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Asymmetric orientation of phage M13 coat protein in *Escherichia coli* cytoplasmic membranes and in synthetic lipid vesicles

(virus structure/membrane assembly)

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ABSTRACT **At each stage of infection, the major coat protein of coliphage M13 binds to the *E. coli* cytoplasmic membrane with its antigenic site exposed to the cell exterior [Wickner, W. (1975) *Proc. Nat. Acad. Sci. USA* 72, 4749–4753]. This antigenic site is now shown to be at the amino-terminus of the protein. The amino-terminus of M13 coat protein is also found exclusively on the outside of dilauroyl or dimyristoyl lecithin vesicles, formed with coat protein by the cholate dilution technique [Racker, E., *et al.* (1975) *FEBS Lett.* 57, 14–18] near the lipid phase transition temperature. The basic carboxyterminus of the coat protein is exclusively on the inside of these vesicles. Vesicles of M13 coat protein and dimyristoyl lecithin when formed below the lipid phase transition temperature have both ends of the coat protein exposed to the vesicle exterior. The asymmetry of a membrane protein can, therefore, be established in the absence of other proteins and of lipid asymmetry; it depends on the physical state of the lipid phase. The factors which cause asymmetry in this model system may affect the distribution of proteins in biological membranes.**

Asymmetric distribution of proteins with respect to the plane of the lipid bilayer appears to be a fundamental property of biological membranes (1). The mechanism which establishes this distribution is a central problem of membrane assembly. The major coat protein of the filamentous coliphage M13 (2) has several advantages for the study of this problem. This protein, whether brought to the cell by infecting virus or produced within the infected *Escherichia coli*, is found firmly bound to the cytoplasmic membrane (3–6). The amino acid sequence of M13 coat protein is known (7, 8); it has 50 amino acids, comprising an acidic NH_2-terminus, a basic COOH-terminus, and a central hydrophobic region. X-ray and circular dichroism studies have led to a model of virus structure (9) in which the DNA, at the center of the filament, is surrounded by largely α-helical coat protein molecules in a shingled array. In this model, the basic COOH-terminus interacts electrostatically with the DNA, whereas the more acidic NH_2-terminus is exposed to the solvent.

As reported previously (10), purified antibody to M13 coat protein can bind to the external membrane surface of M13-infected cells but does not appear to recognize antigen on sonicated, inverted vesicles from these same cells. This asymmetry is characteristic of the infecting, parental coat protein as well as the newly synthesized, progeny coat protein. In this report, this same antibody preparation is shown to recognize only the first eight amino acid residues from the NH_2-terminus of M13 coat protein. Since antibody was purified by combination with virus, this result supports the contention that the NH_2-terminus of coat protein is exposed in the virion. It also implies that the NH_2-terminus of the coat protein is on the exterior surface of the cytoplasmic membrane. Marvin and Wachtel (9) have proposed that the COOH-terminus of coat protein is on the interior of the cell membrane, where it may bind to the progeny viral DNA and aid in its passage through the bilayer.

In support of such an orientation, M13 coat protein is now found to assemble asymmetrically into lipid bilayer vesicles composed of dilauroyl or dimyristoyl lecithin. In such vesicles, prepared near the lipid phase transition temperature (T_m), the amino-terminus is completely accessible to protease from the solvent, whereas the carboxy-terminus is completely inaccessible to protease. In dimyristoyllecithin vesicles prepared well below the T_m, both ends of the coat protein are exposed to solvent. These data suggest that asymmetric orientation is governed by the structure of the protein and the physical state of the lipid and can arise in an otherwise symmetric lipid bilayer in the absence of other proteins.

MATERIALS AND METHODS

Materials. α-Chymotrypsin (type II) and cholate were purchased from Sigma, trypsin treated with L-1-tosylamido-2-phenylethyl chloromethyl ketone was from Worthington, and lecithins were from Calbiochem.

Methods. Growth and isolation of M13 virus were by previously published techniques (10). M13 coat protein was purified from the virus by the phenol extraction method of Knippers and Hoffmann-Berling (11). Where indicated, coat protein was solubilized by incubation in 0.01 M $NaHCO_3$ (pH 9) buffer with 5% sodium deoxycholate at 37° for 30 min. Undissolved material was removed by centrifugation. Antibody to M13 coat protein was prepared in rabbits and purified by combination with virus by published methods (10).

RESULTS

The M13 coat protein (Fig. 1) has 50 amino acids, comprising an acidic NH_2-terminus, a hydrophobic central region (residues 21–39), and a basic COOH-terminus (Fig. 1). In much of the work that follows, coat protein was isolated from virus grown with specific radioactive supplements. $^{35}SO_4$ labels methionine 28, providing a marker for the central hydrophobic region. [^{3}H]Proline labels position 6 at the NH_2-terminus, whereas [^{3}H]lysine primarily labels the COOH-terminus.

Proteolysis. In order to localize the site of antibody recognition, Woolford and Webster's technique of trypsin and chymotrypsin proteolysis (12) was used to generate frag-

Abbreviation: T_m, lipid phase transition temperature.

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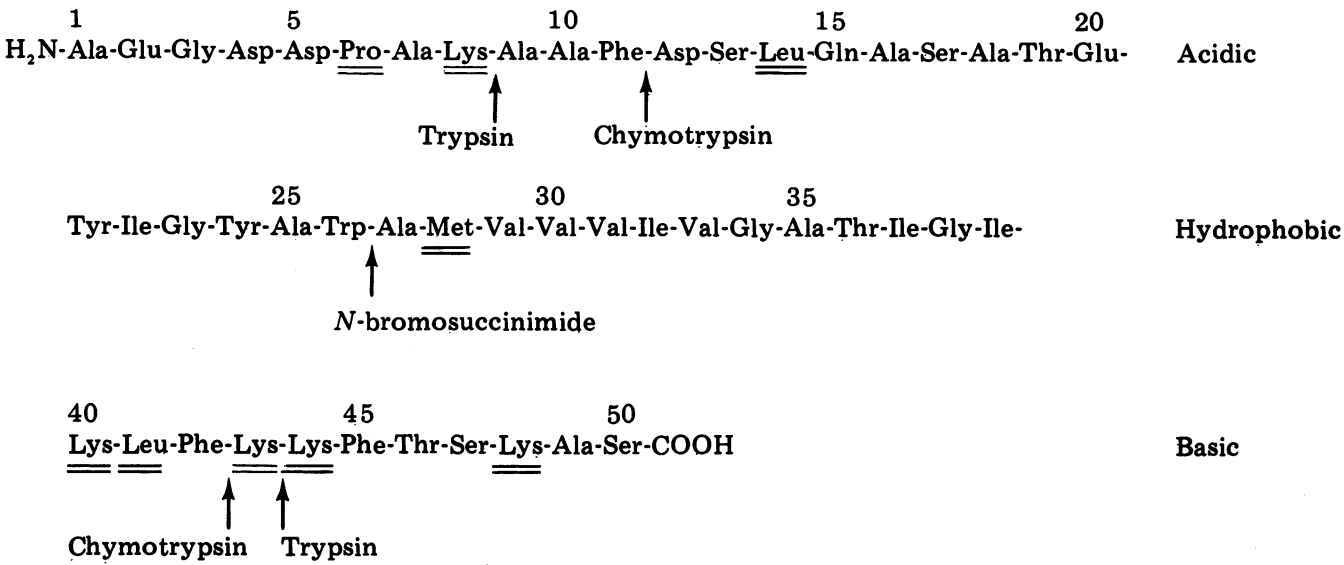

FIG. 1. Amino acid sequence of the M13 coat protein (7, 8). Amino acids which were labeled in the current study are underlined. Positions of cleavage by trypsin, chymotrypsin, and *N*-bromosuccinimide are indicated by arrows.

ments of coat protein. Fragments were isolated by Sephadex G-150 gel filtration in the presence of 1% deoxycholate. Under these conditions, coat protein dimers were resolved from larger aggregates and from smaller species (ref. 12 and Fig. 2A). When deoxycholate-solubilized coat protein labeled with ^{35}S at residue 28 and [^{3}H]proline at residue 6 was digested with trypsin and filtered, the [^{3}H]proline-labeled peptide was completely separated from the central ^{35}S-labeled protein (Fig. 2B). A series of such digestions with trypsin, chymotrypsin, and *N*-bromosuccinimide is summarized in Table 1. In agreement with the more extensive study of Woolford and Webster (12), the central methionine appears to be protected by the deoxycholate from digestion. Thus, chymotrypsin releases all of the [^{3}H]proline-labeled NH_2-terminal peptide and several of the COOH-terminal lysines but fails to release the two [^{3}H]leucines which bracket the central hydrophobic region (Table 1). Trypsin also efficient-

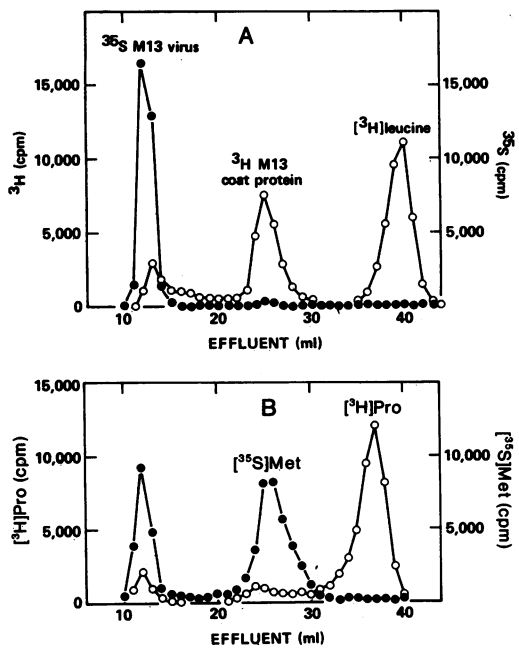

FIG. 2. Gel filtration of coat protein digests (12). (A) Column calibration. [^{3}H]Leucine, [^{3}H]leucine-labeled M13 coat protein, and ^{35}S-labeled M13 (4×10^4 cpm of each) were mixed in 1 ml of 1% sodium deoxycholate, 0.02 M Tris-HCl (pH 8.8), 1.0 mM EDTA and applied to a 1.5 × 27 cm column of Sephadex G-150 Superfine (Pharmacia) equilibrated with the same buffer at room temperature. Fractions (1 ml) were assayed for ^{3}H (○) and ^{35}S (●). (B) Trypsin digestion. ^{35}S- and [^{3}H]proline-labeled coat protein (1 mg in 0.3 ml, solubilized in deoxycholate as described in *Materials and Methods*) was mixed with six 5 μl portions of chymotrypsin (5 mg/ml) at hourly intervals at 37°. The digest was filtered and assayed as described above.

Table 1. Degradation of M13 coat protein with trypsin, chymotrypsin, and *N*-bromosuccinimide

Digestion	Labeled amino acids	% Of label recovered in: Protein	Peptide
Trypsin	1. [^{35}S]Met	100	0
	[^{3}H]Pro	3	97
	2. [^{35}S]Met	100	0
	[^{3}H]Lys	38	62
Chymotrypsin	1. [^{35}S]Met	100	0
	[^{3}H]Pro	2	98
	2. [^{35}S]Met	100	0
	[^{3}H]Lys	19	81
	3. [^{35}S]Met	100	0
	[^{3}H]Leu	100	0
N-Bromosuccinimide	1. [^{35}S]Met	100	0
	[^{3}H]Pro	68	32

Coat protein (1 mg), labeled with approximately 10^4 cpm of the indicated amino acids, was digested with either chymotrypsin or trypsin and analyzed by gel filtration as described in Fig. 2. Results are expressed as percent of total cpm in protein peaks (fractions 10–15 and fractions 23–30, Fig. 2) or in the peptide peak (fractions 33–42, Fig. 2). *N*-Bromosuccinimide digestion: coat protein (5 mg in 2 ml with 10^4 cpm each of [^{35}S] and [^{3}H]proline label, solubilized in deoxycholate as described in *Methods*), was mixed at room temperature with 13 portions (20 μl each) of 4 mM *N*-bromosuccinimide at 1 min intervals, then filtered as above. Forty percent of the absorbance at 280 nm was lost during the digestion.

Table 2. Antibody to amino-terminus of M13 coat protein

Coat protein fraction	Antibody precipitation (% of undigested control)
Undigested (1–50)	100
Trypsin	
NH_2-terminus (1–8)	80
Rest of protein (9–43)	2
Chymotrypsin	
NH_2-terminus (1–11)	73
Rest of protein (12–42)	11
N-Bromosuccinimide	
NH_2-terminus (1–26)	76

This antibody to coat protein is specific for its amino-terminus. Undigested M13 coat protein [5 μg (1.0 nmol) labeled with ^{35}S] or the indicated fragment of coat protein (1.0 nmol or less, prepared as described in Table 1) was incubated for 8 min at 37° with 160 μg of either gamma globulin from a nonimmunized rabbit or affinity-purified antibody to M13 coat protein (10) in 0.8 ml containing bovine-serum albumin (0.8 mg) and Tris·HCl (0.1 M, pH 7.5). NaCl (0.16 ml of 5 M) and sodium deoxycholate (0.1 ml of 0.1 g/ml) were added, followed by 20 mg of gamma globulin from a goat immunized with rabbit gamma globulin. After 15 min at 37°, the suspensions were centrifuged (5 min, 0°, 44,000 × *g*) and the precipitate was assayed for radioactivity. Seventy percent of the undigested coat protein was precipitated. Results are expressed as the percent of radioactivity precipitated by antibody to coat protein relative to this undigested control. Samples which were over 50% precipitated by antibody to coat protein were less than 5% precipitated by gamma globulin from nonimmunized rabbits.

ly releases an NH_2-terminal peptide and catalyzes limited digestion of the COOH-terminus. Partial cleavage with *N*-bromosuccinimide permitted isolation of peptide 1-26. The sites of trypsin, chymotrypsin, and *N*-bromosuccinimide cleavage are indicated in Fig. 1.

Antibody Recognition. To test its site specificity, we incubated antibody with intact coat protein or peptides isolated after the digestions described above. Goat antibody to rabbit gamma globulin was added to facilitate precipitation; the precipitate was assayed for radioactive coat protein fragments. Almost all of the intact coat protein was precipitated by this means. The antibody also precipitated the NH_2-terminal peptides 1-8, 1-11, and 1-26 generated by trypsin, chymotrypsin, and *N*-bromosuccinimide, respectively (Table 2). In contrast, peptides 9-43 and 12-42 were precipitated only to a small extent.

Antibody competition studies were performed to show that all of the antibody to M13 coat protein is specific for the NH_2-terminus. Antibody was incubated with several concentrations of the tryptic NH_2-terminal octapeptide, and then assayed for its residual ability to bind radioactive, undigested coat protein (Fig. 3). Essentially all of the antibody was specific for the NH_2-terminal octapeptide.

Coat Protein in Lipid Vesicles. In order to define the factors necessary for asymmetric assembly of coat protein into membranes, vesicles of pure coat protein and dilauroyl or dimyristoyl lecithin were prepared by the cholate dilution technique of Racker *et al.* (13). Briefly, a clear solution of coat protein and lecithin in cholate was rapidly diluted with buffer. The preparation gradually became turbid, indicating vesicle formation. These vesicles were harvested by sedimentation and characterized by several techniques†. Light and electron microscopy showed vesicles of approximately

† W. Wickner, S. Mabrey, J. Griffith, and R. Simoni, manuscript in preparation.

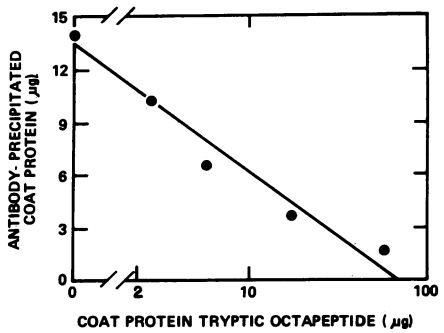

FIG. 3. All of the antibody to M13 coat protein recognizes the NH_2-terminal octapeptide. Antibody to coat protein (200 μg) or gamma globulin from a nonimmunized rabbit was mixed with the indicated amount of coat protein NH_2-terminal tryptic octapeptide (prepared as described in Table 1) in 0.8 ml containing bovine-serum albumin (0.8 mg) and Tris·HCl (0.1 M, pH 7.5). After 10 min at 37°, ^{35}S-labeled coat protein (15 μg, 4×10^3 cpm in 10 μl) was added and the incubation was continued for 10 min. NaCl (0.16 ml of 5 M) and sodium deoxycholate (0.1 ml of 0.1 g/ml) were added, followed by 2.4 mg of gamma globulin from a goat immunized with rabbit gamma globulin. After 15 min at 37°, the suspensions were centrifuged (5 min at 0°, 44,000 × *g*) and the precipitates were assayed for radioactivity. Results are expressed as micrograms of ^{35}S-labeled coat protein specifically precipitated by antibody to coat protein.

0.3 μm diameter, five to ten times the size of vesicles prepared without protein. These vesicles contained two-thirds of the coat protein and three-quarters of the lipid used in their preparation; they are as much as 10% protein by weight and have less than 1 mol of cholate per mol of coat protein. The fluorescence intensity of tryptophan 26 showed an abrupt discontinuity at the lipid phase transition temperature, indicating that the hydrophobic portion of the protein was in intimate contact with the fatty acyl chains. Calorimetry showed a nearly isothermal phase transition at the same temperature where phase transition occurred for vesicles without coat protein. The physical characteristics of these preparations will be reported in detail elsewhere.†

Dilauroyl lecithin vesicles, prepared with [^3H]proline-labeled coat protein at 0° [the T_m of dilauroyl lecithin (14)], were excluded from Sephadex G-150 beads during gel filtration (Fig. 4A). Treatment of these vesicles with trypsin or chymotrypsin released over 95% of the proline label, indicating that the amino termini of almost all of the coat protein peptides were exposed to the enzyme. In contrast, only one-fifth of the label was released by chymotrypsin from vesicles prepared with [^3H]lysine-labeled coat protein (Fig. 4B). Because the NH_2-terminal chymotryptic peptide, which has one of the five lysine residues (Fig. 1), was released completely, it is concluded that essentially none of the COOH-terminal lysines were removed. Vesicles with internal, trapped chymotrypsin proteolyzed 60% of their lysine residues when incubated at 37° (data not shown). This control experiment and experiments with dimyristoyl lecithin presented below demonstrate that the failure of chymotrypsin to attack the COOH-terminal lysines is due to their inaccessibility to the protease. The coat protein associated with these vesicles is therefore proposed to have external NH_2-termini, hydrophobic central regions in contact with the hydrocarbon core of the lipid bilayer, and internal COOH-termini (Fig. 5A).

Vesicles were prepared by the cholate dilution technique with dilauroyl or dimyristoyl lecithin at either 0° (the T_m of dilauroyl lecithin), 23° (the T_m of dimyristoyl lecithin), or

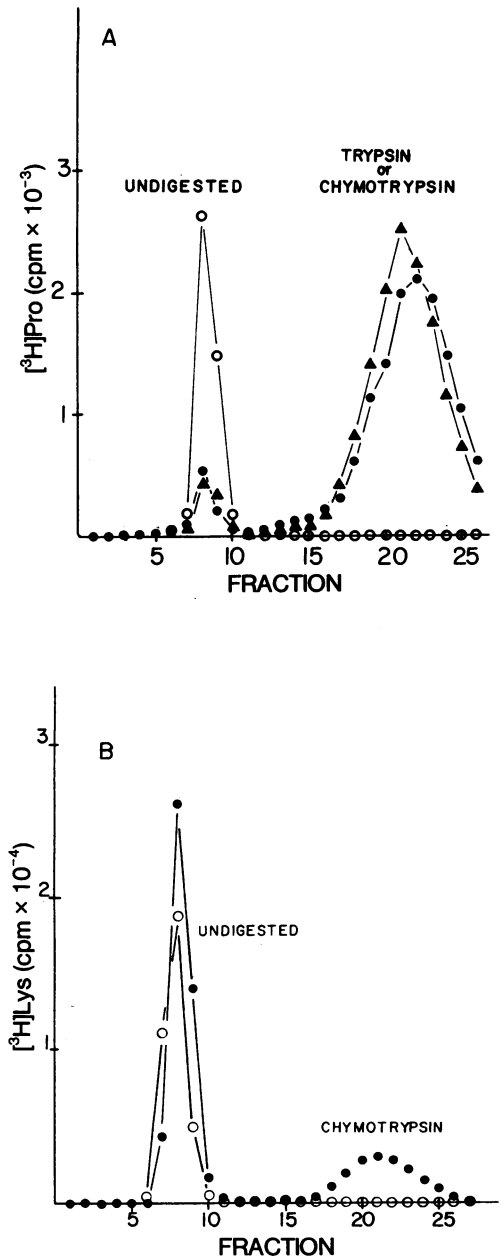

FIG. 4. M13 coat protein, bound to lecithin vesicles, has external amino-termini and buried carboxytermini. Vesicles were prepared essentially by the procedure of Racker *et al.* (13). (A) [^{3}H]Proline-labeled coat protein (1 mg, 10^5 cpm suspended in 0.5 ml of 0.02 M $NaHCO_3$, pH 8.5) was incubated for 30 min at 37° with sodium cholate (0.05 ml of 0.1 g/ml) and undissolved material was removed by centrifugation (0°, 20 min, 44,000 × *g*). This solution was mixed with dilauroyl lecithin (0.2 ml of 40 mg/ml, suspended in 0.1 M KP_i at pH 7.0 and sonicated for 1 min) at room temperature, then quickly transferred to 0°. After 30 min, 30 ml of ice-cold buffer (0.1 M KP_i, pH 7.0) was added. During the subsequent 60 min incubation at 0°, the clear solution gradually became turbid. Vesicles were collected by centrifugation (0°, 10 hr, 44,000 × *g*) and suspended in 1.0 ml of buffer (0.1 M NaCl, 0.05 M Tris·HCl, pH 7.5). An aliquot of these vesicles (O——O) was applied to a Sephadex G-150 column (12 ml, equilibrated with 0.1 M KP_i, pH 7.0, at room temperature) and 0.5 ml fractions were assayed for ^{3}H. Other aliquots (0.2 ml each) were digested with trypsin (●——●, 10 μl of 5 mg/ml) or chymotrypsin (▲——▲, 10 μl of 5 mg/ml) for 60 min at 37°, then applied to the same gel filtration column. (B) Vesicles were prepared and analyzed as above, but with [^{3}H]lysine-labeled coat protein. Undigested vesicles, O——O; chymotrypsin-digested vesicles, ●.

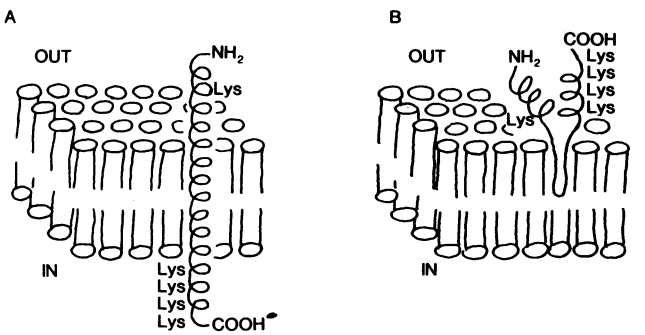

FIG. 5. Membrane-bound forms of M13 coat protein.

30° (Table 3). Incorporation of coat protein into vesicles was maximal near the lipid T_m, where 87% of the NH_2-termini, bearing the [^{3}H]proline label, were in each case accessible to external chymotrypsin (as in Fig. 4A). At the T_m of each lipid, only 27% of the [^{3}H]lysine label was accessible to chymotrypsin, and most of this label is from the lysine in the NH_2-terminal chymotryptic peptide (as in Fig. 4B). The COOH-terminus, with four of the five lysines, is therefore not exposed to the added protease in these vesicles.

In contrast, when coat protein was incorporated into dimyristoyl lecithin vesicles at 0°, well below the T_m, 75% of the [^{3}H]lysine label was accessible to proteolysis by added chymotrypsin (Table 3). Since one of the five lysines, that at position 40, is not cleaved from the protein by chymotrypsin (Table 1 and Fig. 1), this represents nearly quantitative removal of both ends of the vesicle-bound protein. This was confirmed for the NH_2-terminus by the finding that 90% of [^{3}H]proline label was released by chymotrypsin. The proposed structure of coat protein in these vesicles is shown in Fig. 5B.

The orientation of M13 coat protein in dimyristoyl lecithin vesicles, prepared at either 0 or 23°, did not vary over a 20-fold range of coat protein concentration (unpublished observations). Even at these lowest levels of coat protein tested, antibody to coat protein precipitated all the vesicles, whereas control antibody did not cause precipitation.

DISCUSSION

The question of how the asymmetric orientation of proteins in biological membranes is established and maintained is at the center of an understanding of membrane assembly. Asymmetry is maintained by very low rates of diffusion of hydrophilic regions of proteins across the hydrocarbon core of the bilayer (1). The means by which the exterior hydrophilic domains of membrane proteins first cross the hydrophobic barrier during assembly is still not known. The factors which determine membrane protein asymmetry might include: (*i*) lipid asymmetry, (*ii*) pre-existing asymmetry of other membrane proteins, (*iii*) special catalytic "flippases," (*iv*) specific ribosomes or ribosomal sites for membrane protein synthesis, and (*v*) coordinate folding of nascent proteins as they first interact with the membrane.

In this report, and a previous one (10), the coat protein of M13 virus has been shown to be oriented asymmetrically in the membrane, with its NH_2-terminus facing the cell exterior. This asymmetry has now been reproduced in an artificial but relatively simple membrane, formed from single lipid and protein species by cholate dilution. Two factors have so far been found to be involved in establishing asymmetry: the structure of the protein and the physical state of the lipid

Table 3. Chymotrypsin digestion of vesicles of M13 coat protein and lipid

Lipid	Temperature of vesicle preparation	Coat protein ^3H label	% Coat protein incorporated into vesicles	% Release of ^3H by chymotrypsin
Dilauroyl lecithin	0°	Proline	85	87
		Lysine	87	27
	23°	Lysine	2	—
Dimyristoyl lecithin	0°	Proline	11	90
		Lysine	11	75
	23°	Proline	99	87
		Lysine	100	27
	30°	Lysine	1	—

Vesicles were prepared from the indicated lipid and [^3H]lysine- or proline-labeled coat protein as described in Fig. 4. The temperature was maintained at 0, 23, or 30° throughout vesicle preparation and isolation. After the percent of coat protein incorporated into vesicles was determined by assay for ^3H, vesicles were digested with chymotrypsin as described in Fig. 4. A 175 μl portion of the digest was then centrifuged for 15 min at 23°, 160,000 × g, in a Beckman Airfuge and the supernatant and pellet were assayed for ^3H.

during assembly. It is of particular interest that the maximum coat protein incorporation occurs near the lipid T_m, and that the protein spans the bilayer under these circumstances. It is reasonable to hypothesize that this is due to the enhanced lateral compressibility of the lipid during phase transition (15). The symmetric nature of this lipid bilayer and the lack of flippases, other proteins, or even pre-existing vesicle structure make this an attractive model for the study of asymmetric membrane assembly. Whether or not this model is an accurate reflection of the *in vivo* process, understanding this simple assembly may provide a basis for studies of more physiologic processes.

Stimulating discussions with Dr. Robert Simoni and Dr. Robert Webster are gratefully acknowledged. This work was supported by grants from the National Science Foundation and the American Cancer Society; the author is a fellow of the Mellon Foundation.

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