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ASSOCIATION OF THE MAJOR COAT PROTEIN OF fd BACTERIOPHAGE WITH PHOSPHOLIPID VESICLES

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Summary

The association of the major coat protein of fd bacteriophage with a phospholipid bilayer was investigated by analyzing the protein's susceptibility to proteolysis and its circular dichroism spectrum when incorporated into single-walled phospholipid vesicles. In the limits tested, this association appeared to be independent of the mass ratio of protein to lipid and of vesicle size, phospholipid composition, and method of preparation. The circular dichroism data are consistent with a similar "membrane-bound" conformation for all cases of vesicle-associated coat protein and for deoxycholate micelle-associated coat protein. Proteolysis of coat protein associated with deoxycholate micelles and with phospholipid vesicles defined the central hydrophobic core presumed to represent that portion of the protein which associates with membrane bilayers in vivo. The isolated core, which assumed a predominantly β-type conformation in detergent solution, maintained a β conformation when associated with a vesicle phospholipid bilayer.

Introduction

In its subcellular localization, mode of synthesis, primary sequence, and interactions with detergents and phospholipids, the major coat protein of fd

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Abbreviation: TPCK, L-1-tosylamide-2-phenylethylchloromethyl ketone.

bacteriophage exhibits properties expected for a membrane protein. In vivo, both coat protein from the infecting phage and newly synthesized coat protein are found almost exclusively associated with the host *Escherichia coli* membranes after infection with either wild-type phage or amber mutants [1—3]. In an *E. coli* PlsB glycerol auxotroph, coat protein synthesis is correlated with membrane synthesis, and inhibition of phospholipid synthesis leads to decreased coat protein synthesis while permitting synthesis of a soluble phage-specific protein to continue [4]. This effect is similar to that observed for the host membrane and soluble proteins [5]. Sequence analysis [6,7] has shown that the coat protein contains a central core of 19 hydrophobic amino acid residues, bounded by hydrophilic sequences at both termini. This arrangement is similar to the amino acid sequence of the trans-membrane M,N-glycoprotein of the human erythrocyte [8,9]. The hydrophobic region of the coat protein is capable of forming a specific binding site for detergents in a manner expected for a membrane protein [10,11]. The circular dichroism spectrum [12] and chymotryptic digestion [13,14] of phospholipid vesicle-associated coat protein have suggested a conformation similar to that of detergent micelle-associated protein.

Furthermore, insertion of the coat protein into *E. coli* membranes in vivo alters the host phospholipid metabolism in a manner suggesting the existence of a molecular association between the coat protein and cardiolipin in the membrane [15]. This protein therefore represents an attractive system for studying how a particular membrane protein can associate with a phospholipid bilayer and interact with other membrane components. In order to delineate better the association between the coat protein and the phospholipid bilayer, we have further examined the susceptibility to proteolysis and the circular dichroism spectrum of the coat protein when incorporated into phospholipid vesicles.

Materials and Methods

Materials. L-[U-^{14}C]Lysine, L-[*Me*-^{3}H]methionine, L-[U-^{14}C]phenylalanine, L-[U-^{14}C]proline, L-[G-^{3}H]proline, L-[U-^{14}C]serine, L-[G-^{3}H]serine, L-[G-^{3}H]-tryptophan, and L-[3,5-$^{3}H_2$]tyrosine were purchased from New England Nuclear. Lipids used were phosphatidylcholine (egg lecithin) from Serdary Research Laboratories and bacterial phosphatidylglycerol and cardiolipin from Supelco. α-Chymotrypsin (50.9 units/mg) and trypsin-TPCK (266 units/mg protein) were obtained from Worthington. Pronase, B grade (45 000 P.U.K./g) was purchased from Calbiochem. TPCK and the sodium salt of cholic acid were from Sigma. Sodium deoxycholate was from Fisher Scientific. Cholate and deoxycholate were recrystallized from acetone [16]. [*Carboxy*-^{14}C]Deoxycholic acid, sodium salt was purchased from Amersham/Searle. Ultrafiltration equipment was from Amicon, and Sephadex and Sepharose resins were obtained from Pharmacia.

Incorporation of coat protein into sonicated phospholipid vesicles. Radioactive fd phage were prepared in a manner similar to that previously described [10], and coat protein was incorporated into sonicated phospholipid vesicles

by a modification of the procedure of Huang [17]. Coat protein concentrations were determined as described previously [12], using a coat protein content of 87%. Typically, 29—57 μg of phage (25—50 μg of coat protein) in 0.01 M Tris · HCl, pH 7.8 (buffer A) were mixed with 10 μl of $CHCl_3$ and evaporated under N_2. 10 mg of phospholipid in chloroform was added, and, after mixing, the solution was evaporated with N_2. 3 ml of 0.1 M NaCl/0.01 M Tris · HCl, pH 8.5 (buffer B) was added, and the solution was incubated 4 h under a N_2 atmosphere at 4° C with intermittant mixing. The mixture was subjected to 10—12 bursts of 4 min duration at 50 W output with a Branson Model W185 Sonifier Cell Disruptor using a microtip. Sonication was performed at 4° C under a N_2 atmosphere, with 1—2-min intervals between bursts to reduce heating. After sonication, the solution was centrifuged at 27 000 × *g* for 1 h to remove debris. The supernatant was pressure concentrated with N_2 to approx. 0.7 ml using an Amicon PM10 ultrafiltration membrane and applied to a Sepharose 4B column (1.5 × 56 cm) equilibrated and eluted with buffer B. The ultrafiltration apparatus and the column were presaturated with sonication phospholipid vesicles to reduce sample loss by adsorption. The elution position of coat protein was determined by liquid scintillation analysis, and the elution position of the vesicles was monitored by the absorbance at 310 nm and corroborated at times by a phosphorus determination [18]. Fractions from the vesicle peak were pooled and concentrated to approx. 0.7 ml using a PM30 membrane.

For circular dichroism studies, where a higher protein mass was required, radioactive phage was mixed with excess unlabelled phage, and the above procedure was follwed using 500 μg of phage. The vesicle peaks from two such preparations were pooled and concentrated for analysis.

Incorporation of coat protein into detergent dialysis vesicles. Coat protein solubilized by modifications of the deoxycholate solubilization procedure [10,11] was incorporated into phospholipid vesicles by an adaptation of the cholate dialysis procedure [19]. For cholate solubilization, approx. 1 mg of phage in 0.15 ml of buffer A was mixed with 0.05 ml of 0.12 M sodium cholate, 0.2 M KH_2PO_4, pH 7.0, and 20 μl of $CHCl_3$. After incubation for 1 h at 37° C with vigorous agitation, the sample was applied to a G-150 SF column (0.9 × 52 cm) equilibrated and eluted with buffer C (0.1 M KH_2PO_4 (pH 7.0) containing 8 mM sodium cholate. Those fractions corresponding to the major peak of radioactivity (coat protein) were pooled. For deoxycholate solubilization, approx. 1 mg of phage in 0.1 ml of buffer A was mixed with 0.1 ml of buffer D (0.1 M $NaHCO_3$, pH 9.0) containing 70 mM deoxycholate and with 20 μl of $CHCl_3$. The sample was processed exactly as above using an identical column equilibrated and eluted with buffer D containing 8 mM deoxycholate.

To prepare vesicles, phospholipid (approx. 0.6 mg/mg detergent) was evaporated in a tube, detergent-solubilized protein was added, and the solution was incubated at 0° C with mixing until no film of phospholipid remained on the tube. The solution was dialyzed at 4° C for 55—60 h in Union Carbide 8 dialysis tubing (0.39 inch wide, 0.002 inch wall thickness) against 500 volumes of either buffer C (cholate solutions) or buffer D (deoxycholate solutions). The dialysate was changed every 14—18 h. This procedure removed 99.7% of the detergent when the coat protein was solubilized with doexycholate containing tracer deoxy[^{14}C]cholate. Vesicles were collected by centrifugation at 60 000

rev./min in a Beckman type 65 rotor for 3 h and resuspended in a small volume of the appropriate buffer.

Incorporation of coat protein into cholate dilution vesicles. Procedures were modifications of those for vesicle formation [20] and the application to coat protein incorporation and digestion from within [13,14]. After 4 mg of phospholipid were evaporated under N_2, 1 ml of cholate (8 mM)-solubilized coat protein was added, and the solution was incubated at 0°C for 30 min with occasional mixing. The sample was split into two tubes for a Beckman type 60 Ti rotor. 28 ml of ice-cold buffer C was added to one, and 27.5 ml of the same buffer containing 25 μg/ml α-chymotrypsin was added to the other. After 10 min at 0°C, 0.5 ml of buffer C containing 30 mg/ml lysozyme and 4 μg/ml TPCK was added to the sample containing chymotrypsin to reduce digestion of external residues during centrifugation. Both samples were centrifuged 2 h at 60 000 rev./min at 1°C in a Beckman type 60 Ti rotor. Vesicles with trapped chymotrypsin were resuspended in 0.2 ml of buffer C containing 4 μg/ml TPCK. Vesicles without chymotrypsin were resuspended in 0.2 ml of buffer. Approx. 50—65% of the coat protein radioactivity was recoverable in the pellets.

Proteolytic digestions. Three hourly 5-μl aliquots of either chymotrypsin (5.24 mg/ml) or trypsin (1.27 mg/ml) were added to 0.25 ml of sonicated vesicles containing 5—10 μg of coat protein, and the mixture was incubated for a total of 4 h at 37°C with vigorous shaking. For sequential chymotrypsin and pronase digestions, the chymotrypsin was followed by three 5-μl aliquots of pronase (8 mg/ml) at 1.5-h intervals. All enzymes were dissolved in buffer B. To 0.25 ml of cholate dialysis vesicles containing 40—50 μg of coat protein, 3 hourly 10-μl aliquots of either chymotrypsin (7.86 mg/ml) or trypsin (1.90 mg/ml) were added. For sequential digestions, three 10-μl aliquots of pronase (8 mg/ml in 0.1 M NaCl/0.01 M Tris · HCl, pH 7.5) followed the chymotrypsin. Chymotrypsin and trypsin were dissolved in buffer C, and all incubations were as described above. To 0.2 ml of cholate dilution vesicles, two 10-μl aliquots of 4 μg/ml TPCK in buffer C were added at 3-h intervals to vesicles with trapped chymotrypsin. Two 10-μl aliquots of chymotrypsin (6.88 mg/ml in buffer C) were added to vesicles without trapped chymotrypsin. Incubations were for 6 h at 37°C.

Digestion at the external surface of phospholipid vesicles was ascertained by gel chromatography of the reaction mixtures on a column (0.9 cm diameter) consisting of 31 cm of G-150 SF layered atop 66 cm of G-15, equilibrated with 0.1 M NH_4HCO_3, pH 7.75. The column was eluted with the same buffer, and fractions were assayed for radioactivity. Undigested portions of the coat protein eluted with the vesicles at the void volume, while release peptides eluted through the partially included volume to beyond the internal volume (due to adsorption of hydrophobic residues). In addition, sonication vesicles were rechromatographed after digestion on the Sepharose 4B column described previously for vesicle preparation. No change in the elution position of the vesicles and the associated undigested portion of the coat protein could be detected, indicating that vesicle integrity had been maintained and that fusion had not occurred during the digest.

To assess chymotrypsin digestion from within, the reaction mixture was

cooled to 0° C, and 25 μl of buffer C containing 500 mg/ml cholate and 4 μg/ml TPCK was added. After a 5 min incubation at 0° C to permit vesicle dissolution, the sample was applied to the column used to prepare cholate-solubilized coat protein. Digestion was assessed in a manner similar to that previously described [11].

To determine the susceptibility of deoxycholate-associated coat protein to pronase, 0.3 ml of deoxycholate (8 mM)-solubilized coat protein (approx. 200 μg) was incubated with 10 μl of pronase (1 mg/ml in buffer B) at 37° C. After 6 h, a second 10 μl aliquot of pronase was added, and the incubation was continued for an additional 16 h. The reaction mixture was applied to the column used to prepare deoxycholate- solubilized coat protein, and digestion was assessed by determining the radioactivity in the released residues (eluting near the internal volume) and in the aggregated undigested core (eluting at the void volume).

Circular dichroism. Spectra were measured using a Cary 60 spectropolarimeter with circular dichroism attachment or with a Jobin Yvon Dichrographie III. A mean residue weight of 105 was used for both intact and pronase-digested coat protein, as calculated from the amino acid composition and the estimated release of amino acid residues by pronase. The resulting spectra were corrected for any distortion due to light scattering properties of the vesicular preparation by subtracting appropriate blanks. Vesicles were formed without coat protein present and adjusted to the same absorbance at 350 nm as the similarly prepared vesicles containing coat protein, and the circular dichroism spectra of the blanks were substracted from the spectra of vesicles containing coat protein. All spectra present in Fig. 6 have been corrected in this manner.

Electron microscopy. Vesicles were negative stained with 0.5% uranyl acetate in 0.1 M ammonium acetate on a 400 mesh grid coated with a glow discharged carbon film. For thin sectioning, vesicles were fixed for 24 h at 4° C in 0.1 M sodium cacodylate, pH 7.5, and 0.8% osmium tetroxide. The vesicles were collected by centrifugation and stained for 1.5 h in 0.5% uranyl magnesium acetate in 0.85% NaCl. The vesicles were dehydrated, embedded in Epon-Luft medium [21] and cut in sections approx. 400 Å thick. The sections were stained first with uranyl acetate [22] and then lead citrate [23]. All grids were examined using a Joel 100C electron microscope.

Results

Incorporation of coat protein into phospholipid vesicles

Fig. 1 is an elution profile representing incorporation by sonication. In the fraction collected, the radioactivity of the coat protein coincided with the turbidity of the single-walled vesicles and separated from the void volume and the DNA of the disrupted phage. This preparation was used in optical studies and contained more protein than was typical for protelytic digestion. As determined from protein and lipid concentrations and from vesicle size, various preparations ranging from 0.3 to 34 coat protein molecules per vesicle were made. Both the apparent diameter of the single-walled vesicles and the proportion of radioactivity at the void volume increased with increasing mass of protein incorporated. In the high specific activity, low mass preparations for pro-

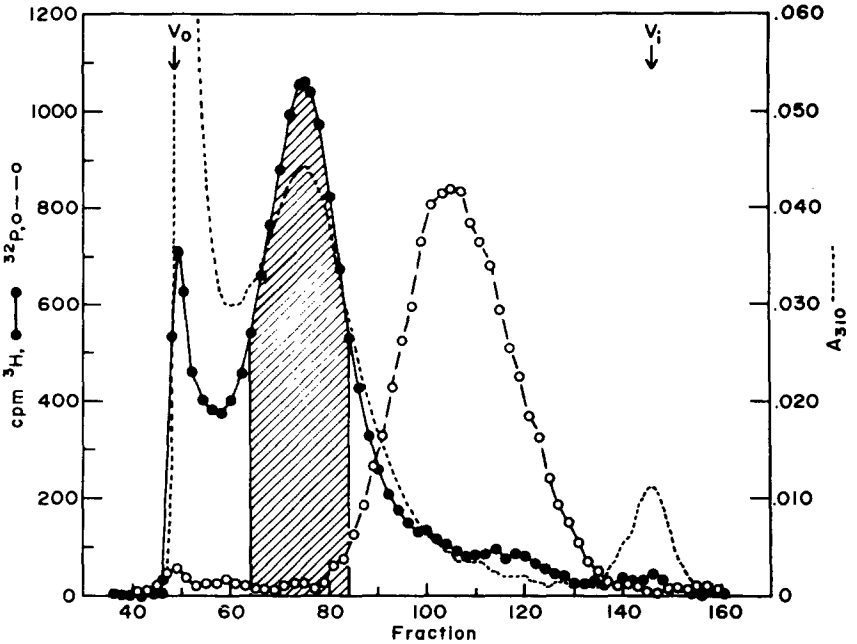

Fig. 1. Gel chromatography of coat protein incorporated into sonicated phospholipid vesicles. [^{3}H]Tyrosine-$^{32}P_i$-labeled fd phage and phospholipid were evaporated, dispersed, and co-sonicated as described in Materials and Methods. After centrifugation and concentration, the sample was applied to a Sepharose 4B column as described in Materials and Methods. The eluate was monitored for light scattering by phospholipid vesicles (A_{310nm}) and for radioactivity in ^{3}H-labeled protein and [^{32}P]DNA. Fractions in which coat protein radioactivity was coincident with single-walled vesicle turbidity (shaded fractions) were pooled for further analysis. The void and internal volumes of the column are indicated by V_0 and V_i, respectively. ●——●, ^{3}H-labeled protein; ○——○, [^{32}P]DNA; - - - - -, A_{310nm}.

teolysis, coat protein in the void volume was greatly reduced, and 95% of the eluate radioactivity could be recovered in the single-walled vesicle peak. The relative elution position indicated that these vesicles were essentially the same size as those prepared from phospholipid alone, approx. 210 Å in diameter [24].

Vesicles produced by dialysis or dilution of detergent-solubilized coat protein and phospholipids were much larger, and typically contained 200—400 coat protein molecules per vesicle. Fig. 2 presents examples of negatively stained and of thin-sectioned vesicles produced by these techniques. The vesicles appeared single-walled in all cases. Cholate dialysis produced the most uniform population, with diameters in negatively stained electron micrographs ranging from 630 to 940 Å and averaging 750 Å. The average diameter of cholate dilution vesicles was similar (720 Å), with diameters varying from 500 to 1060 Å. Deoxycholate dialysis produced the least uniform population, with vesicle diameters ranging from 440 to 1900 Å.

In all cases, the coat protein appeared to be tightly associated with the vesicle bilayer. When vesicles produced by any technique were subjected to equilibrium density centrifugation in sucrose, coat protein radioactivity and lipid phosphorus banded together at a density intermediate between those of pure phospholipid and pure protein (Fig. 3). The coat protein appeared to bear no

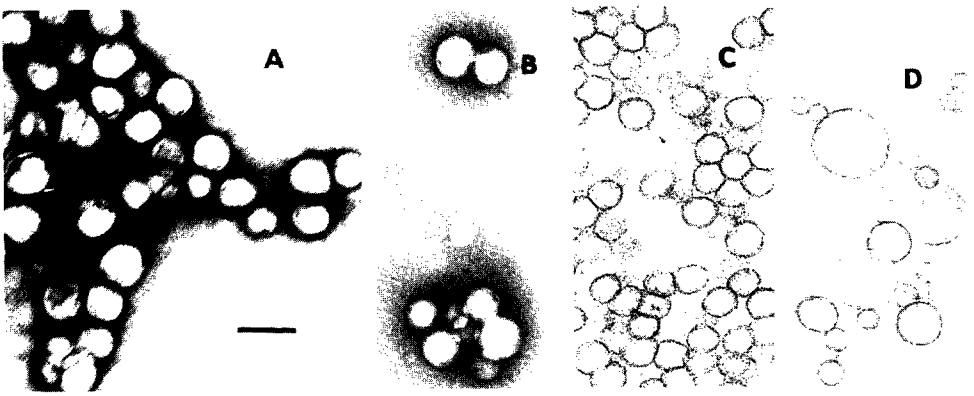

Fig. 2. Electron microscopy. (A) Negatively stained cholate dialysis vesicles. (B) Negatively stained cholate dilution vesicles. (C) Thin-sectioned cholate dialysis vesicles. D. Thin sectioned deoxycholate dialysis vesicles. The black bar in A represents 1000 Å. All examples are at the same magnification.

adhesive properties toward the external surface of such vesicles; if either isolated coat protein in the absence of detergent or sonicated $CHCl_3$-treated phage were mixed with preformed phospholipid vesicles, no significant incorporation of coat protein into vesicles was detected. Nor was the protein associated with these vesicles merely trapped within the aqueous vesicle interior. Prior to banding together in sucrose, 90% of the coat protein radioactivity could be recovered with the vesicles after pressure dialysis or centrifugation to remove external buffer. Since the vesicles contained less than 1% of the total volume of the solution in which the vesicles were formed, these experiments minimized the possbility that a significant percentage of the coat protein is distributed in the aqueous buffer, and thus precluded trapping of the protein within the vesicle interior.

Analysis of proteolytic digestions. Since the amino acid sequence of the coat protein is known (Fig. 4), radioactivity could be introduced at defined positions by growing phage in media containing radioactive amino acids. Specifically labeled coat protein could then be inserted into phospholipid vesicles and these vesicles mixed with proteases to determine the extent to which the protein is exposed at the vesicle surface and susceptable to proteolysis. Quantitation of specific cleavages was achieved through partial resolution of the product peptides by gel exclusion chromatography. Fig. 5 represents a chymotryptic digest of [^{3}H]proline-[^{14}C]lysine-labeled coat protein incorporated into cholate dilution vesicles. Undigested portions of the protein eluted with the vesicles at the void volume, while labelled peptides eluted at positions I, II and III. The chymotryptic peptide containing residues 1—11 was assigned to peak I since it was the largest soluble peptide containing proline and lysine (Fig. 4). Consistent with this interpretation was the finding that this peak also contained phenylalanine but not tyrosine when the same experiment was performed on vesicles containing coat protein labelled with these two amino acids. Using this same reasoning, peaks II and III were assigned to peptides containing residues 46—50 and 43—45, respectively (Table I). Positions of other chymotryptic peptides detected with other radioactive amino acids are indicated by A, B and C.

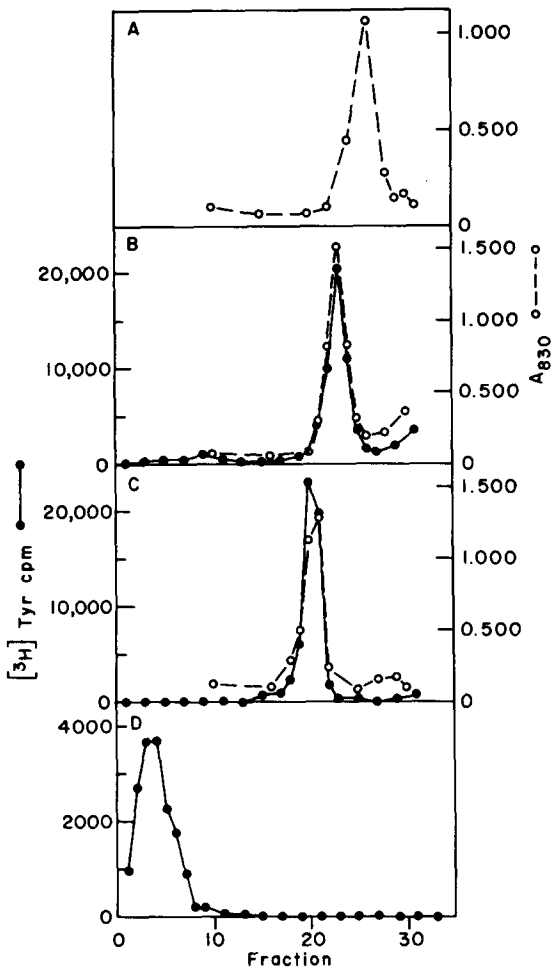

Fig. 3. Sucrose density centrifugation. Linear sucrose gradients (4—14% (w/v) in 0.1 M $NaHCO_3$, pH 9.0) were prepared in 4.5-ml centrifuge tubes atop a 60% (w/v) sucrose shelf. Samples (0.3 ml) were layered on top, and the gradients were centrifuged for 48 h at 40 000 rev./min in a Beckman SW56 rotor. Fractions of approx. 0.14 ml were collected from the bottom of the tubes, and aliquots were assayed for coat protein radioactivity and lipid phosphorus. (A) Phospholipid vesicles prepared by dialysis of deoxycholate-phospholipid mixed micelles. (B) Intact coat protein incorporated into vesicles by the deoxycholate dialysis procedure. (C) Central core of pronase-digested coat protein incorporated into vesicles by deoxycholate dialysis. (D) Intact deoxycholate-solubilized coat protein. •———•, [^{3}H]tyrosine coat protein; ○———○, A_{830nm} indicating relative phosphorus content as determined by the procedure of Bartlett [18].

Table I summarizes the amino acids detected in these peaks and the peptide assignments based upon the amino acids present.

It must be pointed out that some peptides did not resolve into distinct peaks when radiochemically labeled with certain amino acids (e.g. serine in peaks A and II). However, there was at least one unambiguous label for each resolvable peak (e.g. proline in peak I, tyrosine in peaks A and B, and phenylalanine in peaks I, III and C) which could be used to ascertain release of that peptide. Peak II was not completely resolvable either from peak I or from peak III, but

10
Ala-Glu-Gly-Asp-Asp-PRO-Ala-LYS-Ala-Ala-PHE-Asp-SER-Leu-Gln-Ala-SER-

20 30
Ala-Thr-Glu-TYR-Ile-Gly-TYR-Ala-TRP-Ala-MET-Val-Val-Val-Ile-Val-Gly-

40 50
Ala-Thr-Ile-Gly-Ile-LYS-Leu-PHE-LYS-LYS-PHE-Thr-SER-LYS-Ala-SER

Fig. 4. Amino acid sequence of the fd major coat protein [6,7]. Residues in boldface type are those which were radioactively labeled in this study. The underlined residues 26—40 are those which are unavailable for proteolysis when the coat protein was associated with phospholipid vesicles. The extent of the protected region may include those residues with a dashed underline, since Ala-25 was not labeled in this study and the availability of Phe-42 varied among vesicle preparations.

release of this peptide could be determined from a digestion of [^{3}H]proline-[^{14}C]lysine coat protein as in Fig. 5. The lysine in peak I was calculated assuming a constant ^{3}H/^{14}C ratio for the peak since the peptide containing residues 1—11 had an equimolar ratio of proline and lysine. The remainder of the [^{14}C]-

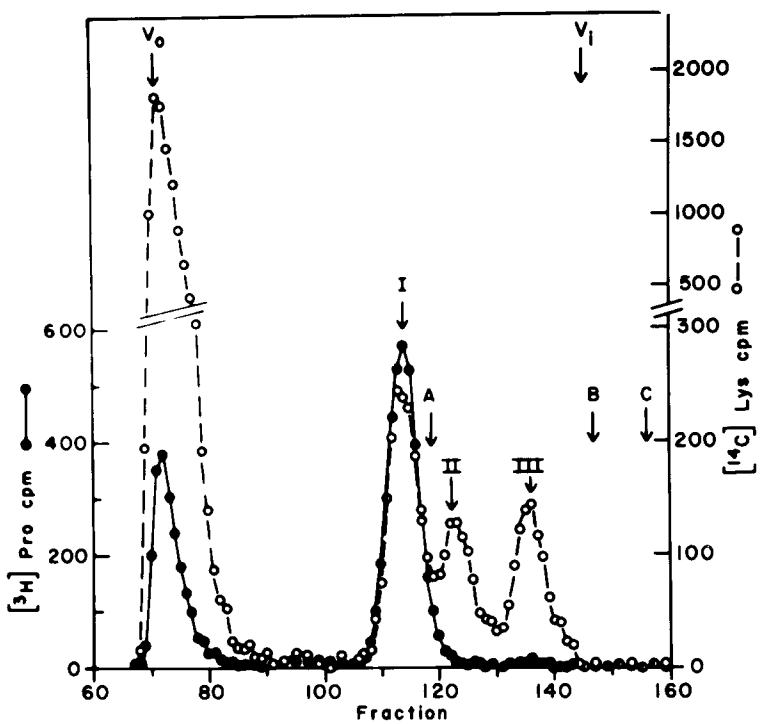

Fig. 5. Gel chromatography of the digestion products of a chymotryptic digestion of [^{3}H]proline-[^{14}C]-lysine coat protein incorporated into cholate dilution vesicles. Cholate-solubilized coat protein was incorporated into 80% phosphatidylcholine, 20% phosphatidylglycerol (w/w) vesicles and subsequently digested with chymotrypsin as described in Materials and Methods. The reaction mixture was applied to the dual Sephadex column described in Materials and Methods, and the eluate was assayed for radioactivity. The void and internal volumes of the column are indicated by V_0 and V_i, respectively. The elution position of product peptides containing either [^{3}H]proline or [^{14}C]lysine are indicated by I, II and III. The elution positions of chymotryptic peptides detected when the coat protein was labeled with other amino acids are indicated by A, B and C. ●———●, [^{3}H]proline; ○———○, [^{14}C]lysine.

TABLE I

IDENTIFICATION OF CHYMOTRYPTIC PEPTIDES

Peptide peak	Amino acids detected	Residues included in peptide assignment
I	Pro, Lys, Phe	1—11
A	Ser, Tyr	12—21 *
II	Ser, Lys	46—50
III	Lys, Phe	43—45
B	Tyr	22—24
C	Phe	Phe—42

* It is conceivable that cleavage additionally occurred at leucine-14. The only radioactive amino acid which would be present in the product tripeptide (Asp-Ser-Leu) is serine, and if it were present it would have to elute with peaks A and II since no additional serine peaks were detected. In that case, peak A would contain the glutamine-15 to tyrosine-21 peptide. No attempt was made to determine if such cleavage did occur since it would in no way affect interpretation of the data.

lysine in the overlapping fractions was assigned to peak II. The distribution of radioactivity between peaks II and III was estimated from a best Gaussian fit for the two peaks. Assignments of peptide release by this method were checked by comparison with the unambiguous release of proline and phenylalanine in peak I and of phenylalanine in peak III. An additional check correlated the total serine release with the calculated value for release of peak A (based on tyrosine) plus that of peak II (based on lysine).

Analysis of tryptic digestions using the same chromatography was less direct. It appeared that the major COOH-terminal peptide (residues 49—50) eluted at the same position as the NH_2-terminal peptide (residues 1—8) since the major peak contained proline, lysine and serine. A minor peak containing serine, phenylalanine, and lysine eluted later and was correlated with the peptide containing residues 45—48 plus free lysine (residue 44). Because of these difficulties, digestion at the NH_2 terminus was assessed by proline release, and release of the COOH-terminal products was assessed by the best fit of the released radioactivity to these constraints: (a) lysine remaining after accounting for NH_2-terminal digestion must be divided between the residue 45—48 peptide and free lysine-44; (b) serine must be divided between the peptide containing residues 45—48 and that with residues 49 and 50; and (c) all released phenylalanine must be in the residue 45—48 peptide.

Tryptic and chymotryptic digestions

Table II presents results of tryptic and chymotryptic digestions of coat protein incorporated into phospholipid vesicles. Vesicles formed from phosphatidylcholine, from phosphatidylcholine and 10% (w/w) cardiolipin, or from phosphatidylcholine/phosphatidylglycerol mixtures with phosphatidylglycerol content ranging from 12.5 to 25% (w/w) yielded similar digestion patterns. Vesicles containing 20% (w/w) phosphatidylglycerol were utilized for most experiments since they formèd solutions of low turbidity preferable for circular dichroism studies. The digestion pattern also appeared to be relatively independent of the method of vesicle formation. Chymotryptic release of the most available NH_2- (residues 1—11) and COOH- (residues 46—50) terminal peptides was essentially identical for vesicles formed by sonication, cholate dilution, or

TABLE II

TRYPTIC AND CHYMOTRYPTIC DIGESTIONS OF COAT PROTEIN IN PHOSPHOLIPID VESICLES

Data is the range of release of peptides determined as described in the text. Numbers in parentheses indicate the percent efficiency of peptide release assuming only 2/3 of the NH_2 and 1/3 of the COOH termini are exposed at the vesicle surface.

Digestion products	Percent of peptide released from vesicle	
	Sonication *	Cholate dilution **
Tryptic peptides		
1—8 (NH_2)	62—63 (93—95)	
49—50	21 (63)	
45—48 (COOH)	2 (6)	
44	2 (6)	
Chymotryptic peptides		
1—11	63—65 (95—98)	61—66 (92—99)
12—21 (NH_2)	11—13 (16—20)	
22—24	7—9 (10—14)	
46—50	21—33 (63—99)	30—34 (90—102)
43—45 (COOH)	2 (9)	18—20 (54—60)
42	3 (9)	4—6 (12—18)

* Coat protein was incorporated into 80% phosphatidylcholine, 20% phosphatidylglycerol (w/w) sonication vesicles. Data was similar for vesicles formed from phosphatidylcholine alone and for other combinations of phosphatidylglycerol or cardiolipin with phosphatidylcholine.

** Coat protein was incorporated into 80% phosphatidylcholine, 20% phosphatidylglycerol (w/w) cholate dilution vesicles.

cholate dialysis (data not shown). However, as indicated in Table II, release of less accessible portions such as the chymotryptic peptide containing residues 43—45 could be affected by the method of preparation. In no case could any methionine be released with either trypsin or chymotrypsin.

If it is assumed that the maximum release is indicative of the availability at the vesicle surface, then ohly $\frac{2}{3}$ of the NH_2 termini (based upon 16 separate determinations of proline release) and $\frac{1}{3}$ of the COOH termini (based upon 8 determinations of lysine and 4 of serine) are exposed. Further release could not be achieved by simultaneously increasing the amount of enzyme 6-fold and the length of incubation 5-fold over standard conditions. Furthermore, the amount of coat protein incorporated into sonicated vesicles was varied 100-fold with no significant alteration in this digestion pattern. The values in parentheses in Table II indicate the efficiencies at which trypsin or chymotrypsin would release the peptides assuming this orientation. These values are in good agreement with coat protein digestion in 5% deoxycholate, where trypsin is capable of hydrolysis at lysine-8 with 97% efficiency, and chymotrypsin can cleave at phenylalanines-11 and -45 with 98—100% efficiency [13]. Also consistent with digestions in deoxycholate [11,13], trypsin is less efficient at COOH-terminal cleavage.

Attempts were also made to determine the stoichiometry of NH_2 and COOH termini within the vesicle interior by trapping chymotrypsin inside during cholate dilution vesicle formation. These vesicles were isolated by centrifugation in

the presence of TPCK and lysozyme to inhibit digestion by any remaining exterior chymotrypsin and then incubated at 37°C in the same buffer to allow cleavage of peptides exposed on the inside of the vesicle (see Materials and Methods). The vesicles were dissolved in cholate buffer, and the released peptides were separated from the complex of cholate and hydrophobic coat protein residue by gel filtration. Quantitation of individual peptides was not possible since no combination of resins attempted satisfactorily resolved the product peptides in the detergent buffer required for analysis. Nevertheless, the relative amounts of NH_2 and COOH termini inside and outside the vesicles could be compared by evaluating proline and lysine release by chymotrypsin. Cleavage at the NH_2 terminus will release the single proline and one of five lysine residues, and cleavage at the COOH terminus will release additional lysines. Thus, the ratio of lysine release to proline release will increase as the relative digestion at the COOH terminus increases. Similarly, for coat protein labeled with radioactive proline and phenylalanine, the ratio of phenylalanine release to proline release will increase with increasing COOH-terminal digestion.

Although the results of digestion from within varied considerably from experiment to experiment, analysis of parallel digestions from within and from the outside indicated that the relative COOH- to NH_2-terminal digestion was greater inside the vesicle than it was on the outside. Data from five such experiments indicated release of 0.7—1.5 more lysines per proline from inside digestions than from outside digestions. For example, in one experiment, digestion from within released 46.6% of the proline and 25.4% of the lysine (corresponding to 2.7 lysines per proline), while outside digestion released 64.0% of the proline and 25.6% of the lysine (2.0 lysines per proline). A single experiment indicated release of 0.8 more phenylalanine per proline from the inside compared to that from the outside. The data is consistent with a higher relative release of COOH-terminal peptides from within the vesicle. However, the total proline accountable from inside and outside release in parallel digests varied from 80 to 110%. Thus, it appears that the NH_2 termini unavailable for outside digestion are available from within.

Pronase digestions

Digestion of deoxycholate-solubilized coat protein by pronase is summarized in the top half of Table III. Release of residues was estimated with certain assumptions. In dilute deoxycholate (Approx. 12 mM), chymotrypsin can release 81% of the radioactive lysine but no leucine [13]. Therefore, lysines 8, 43, 44 and 48 are completely released, while lysine-40 is virtually intact. We assumed that the 80.4% release of lysine observed for pronase digestions in 8 mM deoxycholate also reflected total release of the same four residues. This assumption would signify that serines at positions 47 and 50 (two of the four serine residues present in the coat protein) must be completely released (see Fig. 4). Since 90.1% of the total serine radioactivity is released, then 40.1% must be derived from residues 13 and 17. The complete release of proline-6 and lysine-8 by pronase coupled with the complete accessibility of phenylalanine-11 to chymotrypsin cleavage led us to assume that pronase released virtually all of serine-13. Therefore it is concluded that 61% of serine at position 17 was released by pronase to yield a total of 90.1% release of serine radioactivity.

TABLE III

PRONASE DIGESTIONS OF COAT PROTEIN IN DEOXYCHOLATE MICELLES AND IN SONICATED PHOSPHOLIPID VESICLES

Vesicles were 80% phosphatidylcholine, 20% phosphatidylglycerol (w/w). Digestions were as described in Materials and Methods. Release of individual residues was estimated as described in the text. Numbers in parentheses for vesicles digestion refer to the efficiency of residue release assuming only 2/3 of the NH_2 and 1/3 of the COOH termini are exposed at the vesicle surface.

Digested material	Amino acid	Percent released	Estimated release of individual residues
Deoxycholate-solubilized coat protein	Pro	99.8	99.8% of Pro-6, Lys-8, Ser-13
	Ser	90.1	99.8% of Ser-50, Lys-48, Ser-47, Lys-44, Lys-43
	Lys	80.4	61% of Ser 17
	Tyr	27.3	27.3% of (Tyr-21 plus Tyr-24)
	Trp	9.7	9.7% of Trp-26
	Met	4.8	4.8% of Met-28
			2.8% of Lys-40
Coat protein in sonicated vesicles	Pro	64.6—65.2	64—65% (96—98) of Pro-6, Lys-8, Phe-11, Ser-13
	Ser	40.4—41.7	24—33% (72—99) of Ser-50, Lys-48, Ser-47
	Phe	24.0—25.3	39% (58—59) of Ser 17
	Lys	18.9—20.3	12.6—16.1 (19—25) of (Tyr-21 plus Tyr-24)
	Tyr	12.6—16.1	4—8% (12—24) of Phe-45
	Trp	0.0—0.5	3% (9) of Lys-44, Lys-43, Phe-42
	Met	3.1	0—0.5% (0—1) of Trp-26
			3.1% (4—5) of Met-28

From this type of analysis using coat protein containing different radioactive amino acids, it appears that residues 28—40 are protected from digestion when associated with deoxycholate; less than 5% of methionine-28 and lysine-40 could be released *. In contrast to the results of chymotryptic digestions [11, 13], the tyrosine residues are susceptible to pronase.

The bottom half of Table III summarizes the sequential chymotrypsin plus pronase digestions of coat protein incorporated into sonicated phospholipid vesicles. Release of residues was estimated with the assumptions that maximal digestion from the NH_2 terminus extended through serine-13, and that maximal release of serine occurred when serines 13, 47 and 50 were released with 98—99% efficiency. The calculated maximum release of NH_2- (five determinations of proline) and COOH- (three determinations each of lysine and serine) terminal residues is consistent with the hypothesis that only $\frac{2}{3}$ of the NH_2 termini and $\frac{1}{3}$ of the COOH termini are exposed at the vesicle surface, and the calculated efficiencies of release based upon this orientation are given in parentheses.

Circular dichroism analyses

It was previously suggested [12] that the coat protein conformation in its membrane-bound state differs from that in the phage. Amphiphile-solubilized coat protein and coat protein incorporated into sonicated phosphatidylcholine vesicles exhibited spectra which were very similar to each other and differed greatly from the spectrum in the intact phage. Fig. 6A shows that coat protein incorporated into 80% phosphatidylcholine, 20% phosphatidylglycerol (w/w)

* Control experiments indicated that more than 20% of the methionine radioactivity could be released from aggregates of coat protein in the absence of phospholipid or detergent. Exact quantitation was not possible due to difficulties in completely recovering the undigested portion of the aggregate.

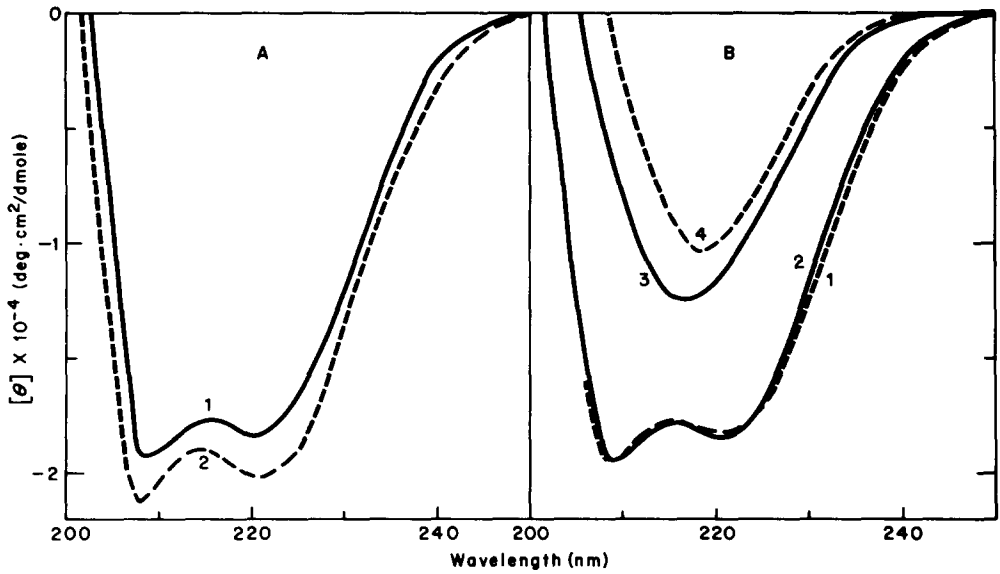

Fig. 6. Circular dichroism spectra of micelle- and vesicle-associated coat protein. (A) Spectra of coat protein incorporated into 80% phosphatidylcholine, 20% phosphatidylglycerol (w/w) vesicles by the cholate (curve 1) and sonication (curve 2) procedures. (B) Spectra of intact (curve 2) and pronase-digested (curve 3) coat protein in deoxycholate micelles were obtained, and the material was then incorporated into 80% phosphatidylcholine, 20% phosphatidylglycerol (w/w) vesicles by dialysis. Curves 1 and 4 represent the spectra of the resultant vesicle-associated intact and pronase-digested coat protein, respectively.

vesicles either by cholate dialyses (curve 1) or sonication (curve 2) also exhibited the "membrane-bound" spectrum. Similar differences in magnitude (less than 10%) could be observed among separate preparations by any one technique and probably represented the inherent inaccuracy in determining protein concentrations. Significantly, the positions and relative intensities of the troughs at 208 and 221 nm were unaltered in the two preparations. Further evidence for a single "membrane-bound" conformation is presented in Fig. 6B (curves 1 and 2). The spectrum of intact coat protein in deoxycholate dialysis vesicles (curve 1) was coincident with that of the deoxycholate-solubilized protein used in vesicle preparation (curve 2), and both curves were superimposable with the spectrum in cholate dialysis vesicles (Fig. 6A, curve 1).

Methods for analysis of circular dichroism spectra [25,26] have proved to be quite reliable of the α-helix content of proteins. From the present data and those published previously [12] we estimate an α-helix content of about 50% for the "membrane-bound" form. For example, using ellipticity at 208 nm, as recommended by Greenfield and Fasman [25], values of 53% helix for curve 1 of Fig. 6A and curves 1 and 2 of Fig. 6B, and 59% helix for curve 2 of Fig. 6A were obtained. Previously published data [12] yielded 49% helix for coat protein in oleyl lysophosphatidylcholine micelles and 46% helix in sonicated lecithin vesicles. Essentially the same results are obtained if data at several wavelengths are combined to estimate the helix content. As noted above, the differences between the various curves are probably largely due to uncertainty in the determination of protein concentration.

On the other hand, circular dichroism data are not reliable indicators of the

secondary structure outside the helical regions, as may be seen from the very different ellipticities assigned to 100% β-structure by Greenfield and Fasman [25] and by Chen et al. [26]. We have used the ellipticity near 202 nm as a rough indication: the α-helix contribution here is small, the β-structure has a large positive ellipticity and randomly coiled polypeptides (and, by inference, unordered regions in folded proteins) have an even larger negative ellipticity. All our curves have $[\theta]$ close to zero at this wavelength, suggesting that the non-helical portions of "membrane-bound" coat protein contained both β-structure and unordered regions, more of the former than of the latter. Using the characteristic ellipticities of Greenfield and Fasman [25] at 202 nm, the overall result of 50% α-helix, 30% β-structure and 20% unordered is in good agreement with the distribution predicted by the method of Chou and Fasman [27,28] which is 46% α, 30% β, 24% unordered. It should be noted that the content of β-structure derived from the experimental data is much less if we base the analysis on curve-fitting in the 208—222 nm region.

Of particular interest is the conformation of that portion of the protein molecule which is intimately associated with the hydrophobic domain of the phospholipid bilayer. Initial attempts to elucidate this structure involved proteolytic digestion of coat protein already incorporated into phospholipid vesicles. But, as discussed earlier, only about $\frac{2}{3}$ of the NH_2 termini and $\frac{1}{3}$ of the COOH termini appeared to be available to the proteolytic enzymes. Furthermore, access to the more internal residues (e.g. lysines 43 and 44) was even more restricted in some vesicles. Accordingly, pronase digestion of coat protein incorporated into sonicated phospholipid vesicles removed only about 25% of the protein mass, primarily from the NH_2 terminus (see Table III). The circular dichroism spectrum of the product was little altered from that of the intact protein incorporated into vesicles.

An alternate procedure involved pronase digestion of the protein in deoxycholate micelles and subsequent incorporation of the undigested product into phospholipid vesicles by dialysis. Based upon the release of various amino acids, it appeared that approx. 50% of the protein mass, constituting a predominant percentage of both hydrophilic termini, could be released from the hydrophobic core (see Table III). As noted previously [11], when both hydrophilic termini are removed, the remaining central region aggregated, even though still in detergent.

The circular dichroism spectrum of this material, shown in Fig. 6B (curve 3), is quite unambiguous. The α-helix content is essentially zero, and the spectrum has the appearance of a typical β-spectrum, with a minimum near 217 nm. The ellipticity becomes positive below 205 nm, suggesting that there is little unordered structure. Using the characteristic ellipticities of Greenfield and Fasman [25], the measured ellipticity at 217 nm corresponds to about 70% β-structure. The measured ellipticity exceeds that of 100% β-structure if the tabulated data of Chen et al. [26] are used.

When this same material was incorporated into phospholipid vesicles, a β-structure was maintained (Fig. 6B, curve 4). The significance of the decreased magnitude and slight shift in the trough are unknown, but these changes may reflect effects of light scattering from the large vesicles formed under these conditions. The banding patterns of protein radioactivity and lipid phosphorus

on sucrose gradients (Fig. 3C) indicated that the residual protein exhibiting the β-structure is tightly associated with the phospholipid bilayer.

Discussion

In the limits tested, the orientation of vesicle-associated fd coat protein appeared to be independent of the mass ratio of protein to lipid and of vesicle size, phospholipid composition, and method of preparation. The similar circular dichroism spectra for the coat protein in the various types of vesicles were consistent with a similar "membrane-bound" conformation in all cases. Significantly, the presence of negatively charged phospholipids such as phosphatidylglycerol or cardiolipin had no apparent effect on the incorporation, orientation, or conformation of the vesicle-associated protein. It has been suggested that negative charges on cardiolipin molecules might facilitate insertion of the coat protein into *E. coli* membranes in vivo [15]. In the in vitro mixing experiments described in this paper and previously [12], coat protein associated equally well with pure phosphatidylcholine vesicles as with vesicles containing negative charges, and it is therefore possible that negatively charged phospholipid is not an absolute requirement for in vivo membrane insertion either. It must be pointed out, however, that in vivo the coat protein is most likely synthesized in the cytoplasm as a larger precursor molecule which is cleaved during membrane insertion [29], and the characteristics of this process might be quite distinct from those for the in vitro mixing experiments with the mature coat protein molecule. The previous data [15] suggesting an association between coat protein and cardiolipin molecules within the membrane was more substantive, and the present data is not in any way inconsistent with such a preferential association with negative charges once the coat protein is in the phospholipid bilayer. It does suggest that such an association would have no gross effect on the protein conformation.

Treatment of micelle- or vesicle-associated coat protein with pronase indicated that the region protected from proteolysis was not that previously indicated. Tryptic and chymotryptic digestions of deoxycholate-solubilized coat protein suggested that the micelle-protected region extended at least from tyrosine-21 to lysine-40 [11,13]. However, pronase digestion of deoxycholate-solubilized coat protein released a substantial amount of tyrosine and some tryptophan (see Table III), and association of the coat protein with a vesicle phospholipid bilayer protected only the sequence from tryptophan-26 to lysine-40, when protection is defined as less than 10% release by any enzyme from any type of vesicle (see Table II and III). The micelle- or bilayer-protected region, consisting primarily of hydrophobic residues, presumably is buried within the hydrophobic interior of the micelle or bilayer, thus accounting for its inaccessibility. By analogy, it is assumed that this portion of the molecule (underlined residues in Fig. 4) contains the residues associated with the *E. coli* membrane in vivo.

The accessibility of residues 22—24 and 42—45 was influenced by phospholipid composition and mode of vesicle preparation. It is likely that these residues are situated near the bilayer surface, and that their availability to proteolytic enzymes is reflective of the dynamic states of the protein and the bilayer.

Fluctuations producing changes in the molecular packing of protein and lipid would be expected to be more prominant at the surface. Since the coat protein probably exists in the cytoplasmic membrane in vivo with its NH_2 terminus exposed on the outside [13,30] the present data predicts that the tyrosine residues can interact with the aqueous environment, and chemical labeling experiments performed on *E. coli* spheroplasts have confirmed this prediction (Grant, R.A. and Webster, R.E., unpublished).

The circular dichroism spectra of the region protected from pronase digestion in deoxycholate suggested a β-structure, both in deoxycholate solution and after subsequent incorporation into phospholipid vesicles. The protein had been extensively digested and might have undergone conformational rearrangement. Consequently, that portion of the intact protein which associates with the membrane bilayer in vivo might not possess such a conformation, but the data indicates that this portion of the molecule can associate with a phospholipid bilayer when in a β-conformation. A β-structured hydrophobic region in the intact coat protein is consistent with the β-structure content of the "membrane-bound" conformation estimated from circular dichroism data (see Results). Also, β-structure has been predicted from the primary sequence for residues 26—37, and alternatively, 27—36 by two separate methods [12]. Our conclusion [12] that the micelle- or vesicle-associated coat protein exhibits the stable conformation of the protein when bound to the host bacterial membrane in vivo has been disputed [31] because the protein can be incorporated into phospholipid vesicles in other states as well. A representative "membrane-bound" conformation should be an equilibrium conformation independent of the initial conformation before insertion into the membrane, but it was demonstrated that the state of the protein in lipid vesicles can depend upon prior treatment [31]. However, it has recently been shown [32] that, in detergent micelles, the "membrane-bound" conformation can be obtained starting from either randomly coiled or fully helical coat protein, indicating that this conformation is in fact the thermodynamically stable conformation, independent of history, in anionic detergents. Although the evidence is not conclusive that this conformation actually prevails in vivo, the suggestion is made that the "membrane-bound" conformation is the thermodynamically stable state in the host membrane as well [32].

If so, the β-structured region could be present in U-shaped conformations, or it could transverse the bilayer with paired antiparallel β-chains (see Fig. 3 of ref. 33). Alternatively, it might be present spanning the bacterial membrane as a parallel "β-barrel" [34] composed of laterally hydrogen-bonded polypeptide chains. If one considers the region within the membrane to be less than the maximum defined by the proteolysis data (residues 26—40), then such a region might not be overly long to span the membrane in a β-structure.

It is not possible to ascribe an absolute gross conformation to be vesicle-associated coat protein. The proteolysis data indicated that approx. $\frac{2}{3}$ of the NH_2 termini and $\frac{1}{3}$ of the COOH termini were available at the vesicle surface, and extreme conformational possibilities are presented in Fig. 7. A continuum of possible combinations of spanning and U-shaped molecules exists between these two extremes, but to achieve the observed ratio of termini available at the vesicle surface, at least $\frac{1}{3}$ of the molecules must span the bilayer with NH_2 ter-

mini oriented outward. It should be emphasized that this data concerns only the possible orientations the coat protein might assume in a phospholipid bilayer in vitro under specified conditions. Incorporation into vesicles does not mimic in vivo membrane insertion, where an asymmetric orientation might be imposed by unidirectional insertion from one side of the bilayer.

The orientation observed in these experiments utilizing phospholipids containing saturated and unsaturated fatty acyl chains of varying length differed from that achieved when coat protein was incorporated into vesicles of dilauroyl or dimyristoyl lecithin near the lipid phase transition temperature [13,14]. In that case, proline release by external chymotrypsin was 82—88%. It was suggested that the protein spanned the bilayer asymmetrically, with NH_2 termini outside and COOH termini inside, but the data is not inconsistent with some combination of orientations as in Fig. 7. The proline radioactivity was not completely released even though chymotrypsin efficiency at the NH_2 terminus was 98% in 5% deoxycholate [13]. Moreover, lysine release in these digestions correspond to 1.5—2 lysines per proline released. Since the NH_2 terminus possesses the sole proline and only one of five lysines, presumably some COOH termini were exposed at the vesicle surface, and, in several experiments, the lysine/proline release was too high to be accountable by any combination of spanning molecules alone.

Thus, the differences between that data and the data in this paper may be of a quantitative, rather than a qualitative, nature. Orientations ranging from all spanning to all U-shaped or various conbinations of these orientations, might possibly exist. Since the digestion patterns of vesicle-associated coat protein were dissimilar only when the state of the fatty acyl chains was altered, the proportions of these conformations might depend upon constraints imposed by the lipid hydrocarbon environment. These constraints could reflect the particular molecular geometry (e.g. saturated versus unsaturated fatty acyl chains), or the lipid phase transition, or both. In vesicles composed of natural lipids, the orientation might be further affected by transmembrane asymmetry of the

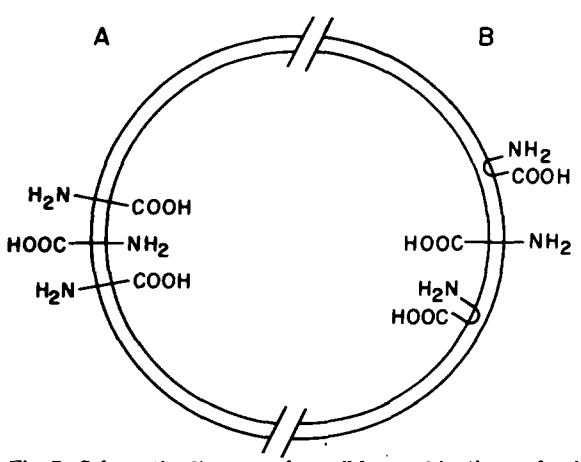

Fig. 7. Schematic diagram of possible combinations of orientations for vesicle-associated coat protein, with a bilayer-spanning conformation either maximized (A) of minimized (B). Alternatively various proportions of extremes A and B might coexist.

phospholipid acyl chains [35]. Although the exact conformation(s) cannot be ascertained, coat protein incorporated into dilauroyl or dimyristoyl lecithin vesicles at the lipid transition temperature [13,14] or into any of the natural lipid vesicles described in this paper appeared to have both NH_2 and COOH termini exposed at the vesicle surface, in proportions depending on the type of fatty acyl chains present. Moreover, the data indicated that a majority of the NH_2 termini, but fewer COOH termini, were available in all cases, suggesting that coat protein spanning the bilayer with the NH_2 terminus outside is a preferred, but not an exclusive, orientation.

Finally, with regard to the lipid phase transition, previous data [13,14] strongly suggested that the coat protein oriented in a U-shape with both termini exposed at the vesicle surface when incorporated into dilauroyl or dimyristoyl lecithin vesicles below the respective transition temperatures of their fatty acyl chains. Additionally, when vesicles were prepared above the transition temperatures, little incorporation of coat protein was detected. We did not ascertain the effect of lipid transition temperature upon coat protein incorporation due to the uncertainties in evaluating either the overall or the local phase transitions in vesicles of mixed phospholipid and fatty acid compositions. However, we were able to efficiently incorporate coat protein into egg lecithin vesicles by the sonication of dialysis procedures at 4°C, well above the reported transition temperature of −15 to −5°C [36].

Although the in vitro conditions in these experiments cannot mimic the in vivo situation, the information garnered is valuable in elucidating possible modes of interaction between a membrane protein and a phospholipid bilayer. Such basic information is not only essential to determining the nature of the interactions which occur in vivo in the *E. coli* membrane, but also might have a more general applicability to the evaluation of other physiological lipid-protein interactions. Furthermore, this data might be directly applicable to designing in vitro membrane protein synthesis experiments in the presence of lipid bilayers.

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