoprecipitated (see *Right*, lane 5). (*Right*) Characterization of the products shown in *Left* by immunoprecipitation. For immunoprecipitation, 10-μl portions were incubated with $NaDodSO_4$ (2% final concentration) for 2 min in a boiling water bath. After the mixtures were cooled to room temperature, 0.2 ml of solution E (150 mM NaCl/50 mM Tris·HCl, pH 7.5/5 mM EDTA/1% Triton X-100/100 units of Trasylol per ml) and 2 μl of a solution containing an immunoselected antibody (3) against CP were added. After incubation at 6°C (cold-room temperature) for 15 hr, 25 μl of packed protein A-Sepharose was added, and incubation was continued at the same temperature for another hour. The protein A-Sepharose with the bound antigen–antibody complexes was then sedimented by centrifugation. The sediment was washed by four cycles of resuspension in solution E and subsequent centrifugation. Antigen and IgG were dissociated from the protein A-Sepharose by resuspension and boiling in a solution of 3% $NaDodSO_4$/50 mM Tris·HCl, pH 7.5/10 mM dithiothreitol. Analysis by electrophoresis and autoradiography was as in *Left*. Lanes: 4, membranes were present during translation; 5, membranes were absent during translation but added post-translationally; 3, as in 4, but incubated with Pronase; 6, as in 5, but incubated with Pronase; 2, ^{14}C-labeled CP from mature phage; 1, samples of 2 and 3 combined. Designation by arrows as in *Left*.

On *a priori* grounds (5) we reasoned that processing of nascent preCP to CP by the membrane fraction is probably coupled with insertion into membrane vesicles. One would then expect that one could examine the location of CP simply by purification and reisolation of the membrane vesicles from the translation mixture. We, therefore, treated the translation products with DNAse I and subsequently with RNase A, and reisolated the vesicles with 500 mM NaCl present which we hoped would minimize nonspecific association of proteins with the membrane. Unfortunately, all of the *in vitro* products, other than the gene 5 protein, cosedimented with the membrane vesicles (Fig. 1 *left*, lane 3) even when the membranes were added post-translationally (lane 4). Thus, this method did not show any specificity in terms of the proteins that were associated with the membrane vesicles and it was, therefore, deemed unsuitable as a test for membrane insertion.

We, therefore, turned to proteolytic enzymes to probe the association of protein with the membrane vesicles. Several proteolytic enzymes were used. Both trypsin and chymotrypsin gave incomplete proteolysis of the translation products (data not shown). However, post-translational incubation with Pronase at 0–4°C for 24 hr caused degradation of most translation products (Fig. 1 *left*, lanes 2 and 5; *right*, lanes 3 and 6) other than CP. The latter, however, was converted to a faster moving derivative, CP′ (Fig. 1 *left*, lane 2; *right*, lane 3), suggesting that a small fragment had been removed from CP by proteolysis. Because there was no CP′ detectable after post-translational proteolysis of a mixture that contained only preCP (Fig. 1 *left*, lane 5; *right*, lane 6), it is likely that CP′ was generated exclusively from CP and not from preCP. The differential sensitivity of CP and preCP to Pronase is not due to intrinsic structural differences between these two species but is clearly the result of a specific association of the newly synthesized CP with the inverted vesicle membrane. This is borne out by the observation that post-translational proteolysis in the presence of Triton X-100 to solubilize the vesicle membrane results in the proteolytic elimination of CP′ as a stable intermediate (Fig. 1 *left*, lane 1).

Three approaches were used to determine the topology of the newly synthesized and inserted f1 CP in the membrane vesicles. First, the product of proteolytic digestion (CP′), which, as shown above, has lost a small fragment, was precipitated with anti-f1 CP serum (Fig. 1 *right*, lane 3). The successful precipitation suggested that the NH_2 terminus of CP′ is intact, because it has been shown that this particular antibody reacts with antigenic determinants located within the first eight amino acid residues at the NH_2 terminus of the CP (3).

The second approach was to determine the partial NH_2-terminal sequence of CP′. CP was synthesized *in vitro* in the presence of the membrane fraction and of appropriate radioactive amino acids. Digestion with Pronase and subsequent immunoprecipitation was carried out as described (Fig. 1 *right*, lane 3), and the product obtained was monitored by subjecting

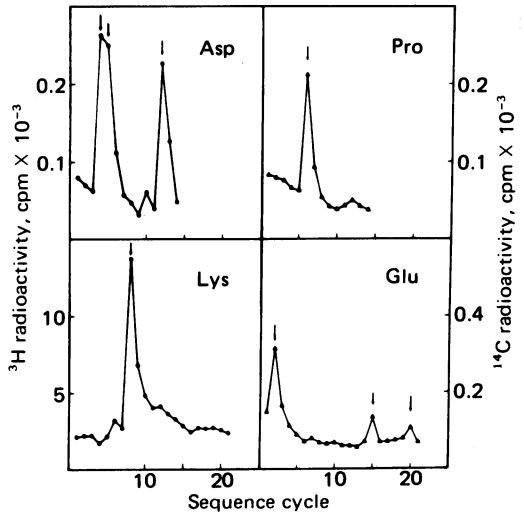

FIG. 2. Partial NH_2-terminal sequence of CP'. Bacteriophage f1 DNA was incubated in the coupled system in the presence of membranes (9.4 A_{280} units/ml) in a total volume of 200 μl containing two pairs of radiolabeled amino acids in separate assays: 75 μCi of [^{3}H]Asp and 60 μCi of [^{14}C]Pro; 100 μCi of [^{3}H]Lys and 15 μCi of [^{14}C]Glu. The translation products were incubated with Pronase and immunoprecipitated as in Fig. 1 *Right*. The immunoprecipitated products (cf. lane 3 of Fig. 1 *Right*) were taken through 14–21 cycles of Edman degradation. The recovered thiazolinones were dried and their radioactivities were determined. Arrows indicate sequence positions of the labeled amino acids.

a portion to gel electrophoresis to ensure that all of the CP had been converted to the digestion product, CP'. A partial NH_2-terminal sequence was then determined by consecutive automated Edman degradations. From the peaks of radioactivity (Fig. 2) it was possible to identify Asp at positions 4, 5, and 12, Pro at position 6, Lys at position 8, and Glu at positions 2, 15, and 20. Alignment of the partial sequence with the NH_2-terminal sequence of authentic CP (2) showed that the two sequences were the same—i.e., that the NH_2 terminus of CP' was protected from proteolysis.

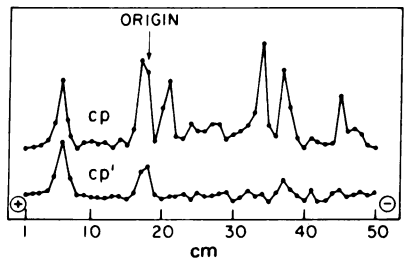

FIG. 3. High voltage paper electrophoresis of [^{3}H]Lys-labeled tryptic peptides of both CP and CP'. Bacteriophage f1 DNA was incubated in the coupled cell-free protein synthesizing system in the presence of both [^{3}H]Lys and membrane vesicle fraction (9.4 A_{280} units/ml). After completion of protein synthesis, half of the translation products were directly immunoprecipitated for gel electrophoresis, and the other half were processed for proteolysis with Pronase and subsequently immunoprecipitated for gel electrophoresis. The [^{35}S]Met-labeled translation products were also prepared and treated in a manner similar to those that were labeled with [^{3}H]Lys. The [^{3}H]Lys-labeled CP and CP' bands from the dried gel were located from the autoradiograph of the corresponding [^{35}S]Met-labeled bands. CP and CP' were eluted separately from the dried gel slices by electrophoresis. The eluted polypeptides were incubated at 37°C for 24 hr with *N*-tosylphenylalanine chloromethyl ketone-treated trypsin in 50 mM NH_4HCO_3, pH 8.0. The lyophilized tryptic peptides were then subjected to electrophoresis on Whatman 3MM paper at pH 3.5, 40 volts/cm, for 2 hr.

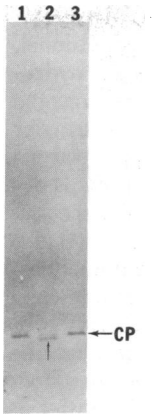

FIG. 4. Asymmetric orientation of the *in vivo* synthesized f1 coat protein on the cytoplasmic (inner) membrane of *E. coli*. *E. coli* strain K 38 was infected with bacteriophage f1 (with an amber mutation in gene 5) in the presence of [^{35}S]Met for 20 min. The inverted membrane vesicle fraction was prepared from these cells and incubated in the absence (lane 1) or presence (lane 2) of Pronase as described in Fig. 1 *Right*. Lane 3 contains ^{14}C-labeled CP from mature phage. Designation by arrows as in Fig. 1.

For the third approach we took advantage of the observation of Snell and Offord (18) that soluble peptides derived from the COOH terminus of filamentous phage protein move away from the origin towards the cathode on high-voltage paper electrophoresis at pH 3.5, whereas those derived from the NH_2-terminal portion of the molecule are basic and move towards the anode (most of the peptides derived from the central portion of the molecule are insoluble under these conditions and are either not transferred to the paper or remain at the origin). Fig. 3 shows that when CP' is subjected to this treatment, most of the basic peptides are lost, whereas the acidic NH_2-terminal peptide survives this treatment. Thus all lines of evidence suggest that the NH_2-terminal portion of the molecule is protected from digestion and is therefore located in the interior of the vesicles, whereas the COOH-terminal region remains accessible on the outside of the vesicles.

Finally, we investigated whether a CP-like fragment can be generated from CP that is synthesized *in vivo* and that is associated with the plasma membrane. Inverted vesicles were isolated from *E. coli* infected with bacteriophage f1 in the presence of [^{35}S]Met. The vesicles were incubated in the absence or presence of Pronase and then subjected to immunoprecipitation as described above (see Fig. 1 *right*). It can be seen from Fig. 4 that incubation with Pronase generates a fragment that is identical to CP' by two criteria: first, it has the same mobility as CP' and, second, it is immunoprecipitable, strongly suggesting that it was generated from CP by the removal of COOH-terminal, not of NH_2-terminal, residues. These data provide experimental support for the proposal (4) that the basic, COOH-terminal sequence of CP extends into the cytoplasm.

DISCUSSION

Our previous demonstration (5) of cotranslational cleavage of nascent preCP by an inverted vesicle fraction derived from the plasma membrane of *E. coli* and our present demonstration of cotranslational integration of CP into this vesicle fraction have contributed to the understanding of the mechanism of protein transfer across the prokaryotic plasma membrane. Taken together with results from other laboratories (19–22), our data suggest that the mode of protein transport across this membrane resembles that across the rough endoplasmic reticulum membrane of eukaryotic cells. An adaptation for the prokaryotic plasma membrane of the proposed models (23) for cotranslational transfer of the nascent chain, either partial or complete, across the rough endoplasmic reticulum membrane is shown in Fig. 5. The NH_2-terminal signal sequence of the nascent chain, after emerging from a space in the large ribosomal subunit, is postulated to interact with signal receptors in the plasma membrane. This is presumed to result in the formation of a proteinaceous tunnel in the membrane and of a functional

ribosome–membrane junction. There is as yet no direct evidence bearing on the existence of such a proteinaceous tunnel, but the observation that proteins containing strongly hydrophilic regions, such as RNase or alkaline phosphatase, cross the membrane would seem to require such a postulate. During or shortly after transfer of the signal sequence into the periplasmic space, it is removed by signal peptidase. This enzyme is depicted in Fig. 5 to be associated with the periplasmic aspect of the plasma membrane. However, rather than an entity that is separated from the postulated plasma membrane tunnel proposed in Fig. 5, the signal peptidase may actually be an integral part of the tunnel. The latter arrangement would permit efficient cleavage of the signal sequence as it passes through the pore and would thereby minimize chances to escape from processing. Cleavage, although tightly coupled to transfer, is however not envisioned as a *conditio sine qua non* for passage. Support for this latter notion comes from the recent demonstration that a single amino acid replacement in the signal sequence of an outer membrane protein inhibited cleavage but not transport (24).

The model shown in Fig. 5 postulates that the initial stages for either complete transfer resulting in secretion (lower part of Fig. 5) or partial transfer resulting in integration of the polypeptide chain into the membrane (upper part of Fig. 5) are identical. At some point during the transfer process, however, the passage of those polypeptides designated to become integral membrane proteins is halted. The information for stopping transfer presumably resides in the portion (or part of it) of the nascent chain that becomes lodged in the membrane. Precisely when transfer stops relative to chain termination and ribosome detachment (before, during, or after) is an interesting question. What is shown here is that the length of the COOH-terminal tail that does not enter the membrane in the case of CP (and is therefore susceptible to protease attack) is less than 10 residues long, and thus shorter than the length of chain that spans the large ribosomal subunit [30–40 residues (25, 26)]. It appears then that the insertion is completed and that the transfer stop occurs when the chain is no longer fully engaged with the ribosome, perhaps after chain termination and ribosome detachment.

The data presented in this and our previous paper (5) are consistent with the model illustrated in Fig. 5. In particular, our observations that cleavage of nascent preCP and "correct" integration of completed CP can occur *only* during translation (not after) provide substantial support to the proposed sequence of events.

It should be noted, however, that our conclusions on the mechanism of insertion of coat protein into the plasma membrane are at variance with those that were reached by Wickner

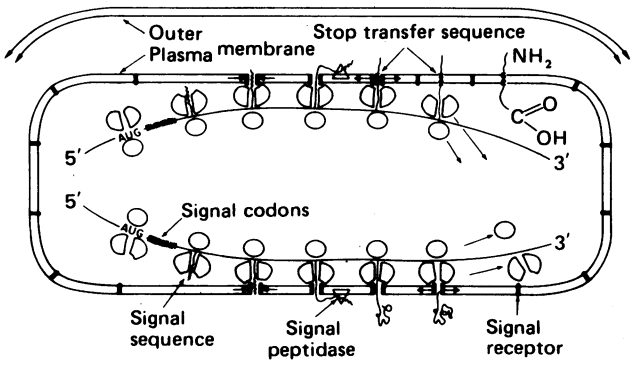

FIG. 5. Adaptation to the prokaryotic plasma membrane of models for cotranslational transfer or cotranslational integration of proteins described for the rough endoplasmic reticulum membrane of eukaryotes (23).

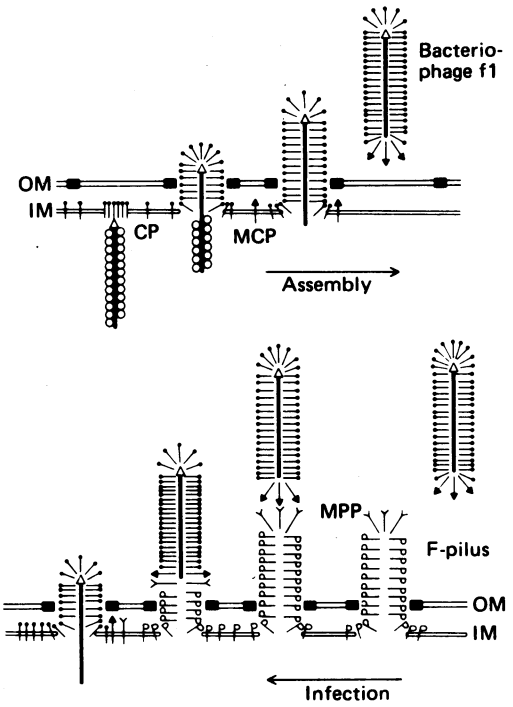

FIG. 6. Hypothetical model for vectorial polymerization and depolymerization of bacteriophage f1 CP in (or near) the plane of the inner membrane of *E. coli* during assembly (upper part) and infection (lower part), respectively. Bacteriophage f1 DNA specifically oriented with respect to one of its two termini (long thick arrow) and encapsulated by gene 5 protein (open circles) approaches a lipid-free patch of CP in the inner membrane (IM). CP is indicated by a bar with a terminal dot to mark its NH_2 terminus. Displacement of gene 5 protein by CP (28) is indicated to initiate "budding." Penetration through the outer membrane (OM) of the nascent phage bud could be mediated by the aggregation into a tightly fitting "annulus" of hypothetical integral proteins of the outer membrane (solid square), in simulation of a conceivably identical mechanism for the budding of pili from the inner membrane. Phage assembly could be terminated by the minor CP (MCP) indicated here as a bilateral integral membrane protein with an arrowhead to mark its NH_2 terminus. The annulus in the outer membrane would disaggregate upon passage of the phage. In infection, MCP would be recognized by a hypothetical minor F-pilus protein (MPP) located at the tip of the F pilus, resulting in alignment of the phage with the F pilus followed by complete depolymerization into the plane of the membrane first of pilin and then of CP and accompanied by the disassembly of the putative annulus in the outer membrane. It should be noted that there are five more phage-coded proteins in addition to the three (CP, MCP, and gene 5 protein) considered here. The role of four of these in the assembly process is presently obscure.

et al. (27). Binding studies of preCP with native membrane vesicles or with liposomes led these authors to conclude that preCP can be integrated post-translationally and suggested that refolding of preCP "as it encounters the bilayer is sufficient to transport large segments of the peptide chain through the apolar hydrocarbon core." Although we observed that preCP binds very strongly to inverted vesicles added after translation is completed, so much so that it remains bound even after centrifugation of the vesicles through sucrose cushions containing EDTA and a high concentration of salt (500 mM NaCl), this bound preCP could be degraded entirely and a stable CP′ intermediate could not be detected. We therefore conclude that completed preCP can bind to the inverted vesicles but cannot be integrated to achieve its physiological orientation.

CP belongs to a group of integral membrane proteins that can be classified as "bilateral." These proteins contain hydrophilic domains on opposite sides of the lipid bilayer in contrast

to "unilateral" integral membrane proteins, which have a hydrophilic domain on only one side of the bilayer. It is likely that bilateral integral membrane proteins that have the same polarity as CP (i.e., periplasmic NH_2 terminus and cytoplasmic COOH terminus) are integrated into the prokaryotic plasma membrane cotranslationally, exactly like CP.

The demonstration here that integration of *de novo* synthesized CP into the plasma membrane can occur only during translation needs to be related to the observation that, in infection, parental CP is integrated into the plasma membrane (28) in an apparently identical orientation (29) and, obviously, not by a cotranslational mode. Considerations of the sort which are illustrated in the model on phage assembly and phage infection shown in Fig. 6 can be invoked to explain how "correct" orientation is achieved. This figure is highly simplified in that it ignores a good deal of what is known of the detailed structure of the phage (30, 31) and that the uncoating of the virion has been shown to be coupled to the formation of parental duplex DNA (32). In this highly speculative model, virus assembly (upper part of Fig. 6) is envisioned essentially as an oriented polymerization process of membrane-integrated CP molecules, which would be initiated in the plane of the membrane and which could be terminated by the interaction of the "minor" CP (gene 3 protein) with the tail of the viral DNA. Virus infection (lower part of Fig. 6) is envisioned essentially as the reverse process, namely, as an oriented depolymerization of parental CP molecules in the plane of the membrane, which would result in the reintegration of CP molecules into the plasma membrane. For this to occur it would be necessary that recognition between the tip of the sex pilus and the minor CP of the infecting phage results in precise docking of these two structures, followed by depolymerization of the pilin (33) (which in Fig. 6 we also suggest to occur in the plane of the membrane) and subsequently of CP. The result would be that the depolymerized parental CP would assume an orientation in the plasma membrane that would be asymmetric and bilateral—i.e., indistinguishable from that of *de novo* synthesized CP. By extrapolation, then, the similarity between the polymerization and depolymerization processes envisioned here for CP and pilin suggests that the latter may be oriented in the plasma membrane in an asymmetric and bilateral orientation with a polarity similar to that of CP and that it may therefore be synthesized and integrated into the membrane by an identical mechanism.

We thank Ms. Helene Desruisseaux for her help with the use of the Beckman sequencer. This work was supported in part by National Institutes of Health Grants CA 12413 and CA 18213 and National Science Foundation Grant PCM 76 11662. C.N.C. is the recipient of National Institute of Health National Research Service Award Postdoctoral Fellowship 1F32 CA05713-01.

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