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Aggregation-Related Conformational Change of the Membrane-Associated Coat Protein of Bacteriophage M13

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ABSTRACT: The state of the coat protein of bacteriophage M13, reconstituted into amphiphilic media, has been investigated. The in situ conformation of the coat protein has been determined by using circular dichroism. Minimum numbers for the protein aggregation in the system have been determined after disruption of the lipid–protein system and subsequent uptake of the protein in cholate micelles. The aggregational state and conformation of the protein were affected by (1) the method of coat protein isolation (phenol extraction vs cholate isolation), (2) the nature of amphiphiles used (variation in phospholipid headgroups and acyl chains), and (3) the ratio of amphiphiles and protein. Under all conditions, phenol-extracted coat protein was in a predominantly β-structure and in a highly aggregated polymeric form. Cholate-isolated coat protein was initially oligomeric and contained a substantial amount of α-helix. Below an aggregation number of 20, this protein showed a reversible aggregation with no change in conformation. Upon further aggregation, a conformational change was observed, and aggregation was irreversible, resulting in predominantly β-structured coat protein polymers. This effect was observed upon uptake in phospholipids at low lipid to protein molar ratios (L/P ratios) and with phosphatidylcholines (PC) and phosphatidic acids (PA) containing saturated acyl chains. After reconstitution in phospholipds with unsaturated acyl chains and with phosphatidylglycerols (PG) at high L/P ratios, the original α-helix-containing state of the coat protein was maintained. Cross-linking experiments demonstrated that the β-polymers are able to form reversible superaggregates within the vesicle system. An aggregation-related conformational change mechanism for the coat protein in phospholipid systems is proposed.

M13 bacteriophage is an *Escherichia coli* specific filamentous phage. The rod-shaped virion is composed of a cylindrical protein capsid, which mainly consists of the gene 8 product: the major coat protein [see reviews by Ray (1977), Makowski (1984), and Rashed and Oberer (1986)]. During infection, the viral DNA is released into the cytoplasm of *E. coli*, while its coat protein is inserted into the cytoplasmic membrane (Pratt et al., 1969). It is generally assumed that, in vivo, parental as well as newly synthesized coat protein is stored oriented as an integral (trans)membrane protein (Smilowitz, 1974; Wickner, 1975; Ohkawa & Webster, 1981). During the combined assembly–extrusion process, the major coat protein is assembled around the viral DNA.

The primary structure of the coat protein [molecular weight (MW)[1] 5240] is given in Figure 1. Three specific domains can be distinguished: an acidic N-terminus (residues 1–20)

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[1] Abbreviations: DMPC, dimyristoylphosphatidylcholine; DPPC, dipalmitoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; PC, phosphatidylcholine; PE, phosphatidylethanolamine; DMPG, dimyristoylphosphatidylglycerol; DPPG, dipalmitoylphosphatidylglycerol; DOPG, dioleoylphosphatidylglycerol; PG, phosphatidylglycerol; DMPA, dimyristoylphosphatidic acid; DOPA, dioleoylphosphatidic acid; PA, phosphatidic acid; SDS–PAGE, sodium dodecyl sulfate–polyacrylamide gel electrophoresis; L/P ratio, lipid to protein molar ratio; CD, circular dichroism; MW, molecular weight; DMA, dimethyl adipimidate; DMS, dimethyl suberimidate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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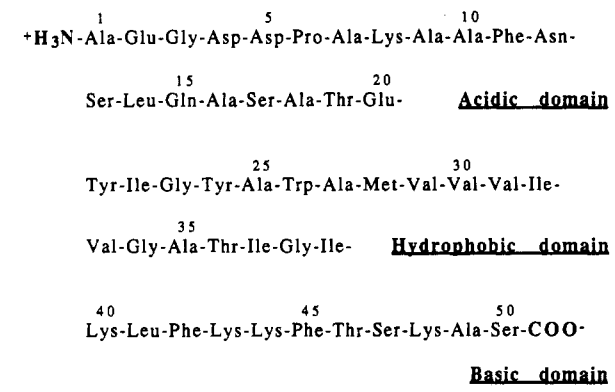

FIGURE 1: Primary structure of the M13 coat protein. The amino acid sequence of M13 coat protein differs from that of fd and f1 coat proteins at position 12 where fd and f1 coat proteins contain aspartic acid instead of an asparagine (Beck et al., 1978; Nakashima & Konigsberg, 1974; van Weezenbeek et al., 1980).

containing glutamic acid and aspartic acid, which in the phage is in contact with the solvent; a basic C-terminus (residues 40–50), which contains four lysines and interacts with the negatively charged DNA phosphate backbone; a hydrophobic core (residues 21–39), which has a possible role in the protein–protein interactions in the phage and in the hydrophobic protein–lipid interactions when the coat protein is in the cytoplasmic membrane.

The coat protein can be incorporated into phospholipid bilayers by various techniques (Chamberlain et al., 1978; Bayer & Feigenson, 1985), and some information has been obtained about the conformation of the coat protein in the phage and after reconstitution into membranes (Nozaki et al., 1976; Williams & Dunker, 1977; Fodor et al., 1981). As compared to the mainly α-helical conformation (the so-called "a" conformational state) of the coat protein in the virion, a remarkable decrease in α-helix content and concomitant increase of the amount of β-structure are observed when the coat protein is reconstituted into amphiphilic media (Nozaki et al., 1976; Williams & Dunker, 1977; Dunker et al., 1981). A conformational change can be expected for two reasons: (1) the microenvironment of the coat protein alters drastically upon incorporation into amphiphilic media; and (2) the binding between the basic C-terminus and the viral DNA is lost (Chou & Fasman, 1974; Vriend et al., 1986). In amphiphilic media, the coat protein adopts two specific conformations, which are indicated as the b and c states. The b-state protein contains a substantial amount of β-structure and α-helix. The c state of the coat protein is characterized by an even higher amount of β-structure and a lack of α-helix content (Williams & Dunker, 1977; Fodor et al., 1981).

The α-helix content of the b-state protein has been assigned to the hydrophilic termini, the hydrophobic core adopting a predominantly β-structure (Chamberlain et al., 1978). A β-structure for integral membrane proteins is remarkable, since many membrane proteins are known to span the membrane by an α-helix (Bloom & Smith, 1985; Marsh, 1985). However, a β-structure of the coat protein of M13 or the closely related fd phage has been observed before in model membranes (Datema et al., 1987a; Williams & Dunker, 1977; Chamberlain et al., 1978; Fodor et al., 1981). The significance of the b and c states has been discussed previously; however, factors affecting the conformational change from the b to the c state have not been well understood (Dunker et al., 1981; Nozaki et al., 1978; Wilson & Dahlquist, 1985).

In the present work, the secondary structure of the coat protein of M13 in various phospholipid systems is studied to obtain information about the transition from the b to the c conformational state. The aggregational state of the coat protein is determined with special interest, since an aggregated state of the reconstituted protein has previously been suggested (Datema et al., 1988; Dettman et al., 1982; Bayer & Feigenson, 1985; Williams & Dunker, 1977) or rejected (Kimelman et al., 1979; Wolber & Hudson, 1982; Wilson & Dahlquist, 1985).

Furthermore, attention is paid to the effects of phenol-extracted coat protein and cholate-isolated coat protein reconstituted into stable phospholipid bilayer systems consisting of PC, PG, and PA, using sodium cholate as an amphiphilic intermediate. In addition, the length of the phospholipid acyl chain and the degree of saturation are varied.

Materials and Methods

Chemicals. E. coli K37 and M13 bacteriophage (wild type) were a gift of Dr. Harmsen, University of Nijmegen. DMPC, DPPC, DOPC, egg yolk PC, DMPG, DPPG, DOPG, egg yolk PG, DMPA, DOPA, egg yolk PA, sodium cholate, and globular molecular weight markers (range 12×10^3 to 2×10^6) were purchased from Sigma Chemical Co., St. Louis, MO. All lipids were of 98–99% purity and were used without further purification. Sodium dodecyl sulfate was obtained from BDH Chemicals Ltd., Poole, England. ^{14}C-Labeled cholate was obtained from Dr. A. Watts, University of Oxford. Sepharose 6B-CL and the Superose 6 (HR 10/30) column were purchased from Pharmacia/LKB. Octadecyl Rhodamine B was purchased from Molecular Probes, Inc. DMA and DMS were purchased from Pierce Chemical Co., Rockford, IL. All other materials were of pro-analytical grade.

Bacteriophage Growth and Purification. E. coli K37 was grown in 40 L of M9 minimal medium, supplemented with 0.8% (w/v) glucose and 0.33% (w/v) casamino acids at 38 °C, with vigorous stirring and aeration. *E. coli* was infected at a density of 2×10^8 cells/mL with M13 bacteriophage. The multiplicity of infection was approximately 10. At a density of approximately 5×10^9 cells/mL, the infected cells were collected by centrifugation (20 min at 10000*g*). M13 bacteriophage was precipitated at 4 °C by adding 5% (w/v) poly(ethylene glycol) 6000/0.5 M NaCl and was harvested by 20-min centrifugation at 10000*g*. The pellet was resuspended in 100 mM Tris-HCl/1 mM EDTA, pH 8.0, and the PEG precipitation was repeated under the same conditions. The phage precipitate was taken up in 100 mM Tris-HCl/1 mM EDTA, pH 8.0, layered over a 3-fold volume of CsCl solution (final density 1.29 g/mL), and centrifuged at 150000*g* for 24 h. The bacteriophage bands were collected, dialyzed against the same buffer, and stored frozen at −30 °C. The purification of the virus was checked by using urea/SDS–PAGE, as described by Simons et al. (1979). The amount of bacteriophage was measured spectrophotometrically using A_{260} = 3.80 (0.1% w/v, 1-cm path length) (Nozaki et al., 1976; Berkowitz & Day, 1976).

Preparation and Purification of M13 Coat Protein. (1) Phenol-extracted coat protein was obtained by the procedure of Knippers and Hoffmann-Berling (1966) and subsequent extensive dialysis against distilled water. After lyophilization, the coat protein was obtained as a white powder and stored at 4 °C.

(2) Cholate-isolated coat protein was prepared by a modification of the procedure of Makino et al. (1975). Typically, 3 mL of bacteriophage solution (concentration 10–15 mg/mL) was mixed with 6 mL of 120 mM cholate in 10 mM Tris-HCl, 0.2 mM EDTA, pH 8.0, and chloroform (final concentration 2.5% v/v). The suspension was incubated at 37 °C with

occasional mixing until a clear nonopalescent solution was obtained. The dissociated virus was then applied on a Sepharose 6B-CL column (3.7 × 45 cm) and eluted with 10 mM cholate in 10 mM Tris-HCl/0.2 mM EDTA, pH 8.0, to separate the major coat protein from the viral DNA. Fractions with an absorbance ratio A_{280}/A_{260} greater than 1.5 were collected and stored at 4 °C.

Reconstitution into Micelles. (1) Phenol-extracted coat protein was solubilized in cholate or SDS micelles by incubation at 55 °C and vigorous mixing or sonication with a Branson B15 sonifier (2–10 min, duty cycle 50%, 75-W output). Using cholate, only concentrations higher than 50 mM were capable of dissolving the coat protein.

(2) In the case of cholate-isolated coat protein, the cholate concentration was adjusted to the desired concentration.

Reconstitution into Phospholipid Bilayers. (1) Phenol-extracted coat was mixed with protein and phospholipids in 8 M urea, 2% w/v cholate, and 20 mM ammonium sulfate in 10 mM Tris-HCl/0.2 mM EDTA, pH 8.0, and incubated at 55 °C with vigorous vortexing and sonication, and subsequent dialysis for 48 h (buffer changes every 12 h) against 100 times excess of buffer (10 mM Tris-HCl/0.2 mM EDTA, pH 8.0) at room temperature (Hagen et al., 1978). Experiments with ^{14}C-labeled cholate yielded very low levels of remaining cholate after dialysis. Depending on dialysis temperature and protein content, lipid/cholate ratios in the range 75 (at L/P 5) to 150 (at L/P 50) were obtained at room temperature and at least 5-fold lower at 4 °C.

(2) Cholate-isolated coat protein and phospholipids were mixed in 50 mM cholate in 10 mM Tris-HCl/0.2 mM EDTA, pH 8.0, and dialyzed as described for the phenol-extracted protein.

Determination of Coat Protein Incorporation and Sample Homogeneity. After reconstitution, an aliquot of the resulting vesicle suspension (ca. 2–5 mg of material) was layered over a linear 0–40% w/w sucrose gradient and centrifuged in a swing-out rotor at 100000*g* at 10 °C for 16 h. Sample homogeneity was checked visually. In the case of less opalescent samples, octadecyl Rhodamine B (0.5% mol/mol), which dissolved in the lipids with high specificity, was added before sample preparation, enabling the visualization of the lipid–protein complexes.

Determination of L/P Ratio. L/P ratios were determined after sample preparation (Datema et al., 1988). The lipid content was measured by the procedure of Bartlett (1959) with inorganic phosphate as a standard. The coat protein content was determined by the procedure of Peterson (1977) with bovine serum albumin as a standard (A_{280} = 0.666, 1 mg/mL, 1-cm path length).

Circular Dichroism Measurements. CD experiments were performed directly after sample preparation. When vesicle aggregation occurred, the samples were diluted and measured after brief sonication (1 min, 50% duty cycle, 50-W output). CD spectra were recorded at room temperature on a Jobin–Ivon Auto Dichrograph Mark V in the wavelength range 250–200 nm, using a sample cell with 0.1-cm path length. CD spectra were the average of 10 scans and were corrected for the coat protein content and for the lipids in the sample by subtracting the CD spectra of lipid vesicle samples. To determine the secondary structure of the coat protein from experimental data, the CD spectra were fitted to the reference spectra of Greenfield and Fasman (1969) by a least-squares fitting procedure using spectral points in the 250–200-nm range with 5-nm steps.

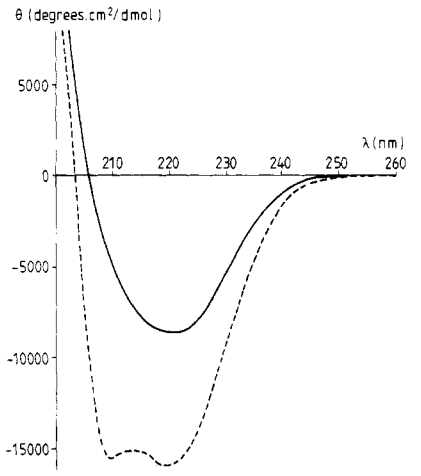

FIGURE 2: Typical CD spectra of the two conformational states of the cholate-solubilized M13 coat protein: phenol-extracted coat protein (c state; —) and cholate-isolated coat protein (b state; - - -) solubilized in 50 mM cholate, 10 mM Tris-HCl, and 0.2 mM EDTA, pH 8.0.

Determination of the Aggregate State of the Coat Protein. Determination of the aggregation numbers of the coat protein was performed by using gel filtration chromatography on an LKB Ultrochrom GTi Bioseparation system equipped with a Superose 6 column (1.0 × 30 cm). This column can be used in the pH range 1–14 and is suitable for separations in the protein MW range 5 × 10^3 to 5 × 10^6. Samples were preincubated in the presence of 50 mM cholate in 10 mM Tris-HCl/0.2 mM EDTA, pH 8.0, for 1.5 h at room temperature, unless stated otherwise; 50 mM cholate/10 mM Tris-HCl/0.2 mM EDTA (pH 8.0), degassed with helium gas, was used as eluent. The flow was adjusted to 0.25 mL/min. The system was calibrated with globular molecular weight markers (Sigma Chemical Co.) in the range 12 × 10^3 to 2 × 10^6. Assuming a binding ratio for cholate of 9 mol/mol of coat protein for the less aggregated (oligomeric) coat protein and 1 mol/mol of coat protein for the polymeric coat protein (Makino et al., 1975; Cavalieri et al., 1976), the aggregation numbers of the protein were calculated.

Cross-Linking of Coat Protein in Phospholipid Bilayers. Samples, prepared in the absence of Tris-HCl, were incubated in the presence of DMA or DMS (concentration 25 mM) at pH 8, room temperature, for 6 h. The cross-linking reaction was stopped by adding glycine (final concentration 0.1 M).

Other Methods. Coat protein concentrations in micelles were determined spectrophotometrically, using A_{280} = 1.60, 1 mg/mL, 1-cm path length (Knippers & Hoffmann-Berling, 1966).

RESULTS

Cholate-Solubilized Coat Protein. Figure 2 shows the CD spectra of the phenol-extracted (solid line) and the cholate-isolated (dashed line) major coat protein of the M13 bacteriophage when associated with cholate. According to the fitting procedure used, the conformation of the cholate-isolated protein results in approximately 30% α-helix, 50% β-structure, and 20% unordered. This conformation is referred to as the b state. The phenol-extracted coat protein contains no α-helix, ca. 75% β-structure, and 25% random coil and is referred to as c-state protein. These secondary structural data are comparable to data found previously (Nozaki et al., 1976, 1978; Williams & Dunker, 1977; Chamberlain et al., 1978; Fodor et al., 1981). No changes in conformational state are observed upon variation of the concentration of cholate (Table I).

Figure 3 shows two typical elution patterns of the M13 coat protein, solubilized in cholate, on a Superose 6 column.

Table I: Conformational and Aggregational State of the Phenol-Extracted and Cholate-Isolated Coat Protein Solubilized into Cholate Micelles upon Additional Treatments

coat protein isolation method	treatment	[cholate] (mM)	conformational state	aggregation no. (av)
phenol extraction		10	c	>880[b]
		50	c	>880[b]
		100	c	>880[b]
	8 M urea	50	c	280
cholate isolation	freshly prepared	10	b	4
	after storage	10	b	20
	after storage and preincubn, 50 mM[a]	10	b	12
	after storage and preincubn, 100 mM[a]	10	b	8
	freshly prepared	50	b	4
	freshly prepared	100	b	2
	temp 55 °C	10	c	440
	temp 55 °C	50	b	4

[a] Aggregated b-state coat protein (after 2-weeks storage at 10 mM cholate with aggregation number 20) was preincubated in the given cholate concentration for 1.5 h. [b] Protein elutes predominantly in the void volume; no precise aggregation numbers can be determined.

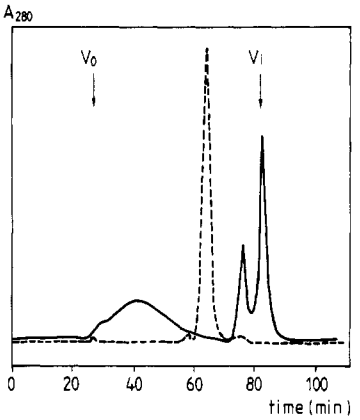

FIGURE 3: Elution pattern of cholate-solubilized M13 coat protein on a Superose 6 column (see Materials and Methods). Phenol-extracted coat protein (—) previously dissolved in elution buffer containing 8 M urea. Cholate-isolated coat protein (---) in elution buffer directly after preparation. The arrows indicate the void volume (V_0) and internal volume (V_i). Cholate micelles elute from the column after 75 min.

Phenol-extracted protein is found in a very broad peak, ranging between 26 and 55 min, with a maximum at 40 min. The cholate-isolated coat protein is found after 64 min. These retention times correspond to molecular weights of 1.6×10^6 for the phenol-extracted protein and 36×10^3 for the cholate-isolated protein, respectively. Calculations of the average aggregation numbers yield 280 for the phenol-extracted protein and 4 for the cholate-isolated coat protein.

By variation of the concentration of cholate in the eluent and protein solution, the phenol-extracted coat protein elutes predominantly in the void volume (Table I). Therefore, these coat protein polymers are very large (average aggregation number >880) and do not or only slightly dissociate. In the presence of 8 M urea, which is thought to prevent the possible self-aggregation of the coat protein (Hagen et al., 1978; Datema et al., 1988), smaller polymers with an average aggregation number of 280 are obtained. Even in a solution containing 8 M urea and 2% SDS, which allows the formation of coat protein monomers (Simons et al., 1979), dissociation of the polymers is not observed (data not shown). Phenol-extracted coat protein appears to be polymeric and in the c state, under all conditions tested.

In contrast, the cholate-isolated coat protein can be obtained in various oligomeric states, depending on the preparation conditions (Table I). Coat protein oligomers in the range of 2–20 units per aggregate can easily dissociate and associate. The b state of these oligomers is retained under all conditions, also at 10 mM cholate, which is below the critical micelle concentration of cholate (13–15 mM; Helenius & Simons, 1975). However, at these sub-cmc levels, the secondary structure of the coat protein changes to the c state upon a temperature increase to 55 °C during 4 h (Table I). Gel filtration chromatography demonstrates that in this case the coat protein has aggregated strongly. A temperature increase to 55 °C is used to accelerate the solubilization of phenol-extracted coat protein (Hagen et al., 1978). At higher cholate concentration levels (50 mM), the coat protein is not sensitive to a temperature increase, and no conformational and concomitant aggregational change is observed.

Coat Protein Reconstituted into Diacylphospholipid Vesicles. After M13 major coat protein is reconstituted into diacylphospholipids using the cholate dialysis method, the conformational state of the coat protein can be measured in situ, using CD. However, to determine the aggregational state, the coat protein should be extracted from the vesicles. Therefore, a method is developed in which the coat protein is transferred from the lipid environment to cholate micelles and subsequently sized by using gel filtration chromatography. It is expected that the protein will not further aggregate upon the uptake in cholate, and therefore minimum numbers for the aggregation in situ are obtained. Since gel filtration chromatography is performed in 50 mM cholate, the oligomeric aggregated coat protein is observed as tetramers (Figure 3 and Table I). The aggregation number of the polymeric protein is not influenced by different cholate concentrations in the extraction and elution procedure (data not shown).

Reconstitution experiments are performed with phenol-extracted and cholate-isolated coat protein solubilized in the buffer systems mentioned under Materials and Methods. It should be noted that the phenol-extracted coat protein under these conditions is already in the c state and polymeric (average aggregation number about 280) whereas the cholate-isolated coat protein is in the b state and tetrameric (see Table I). In case of phenol-extracted coat protein, reconstituted into DOPC or into DOPC/DOPG (80/20 w/w) mixed bilayers, only c-state CD spectra are found (Table II), similar as in cholate micelles (Table I). Gel filtration chromatography of the protein extracted from the vesicles yields elution patterns similar to those shown in Figure 3 (solid line), illustrating the polymeric nature of the phenol-extracted coat protein. Table II (upper part) shows the aggregational state of the phenol-extracted coat protein in more detail, after reconstitution and successive extraction. Upon an increase of the L/P ratio, the average aggregation numbers are decreased. Coat protein extracted from the DOPC/DOPG (80/20 w/w) bilayers appears to be aggregated to a smaller extent than the protein extracted from the pure DOPC system.

Table II: Conformational State and Aggregation Numbers of Phenol-Extracted and Cholate-Isolated M13 Coat Protein Reconstituted into Pure DOPC and Mixed DOPC/DOPG (80/20 w/w) Lipid Systems at Various L/P Ratios

lipids	isolate	L/P ratio	aggregation no. (av)	conformational state
DOPC	phenol	8	443	c
		35	279	c
		72	176	c
		137	90	c
DOPC/DOPG	phenol	8	176	c
		41	70	c
		62	62	c
		117	53	c
DOPC	cholate	10	328	c
		47	53/4	c/b
		87	4	b
		188	4	b
DOPC/DOPG	cholate	8	111	c
		46	4	b
		82	4	b
		169	4	b

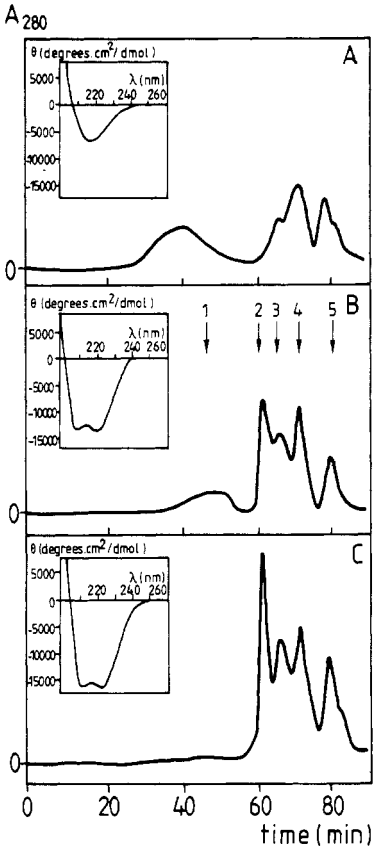

FIGURE 4: Elution patterns and CD spectra (insets) of cholate-isolated M13 coat protein, reconstituted into DOPC, as a function of the L/P ratio. Measurements are performed directly after sample preparation under conditions outlined under Materials and Methods. (A) L/P 10; (B) L/P 47; (C) L/P 87 and 188 (identical). Peak assignments as indicated in (B): peak 1, polymeric coat protein; peak 2, tetrameric coat protein; peak 3, cholate-solubilized DOPC; peak 4, cholate micelles; peak 5, internal volume.

Figure 4 shows the CD spectra of cholate-isolated coat protein, reconstituted into DOPC at several L/P ratios, as well as the elution patterns of the protein extracted from these systems. After reconstitution at L/P 10 (Figure 4A), the coat protein is strongly aggregated into coat protein polymers. These polymers are observed in a very broad peak which demonstrates the large spread in aggregate size. Analysis of the CD spectrum yields the c state. After reconstitution at L/P 47 (Figure 4B), chromatography of the protein extracted from this system results in two peaks, corresponding to coat protein polymers and coat protein tetramers (at 64 min). Analysis of the CD spectrum yields 19% α-helix, 56% β-structure, and 23% random coil, which is interpreted as an intermediate situation in which one part of the protein population is in the b state and the other part is in the c state. Samples with L/P 87 and L/P 188 (Figure 4C) both yield tetrameric and b-state coat protein. It should be noted that all these experiments have been carried out directly after sample preparation. After aging, the elution patterns change toward more aggregated protein and larger aggregates.

Table III: Conformational State and Aggregation Number of Cholate-Isolated M13 Coat Protein in Various Pure Phospholipid Bilayer Systems, as a Function of L/P Ratio

	L/P 66 ± 10		L/P 9 ± 1	
lipid system	conformational state	aggregation no. (av)	conformational state	aggregation no. (av)
DMPC	c	280	c	440
DPPC	c	280	c	395
DOPC	b	4	c	340
egg PC	b	4	c	340
DMPG	b	4	c	39
DPPG	b	4	c	39
DOPG	b	4	c	35
egg PG	b	4	c	39
DMPA	c	28	c	35
DOPA	b	4	c	62
egg PA	b	4	c	44

The conformational state of the cholate-isolated coat protein incorporated into the mixed DOPC/DOPG (80/20 w/w) system is also listed in Table II. As compared to the pure DOPC, the amount of aggregated coat protein and also the aggregation number are reduced.

The conformation and aggregational state of the initially b state and tetrameric cholate-isolated coat protein as a function of the nature of the phospholipid and L/P ratio are given in Table III. After reconstitution at L/P 9, the c state is observed for all phospholipids examined. A c state is also found in systems with L/P 66 for PC and PA with saturated acyl chains (DM and DP). In pure DMPG and DPPG, however, the b-state conformation is maintained. When the coat protein is reconstituted into systems containing unsaturated acyl chains (DO and egg yolk material), the b state of the coat protein is maintained for L/P 66.

In agreement with previous observations, c-state coat protein extracted from the lipid–protein system is found to be polymeric while the extracted b-state coat protein yields tetrameric coat protein (Table III). At L/P 9, the aggregation number in PC is about 400. This is much higher than the aggregational state in PG and PA which give a value of about 40.

Coat protein reconstituted in DMPC (L/P 80) at 4 and at 35 °C, well below and above the gel-to-liquid-crystalline phase transition temperature of DMPC (23.8 °C), appeared to be in the c state and polymeric (data not shown). For this reason, no further attention has been paid to the effect of temperature on the conformational and aggregational properties of the coat protein.

Cross-Linking of the Coat Protein in a Lipid Environment. To elucidate the in situ aggregational state of the protein in the lipid bilayer, cross-linking of the amide groups of the lysine residues is performed (Staros, 1988). In Figure 5, elution patterns are shown of cholate-isolated coat protein extracted from the DMPC/DMPG (80/20 w/w) system (L/P approximately 80), before and after treatment with the cross-linking agent DMA. As can be seen from the elution pattern

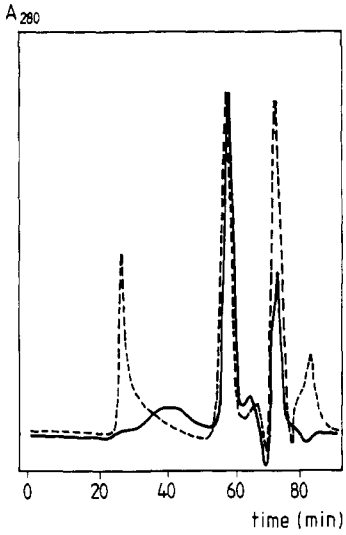

FIGURE 5: Elution patterns of cholate-isolated M13 coat protein incorporated into DMPC/DMPG (80/20 w/w) at L/P 80 before (—) and after (---) treatment with the cross-linking agent DMA. The Superose 6 column was eluted with 2% SDS in 10 mM Tris-HCl/0.2 mM EDTA, pH 8.0, with a flow of 0.25 mL/min.

before treatment, polymeric as well as oligomeric coat protein is observed in this lipid system. Therefore, various aggregational intermediates can be expected. Instead of cholate, SDS is used in the elution buffer, enabling the disruption of tetramers, if present (Knippers & Hoffmann-Berling, 1966; Makino et al., 1975; Bayer & Feigenson, 1985). The amount and the size of the oligomeric protein (aggregation number <20) are hardly changed after cross-linking, but the coat protein polymers (aggregation number ≫20) elute predominantly in the void volume of the column. Identical results are obtained with DMS.

Discussion

The dimeric state as well as the polymeric state of the coat protein of M13 bacteriophage and the closely related fd/f1 bacteriophage (Figure 1) have been described in the literature (Makino et al., 1975; Cavalieri et al., 1976; Williams & Dunker, 1977; Nozaki et al., 1978), although no detailed and combined aggregational and conformational change mechanism has been proposed before. In this work, for the various coat protein conditions, both the conformational states as well as the minimum aggregation numbers have been determined (Tables I–III), enabling a relationship between the aggregation state and the conformation of the coat protein to be established.

Cholate Micelles. In cholate micelles, both the conformation and also the aggregational state of the solubilized coat protein have been determined in situ. From the results stated in Table I, a relation is found between the aggregational state and the conformation of the coat protein. We can distinguish between irreversibly associated coat proteins with a c conformation (β-polymers) and reversibly aggregated coat proteins with a b conformation (α-oligomers). These two states are well illustrated by phenol-extracted coat protein [which is found to be in the c-conformational state (Figure 2) and aggregated into large polymers (Figure 3)] and cholate-isolated coat protein (which is obtained as b-state oligomers with aggregation numbers below 20). β-Polymers are formed irreversibly since all efforts to manipulate the polymers into α-oligomers were unsuccessful. The α-oligomeric coat protein could be reversibly manipulated into different aggregational states depending on storage conditions and cholate concentration and irreversibly into β-polymers upon a temperature

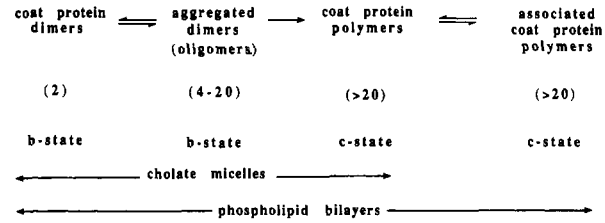

FIGURE 6: Schematic representation of the aggregation and conformational states of the M13 bacteriophage major coat protein, as found after sizing of the aggregates in cholate micelles or after extraction from lipid–protein systems. The approximate aggregation numbers are shown in parentheses.

increase to 55 °C at low cholate concentration (Table I).

The relation between the conformation and the aggregational state of the coat protein and the aggregation mechanism of the coat protein is illustrated in Figure 6. It is assumed that the coat protein dimer is the smallest aggregate (Makino et al., 1975; Knippers & Hoffmann-Berling, 1966; Bayer & Feigenson, 1985). Therefore, coat protein aggregation is thought to be a process in which protein dimers are joined together. The maximum size of the b-conformational oligomer is reached when approximately 20 coat protein units are aggregated, since this is the maximum number obtained for the b-state coat protein in cholate micelles (Table I). Further aggregation of the coat protein is concomitant with a conformational change, resulting in the irreversibly aggregated c-state polymers. Since the coat protein polymers appear on gel filtration chromatography as very broad peaks, indicating a large distribution in aggregate size, the polymerization process is an aspecific, uncontrolled process. Apart from this, the absolute size of the polymers may be overestimated by using gel filtration chromatography, since aggregates with a nonspherical shape have a higher Stokes radius, so that they will elute relatively fast from the column (Siegel & Monty, 1966).

Concerning the protein association, two kinds of interactions between the coat proteins can be distinguished: (1) weak hydrophobic interactions between amino acids residues, which can be disrupted by detergents, are responsible for the reversible aggregation in the oligomeric range; (2) strong hydrogen bonds between the backbones of the coat proteins. These bonds, which cannot be disrupted by detergents, require a close proximity of adjacent polypeptide chains (i.e., an aggregated state of the coat protein) and result in interprotein β-sheets (Birktoft et al., 1972; Richardson et al., 1972; Nozaki et al., 1978).

Model Membranes. The relation between the aggregational state and conformational state of the coat protein reconstituted in lipids is also examined. Instead of the in situ determination of the coat protein conformation, sizing of the aggregates using gel filtration chromatography necessitates an extraction of the coat protein from the lipid–protein systems, thereby possibly changing the actual aggregational state. Incidentally, cross-linking experiments are performed before extraction and successive sizing (Figure 5) to conserve the in situ aggregational state (Staros, 1988).

According to previous results obtained in cholate micelles (Table I) and using extraction and subsequent sizing without cross-linking, it is possible to distinguish between c-state coat protein polymers (aggregation numbers higher than 20) and b-state oligomers (aggregation numbers below 20). Under our experimental conditions, the b-state oligomeric coat proteins are observed as tetramers.

Phenol-extracted coat protein, which is already aggregated in c-state polymers before reconstitution, maintains this state

after successive extraction (Table II). The reduction of the average aggregation numbers with increasing L/P ratios, which is observed as a slight fragmentation of the separate polymers, can be explained by better solubilizing capacities in the case of phospholipids at high L/P ratios, as compared to cholate, but takes place without conformational change.

Cholate-isolated coat protein, which is initially in the form of b-state tetramers in the cholate micelles, maintains this state at high L/P ratios in the DOPC system but shows c-state polymers at L/P 10 (Figure 4, Tables II and III). Presumably, at low L/P ratios, there are too few lipid molecules to shield the separate coat protein oligomers, resulting in an increased aggregated state and conformational change.

The polymerization process yields separate c-state polymers with large differences in size, as reflected by the broad polymer peaks. However, these polymers are able to associate reversibly, as is illustrated in Figure 6. This intravesicle polymer association is normally disrupted after the extraction of the coat protein from the vesicles but is detected after cross-linking (Figure 5). Separate c-state polymers can be cross-linked very effectively and yield huge coat protein superaggregates, indicating that they are arranged in rather close proximity in the plane of the membrane. However, cross-linking does not affect the peak in Figure 5, related to the coat protein oligomers. In addition, the polymerization process is not accelerated by the presence of cross-linking agents. This result can be explained in two ways: (1) The cross-linking agents are reactive, indicating the presence of *isolated* coat protein dimers in the lipid system. These dimers are then cross-linked by the agent, but interdimer cross-linking is not possible. (2) A more reasonable explanation is that the cross-linking agents are not reactive due to the fact that they cannot reach the lysine residues of *oligomeric* protein because of conformational reasons in combination with the sticking of the positively charged lysine residues on the negatively charged membrane surface (Ohkawa & Webster, 1981). However, polymeric protein is accessible for the agents.

Questions arise about the configuration of the β-structured parts of the dimeric coat protein, since these β-structures are only thermodynamically stable when a sufficient number of hydrogen bonds between the protein backbones can be formed. For β-structural parts of the oligomeric coat protein, a U-shape configuration may be proposed, although this U shape disagrees with the assumption that the coat protein is stored as an integral (trans)membrane protein (Smilowitz, 1974; Wickner, 1975; Chang et al., 1979). Also for dimensional reasons a U shape is possible since it is the only way to store a β-structured hydrophobic core of 20 amino acids of the M13 coat protein in a 3-nm hydrocarbon layer of the membrane (Tanford & Reynolds, 1976), without severe distortions of the bilayer structure (Datema et al., 1988).

Effects of Lipid Composition. For both the cholate-isolated and phenol-extracted coat protein, remarkable effects are found with respect to phospholipids with PG headgroups and/or (poly)unsaturated acyl chains. Both the PG headgroup and unsaturated acyl chains stabilize the oligomeric b state of the cholate-isolated coat protein and also show reduced aggregation numbers for the polymeric c-state phenol-extracted coat protein, as compared to systems lacking PG or unsaturated acyl chains (Tables II and III). This is in agreement with aggregation properties of the coat protein deduced from tryptophan quenching measurements on phenol-extracted M13 coat protein reconstituted in mixed DMPC/DMPG systems (Wolfs et al., 1989).

These distinct phospholipid properties are particularly interesting in view of the lipid composition of the *E. coli* cytoplasmic membrane, which is 19% PG, 74% PE, and 3% cardiolipin, with predominantly unsaturated acyl chains (Burnell et al., 1980). As compared to PE and PC, PG has been found to have an increased binding specificity to the coat protein (Datema et al., 1987b). Therefore, it is proposed that the coat protein aggregation is prevented by the binding of PG to the coat protein. In the case of PA, which is found to have an even higher binding specificity for the M13 coat protein (Datema et al., 1987b), the b-state oligomeric coat protein is only maintained with species containing unsaturated acyl chains. However, the aggregation numbers of the c-state polymers observed in PA are comparable to those observed in PG and are only one-tenth of the values found for PC systems. This suggests a similar type of interaction of PG and PA. The b-state-preserving properties of the unsaturated acyl chains may also be explained by a more effective shielding of the coat protein by the phospholipid species involved. This shielding effect is related to the binding specificity of the lipid to the coat protein and might be enhanced with phospholipids with space-occupying unsaturated fatty acyl chains. In this view, separate coat protein units are shielded by the lipids, preventing the self-aggregation of the protein. Also, the reduced aggregation numbers of the c-state phenol-extracted coat protein (Table II) are best explained by an increased binding of the phospholipids to the protein.

Biological Applications. On the basis of our experiments and the presence of PG and unsaturated alkyl chains in the *E. coli* cytoplasmic membrane, it is unlikely that the coat protein in vivo is polymeric. Probably the nonaggregated state of the coat protein is the functional state in vivo and effective in the assembly process. This idea is supported by the monomeric membrane insertion mechanism of the gene 8 protein proposed by Kuhn et al. (1986) and the assembly process proposed by Boehler-Kohler and Rashed (1987), in which the binding of monomeric coat proteins around the DNA is triggered by the gene 5 protein. In addition to this, it should be noted that in vivo the orientation of the coat protein in the cytoplasmic membrane will be parallel (Ohkawa & Webster, 1981), whereas in reconstituted systems the orientation is random (Wickner, 1976; Chamberlain et al., 1978; Bayer & Feigenson, 1985). Therefore, in a random situation, electrostatic attraction may occur between the negatively charged N-terminus and the positively charged C-terminus of the coat protein, resulting in antiparallel dimers. In the parallel, oriented situation in vivo, these interactions will be mainly repulsive, suggesting again a reduction of the aggregation properties of the coat protein in this case.

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