# The in Situ Aggregational and Conformational State of the Major Coat Protein of Bacteriophage M13 in Phospholipid Bilayers Mimicking the Inner Membrane of Host *Escherichia coli*

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ABSTRACT: The major coat protein of bacteriophage M13 has been reconstituted into phospholipids with a composition comparable to that found in the host (*Escherichia coli*) inner membrane. Reconstitution experiments have revealed conditions in which the $\alpha$-oligomeric state is favored over the $\beta$-polymeric state. Discrimination between the two states of the membrane-bound coat protein ($\alpha$-oligomeric and $\beta$-polymeric states) has been achieved using high-performance size-exclusion chromatography and circular dichroism. Interprotein electrostatic interactions, probably induced by head–tail binding, are initiating and facilitating the aggregation-related conformational change process, in which $\alpha$-oligomeric coat protein is converted into $\beta$-polymeric coat protein. A model for this $\beta$-polymerization process of the coat protein is presented. The $\alpha$-helical protein has been studied by the in situ Trp fluorescence quantum yield. This shows that the average distances between coat proteins decrease upon lowering the L/P ratio. In situ cross-linking reactions of the coat protein at high L/P ratios reveal a monomeric state, thus excluding specific aggregation of the coat protein. A monomeric state of detergent-solubilized coat protein is also observed using SDS–PAGE and SDS–HPSEC. On the basis of these results, the smallest in situ aggregational entity of the coat protein is proposed to be a monomer. This finding is discussed in relation to the functional state of the M13 coat protein in the membrane-bound assembly and disassembly processes during infection.

M13 bacteriophage and closely related phages f1 and fd are *Escherichia coli* specific filamentous phages belonging to the genus *Inovirus*. The virion consists of a circular single-stranded DNA molecule of about 6408 nucleotides encapsulated in a long cylindrical protein coat. The protein coat is composed of a few copies of some minor coat proteins and about 2700–3000 copies of the major coat protein, the product of gene VIII [for reviews, see Denhardt (1975), Ray (1977), Webster and Lopez (1985), Rasched and Oberer (1986), and Model and Russel (1988)].

During infection, the major coat protein is involved in four processes located at the *E. coli* inner or cytoplasmic membrane: (1) the parental coat proteins are inserted in the membrane while the viral DNA is released in the cytoplasm (Pratt et al., 1969); (2) newly synthesized coat proteins are inserted into the membrane and processed by a leader peptidase (Chang et al., 1978; Mandel & Wickner, 1979); (3) parental as well as newly synthesized coat proteins are stored oriented and transmembrane at high local levels (Smilowitz et al., 1972; Wickner, 1975; Ohkawa & Webster, 1981); and (4) parental as well as newly synthesized coat proteins are assembled around the viral DNA during the combined assembly–extrusion process at membrane-bound assembly sites (Trenker et al., 1967; Smilowitz, 1974; Armstrong et al., 1983; Bayer & Bayer, 1986).

The phospholipid composition of the inner membrane of *E. coli*, which is about 70% phosphatidylethanolamine (PE),[1] 25% PG, and 5% CL (Woolford et al., 1974; Burnell et al., 1980), is suitable to accommodate the major coat protein of the bacteriophage as well as many other bacterial proteins in a proper way. During the normal development of the infection process in the case of a wild-type bacteriophage, the phospholipid composition of the cell envelope is little affected in favor of the charged phospholipids PG and especially CL (Ohnishi, 1971; Chamberlain & Webster, 1976; Pluschke et al., 1978). However, as has been shown for abortively infected cells employing various amber mutants of the bacteriophage, accumulation of high amounts of the major coat protein in the inner membrane resulted in significantly increased levels of CL and PG and a compensating decline in PE (Ohnishi, 1971; Woolford et al., 1974; Pluschke et al., 1978). This strongly suggests a role of CL and PG in conserving the functional state of the membrane-bound coat protein.

The major coat protein (MW 5240) is composed of three specific domains (Figure 1): a 19 amino acid long hydrophobic core is flanked by an acidic N-terminal part (residues 1–20) and a basic C-terminal part (residues 40–50). When associated in vitro with lipid bilayers or detergents, the coat protein can adopt two states: the $\alpha$-oligomeric and $\beta$-polymeric states (see Figure 2). This has been revealed by studying the protein conformation (Nozaki et al., 1976, 1978; Williams & Dunker, 1977; Chamberlain et al., 1978; Fodor et al., 1981; Spruijt et al., 1989) and its minimal, dimeric aggregation state (Knippers & Hoffmann-Berling, 1966; Makino et al., 1975; Cavalieri et al., 1976; Nozaki et al., 1978; Bayer & Feigenson, 1985; Henry & Sykes, 1990). The $\alpha$-oligomeric state of the coat protein is characterized by a debatable but substantial amount of $\alpha$-helix (50–90%) (Nozaki et al., 1976; Fodor et al., 1981; Sanders et al., unpublished results; Shon et al., 1991; Nambudripad et al., 1991) and the ability to undergo a reversible protein aggregation (Spruijt et al., 1989), while the $\beta$-poly-

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[1] Abbreviations: MW, molecular weight; *E. coli*, *Escherichia coli*; Trp, tryptophan; PC, phosphatidylcholine; PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; SDS, sodium dodecyl sulfate; HPSEC, high-performance size-exclusion chromatography; PAGE, polyacrylamide gel electrophoresis; L/P, lipid to protein molar ratio; CD, circular dichroism; DMA, dimethyl adipimidate; Tris-HCl, tris(hydroxymethyl)aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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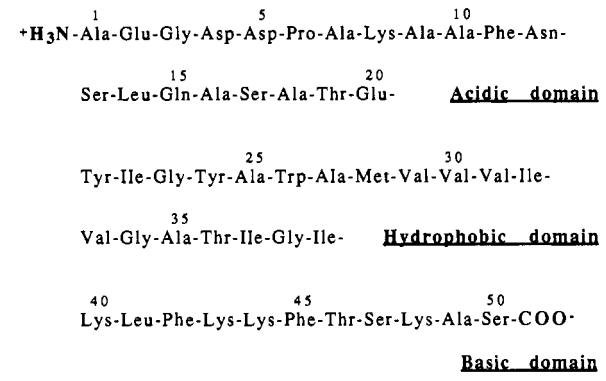


FIGURE 1: Primary structure of the M13 coat protein. The amino acid sequence of M13 coat protein differs from that of fd and f1 coat proteins at position 12 where fd and f1 coat proteins contain aspartic acid instead of an asparagine (Van Wezenbeek et al., 1980). Note that the presence of amine groups on both hydrophilic termini of the coat protein is essential for performing cross-linking assays.

meric state is strongly aggregated in an irreversible way, and the corresponding conformation is characterized by a high amount of $\beta$-sheet (>70%) and a lack of $\alpha$-helix.

During the last decade, the major coat proteins of Ff coliphages have been used extensively as model system for studying lipid–protein interactions using predominantly biophysical methods. Less attention has been paid to the role of protein aggregation and protein conformation and the regulatory role of phospholipids in providing suitable storage conditions for the coat protein in the model membranes. Therefore, many studies described in the literature are incomplete, incomparable, or even contradictory due to different protein isolation procedures, reconstitution methods, amphiphiles employed, and conditions applied (Wickner, 1976; Williams & Dunker, 1977; Chamberlain et al., 1978; Hagen et al., 1978; Kimelman et al., 1979; Smith et al., 1980; Bayer & Feigenson, 1985; Wilson & Dahlquist, 1985; Datema et al., 1987; Leo et al., 1987; Spruijt et al., 1989; Johnson & Hudson, 1989; Sanders et al., 1991b).

In this work, the conditions to store the coat protein in artificial phospholipid bilayers, mimicking the lipid composition of the *E. coli* membrane inner membrane, are examined to understand the lipid–protein interactions during infection. The aggregational and in situ conformational state of the coat protein in PE/PG/CL-containing bilayers is therefore determined as a function of acyl chains (length, saturated or unsaturated), presence and amounts of the charged phospholipids PG and CL, L/P ratio, and ionic strength of the buffer. In addition, the state of the coat protein solubilized in detergents has been investigated.

## Materials and Methods

*Chemicals. E. coli* K37 and M13 bacteriophage (wild type) were a gift of Dr. Harmsen, University of Nijmegen. Phospholipids were obtained from Avanti Polar Lipids Inc., Alabaster, AL, and were used without further purification. Sodium cholate was purchased from Sigma Chemical Co., St. Louis, MO. Materials used for chromatography were obtained from Pharmacia–LKB Biotechnology, Uppsala, Sweden. DMA and the BCA assay were purchased from Pierce Chemical Co., Rockford, IL. Octadecyl Rhodamine B was purchased from Molecular Probes Inc., Eugene, OR. Sodium dodecyl sulfate and all other chemicals of proanalytical grade were obtained from Merck, Darmstadt, F.R. Germany.

*Preparation of M13 Coat Protein.* Bacteriophage growth and purification were as described earlier (Spruijt et al., 1989). The major coat protein was isolated from the bacteriophage by a modified cholate extraction procedure (Makino et al., 1975) as described (Spruijt et al., 1989). The employment of saline conditions during the coat protein preparation procedure resulted in an enhanced chromatographic resolution. The cholate concentration of the stock solution of the coat protein was raised to 50 mM prior to storage at 4 °C. Under this condition, the coat protein was found to be stable for weeks with respect to its aggregational and conformational state.

*Reconstitution into Micelles.* In the case of cholate, the concentration of the cholate was adjusted to the desired level by either dialysis or mixing procedures. In the case of SDS, the cholate from the coat protein stock solution was exchanged for SDS. Unless stated otherwise, the buffer was composed of 10 mM Tris-HCl and 0.2 mM EDTA, pH 8.0, and, if mentioned, the ionic strength was provided by 150 mM NaCl.

*Reconstitution into Phospholipid Bilayers.* Phospholipids were mixed in chloroform and dried under a flow of nitrogen gas and subsequently in vacuum to remove all traces of chloroform. The phospholipids were solubilized in 50 mM cholate in buffer [10 mM Tris-HCl and 0.2 mM EDTA (and if desired 150 mM NaCl), pH 8.0] by brief and mild sonication. After addition of coat protein stock solution, the mixtures of phospholipids and coat protein were dialyzed at room temperature against at least 100 times excess of buffer during 48 h with buffer changes every 12 h. Aliquots were taken after sample preparation to determine the protein content (Smith et al., 1985) and phospholipid content (Bartlett, 1959) to give the L/P ratios of the samples. The homogeneity in L/P ratio was checked by sucrose density gradient centrifugation as described earlier (Spruijt et al., 1989) after the samples were doped with a small amount of octadecyl Rhodamine B to enhance the visualization of the lipid–protein complexes.

*Discrimination between the $\alpha$-Oligomeric and $\beta$-Polymeric States of the Coat Protein after Reconstitution in Phospholipids.* After extraction of the coat protein from the phospholipid bilayer systems, the molecular weight of the resulting solubilized coat protein was determined using HPSEC on an LKB Ultrochrom GTi Bioseparation system equipped with a Superose 6 column (HR 10/30). For details, see Spruijt et al. (1989). To provide an optimal solubilization of the phospholipids involved, we used 25 mM SDS, 10 mM Tris-HCl, 0.2 mM EDTA, and 150 mM NaCl, pH 8.0, as elution buffer. Samples were preincubated in the same buffer except that the SDS concentration was 50 mM. Under these conditions, it was found by CD measurements that the conformational state of the coat protein did not change upon extraction and was conserved for a long time. Other conditions and the calibration procedure were as described earlier (Spruijt et al., 1989) or stated in the figure legends.

*Circular Dichroism Measurements.* CD experiments were performed in situ on the coat protein reconstituted into phospholipid vesicles, as described earlier (Spruijt et al., 1989).

*Fluorescence Measurements.* The fluorescence properties of the single tryptophan residue of the major coat protein, reconstituted either into phospholipids or into detergents, were recorded in situ on a Perkin Elmer LS-5 luminescence spectrophotometer. Excitation was at 295 nm (to avoid emission due to the tyrosines), and emission scans were recorded from 310 to 360 nm. Excitation and emission slits were set at 5 nm. Measurements were performed in a quartz tube with a internal diameter of 1.5 mm placed in a temperature-controlled cuvette housing (20 °C). The overall concentration of coat protein was about 0.1 mg/mL; the optical density of the samples at the excitation wavelength never exceeded 0.1. The fluorescent

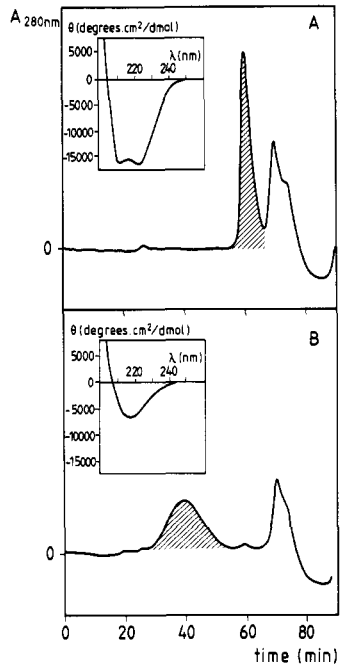


FIGURE 2: Typical HPSEC elution patterns and corresponding CD spectra (insets) of the two almost pure states of the membrane-bound form of M13 coat protein: the α-oligomeric state (A) and the β-polymeric state (B). The α-oligomeric coat protein is characterized by a high content of α-helix and can aggregate reversibly. The β-polymeric coat protein contains about 70% β-sheet and is strongly and irreversibly aggregated. The protein peaks are indicated by the hatched areas.

quantum yield was corrected for the exact coat protein content, calculated to a fix scale of 1.0, and corrected for phospholipid or detergent background contributions.

*Cross-Linking of Coat Protein.* Samples, prepared in the absence of Tris-HCl, were incubated in the presence of DMA (final concentration 100 mM) at pH 8.5 at room temperature. The incubation time was kept as short as possible. The cross-linking reaction was stopped by adding concentrated Tris-HCl buffer up to 0.2 M. Samples were subsequently mixed with an equal volume of 100 mM SDS, 10 mM Tris-HCl, 0.2 mM EDTA, and 150 mM NaCl (pH 8.0) to conserve the state of the coat protein prior to analysis.

## Results and Discussion

*Reconstitution Experiments.* The aggregational and conformational state of the coat protein of bacteriophage M13 reconstituted into phospholipid bilayers is determined using both HPSEC and CD, as described previously (Spruijt et al., 1989). Figure 2 shows typical HPSEC elution patterns and CD spectra of both states of the coat protein. The α-oligomeric coat protein (Figure 2A) is eluting from the column after 59 min, and the corresponding CD spectrum indicates the presence of α-helix conformation. The coat protein in this conformation easily aggregates and dissociates upon changes in its amphiphilic environment or changes in protein concentration. Figure 2B shows the elution pattern of the β-polymeric coat protein. The large spread in the sizes of the aggregates is reflected by a very broad peak between 35 and 55 min. The CD spectrum of the irreversible aggregated protein polymers indicates the presence of predominant β-structure. Thus, the performance of both HPSEC and CD techniques enables us to discriminate between the α-oligomeric and the β-polymeric state of the membrane-bound coat protein.

Prior to the reconstitution procedure, the coat protein is solubilized in sodium cholate. In this amphiphilic intermediate, the state of the protein is found to be completely α-oligomeric. A possible change of the aggregation-related conformational state of the protein observed after reconstitution, i.e., the formation of β-polymers, can thus be assigned to a changed amphiphilic environment of the coat protein.

Table I: Amounts of α-Oligomeric Coat Protein, As Determined from HPSEC Elution Patterns, Left after Reconstitution into Various PE/PC (3/1 mol/mol) and PE/PG (3/1 mol/mol) Systems at Low Ionic Strength

| lipid system | % α-oligomeric protein (±5%) L/P 10 | L/P 100 |
|---|---|---|
| DLPE/DLPC | 20 | 15 |
| DMPE/DMPC | 10 | 10 |
| DPPE/DPPC | 20 | 10 |
| POPE/POPC | 10 | 80 |
| *coli* PE/egg PC | 10 | 70 |
| DOPE/DOPC | 10 | 90 |
| DMPE/DMPG | 45 | 10 |
| DPPE/DPPG | 60 | 35 |
| DSPE/DSPG | 75 | 50 |
| POPE/POPG | 35 | 90 |
| *coli* PE/egg PG | 10 | 90 |
| DOPE/DOPG | 20 | 100 |

Due to the difficulty in handling pure PE, the major phospholipid component of *E. coli* membranes, concerning solubilization, hydration, and formation of nonbilayer structures (Cullis & De Kruijff, 1978; Van Duijn et al., 1986), we have employed PE in combination with the bilayer-stabilizing phospholipids PC and PG. As an additional advantage, it is possible to vary the net membrane charge in these mixed systems.

The upper part of Table I shows the amounts of α-oligomeric coat protein left after reconstitution into various net neutrally charged PE/PC phospholipid bilayer systems as a function of L/P ratio and length and degree of unsaturation of the acyl chains involved. The α-oligomeric state of the coat protein is maintained only in lipid systems containing at least one unsaturated acyl chain and then only at high L/P ratios. The data shown are calculated from HPSEC patterns. There is no relation found between the maintenance of the α-oligomeric state and the length of the acyl chains, which could possibly affect the hydrophobic matching of the coat protein in the hydrophobic interior of the bilayer (Lee, 1987). Control CD measurements performed to determine the in situ conformation of the coat protein in the various phospholipid systems indicate an amount of α-helix directly related to the amount of oligomeric coat protein. These results are in agreement with the results obtained by Fodor et al. (1981) and in our laboratory (Spruijt et al., 1989). The amounts of α-oligomeric coat protein generally decrease upon aging, due to a continual and irreversible change into β-polymers. Depending on the conditions, the rate constant of this, first-order, polymerization process ranges between approximately $10^{-4}$ and $10^{-8}$ $s^{-1}$. These numbers imply complete polymerization after approximately 2 h to 1.4 years, respectively. However, by employing suitable conditions, we can denote the α-oligomeric state of the coat protein as stable.

Analogous results are obtained after reconstitution of the coat protein into the various net negatively charged PE/PG phospholipid bilayer systems (Table I, lower part). As compared to the PE/PC system, we observe increased amounts of α-oligomeric coat protein after reconstitution into PE/PG, both at low and at high L/P ratios and also in systems with saturated and/or unsaturated acyl chains (in the gel phase and in the liquid-crystalline phase, respectively).

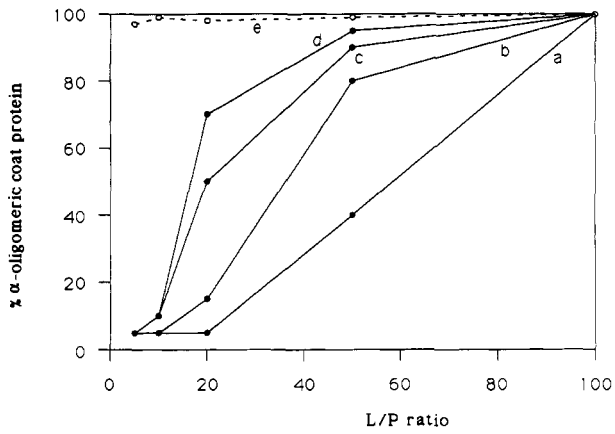


FIGURE 3: Amounts of α-oligomeric coat protein left after reconstitution into *E. coli* PE/egg yolk PG/*E. coli* CL (varied compositions with respect to the amount of charged phospholipids PG and CL) as a function of the L/P ratios in the absence of NaCl (solid lines) or in the presence of 150 mM NaCl (dashed line). The percentages of α-oligomeric coat protein are calculated from HPSEC elution patterns and are accurate within 5% variation. (a) PE/PG (75/25 mol %); (b) PE/PG/CL (65/25/10 mol %); (c) PE/PG/CL (55/25/20 mol %); (d) PE/PG/CL (45/25/30 mol %) and PE/PG/CL (45/45/10 mol %), identical results; (e) average of percentages of α-oligomeric coat protein left after reconstitution in the same phospholipid systems as a–d, but prepared in the presence of 150 mM NaCl.

The effect of (negatively) charged phospholipids on the conservation of the α-oligomeric state of the coat protein is investigated in more detail by reconstituting the coat protein into PE/PG phospholipid bilayer systems with varying amounts of the negatively charged phospholipids cardiolipin or PG. Figure 3 shows the amounts of α-oligomeric coat protein as a function of the L/P ratio of the various PE/PG/CL lipid systems. Apart from an increased amount of conserved α-oligomeric coat protein at increasing L/P ratios, an additional α-helix-conserving effect is observed when the molar content of charged phospholipids is raised. The increased level of especially CL in the stressed cytoplasmic membrane, as a result of a blocked phage assembly process (Ohnishi, 1971; Woolford et al., 1974), can thus very well be a mechanism of the *E. coli* cell to store huge amounts of the coat protein without severe distortions of membrane functioning.

The preservation of the α-oligomeric state of the coat protein is enhanced by an increased ionic strength of the buffer. Reconstitution experiments performed in buffer systems containing 150 mM NaCl, surprisingly, yield a complete maintenance of the α-oligomeric state (Figure 3). For all systems shown in Table I, but in the presence of 150 mM NaCl, an almost complete (95–100%) α-oligomeric state conserving effect is observed, independent of the composition of the phospholipid bilayer and the protein content (data not shown). Similar effects are obtained when the ionic strength is provided by KCl or inorganic phosphate (data not shown). Even in the absence of amphiphiles, the α-oligomeric state is observed; i.e., the coat protein appears to be strongly aggregated as determined by HPSEC, as observed by an increased turbidity and in agreement with previous observations (Cavalieri et al., 1976; Rasched et al., 1980), but these aggregates easily dissociate in the presence of detergents, and the conformation of the coat protein is not changed.

The molecular mechanism of the β-polymerization process is still not well understood. The β-sheet conformation of proteins, as formed by strong hydrogen bonds between adjacent peptide backbone chains, is only stabilized by a sufficient number of peptide chains involved (Birktoft & Blow, 1972; Tanford & Reynolds, 1976; Nozaki et al., 1978). In the case of small (50 amino acids) coat proteins, this condition is only provided by an aggregated state of the proteins (Nozaki et al., 1978; Spruijt et al., 1989). The aggregational state of the α-helical coat protein is affected by the protein concentration (i.e., the L/P ratio). This is illustrated by a decreased amount of β-polymers observed after reconstitution at high L/P ratios [Table I; Figure 3; (Spruijt et al., 1989)] and a decreased amount of steady-state fluorescence quantum yield at lower L/P ratios (see next section).

However, apart from the required aggregation state of the coat protein prior to a possible conformational change, more factors affect the β-polymerization process. As can be concluded from the results observed in the presence of the net negatively charged phospholipids PG and CL, or under saline conditions (Table I), electrostatic interactions play a crucial role in the initiation of the β-polymerization process. Interprotein electrostatic interactions provided by head–tail binding (see Figure 1) occur due to randomly oriented but transmembrane coat proteins. The close proximity of oppositely charged protein residues can facilitate the β-polymerization process, i.e., by lowering the energy barrier of the process (Sanders et al., 1991a), which is in agreement with a heat-induced increase of the process rate (Spruijt et al., 1989), and by allowing direct backbone–backbone interactions, which result in the formation of interprotein hydrogen bonds. Starting with the hydrophilic protein termini, the entire proteins can cooperatively form interprotein hydrogen bonds and change their conformations from α-helix into β-sheet according to a zip-fastener model. As a consequence, electrostatically initiated β-polymerization implies an antiparallel β-sheet arrangement (Sanders et al., 1991a). Shielding of the charged residues by charged phospholipids or at high ionic strength inhibits the aggregation and thus the conformational change from the α-helix to the β-sheet of the coat protein.

Apart from stabilizing the α-oligomeric state of the coat protein, we have observed additional advantages of the presence of salt in relation to the sample preparation procedure. This finds expression in an increased solubilization of the phospholipids, especially PE, used prior to the cholate dialysis step and in the increased homogeneity in L/P ratio on a sucrose gradient. The presence of salt influences the headgroup properties of PE by preventing the formation of a compact lattice via electrostatic interactions and hydrogen bonds between the phosphate oxygens and ammonium nitrogens, and avoiding, together with the bilayer-stabilizing co-phospholipids PC or PG, the formation of hexagonal $H_{II}$ structures (Nir et al., 1983; Sanders et al., 1991b). Moreover, the saline conditions employed reflect the ionic strength of the in vivo situation, where high levels of potassium and sodium (up to 200 mM) are characteristic for the interior of energized *E. coli* cells and their growing medium (Ugurbil et al., 1979).

In conclusion, from these reconstitution experiments, suitable conditions can be defined, in which the α-helical state of the coat protein is stabilized and, thus, the formation of the β-polymerized state of the coat protein is inhibited. The properties of the α-helix-containing coat protein are further discussed in the following sections.

*Fluorescence Measurements on the α-Helix-Containing Coat Protein.* Using HPSEC, it is not possible to determine the in situ aggregational state of the coat proteins, when these coat proteins are embedded in phospholipid bilayers of vesicles. Size-exclusion chromatography employed in the absence of detergents just offers information about the size of the entire

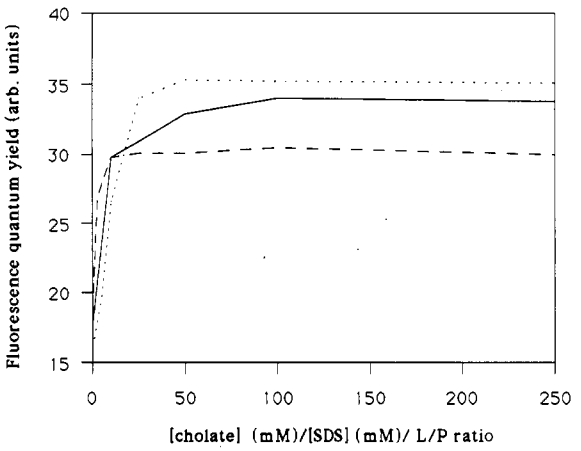


FIGURE 4: Fluorescence quantum yield (arbitrary units) measurements on the $\alpha$-helical M13 coat protein reconstituted into cholate (solid line), SDS (dashed line), and into phospholipid bilayers (dotted line) as a function of the amphiphile content. The phospholipids are composed of *E. coli* PE/egg yolk PG (75–25 mol %) or *E. coli* PE/egg yolk PG/*E. coli* CL (65/25/10 mol %). Samples are prepared and measured in the presence of 150 mM NaCl to avoid the formation of $\beta$-polymers.

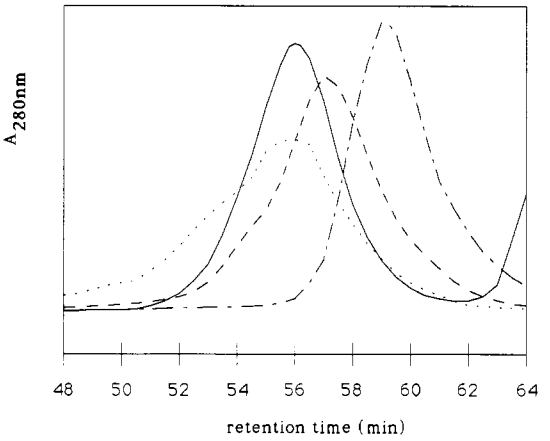


FIGURE 5: SDS–HPSEC elution patterns of $\alpha$-helical M13 coat protein extracted from various described phospholipid bilayer systems after reaction with the cross-linking agent DMA: *E. coli* PE/egg yolk PG/*E. coli* CL (65/25/10 mol %), L/P 100 (solid line); egg yolk PC/egg yolk PG/*E. coli* CL (65/25/10 mol %), L/P 100 (dashed line); *E. coli* PE/egg yolk PG/*E. coli* CL (65/25/10 mol %), L/P 10 (dotted line). The elution pattern of the untreated coat protein is indicated by (–·–). For an example of a completely shown and comparable elution pattern of the untreated coat protein, see Figure 2A. Samples are prepared and measured under saline conditions to avoid the formation of $\beta$-polymers. Calibration of the Superose 6 column has been carried out as described under Materials and Methods.

lipid–protein complex, i.e., the size of the vesicle (Tanford & Reynolds, 1976). On the other hand, the presence of detergents in the elution buffer results in disrupted lipid–protein complexes and extracted coat protein, thereby probably changing the original aggregation state of the coat protein (Spruijt et al., 1989).

We have used the presence of the single, fluorescent tryptophan residue at position 26 of the coat protein as well as the known aggregation states of the coat protein in micelles to obtain an indication of the in situ aggregation state of M13 coat protein in phospholipid bilayers. In all cases, the emission spectra show identical characteristics concerning the line shape and position of emission maximum. The wavelength of the emission maximum has been found to be approximately 330 nm, indicating that the tryptophan residue is buried in a hydrophobic environment (Burstein et al., 1973; Lakowicz, 1983), independent of the L/P ratio and lipid type.

By using cholate, we are able to perform both steady-state fluorescence measurements and also HPSEC determinations within one system. Figure 4 (solid line) shows the steady-state fluorescence quantum yield measurements performed for the coat protein reconstituted into various concentrations of cholate. The quantum yield observed is low at low concentrations of cholate, increases at higher concentrations, and reaches the maximum level above 100 mM cholate. The aggregation numbers of the coat protein, as determined by HPSEC at the same concentrations of cholate, are found to be mainly 8, 4, and 2 at 10, 50, and 100 mM cholate, respectively. In the absence of cholate, the coat protein is in a strongly aggregated state. Considering the gradual, continual increase of the quantum yield observed in cholate, which is concomitant with a gradual decrease in numbers of coat protein per micelle, these measurements do not distinguish between cholate micelles containing less than four coat protein monomers, but give information about the average distances between the tryptophan residues (Lakowicz, 1983).

Quenching results obtained for the coat protein reconstituted into varying concentrations of SDS are also shown in Figure 4 (dashed line). Although the shape of this curve is similar, the maximum level is lower. Since HPSEC studies reveal a monomeric state of the coat protein at high SDS/protein ratios (see next section), this difference in fluorescence should be explained by differences in the amphiphilic surrounding. This implies that the maximum levels obtained in various amphiphiles cannot directly be translated into the in situ aggregation states of the coat protein.

The fluorescence quantum yield of the detergent-solubilized coat protein of bacteriophage M13 can be related to its aggregational state, in agreement with previously obtained results (Talbot et al., 1979; Wolfs et al., 1989; Killian et al., 1990). At low concentrations of detergents, the coat proteins are forced together, allowing interprotein tryptophan–tryptophan interactions, such as energy transfer between the indole rings within an aggregate as demonstrated for aromatic dimers (Visser et al., 1983). As a consequence, self-quenching of the tryptophan fluorescence occurs, which is monitored as a lower quantum yield.

The steady-state fluorescence quantum yield measurements performed for the coat protein reconstituted into the phospholipid bilayer systems *E. coli* PE/egg yolk PG (75/25 mole fractions) and *E. coli* PE/egg yolk PG/*E. coli* CL (65/25/10 mole fractions) as a function of the L/P ratio are shown in Figure 4 (dotted line). In both bilayer systems, the change of fluorescence quantum yield at higher L/P ratios is analogous as observed in detergents. This again suggests strongly decreased interprotein distances at low L/P ratios. Since the increase of the Trp fluorescence quantum yield at higher L/P ratios can be explained by larger distances between the coat proteins, it can be concluded that the average inter-coat protein distance exceeds the maximum quenching working range above L/P 25. In addition, the fact that the fluorescence quantum yield above L/P 25 does not further increase upon a 10-fold dilution of the coat protein is indicative for coat protein which is homogeneously distributed in the membrane.

*Cross-Linking Experiments.* To elucidate the in situ aggregational state of the $\alpha$-helix containing membrane-bound coat protein within the oligomeric range, cross-linking of the amine groups of the lysine residues or the N-termini (see Figure 1) has been performed (Staros, 1988). Using this approach, the in situ aggregational state of the coat protein in the phospholipid bilayer is fixed almost instantaneously by adding an overdose of a cross-linking agent, thereby resulting

in a "snapshot" of the aggregational state. Figure 5 shows partial SDS–HPSEC elution patterns of coat protein, extracted from *E. coli* PE/egg yolk PG/*E. coli* CL bilayers at L/P ratios of approximately 100, before and after treatment with the homobifunctional cross-linking agent DMA. Calculation of the MW of the untreated coat protein with retention times of about 59 min reveals a MW of about 5600. This MW is very close to the MW of coat protein monomers (5240), as calculated from the nucleotide sequence (Van Wezenbeek et al., 1980). However, analysis of the coat protein extracted from the lipid complexes after application of cross-linking assays results in decreased retention times of about 56 min, corresponding to a MW of about 9200 and indicating an increased MW of the coat proteins. In addition, the MW's as estimated from SDS–PAGE patterns, obtained under similar nonchaotropic conditions, are found to increase from approximately 4000 to 6400 upon treatment with DMA (data not shown). Thus, HPSEC and SDS–PAGE both clearly indicate the predominant existence of the monomeric state of the untreated coat protein in SDS detergent, and an increase in MW of about 3000 for the coat protein treated with DMA.

Questions arise about the origin of this increase in MW. Three factors can be responsible for the increased MW: (1) a coat protein monomer carries one-side-bound DMA; (2) a coat protein monomer carries cross-linked PE molecules (since PE also contains a reactive quaternary ammonium group); (3) two coat protein monomers are cross-linked (dimer) and carry cross-linked DMA and/or PE.

To distinguish between these possibilities, we have determined the MW of the protein complex after applying cross-linking reactions in case of coat protein reconstituted into phospholipid bilayers (L/P 100), in which the PE fraction has been replaced by PC molecules, that do not have reactive ammonium groups (Figure 5, dashed line). The coat protein extracted from the PC-containing system eluted as a double peak with retention times of about 54/55 and 57 min, corresponding to MW's of about 14000 and 7700, respectively. These peaks are assigned to (cross-linked) dimeric and monomeric coat protein carrying only one-side-bound DMA. The amount of dimeric coat protein has been estimated to be approximately 10% and is due to the time course necessary to carry out the "snapshot-assay" (which is about 5 min).

Determination of the phospholipid content of the isolated coat protein fraction reveals the presence of about 4 mol of phosphorus per mole of protein extracted from the PE-containing system (L/P 100) and less than 1 mol for the coat protein extracted from the PC-containing system. Calculation of the MW of such a protein complex yields about 8800, which corresponds well to the experimentally obtained value of 9200.

In addition, we have performed the cross-linking assay in a protein/lipid system at low L/P ratio (*E. coli* PE/egg yolk PG/*E. coli* CL, L/P 10). Under this condition, the probability to observe coat protein aggregates is strongly enhanced, and the probability for the coat protein to react with PE is reduced. HPSEC analysis of the reaction products (Figure 5, dotted line) results in the observation of (monomeric) coat protein–PE complexes (retention time about 56 min) plus the observation of a small amount (about 10%) of cross-linked, dimeric coat proteins (retention time about 54/55 min, MW about 14000) and even some trimeric and tetrameric coat protein (48–52 min).

The increase of the MW by treatment with DMA is predominantly based on cross-linked phospholipids instead of cross-linked coat protein. The observations, by this assay, of the "induced aggregation state" at low L/P ratios (the coat proteins are forced together) clearly shows that the cross-linking assay has been carried out well and is in agreement with the results described in previous sections. In conclusion, interprotein cross-linking at high L/P ratios has hardly taken place. The possibility to cross-link phospholipids surrounding the coat protein excludes the presence of specific coat protein aggregates or artificial head–tail dimers, which could arise from the probably random, transmembrane orientation as a result of our reconstitution method. This suggests a monomeric state of the coat protein under the conditions employed.

*In Situ Aggregational Entity of the Major Coat Protein and Its Biological Implication.* By employing suitable conditions, several techniques described in this work indicate the presence of monomeric $\alpha$-helix-containing coat protein in SDS micelles, but also in phospholipid bilayer systems. A monomeric state of the coat protein in the absence of chaotropic agents has not been reported before and is in contrast with the reported dimeric state (Knippers & Hoffmann-Berling, 1966; Makino et al., 1975; Cavalieri et al., 1976; Nozaki et al., 1978; Bayer & Feigenson, 1985). Apparently, our observation of coat protein monomers is due to the conditions used, which favor the monomeric state of the coat protein (i.e., low protein concentration with respect to the amphiphilic content and the presence of salt), and the improvement of the analytical methods. Recent biophysical studies support our finding of a monomer being the smallest entity in a membranous environment (Henry & Sykes, 1990; Sanders et al., unpublished results).

The $\alpha$-helix-containing coat protein is suggested to be the membrane-bound form, which is functional in in vivo situations. This statement is based on an $\alpha$-helical state conserving effect by natural occurring phospholipids and saline conditions, and the recently described protein properties, allowing reversible aggregation of the $\alpha$-helical coat protein (Spruijt et al., 1989). In addition, an $\alpha$-helical membrane-spanning protein portion is also found for other cytoplasmic membrane-spanning proteins (Warren, 1981; Unwin & Henderson, 1984; Lee, 1987; Schiksnis et al., 1987; Shon et al., 1991). On the other hand, the $\beta$-sheet-containing polymeric coat protein has to be considered as an artificial and denatured form of the coat protein.

To realize storage of the coat protein at high levels, i.e., in vitro in artificial bilayer systems at low L/P ratios and in vivo around the assembly site (prior and during phage assembly), a dynamic protein–lipid network is proposed which can accommodate high amounts of the $\alpha$-helical coat protein in a proper way, i.e., in the absence of formation of the $\beta$-polymeric form of the coat protein. The dynamic coat protein–lipid network is characterized by the absence of a preferential association between coat proteins and/or lipids. The network is based (on average scale) on coat protein monomers surrounded by five to six lipids in each leaflet of the lipid bilayer (Wolfs et al., 1989), and lipids can be shared by more proteins. The reversible contacts between $\alpha$-helical coat proteins are especially characterized by short contact times via their (hydrophobic or hydrophilic) amino acid residues, and less by their average backbone–backbone distances that are dependent on the L/P ratio. In fact, the coat protein molecules fit and behave as their surrounding lipids in the membranous environment, in agreement with results described (Smith et al., 1980; Sanders et al., 1991b; Sanders et al., unpublished results).

In conclusion, this investigation has revealed that the smallest aggregational entity of the $\alpha$-helical membrane-bound major coat protein is a monomer. This finding excludes ar-

tificial head–tail dimers due to the probable randomly oriented transmembrane state, and emphasizes the reversible nature of this "aggregational" state. Therefore, the $\alpha$-helical state is thought to be effective in the assembly process. This is in agreement with the previously presented idea of monomeric coat protein insertion and processing of newly synthesized coat protein (Kuhn et al., 1986), the exchange interaction between gene V proteins and monomeric coat protein on the viral DNA (Boehler-Kohler & Rasched, 1987), and the commonly accepted idea that parental coat protein can be re-used in the assembly process (Armstrong et al., 1983), indicating reversible uncoating and encapsulating processes.

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# Membrane Fluidity and Lipid Hapten Structure of Liposomes Affect Calcium Signals in Antigen-Specific B Cells

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ABSTRACT: Antigen-specific B-cell clones directed against a 2,4,6-trinitrophenyl (TNP) hapten have been established [Hamano et al. (1990) *J. Immunol. 144*, 811–815]. We measured here the cytosolic free calcium ion concentration ($[Ca^{2+}]_i$) in these B-cell clones after antigen stimulation. Trinitrophenylated liposomes with different length spacers between TNP and phosphatidylethanolamine (TNP-$C_n$-PE) increased cytosolic free calcium concentration in TNP-specific B cells (clone TP67.21). The magnitude of calcium signals depended on the length of the spacer. TNP-$C_6$-PE in dipalmitoylphosphatidylcholine (DPPC) liposomes triggered larger calcium signals in B cells than TNP-$C_n$-PE with $n$ = 0, 4, 8, or 12. The magnitude of the calcium signals was strongly dependent on the fluidity of the liposome membranes. TNP-$C_6$-PE in the solid DPPC liposomes triggered the calcium signals in B cells 50–100 times as efficiently as TNP-$C_6$-PE in the fluid dimyristoylphosphatidylcholine liposomes. The difference between the solid liposomes and the fluid liposomes was more pronounced in triggering calcium signals in B cells than in antibody binding to these liposomes.

Membrane forms of immunoglobulin (mIg) serve as antigen receptors on B lymphocytes. Following antigen binding, the membrane immunoglobulin transduces transmembrane signals into the B cells. These signals play important roles in the inactivation of immature B cells, a process that contributes to tolerance to self, and in the activation of mature B cells to produce antibodies (Defranco et al., 1989; Cambier & Ransom, 1987; DeFranco, 1987). However, antigen-specific B cells are difficult to isolate and utilize because of their small numbers. Thus, many studies on the B cell signal transduction have been done by examining the effects of anti-immunoglobulin antibodies (Cambier & Ransom, 1987; DeFranco et al., 1989).

Recently, Hamano et al. established antigen-specific B-cell clones directed against 2,4,6-trinitrophenyl (TNP)[1] hapten (Hamano et al., 1987, 1990). These B-cell hybridomas have served as good models for the study of B-cell activation and differentiation. These antigen-specific B-cell clones are also attractive models of membrane-bound antigen recognition by B cells. Liposomes sensitized with lipid haptens have been extensively studied by many authors (Six et al., 1973; Brulet & McConnell, 1977; Dancey et al., 1979; Balakrishnan et al., 1982; Ho & Huang, 1985; Kimura et al., 1990). However, most studies have focused on the interaction between the hapten-sensitized liposomes and antibody molecules. Unfortunately, there has been no systematic study of the interaction between liposomes bearing lipid haptens and their membrane-bound receptor molecules (membrane immunoglobulins) in B lymphocytes.

In this paper it will be shown that trinitrophenylated liposomes increase the cytosolic free calcium concentration in TNP-specific B cells. The magnitude of the calcium signals was dramatically dependent on both the membrane fluidity and the spacer lengths of the lipid haptens.

## Materials and Methods

*Materials.* L-α-Dimyristoylphosphatidylcholine (DMPC) and L-α-dipalmitoylphosphatidylcholine (DPPC) were purchased from Avanti (Birmingham, AL). 2,4,6-trinitrophenylated (TNP) phosphatidylethanolamine with different length spacers were prepared by a previous method (Okada et al., 1982).

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[1] Abbreviations: DMPC, L-α-dimyristoylphosphatidylcholine; DPPC, L-α-dipalmitoylphosphatidylcholine; TNP, 2,4,6-trinitrophenyl; PE, L-α-dipalmitoylphosphatidylethanolamine; TNP-$C_0$-PE, TNP-PE; TNP-$C_4$-PE, TNP-aminobutyryl-PE; TNP-$C_6$-PE, TNP-aminocaproyl-PE; TNP-$C_8$-PE, TNP-aminocapryl-PE; TNP-$C_{12}$-PE, TNP-aminolauryl-PE; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; PBS, phosphate-buffered saline; FCS, fetal calf serum; BSA, bovine serum albumin; KLH, keyhole limpet hemocyanin.

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