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Reconstitution of M13 bacteriophage coat protein. A new strategy to analyze configuration of the protein in the membrane

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The configuration of M13 bacteriophage coat protein in a model membrane was analyzed using protease digestion followed by gel permeation chromatography on Fractogel TSK in formic acid/ethanol. Important information is contained in the chromatographic patterns of the membrane-bound fragments, as well as of the fragments released by the digestion. A new reconstitution was thereby developed which involves adding a small volume of a concentrated solution of cholate-solubilized coat protein to preformed vesicles (with the amount of detergent added being less than that required to solubilize the vesicles), freezing in liquid nitrogen, thawing, followed by dialysis to remove excess detergent. The coat protein is incorporated with high efficiency (95 percent) making subsequent fractionation unnecessary. In addition, the incorporated protein is not aggregated, and is incorporated with most molecules spanning the membrane, oriented in the same manner as in vivo (N-terminus outwards). Two previously described reconstitutions, using sonication or cholate dialysis, are analyzed and found to be less satisfactory.

Introduction

Because of its size, single tryptophan residue, structure, and known sequence, M13 coat protein is a good model protein for physical studies of protein-lipid interactions. It is readily obtained in large quantities from the phage, which is 88 percent coat protein by weight. In vivo, coat protein incorporated into the host cell (*Escherichia coli*) membrane either from infecting phage or from newly synthesized coat protein spans the membrane with the N-terminus outwards, the C-terminus inwards [1,2]. We seek to study the protein in reconstituted membranes which preserve the native orientation and conformation.

The coat protein is 50 amino acid residues in length, and has been sequenced [3]. The N-terminus contains a number of acidic amino acids, the C-terminus contains a number of basic amino acids, while the center of the protein is hydrophobic. Proteolytic digests of deoxycholate-solubilized coat protein reveal that the central hydrophobic portion of the protein is inaccessible to protease [4].

The protein can be incorporated into phospholipid vesicles by several methods, including cholate and deoxycholate dilution, cholate and deoxycholate dialysis, and sonication. However, it has been suggested that the conformation of the resulting reconstituted protein might be dependent both on the lipids used and on how the protein was reconstituted [5,6]. In an interesting case described by Wickner [1] using cholate dialysis reconstitution into DMPC vesicles at a temperature below the gel-to-liquid crystal phase transition temperature of the lipid, the protein was incorporated into vesicles in a U-shaped configuration, i.e.

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both termini of a protein molecule at one interface. An extensive study of the configuration of incorporated coat protein in phospholipid vesicles was carried out by Chamberlain et al. [7]. Using protease digests of coat protein incorporated by sonication, deoxycholate or cholate dialysis, or deoxycholate or cholate dilution, they obtained the universal result that two-thirds of the N-termini and one-third of the C-termini were exposed on the outside of the bilayer, while one-third of the N-termini and two-thirds of the C-termini were in the vesicle interior, raising the possibility that a large fraction of incorporated protein was not spanning the membrane, but may be incorporated in the U-shaped configuration. Digestions were analyzed by gel chromatography on Sephadex G-150/G-15, which allowed partial resolution of peptides released from the core, but no separation of the remaining fragments containing the undigested hydrophobic core.

As long as a large percentage of each terminus is accessible on each half of the bilayer, the fraction of protein actually spanning the membrane rather than in the U-shape cannot be determined from the peptides released. Rather, this information resides in the portion of the protein remaining in the bilayer, which will have none, one, or both ends removed. We describe here a chromatographic system which partially resolves these membrane-bound peptides and thereby significantly improves the analysis of the configuration of the protein in the membrane. We use this improved analysis to develop a new membrane reconstitution procedure for the coat protein.

Methods have recently been developed in Khorana's laboratory [8,9] for separating hydrophobic peptides which result from protease digests of bacteriorhodopsin or of cytochromes. Gel permeation chromatography on Sephadex LH-20 or LH-60, and HPLC in formic acid/ethanol allowed sufficient resolution of hydrophobic fragments to make sequencing possible. In order to resolve the hydrophobic peptides from protease digests of M13 coat protein, we have used the same solvent system together with a new gel permeation material, Fractogel TSK.

Experimental Procedures

Materials. Egg PC (1,2-diacyl-*sn*-glycero-3-phosphocholine derived from hen egg yolks) and egg PA (lyophilized disodium form) from Avanti were used without further purification. [2,4-^{3}H]Cholic acid, L-[4,5-^{3}H(N)]lysine, L-[^{14}C (U)]methionine, L-[4,5-^{3}H(N)]proline, and L-[^{35}S] methionine were obtained from New England Nuclear. Protease type XI (proteinase K), protease type XIV (pronase E), carboxypeptidase A-PMSF, carboxypeptidase B-DFP, carboxypeptidase Y, alpha-chymotrypsin, DPPC-treated trypsin, and hexa-L-alanine were from Sigma. Formic acid (88 percent) was from Mallinckrodt, absolute ethanol was from U.S. Industrial Chemicals Co. Cholic acid from Sigma was decolorized with activated charcoal and recrystallized from 70 percent ethanol. Hepes buffer was used throughout because of its lack of interaction with the divalent cations used in further studies using reconstituted coat protein (Bayer, R. and Feigenson, G.W., unpublished experiments). All other chemicals were reagent grade. Water was purified using a Milli-Q water purification system (Millipore).

Phage growth. Labelled and unlabelled phage were both grown in M9 medium + 10 mM $MgCl_2$ [10]. M13 bacteriophage strain mp2 and *E. coli* strain 71–18 were both obtained from Dr. Ray Wu of Cornell University. Unlabelled phage purification is essentially according to the method of Wickner [1]. Labelled phages are prepared by inoculating 50 ml M9 with all amino acids at 20 μg/ml (except those to be labelled) together with thiamine at 10 μg/ml in a 250 ml fluted erlenmeyer flask with 0.5 ml of an overnight culture of *E. coli* 71–18. After 70 min of growth at 37°C at 250 rpm, 0.5 ml of phage stock ($1.6 \cdot 10^{11}$/ml) is added, and very slow shaking continued for 15 min. Then, labelled amino acids plus unlabelled supplement to a total of 20 μg/ml are added, and growth continued an additional 5 h at 37°C with shaking at 250 rpm. Cells are then centrifuged in a Sorvall SS-34 rotor at 10000 rpm for 10min to pellet cells, and polyethylene glycol (Sigma, approx. M_r = 6000) added to 5 percent w/v, NaCl to 0.5 M. The supernatant immediately becomes turbid. After two days at 4°C, the phage precipitate is collected by centrifugation (SS-34 rotor, 15 min at 10000

rpm at 4°C) and taken up in CsCl (Sigma, optical grade) in buffer A (10 mM Hepes/0.1 M KCl/0.1 mM EDTA (pH 7.0)) at a density of 1.29 g/ml and centrifuged at 42000 rpm at 4°C in a Beckman 50Ti rotor for 33 h. A distinctly visible phage band forms in the center of the tube. Phage bands are pooled, then dialyzed against buffer A (four times 500 ml changes at 12-h intervals). Phage is stored frozen.

Coat protein. Cholate-solubilized coat protein was prepared essentially by the method of Chamberlain et al. [7]. In each of two 13 × 100 mm tubes, 1mg phage in 0.3 ml buffer A plus 0.1 ml 0.12 M cholate, 0.2 M Hepes, 0.1 M KCl pH 7.0 and 10 μl chloroform are vortexed and incubated with occasional mixing at 37°C for one h. This mixture is then applied to a 1.5 × 45 cm Sephacryl S-200 column and eluted with buffer B (buffer A + 12 mM cholate) to separate solubilized coat protein from DNA. Coat protein-containing fractions with an absorbance ratio A_{280}/A_{260} greater than 1.5 are combined and concentrated to 2.5 mg/ml by ultrafiltration (20–30 lb/in^2 (138–207 kPa), Millipore PTGC membrane).

Reconstitution by sonication. Phage (0.31 mg) in buffer A + 10 μl chloroform are dried in vacuo (mechanical pump), 9.5 μmol egg PC in chloroform added and then dried in vacuo, 0.3 ml buffer A added, the mixture incubated 4 h on ice with occasional mixing, and then sonicated to clarity under argon using a bath sonicator [7].

Reconstitution by cholate dialysis. 7.7 μmol egg PC are dried on the walls of a test tube (first under a stream of nitrogen and then in vacuo 3–4 h), 250 μg coat protein in 0.3 ml 0.12 M cholate in buffer A is added, and the mixture incubated, with mixing, 1 h on ice. The solution is then dialyzed for 48 h against 0.5 l buffer A at 4°C (buffer changes every 12 h) [7].

Freeze-thaw (FT)-cholate reconstitution. Coat protein (0.15 ml of 2.5 mg/ml) in buffer B is added to 0.25 ml of 27 mM sonicated egg PC vesicles in buffer A, and the solution is vortexed. Buffer A (0.6 ml) is added, the solution then frozen in liquid nitrogen, thawed slowly to room temperature, and then sonicated for 30 s in a bath sonicator. The vesicles are then dialyzed against four 12-h changes of buffer A (0.5 liter) at 4°C to remove cholate. A reconstitution using tracer [^{3}H]cholate showed that dialysis removes 93 percent of cholate (1.7 molecules cholate per coat protein molecule remain) in either egg PC vesicles or in 50 percent egg PC/50 egg PA vesicles.

Fractionation. Vesicles (0.3 ml) are mixed with 0.4 ml 70 percent sucrose in a 1.5 ml polypropylene microfuge tube, overlayed with 0.6 ml 25 percent sucrose, and then with 0.2 ml buffer A. The microfuge tube is then floated in sucrose and centrifuged 4 h at 25000 rpm in a Beckman SW27 rotor, and the vesicle band at the topmost interface is harvested. Fractionation was not performed on FT-cholate reconstituted coat protein, because 95 percent of the protein is included in the vesicle fraction using this method, as assayed by freezing and slicing the microfuge tube after fractionation of labelled coat protein and then assaying for protein radioactivity. The other 5 percent of the protein could be in either small vesicles or in aggregates.

Protease digestions and analysis of resulting peptides. Proteinase K solutions were kept frozen in the buffer to be used in the digest at −20°C, thawed just before use, and then rapidly refrozen in liquid nitrogen to prevent self-digestion. Other protease solutions were prepared just before use. Samples were incubated with proteases at 37°C for various times, and then evaporated to dryness in a Savant Speedvac vacuum centrifuge. No protease inhibitors were added to stop digestion since the samples froze quickly while being dried. Digests were taken up in 0.21 ml formic acid, 0.49 ml ethanol added, and the sample loaded on a 0.9 × 95 cm Fractogel TSK HW40(s) column. Freshly prepared and degassed 88% formic acid/ethanol (30 : 70, v/v) is pumped through the column at a flow rate of 10 ml/h. The column is washed for 3 h at this flow rate before the sample is injected. The solvent is constantly purged with helium to eliminate degassing of eluant as it emerges from the column, ensuring constant fraction volume. Fractions of 1.2 ml are collected, solvent removed in the vacuum centrifuge, 2.5 ml ACS scintillant added, and the vials are then counted for 20 min each. The background (12 cpm for each channel) is subtracted, and the counts then corrected for crosstalk between ^{3}H and ^{35}S channels.

Other methods. Phage concentration was measured spectrophotometrically using $A_{268} = 3.94$

(1.0 percent w/v, 1 cm pathlength), and coat protein concentrations were likewise determined using $A_{280} = 1.66$ [11]. Concentrations of proteins used as molecular weight standards were determined by the method of Lowry [12].

Results

The following experiments involve protease digestions of coat protein grown on radioactively labelled amino acids to determine the configuration and orientation of coat protein when reconstituted into vesicles. In order to explain the strategy of these experiments we refer to Fig. 1A which shows the amino acid sequence of M13 coat protein as determined by Nakashima and Koningsberg [3]. The hydrophobic region of the protein which lies within the bilayer runs from approximately Trp-26 to Lys-40 [7].

Coat protein was prepared, labelled with [^{35}S]-methionine to mark the hydrophobic core and [^{3}H]proline to mark the N-terminus. Since the half-life of ^{35}S is short (87 days), coat protein was also grown labelled with [^{35}S]methionine alone, which was used to supplement the doubly-labelled protein to obtain a convenient ^{3}H/^{35}S ratio in later experiments.

For determining digestion at the C-terminus, coat protein was also prepared from phage grown on media containing [^{14}C]methionine and [^{3}H]lysine. The coat protein contains five lysines: Lys-8 in the N-terminus, Lys-40 in the hydrophobic core which is inaccessible to protease, and Lys-43, Lys-44, and Lys-48 in the C-terminus.

Cleavage sites for externally added protease are shown in Fig. 1B. Four peptides are expected containing the hydrophobic core (labelled with methionine): intact (inaccessible) coat protein; coat protein with the N-terminus removed; protein with the C-terminus removed; and the hydrophobic core. Peptides containing ^{3}H vary with the protease used.

Calibration of Fractogel TSK column

Separation of proteins and peptides in formic acid/ethanol on Fractogel TSK is primarily by molecular weight, as shown in Fig. 2. However, lysine elutes much later than methionine (fraction 60), so charge is also a factor. The elution position of intact coat protein indicates that in the solvent system used the protein elutes as a dimer.

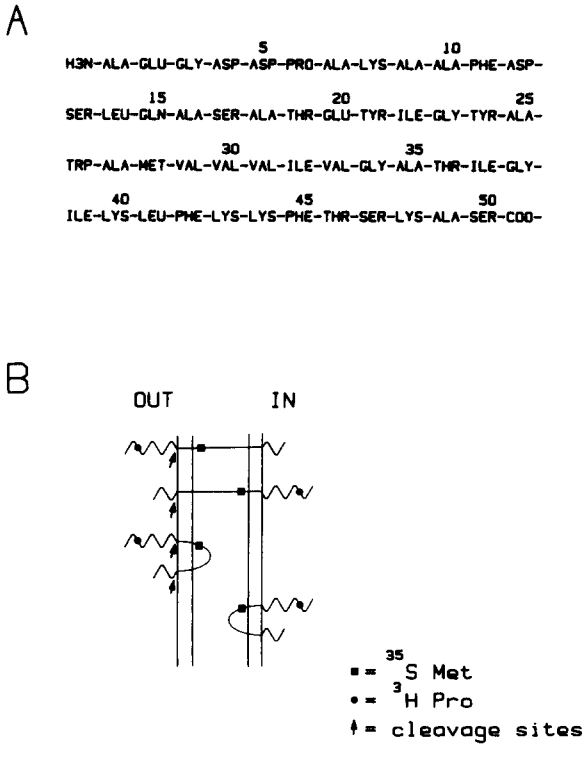

Fig. 1. (A) Sequence of M13 coat protein. (b) Possible configurations and orientations of coat protein in the membrane and sites of proteinase K action (assuming all exposed regions are digested). Coat protein molecules shown are labelled at proline and methionine. Note that actual state of coat protein in membrane is probably dimeric.

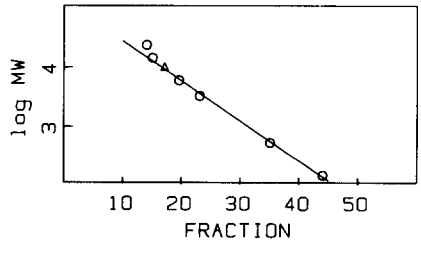

Fig. 2. Molecular weight standards on Fractogel TSK column. Circles represent trypsin (M_r 24000), lysozyme (M_r 14400) insulin (M_r 6000), glucagon (M_r 3350), hexa-L-alanine (M_r 530), and [^{14}C]methionine (M_r 149). Coat protein, represented by a triangle, is plotted at the molecular weight of the dimeric form (M_r 10400).

Reconstituted coat protein on fractogel TSK column

One simple use of the column is to examine the aggregation state of coat protein after various treatments. Column runs of coat protein reconstituted into vesicles but not subjected to protease, as well as cholate-solubilized coat protein are shown in Fig. 3. Both the sonication-incorporated coat protein and cholate dialysis-incorporated coat protein vesicles were fractionated to remove significant amounts of protein which is not associated with lipid. Both cholate-solubilized coat protein and protein incorporated by the FT-cholate reconstitution elute as a single peak. However, both sonication and cholate dialysis yield some incorporated protein in a higher aggregation state as evidenced by the void-volume peak, although in some cases cholate dialysis reconstitutions yield results similar to that of FT-cholate reconstitution.

Protease digests of solubilized coat protein

Experiments were performed to identify products of protease digestions of cholate-solubilized coat protein. Figs. 4A and 4B are chymotrypsin digests of [^{35}S]Met,[^{3}H]Pro-labelled coat protein and [^{14}C]Met, [^{3}H]Lys-labelled coat protein at 0.1 mg/ml coat protein. The largest peak in Fig. 4A

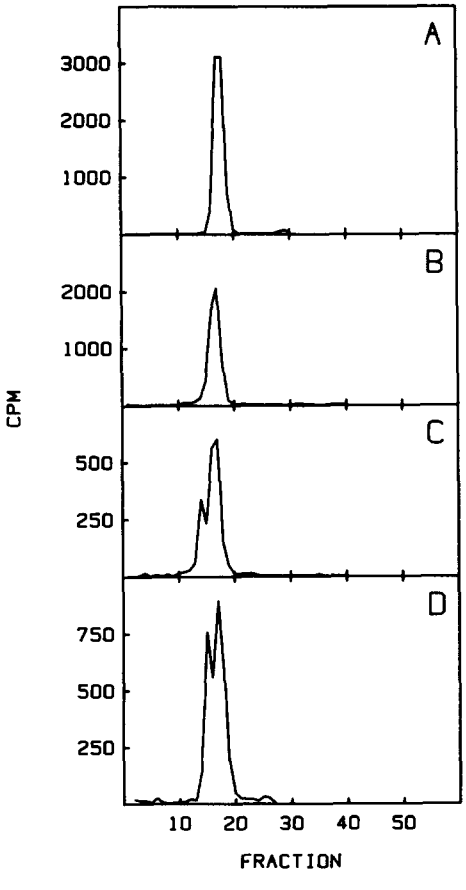

Fig. 3. Fractogel TSK analysis of undigested reconstituted coat protein. (A) Cholate-solubilized coat protein. (B) Coat protein reconstituted by the FT-cholate reconstitution. (C) Coat protein reconstituted by cholate dialysis. (D) Coat protein reconstituted by the sonication method. The *Y*-axis presents total counts.

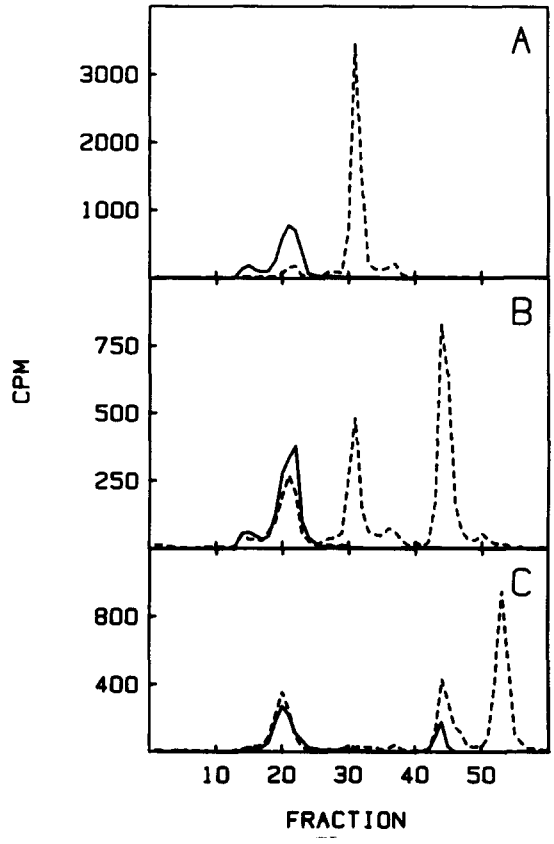

Fig. 4. Chymotrypsin digest of [^{35}S]Met,[^{3}H]Pro coat protein; chymotrypsin and pronase digests of [^{14}C],[^{3}H]Lys coat protein in buffer B. Concentration of coat protein was 0.1 mg/ml in all digests. Elution profiles: (A) is [^{35}S]Met,[^{3}H]Pro coat protein; (B) is [^{14}C]Met,[^{3}H]Lys-labelled coat protein; both were digested with 0.8 mg/ml chymotrypsin as described previously. (C) [^{14}C]Met,[^{3}H]Lys-labelled coat protein digested overnight with 0.02 mg/ml pronase. Solid lines represent [^{35}S]- or [^{14}C]methionine label; broken lines represent ^{3}H label (lysine or proline).

(centered at fraction 31) contains 86 percent of the proline in the sample. The corresponding peak in Fig. 4B contains 22 percent of the lysine. Hence, this peak corresponds to the peptide which contains residues 1 though 11. On the basis of molecular weight (1270 Da), this peptide is expected to elute at fraction 30. The peak centered at fraction 21 has 22 percent of lysine and only 5 percent of proline remaining, identifying this peak as the major hydrophobic core peptide. Hence, this represents coat protein which has been digested at both ends. A peptide containing residues 25–42 (2190 Da) would be expected to elute at fraction 22 on the basis of molecular weight, if it were dimeric.

The amount of material eluting at the void volume is dependent upon the concentration of coat protein during the digest. At high concentration (0.5–2.5 mg/ml) of coat protein, up to 40–50 percent of total methionine is found in the peak centered at fraction 14 (data not shown), similar to results obtained by Woolford and Webster [4].

Since the extent of chymotrypsin digestion at the N-terminus could not be determined from these experiments, the non-specific protease pronase was used to see if a different digestion pattern would be obtained. Fig. 4C shows an overnight digestion of 0.1 mg/ml [^{14}C]Met,[^{3}H]Lys coat protein with 0.02 mg/ml pronase. The main hydrophobic core-containing peptide elutes at fractions 20–21. (Variability in elution position of peaks is generally one fraction or less). The $^{3}H/^{14}C$ ratio in the peak indicates that 62 percent of lysine has been removed. In a parallel digest of [^{35}S]Met,[^{3}H]Pro coat protein (data not shown), the $^{3}H/^{35}S$ ratio in the peak indicated that 87 percent of proline was released. Hence, this represents digestion at the N-terminus at least through Lys-8 and digestion at the C-terminus to Lys-43. Some of the [^{14}C]methionine is released at this protease concentration. Higher levels of pronase can release almost all of the methionine label in detergent-solubilized coat protein (data not shown).

Attempts were made to prepare cholate-solubilized coat protein digested only at the C-terminus with carboxypeptidase Y, and with combinations of carboxypeptidase A and B, both treated to eliminate tryptic and chymotryptic activity. No conditions were found under which the C-terminus was digested more rapidly than release of proline from the N-terminus. However, it is clear from digests of reconstituted coat protein, discussed below, that coat protein with the N-terminus removed elutes at approximately the same position as coat protein with both ends clipped, while coat protein with only the C-terminus removed elutes just after intact coat protein and is not well resolved.

Proteinase K digests

Proteinase K was used to ensure complete digestion of all exposed peptides in reconstituted preparations. Fig. 5A shows an overnight digestion of cholate-solubilized [^{14}C]Met,[^{3}H]Lys coat protein (0.54 mg/ml) with 0.5 mg/ml proteinase K.

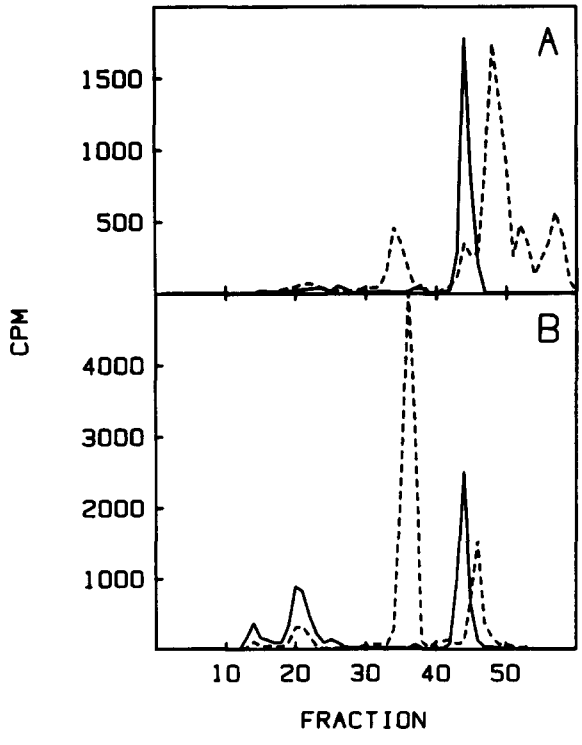

Fig. 5. Proteinase K digests of cholate-solubilized coat protein and of aggregated coat protein. (A) Proteinase K (0.5 mg/ml) added to [^{14}C]Met,[^{3}H]Lys coat protein (0.54 mg/ml) in buffer B and incubated overnight at 37°C. (B) Cholate-solubilized [^{35}S]Met,[^{3}H]Pro coat protein (2.4 mg/ml) dialyzed against buffer without cholate to induce aggregation. Proteinase K was then added to 0.5 mg/ml, and the solution incubated at 37°C overnight. Solid lines represent [^{35}S]- or [^{14}C]methionine label; broken lines represent ^{3}H label (lysine or proline).

In contrast to digestion with chymotrypsin, all methionine is released in cholate-solubilized coat protein. To determine the susceptibility of aggregated protein, cholate-solubilized protein (2.4 mg/ml) was dialyzed against detergent-free buffer to induce aggregation. The turbid suspension was then digested overnight with 0.5 mg/ml proteinase K, and the resulting FTSK analysis is shown in Fig. 5B. Approximately half of the methionine residues are inaccessible and resistant to digestion.

Proteinase K digests of sonication incorporated protein. Figs. 6A and 6B show digestion of [^{14}C]Met,[^{3}H]Lys coat protein reconstituted into vesicles at lipid/protein ratios of 40 : 1 and 160 : 1 (mol/mol). Fig. 6C is [^{35}S]Met,[^{3}H]Pro coat protein reconstituted into vesicles. In all, a significant portion of the protein elutes at the void volume. From the ^{3}H/^{35}S ratio, about half of the proline has been removed, as well as 15–20 percent of lysine (judging from the ^{3}H/^{14}C ratio). The aggregated material eluting at the void volume is not dispersed by heating at 37°C for 30 min, or by sonication. Since only about 50 percent of aggregated nonlipid associated coat protein is digested to free methionine, this may reflect aggregated lipid-associated protein which is protease resistant.

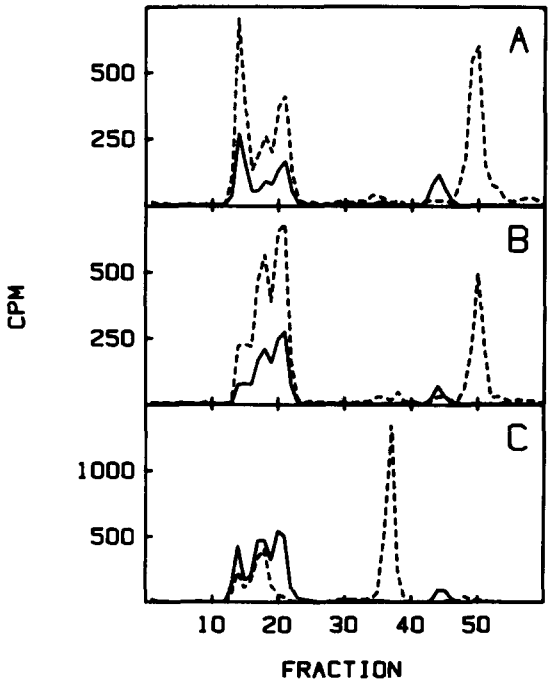

Fig. 6. Proteinase K digests of sonication incorporated coat protein showing the effect of lipid : protein ratio and salt concentration. (A) and (B) Proteinase K (0.5 mg/ml) digests of [^{14}C]Met,[^{3}H]Lys coat protein at 40 : 1 and 160 : 1 (mol/mol) lipid/protein ratio, respectively. (C) A digest of [^{35}S]Met,[^{3}H]Pro coat protein reconstituted at 160 : 1 (mol/mol) lipid/protein ratio. Solid lines represent [^{35}S]- or [^{14}C]methionine label; broken lines represent ^{3}H label (lysine or proline).

The amount of material in this void volume peak is variable, but at higher initial lipid/protein ratios (40 : 1, mol/mol) in the reconstitution, the amount of this material increases. It was not determined if the lipid/protein ratio in the fractionated vesicles reflected the amounts of protein and lipid added to the reconstiution mix. The central peak represents mostly undigested coat protein which was not accessible to protease. In Fig. 6C, 73 percent of proline remains in fractions 17–18, whereas in Fig. 6B 79 percent of lysine remains in the same fractions. The peak at fraction 21 consists mostly of coat protein which spanned the membrane with N-terminus outward: in Fig. 6C, 94 percent of proline is removed from material in fraction 21, while in Fig. 6B 24 percent of lysine is removed. Hence, this establishes the elution position of coat protein with only the N-terminus cleaved. Increasing salt concentration has little effect on this reconstitution, yielding a digestion pattern similar to Fig. 6C (data not shown).

Proteinase K digests of coat protein reconstituted by cholate dialysis. Fig. 7 shows digests of cholate dialysis-reconstituted [^{35}S]Met,[^{3}H]Pro coat protein and [^{14}C]Met,[^{3}H]Lys coat protein at a lipid/protein ratio of 40 : 1. Again, no attempt has been made to verify that lipid/protein ratios in the fractionated vesicles reflect the quantities used in the reconstitution mix. Lipid/protein ratio and brief sonication have little effect on the digestion pattern. Again, the peak at fraction 18 is found to be mostly intact coat protein (85 percent of proline remaining in Fig. 7A, 95 percent of lysine remaining in Fig. 7B), and fraction 21 is coat protein with N-terminus removed (20 percent proline remaining in Fig. 7A, 84 percent of lysine remaining in Fig. 7B).

Proteinase K digests of FT-cholate reconstituted coat protein. In Fig. 8, digestions are shown of coat protein reconstituted by the FT-cholate method using various extents of sonication. Also, results of

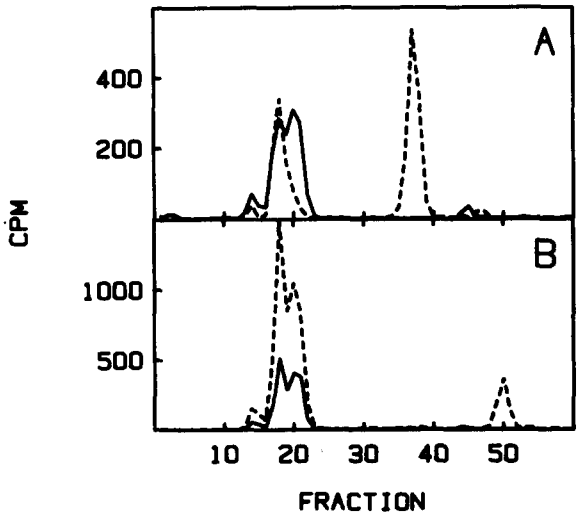

Fig. 7. Proteinase K digests of coat protein reconstituted by cholate dialysis. (A) [^{35}S]Met,[^{3}H]Pro coat protein reconstituted at 40:1 lipid/protein. (B) [^{14}C]Met,[^{3}H]Lys coat protein reconstituted at 40:1. Solid lines represent [^{35}S]- or [^{14}C]methionine label; broken lines represent ^{3}H label (lysine or proline).

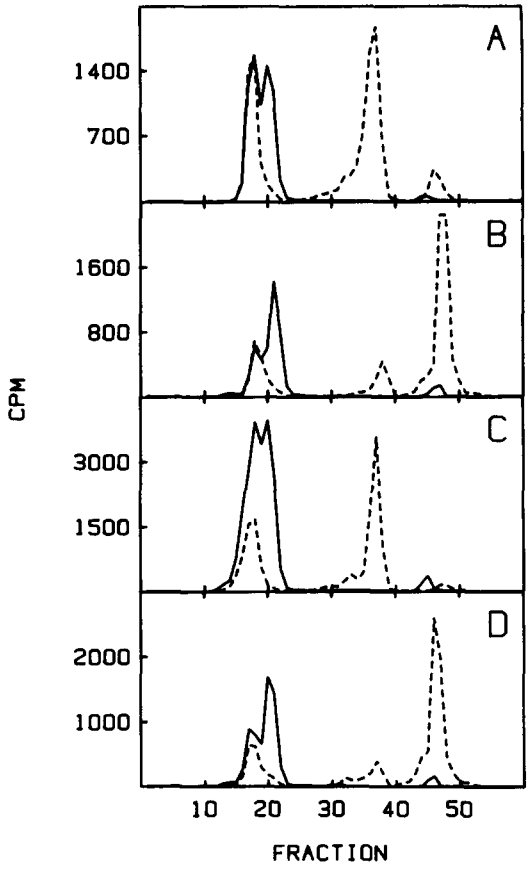

Fig. 8. Proteinase K digests of FT-cholate reconstituted coat protein, showing the effect of sonication. All digests are [^{35}S]Met,[^{3}H]Pro labelled coat protein. (A) Unsonicated; (B) briefly sonicated (30s); (C) extensively sonicated (5 min). Extensively presonicated vesicles were used in (D), which was also sonicated briefly (30 s) after reconstitution. Note that (C) was done at a later date, so ^{3}H/^{35}S ratios are not comparable to the other digests. Solid lines represent [^{35}S]Met label; broken lines [^{3}H]Pro label.

reconstitution starting with extensively presonicated (to clarity) vesicles are shown in Fig. 8D. In contrast to incorporation by the simple sonication method described earlier, there is very little material eluting at the void volume. Brief sonication increases the fraction of coat protein with the N-terminus accessible, but extensive sonication yields a large amount of material eluting earlier, indicating that some aggregation has occurred. Addition of KCl to 0.6 M either with or without sonication increases the percentage of accessible protein (data not shown). Adding coat protein with 30 percent less total cholate greatly increases the amount of inaccessible protein to two-thirds of the total material (data not shown). We note that reproducibility of these digestion results was somewhat variable, with from 50 percent to 80 percent of proline being released.

Fig. 8D shows that extensive presonication of lipid vesicles has little effect. Visually, there is a larger turbidity increase upon freezing when reconstitutions are performed with vesicles sonicated to clarity, indicating a higher level of aggregation. Samples do clarify rapidly upon brief (30 s) sonication in a bath sonicator. In contrast, extensively presonicated vesicles containing egg PC alone which are frozen and thawed require a long period of resonication to clarify. Hence, either coat protein or residual cholate has a substantial effect on vesicle fusion or aggregation.

Fig. 9 shows a proteinase K digest of [^{14}C]Met,[^{3}H]Lys coat protein reconstituted using the FT-cholate procedure. The ^{3}H/^{14}C ratio indicates loss of 25 percent of lysine in fraction 18, 18 percent in fraction 22.

Fig. 10 shows coat protein incorporated into

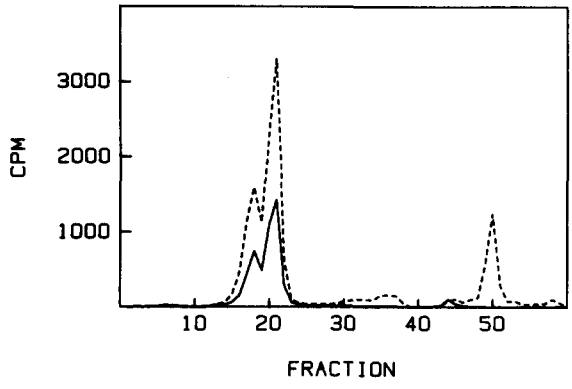

Fig. 9. Proteinase K digest of [^{14}C]Met,[^{3}H]Lys FT-cholate reconstituted coat protein. Protein was reconstituted as described in Methods. Proteinase K was added to 0.5 mg/ml, and digestion was overnight at 37°C. Solid line represents [^{14}C]Met label; broken line is [^{3}H]Lys label.

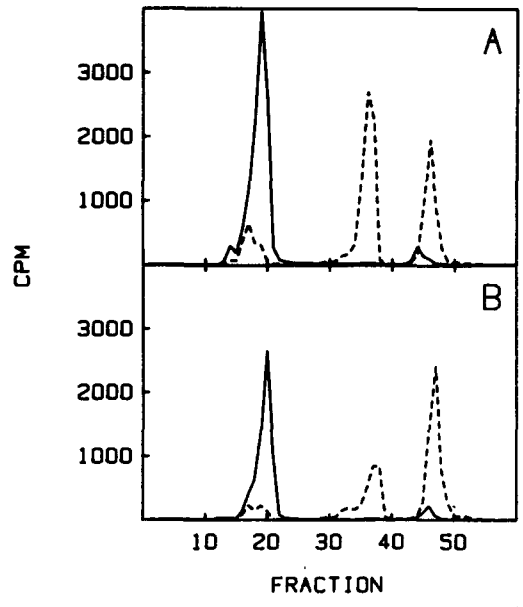

Fig. 10. Proteinase K digests of FT-cholate reconstituted coat protein in 50 percent egg PC/50 percent egg PA. [^{35}S]Met,[^{3}H]Pro coat protein was reconstituted into vesicles composed of 50 percent PC/50 percent PA. In (B), the last dialysis was against buffer A + KCl to 0.3 M. Digestions were then done with proteinase K (0.5 mg/ml) overnight at 37°C. Solid line represents [^{35}S]Met label; broken line is [^{3}H]Pro label.

vesicles composed of 50 percent egg PC/50 percent egg PA. Because of the high negative charge of these vesicles, a higher salt concentration was tested to determine its effect on the extent of digestion, since protease might be repelled from the vesicle surface. In Fig. 10B the last buffer change during dialysis contained 0.3 M KCl. In Fig. 10A, 86 percent of proline is released, whereas in Fig. 10B, 91 percent of proline is released. Because the amount of material in fraction 17 (N-terminus intact) is small, it is possible to see a small overlapping proline peak at fractions 18–19, which probably corresponds to coat protein digested at the C- rather than the N-terminus, and which accounts for about 5 percent of total protein. On the basis of molecular mass, this peptide (8100 Da for the dimeric form) is expected to elute at fraction 18.

In order to determine the number of C-termini accessible on the outside of the vesicle, attempts were made to analyze chymotrypsin digests of [^{14}C]Met,[^{3}H]Lys coat protein reconstituted into vesicles using the FT-cholate procedure. As is apparent from Fig. 4A and 4B, lysine released from the C-terminus is found primarily in fractions 43–44, in contrast to lysine from the N-terminus which is predominantly in the peptide eluting at fraction 31. Hence, the number of N- and C-termini accessible could be determined, in principle, from a single experiment.

Chymotrypsin was added at a final concentration of 0.8 mg/ml to 1 ml of reconstituted vesicles containing 0.37 mg coat protein as for digests of coat protein in detergent. The sample was slightly turbid in formic acid/ethanol. Recovery from the column was only about 25 percent, and much of this material eluted at the void volume. Reducing the concentration of chymotrypsin 4-fold and reducing the amount of sample loaded by 2-fold only increased recovery to about 40 percent. Presumably, a large percentage of the remaining fragments are associated with chymotrypsin in the absence of detergent, and during sample preparation are irreversibly adsorbed to denatured chymotrypsin, and the material not trapped by the column prefilter elutes at the void volume. This may also explain the source of the void volume peak in chymotrypsin digests of coat protein in cholate when the concentration of coat protein is high.

Discussion

The ability to resolve coat protein with the N-terminus intact from protein with the N-terminus cleaved enables the determination of protein orientation and configuration in the membrane after reconstitution using doubly labelled protein. Estimation is possible of the number of molecules incorporated in a U-shape on the outside of the vesicle from the amount of lysine remaining in the peak of material with the N terminus removed (with or without digestion at the C-terminus) centered at fraction 21. Molecules with only the N-terminus removed should have 20 percent of the lysine removed, whereas those digested at both termini should have 80 percent of the lysine removed. In the digest of sonication-incorporated coat protein shown in Fig. 6B, 24 percent of lysine is removed in fraction 21, indicating little or no digestion at the C-terminus. Likewise, for cholate dialysis-reconstituted coat protein (Fig. 7B), 16 percent of lysine is removed. Lastly, for FT-cholate reconstituted coat protein (Fig. 9), 18 percent of lysine is removed from this peak. Hence, according to this criterion, in all of these reconstitutions no significant amount of coat protein is incorporated in a U-shape configuration on the outside of the vesicle.

Likewise, from the $^{3}H/^{14}C$ ratio of the peptides eluting at fraction 17–18 (N-terminus intact), the fraction of coat protein spanning the membrane with the C-terminus outwards can be estimated. Any lysine removed is from the C-terminus. For sonication-incorporated coat protein (Fig. 6B), 79 percent of lysine remains. Since three lysines would be lost from digestion at the C-terminus, 35 percent of this peak, or 12 percent overall (since this peak represents one-third of total material with this incorporation procedure), is from coat protein spanning the membrane with the C-terminus outwards. Similarly, 4 percent of cholate dialysis incorporated protein and 12–14 percent of FT-cholate reconstituted coat protein is spanning the membrane with the C-terminus outwards.

Uncertain, however, is the source of the aggregated material eluting near the void volume in digests of sonication- or cholate dialysis-reconstituted coat protein. The possibility remains that coat protein in membrane-bound form in a U-configuration is aggregated and hence protease resistant. However, because of the small amount of such material in FT-cholate reconstitutions, almost all accessible protein spans the membrane.

In all reconstitutions, a significant (20–50 percent) fraction of coat protein molecules are inaccessible. Possible sources of this material are: coat protein in a U-shaped configuration on the inside face of the bilayer; coat protein trapped within a vesicle; coat protein which is somehow resistant to protease but still located at the vesicle surface; and coat protein present in vesicles trapped within larger vesicles, i.e. multilayers. The possibility of coat protein trapped within the aqueous compartment of a vesicle can probably be discounted since the protein is likely to be aggregated in the absence of amphiphile. Vesicles prepared by the sonication method have been shown to be single-walled of approx. 20 nm diameter [7]. Likewise, cholate dialysis vesicles, although larger (approx. 60–100 nm diameter), were found to be unilamellar in thin-section electron microscopy [7]. In both cases it is possible that protein which is inaccessible to digestion is from U-shaped molecules on the inside face of the bilayer.

Freeze-thawing is known to induce multilayer formation and aggregation, with mostly unilamellar vesicles resulting after brief sonication [13]. The FT-cholate reconstitution described here is similar to the freeze-thaw method of Kasahara and Hinkle [13], except that a detergent is required for successful reconstitution. Higher salt concentration, brief sonication, and using negatively charged phospholipids in the reconstitution all will reduce the fraction of lipid in multilayers, and all of these treatments did result in more coat protein being accessible. Hence, it is possible, although not proven, that most inaccessible protein in FT-cholate reconstituted coat protein is present within multilayers.

The particular chromatographic system used for analyzing peptides from protease digests was chosen over other methods for several reasons. Aggregation of hydrophobic peptides is much more severe a problem in aqueous systems even in detergent. Hence, on SDS gels, many membrane proteins do not enter the gel matrix. Reverse phase HPLC in formic acid/ethanol has been used to separate some membrane protein fragments, but

extremely hydrophobic peptides were lost on the column [8]. Molecular exclusion columns for HPLC which are stable in formic acid/ethanol are not yet available. Hence the present system, which has the advantage of relatively fast flow rates and high recovery, was chosen. Unfortunately, the system is unable completely to resolve all proteolytic fragments containing the core. As with other materials used for gel permeation chromatography, charge effects are significant at low ionic strength. Thus, for material with the N-terminus digested, digestion at the C-terminus results in a smaller peptide which should elute later on the basis of size, but simultaneous loss of charge (3 of the 4 remaining lysines) compensates and the elution position remains constant. Similarly, the peptide which has only the C-terminus cleaved is not well resolved from intact coat protein because of both the small change in size and the loss of charge.

The FT-cholate reconstitution described here appears to be the best available for physical studies where preserving the initial aggregation state and orientation of the protein are important. This reconstitution also incorporates almost all of the protein into vesicles, making fractionation unnecessary, in contrast to the other reconstitution procedure. Unfortunately, it does not allow much latitude in varying the lipid/protein ratio, and thus is not likely to be suitable for preparing vesicles containing a high percentage of protein (e.g. for NMR studies). Reconstitution in other lipid systems of interest should be studied to determine whether the reconstitution is satisfactory. Lastly, because of the observed variability, if experiments require knowing precisely the fraction of accessible protein, the particular sample used should be evaluated using the procedures above.

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References

1 Wickner, W. (1975) Proc. Natl. Acad. Sci. USA 72, 4749–4753
2 Ohkawa, I. and Webster, R. (1981) J. Biol. Chem. 256, 9951–9958
3 Nakashima, Y. and Konigsberg, W. (1974) J. Mol. Biol. 88, 598–600
4 Woolford, J.L. and Webster, R.E., (1975) J. Biol. Chem. 250, 4333–4339
5 Williams, R.W. and Dunker, A. (1977) J. Biol. Chem. 252, 6253–6255
6 Dunker, A.K., Fodor, S.P.A. and Williams, R.W. (1981) Biophys. J. 37, 201–203
7 Chamberlain, B., Nozaki, Y., Tanford, C. and Webster, R. (1978) Biochim. Biophys. Acta 510, 18–37
8 Khorana, H.G., Gerber, G.E., Herlihy, W.C., Gray, C.P., Anderegg, R.J., Nihei, K. and Biemann, K. (1979) Proc. Natl. Acad. Sci. USA 76, 5046–5050
9 Takagi, Y., Gerber, G., Nihei, K. and Khorana, H.G. (1980) J. Biol. Chem. 255, 1536–1541
10 Miller, J.H. (1972) Experiments in Molecular Genetics Cold Spring Harbor Laboratory, Cold Spring Harbor, NY
11 Nozaki, Y., Chamberlain, B., Webster, R. and Tanford, C. (1976) Nature 259, 335–337
12 Lowry, O., Rosebrough, J.S., Farr, A.L. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275
13 Kasahara, M. and Hinkle, P. (1977) J. Biol. Chem. 252, 7384–7390