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STUDIES OF ASYMMETRIC MEMBRANE ASSEMBLY

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Summary

The major capsid protein of M13 bacteriophage is incorporated at each stage of infection into the host plasma membrane with its amino terminus exposed on the outer surface. Purified M13 coat protein is incorporated with the same asymmetry into synthetic phosphatidylcholine vesicles formed near the T_m of the lipid by a cholate dilution technique. We now report that the lipid in the pre-dilution mixture exists as mixed micelles of uniform size. Prior to dilution, the coat protein is present in at least two states of aggregation, both of which behave similarly in the model membrane assembly reaction. No detectable lipid-protein interaction occurs prior to dilution. Upon dilution there is rapid production of small closed vesicles and coat protein is converted to a chymotrypsin-resistant form, presumably reflecting its incorporation into these vesicle bilayers. Formation of large (>6000 Å diameter) vesicles occurs slowly with preservation of coat protein asymmetry and internal volume. A model for this assembly reaction is proposed.

Introduction

The asymmetric orientation of membrane components, particularly integral membrane proteins, is now well known [1]. The question of how this asymmetry is established is a central problem of membrane biosynthesis. One approach to this problem is to seek a simple model for membrane assembly.

The (gene 8) capsid protein of the filamentous coliphage M13 is a 5280 dalton peptide with an acidic N-terminus, a basic C-terminus, and a hydrophobic center [1,2]. This protein is inserted in the host plasma membrane during infection [4—7]. Both this parental coat protein and the newly synthesized coat protein, which is also membrane-bound, are later utilized to produce progeny virus particles [7]. The parental and progeny coat proteins are oriented with their amino-termini exposed to the outer surface of the host plasma membrane [8]. It has been postulated that the basic carboxyl terminus is exposed on the

interior surface of the plasma membrane where it can bind the acidic viral DNA [9].

This orientation is also found when M13 coat protein assembles into phosphatidylcholine vesicles [10] prepared by the cholate-dilution technique of Racker et al. [11]. In this procedure, a clear, non-sedimentable solution of coat protein, lipid, and cholate is diluted below the critical micellar concentration of cholate, resulting in vesicle formation. When vesicles are prepared near the phase transition temperature (T_m) of the lipid fatty-acyl groups, the coat protein amino-terminus is found exposed on the exterior surface of the vesicle while the carboxy terminus is exposed to the interior space of the vesicle [12]. A previous communication from this laboratory [12] showed this orientation to be independent of such factors as pH, ionic strength, the identity of lipid polar head group or the presence of either polar portion of the coat protein sequence. It was suggested that orientation might be the result of hydrophobic interactions between the lipid hydrocarbon core and the hydrophobic protion of the coat protein during the assembly reaction.

We now report the characterization of the molecular species participating in the vesicle-forming reaction and the kinetics of vesicle formation. The coat protein is found to exist in two general forms, a highly aggregated form and a smaller, possibly dimeric, form. The lipid is present as a single molecular weight species, probably in a mixed micellar structure with detergent. No interactions were found between lipid and protein prior to initiation of vesicle formation.

Time course experiments indicated that after dilution there was a rapid incorporation of coat protein into structures in which the coat-protein C-terminal residues were resistent to proteolysis by chymotrypsin. After this reaction was complete, there was a slower formation of large ($\geqslant$6000 Å diameter) vesicles. Under similar conditions, the formation of closed vesicles was initially very rapid and then continued throughout the incubation period at a lower rate.

We suggest that the coat protein is initially inserted asymmetrically into small vesicular structures. Initial asymmetry of the protein-lipid association is mediated by forces which are not well understood but which could include a graded hydrophobicity of the protein's non-polar region and/or physical asymmetries in the protein or bilayer. Once the protein and lipid have formed small vesicles, fusion of these small vesicles, with preservation of protein asymmetry and internal volume, could produce large vesicles.

Materials and Methods

Materials. Bovine α-chymotrypsin was purchased from Sigma, phosphatidylcholine was from Calbiochem. ^{3}H-Labelled and ^{14}C-labelled glucose was purchased from ICN Pharmaceuticals. [^{32}P]ϕX174 Virus and [^{32}P]G4 phage were the generous gifts of Drs. Michael Farber, Jon Govner and Dan S. Ray of this Institute.

Methods. Growth and isolation of M13 virus and purification of M13 coat protein from the virus were by previously published methods [8]. Coat protein was solubilized by incubation for 30 min at 37° C in either $NaHCO_3$ (0.02 M, pH 8.5) with 0.92% sodium cholate, $NaHCO_3$ (0.1 M, pH 9.5) with 2%

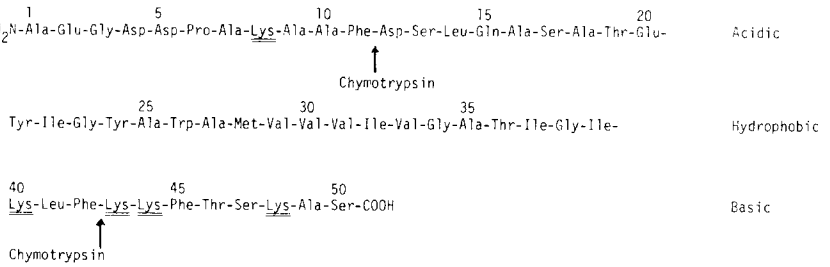

Fig. 1. Amino acid sequence of the M13 coat protein. Lysine, which was tritium-labeled in the current study, is underlined. Points of cleavage by chymotrypsin are indicated by arrows.

sodium deoxycholate or $NaHCO_3$ (0.1 M, pH 9.5) with 1% sodium cholate. Solutions were centrifuged (0°C, 30 min, 44000 × *g*) to remove any undissolved material. Unless otherwise noted, all preparations contained 0.5 mg protein per ml. Dimyristoyl phosphatidylcholine dispersions were formed by sonicating 40 mg dimyristoyl phosphatidylcholine in 1 ml of 0.1 M potassium phosphate, pH 7.0 at room temperature.

Vesicles were produced by the cholate dilution technique of Racker et al. [11]. Typically, 5 parts protein-cholate solution and 2 parts of 40 mg/ml dimyristoyl phosphatidylcholine were mixed and incubated 30 min at room temperature. This mixture was then diluted 30 fold with 0.1 M potassium phosphate, pH 7.0 at 20°C. The diluted mixture was incubated at 20°C for the time indicated.

Incorporation of coat protein into lipid complexes was followed by observing the development of resistant of the C-terminal lysines to chymotrypsin digestion as described previously [10,12]. Briefly, M13 coat protein (Fig. 1) contains 5 lysine residues, one near the N-terminus and 4 near the C-terminus of the protein. Chymotrypsin digestion releases the N-terminal lysine and three of the C-terminal lysines from the detergent-solubilized coat protein [10,13]. Upon incorporation into vesicles, the C-terminal lysines become resistant to chymotryptic digestion [10]. Incorporation of coat protein into large vesicles was measured by sedimentation.

Results

Physical state of the lipid. A sonicated suspension of [^{3}H]dimyristoyl phosphatidylcholine was mixed with sodium cholate in the absence of coat protein. The suspension clarified at once. It was analyzed by gel filtration in the same buffer and detergent. The lipid eluted in a single peak with K_{av}, 0.5 (Fig. 2) as would be expected for a mixed micelle of cholate and lipid.

Physical state of the protein was also measured by gel filtration. Detergent-solubilized M13 coat protein was found in two states of aggregation, one excluded from the resin and one eluting in a peak with K_{av}, 0.5 (Fig. 3A). A greater proportion of the protein was recovered in the included peak, shown by others to be a coat protein dimer [14], at alkaline pH and high detergent concentrations (Fig. 3B). The excluded species (not shown) and the dimer (Fig. 3C) gave single peaks with unchanged K_{av} upon second filtrations. Dimeric pro-

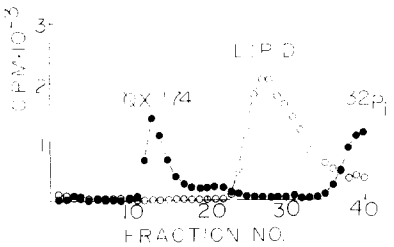

Fig. 2. Gel filtration of dimyristoyl phosphatidylcholine/cholate mixture. ^{3}H-Labeled dimyristoyl phosphatidylcholine (50 μl, 40 mg/ml, 28000 cpm), prepared as in Materials and Methods, was added to 125 μl of 0.92% sodium cholate in 0.02 M $NaHCO_3$, pH 8.5 and incubated for 30 min at room temperature. $H^{32}PO_4$ and [^{32}P]ϕX174 in 0.1 M potassium phosphate, pH 7.0 (5 μl, 10^4 cpm of each) were added as column volume and void volume markers respectively. This mixture was applied to a 1.5 X 9 cm Sephadex G-150SF column equilibrated with a buffer consisting of five parts 0.92% sodium cholate in 0.02 M $NaHCO_3$, pH 8.5 plus two parts of 0.1 M potassium phosphate, pH 7.0. Fractions (0.5 ml) were collected and assayed for ^{3}H (○) and ^{32}P (●).

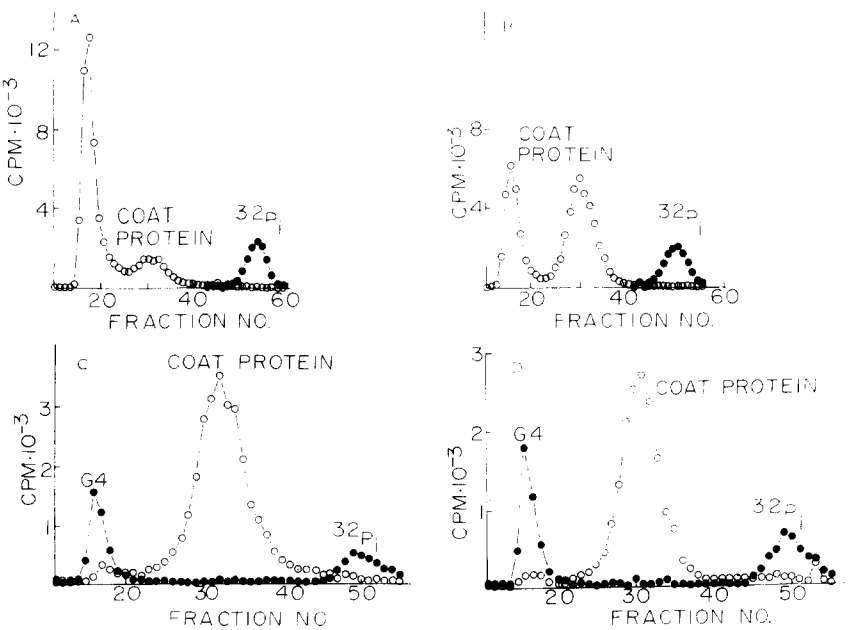

Fig. 3. Gel filtration of M13 coat protein. (A), Coat protein (0.5 mg/ml, 1.5 · 10^6 cpm) was solubilized in 0.5 ml of 0.92% sodium cholate in 0.02 M $NaHCO_3$, pH 8.5 as described in Materials and Methods. Inorganic $H^{32}PO_4$ (3 · 10^5 cpm) was added as a column volume marker and the solution was applied to a 1 X 13 cm Sephadex G-150SF column equilibrated with the same buffer. Fractions (0.2 ml) were collected and 15-μl aliquots were assayed for ^{3}H (○) and ^{32}P (●). (B), coat protein (0.5 mg/ml, 1.5 · 10^6 cpm) was solubilized in 0.5 ml of 2% sodium deoxycholate in 0.1 M $NaHCO_3$, pH 9.5 as described as above. 5 μl of $H^{32}PO_4$ in 0.1 M potassium phosphate, pH 7.0 was added as a column volume marker. This was applied to a 1 X 13 cm Sephadex G-150SF column equilibrated with 1% sodium cholate in 0.1 M $NaHCO_3$, pH 9.5 fractions (0.2 ml) were collected and 15-μl was assayed for ^{3}H (○) and ^{32}P (●); (C), included peak coat protein was prepared by pooling the appropriate fractions described in 3B above. 100-μl of this solution (4.7 · 10^4 cpm) was mixed with 4 μl each of $H^{32}PO_4$ (1600 cpm) and ^{32}P-G4 bacteriophage (2000 cpm) acting as column and void volume markers respectively. This was applied to a 1 X 13 cm Sephadex G-150SF column equilibrated with 1% sodium cholate in 0.1 M $NaHCO_3$, pH 9.5. Fractions (0.2 ml) were collected and assayed for ^{3}H (○) and ^{32}P (●); (D), 50 μl of included peak material was incubated for 30 min at room temperature with 16 μl of dimyristoyl phosphatidylcholine solution (40 mg/ml in 0.1 M potassium phosphate, pH 7.0). ^{32}P-G4 and $H^{32}PO_4$ were added as above and the solution was applied to a 1 X 13 cm column equilibrated with five parts 1% sodium cholate in 0.1 M $NaHCO_3$, pH 9.5 and 2 parts 0.1 M potassium phosphate, pH 7.0. Fractions (0.2 ml) were collected and assayed for ^{3}H (○) and ^{32}P (●).

tein from the experiment shown in Fig. 3B was mixed with dimyristoyl phosphatidylcholine and again analyzed by gel filtration (Fig. 3D). There was no detectable change in the position of protein elution, indicating a failure of protein and lipid to form vesicles in the presence of high detergent concentrations. Both aggregated and dimeric species were 50—80% incorporated into vesicles upon dilution of the detergent-protein-lipid solution (data not shown).

Kinetics of membrane assembly. Protection of [^{3}H]lysyl residues from chymotrypsin was used to assay incorporation of coat protein into closed structures. Both before and after initiation of vesicle formation by dilution, aliquots were heated to 37°C, where coat protein will not be incorporated into vesicles [12]. Chymotrypsin digestion at 37°C was followed by measurement of nonsedimentable ^{3}H that was included in Sephadex G150, representing lysine-bearing unprotected portions of coat protein, and ^{3}H that was either sedimentable or was nonsedimentable but excluded from the gel, representing the protected coat protein. Prior to dilution, chymotrypsin released four-fifths of the lysines (Fig. 4A), consistant with complete accessibility of coat protein [13],

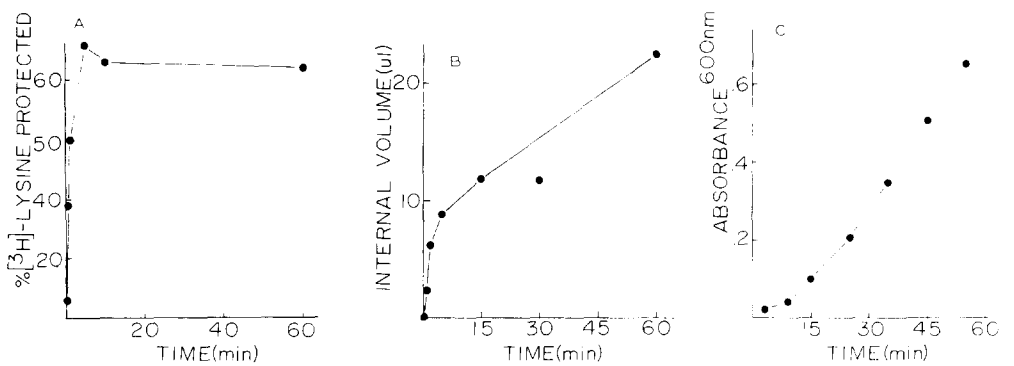

Fig. 4. Kinetics of membrane assembly. (A), Incorporation of M13 coat protein into dimyristoyl phosphatidylcholine vesicles. 0.5 ml of labeled M13 coat protein (0.5 mg/ml, $1.5 \cdot 10^5$ cpm) solubilized in 0.92% sodium cholate, 0.02 M $NaHCO_3$, pH 8.5 was mixed with 0.2 ml dimyristoyl phosphatidylcholine solution prepared as described in Materials and Methods. After incubation at 20°C for 30 min, a 30-µl aliquot was removed (zero time). The remainder was diluted 25-fold with 0.1 M potassium phosphate pH 7.0 at 20°C. At intervals up to 60 min, 1.0-ml aliquots were removed and digested for 30 min at 37°C with chymotrypsin (50 µg/ml). The digests were quickly chilled and centrifuged (0°C, 10 min, 440 000 × *g*). The pellets were assayed for ^{3}H. The supernatants and the zero time sample were applied to 1.5 × 9 cm Sephadex G-150SF columns equilibrated with 0.1 M potassium phosphate pH 7.0 room temperature. Fractions (1 ml) were collected and assayed for ^{3}H. "Protected counts" were defined as activity appearing in the pellet plus that eluting at the void volume of the columns. The percentage of recovered counts that were protected was determined (•); (B), development of closed vesicles. Unlabeled coat protein (0.5 mg/ml in 1% sodium cholate, 0.1 M $NaHCO_3$, pH 9.5) was mixed with dimyristoyl phosphatidylcholine as described in Materials and Methods. To a 175-µl aliquot of this solution, [^{3}H]glucose (10^7 cpm) was added. This and a separate 1.02 ml aliquot of the protein/dimyristoyl phosphatidylcholine mixture were simultaneously diluted 30-fold with 0.1 M potassium phosphate, pH 7.0 at 20°C. At various times after dilution, 5-ml aliquots were removed from the unlabeled vesicle preparation and placed in tubes containing [^{3}H]glucose (10^7 cmp). After removal of the last time point all samples were chilled to 0°C. [^{14}C]Glucose (10^7 cmp) was added, all samples were centrifuged (0°C, 15 min, 35 000 × *g*) and the pellets were resuspended in 1 ml of 1% sodium dodecyl sulfate at 40°C. Both the supernatants and the pellets were assayed for ^{3}H and ^{14}C. Internal vesicle volume was determined by correcting the [^{3}H]glucose radioactivity for external water in the pellet, measured by [^{14}C]glucose radioactivity; (C), large vesicle development. Vesicles were prepared as described in Materials and Methods (0.5 mg/ml coat protein in 0.1 M $NaHCO_3$, pH 9.5 with 1% sodium cholate, 40 mg/ml dimyristoyl phosphatidylcholine in 0.1 M potassium phosphate, pH 7.0). At various times after dilution aliquots were removed and the absorbance at 600 nm was determined (•).

since lysine 40 is not released by protease. Within 5 min of initiating vesicle formation, 62% of the lysines became resistant to chymotrypsin (Fig. 4A). Since lys_8 never became resistant and since incorporation of coat protein into vesicles rarely exceeded 80% at any time, this 62% protection represented essentially complete protection of the incorporated protein's C-termini.

In contrast, the appearance of turbidity, measured as absorbance at 600 nm and representing the formation of vesicles with a diameter comparable to 600 nm, occurred slowly (Fig. 4C). 5 min after the concentrated solution of cholate, dimyristoyl phosphatidylcholine and coat protein had been diluted to initiate vesicle formation, at a time when protection of the coat protein termini from chymotrypsin was completed, less than 5% of the final turbidity was present.

To determine whether coat protein might be rapidly protected from chymotrypsin by incorporation into small vesicles, the kinetics of vesicle internal volume formation were measured. There was a rapid appearance of internal volume (Fig. 4B) followed by a phase of slow volume increase.

Discussion

Membrane protein asymmetry is the characteristic of each of the many copies of a particular membrane protein in a cell having a common orientation with respect to the plane of the bilayer. This asymmetry is apparently ubiquitous in biological membranes [1], yet its origin is, at present, obscure. Proposed explanations have included: (i) Special sites of synthesis of membrane proteins, (ii) catalytic "flippases" which orient membrane proteins according to information in their sequence or in "leader" peptide sequences not present in the mature protein, (iii) orientation as a response to lipid polar head group or fatty acyl chain asymmetry, (iv) pH or other ion gradients and (v) differences in the lateral compressibility of the two halves of the bilayer.

Unfortunately, few data are available which bears on any of these mechanisms. Reconstitution of purified proteins with pre-sealed membrane vesicles of either natural or synthetic origin [15—17] had lead to binding exclusively on the outer membrane face. In recent years, Racker and co-workers have achieved partial reconstitutions of oxidative phosphorylation with partially purified preparations of lipids and proteins. The widely-held assumption that this process requires a physically vectorial distribution of the electron carriers implies that these reconstitution methods are sufficient for establishing membrane protein asymmetry.

We have examined these questions with the aid of a viral probe, the coat protein of coliphage M13. Our previous studies [8] showed that coat protein of the infecting virus and coat protein synthesized de novo within the infected cell have the same orientation across the cytoplasmic membrane. This demonstrated that a membrane protein can be oriented without involvement with the machinery of protein synthesis and favored the non-catalytic models of assembly. Using Racker's cholate-dilution technique, it was found that coat protein could be assembled into synthetic lipid vesicles [10]. The protein in such vesicles had a unique orientation even though the synthetic lipid employed had but a single type of polar head group (phosphorylcholine) and a single type of

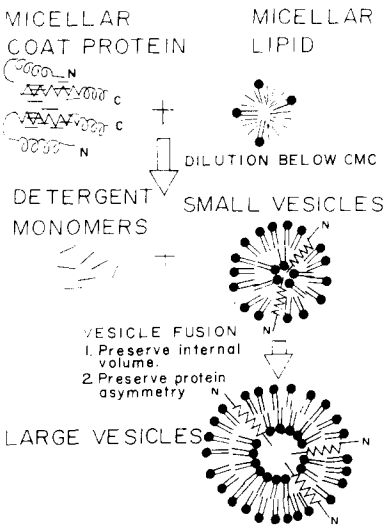

Fig. 5. Model for the formation of asymmetric lipoprotein vesicles by cholate dilution. M13 coat protein and lipid are present prior to dilution in cholate micelles. Upon dilution below the critical micellar concentration of the detergent, small vesicles form with asymmetrically oriented coat protein. These then fuse, preserving both internal volume and protein orientation, to form large vesicles.

fatty acyl chain (lauryl or myristoyl). When this assembly occurred at temperatures which were physiological in being near the lipid fatty acyl T_m, the coat protein spanned the bilayer with the physiological orientation. At lower temperatures, that protein which was incorporated had both ends exposed to the vesicle exterior. In contrast to this pronounced effect of the physical state of the hydrophobic phase, polar interactions seemed to have little role in orientation. We therefore proposed [12] that the asymmetry of assembly in this model reaction was governed by hydrophobic forces.

In this report, we have examined in greater detail the physical state of the reactants prior to vesicle assembly and the kinetics of this assembly.

After vesicle formation was initiated by dilution, there was rapid formation of internal volume (defined experimentally as a space which can trap labelled glucose). Since turbidity (absorbance at 600 nm) developed over a longer period of incubation, the initial internal volume was presumably in small vesicles. Another event occuring soon after dilution is the protection of the lysine-rich C-terminus of coat protein from protease. We propose that this is a result of protein incorporation into these small vesicles during their formation. Vesicles then fuse during subsequent incubation with preservation of internal volume and protein orientation. This working hypothesis is shown schematically in Fig. 5. Such a model is, at best, an oversimplification of the assembly process. Nevertheless, understanding this "simple" asymmetric assembly may provide a foundation for studying the more complex cellular processes.

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