In Situ Aggregational State of M13 Bacteriophage Major Coat Protein in Sodium Cholate and Lipid Bilayers†

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ABSTRACT: The *in situ* aggregational behavior of the bacteriophage M13 major coat protein was determined for the protein isolated in sodium cholate and reconstituted into DOPC lipid bilayers. For this purpose, the cysteine mutants A49C and T36C of the major coat protein were labeled with either a maleimido spin-label or a fluorescence label (IAEDANS). The steric restrictions sensed by the spin-label were used to evaluate the local protein conformation and the extent of protein−protein interactions at the position of the labeled residue. In addition, fluorescent labels covalently attached to the protein were used to determine the polarity of the local environment. The labeled coat protein mutants were examined under different conditions of protein association (amphiphile environment, ionic strength, temperature, and pH). The aggregational state of the major coat protein solubilized from the phage particle in sodium cholate was not dependent on the ionic strength, but was strongly dependent on cholate concentration and pH during sample preparation. At pH 7.0 and high sodium cholate concentration, the protein was in a dimeric form. The unusually strong association properties of the protein dimer in sodium cholate at pH 7.0 were attributed to the inability of sodium cholate to disrupt the strong hydrophobic forces between neighboring protein subunits in the phage particle. Such a "structural protein dimer" was, however, completely and irreversibly disrupted at pH 10.0. Qualitatively the same aggregational tendency was found upon changing the pH for the coat protein reconstituted in DOPC lipid bilayers. This reveals that the dimer disruption process is primarily a protein property, because there are no titratable groups on DOPC in the experimental pH range. The results are interpreted in terms of a model relating the protein aggregational state in the assembled phage to the protein aggregational behavior in sodium cholate and lipid bilayers.

The major coat protein of the bacteriophage M13 is a multifunctional protein. It forms a polymeric protective coat around the viral DNA (Marvin et al., 1994). Alternatively, it inserts into the cytoplasmic membrane of the host *Escherichia coli* during virus disassembly, and it leaves the membrane in a virus assembly−extrusion process (Russel, 1991). The conformational and aggregational states of the coat protein are believed to change from a polymeric form in the virus particle to a monomeric form in the membrane, and back to the polymeric form in a newly assembled phage (Hemminga et al., 1993). Versatility and also reversibility of these processes are remarkable characteristics of the major coat protein.

The conformational and aggregational states of the major coat protein, as studied in various amphiphatic environments, are strongly dependent on the protein history and method of sample preparation. The amphiphile composition, ionic strength, temperature, and pH are the most important factors that shift the chemical equilibrium between different coat protein aggregate sizes (Hemminga et al., 1993). Depending on the sample preparation, two specific conformations of the coat protein were suggested: a predominantly α-helical conformation, where the coat protein has an ability to undergo a reversible aggregation; and a predominantly β-polymeric state, where the coat protein is irreversibly aggregated into large β-sheet complexes (Nozaki et al., 1978). It should be mentioned that the β-polymeric state is considered as an artifact, a denatured form of the coat protein, because it is formed in an irreversible way, unable to convert into an α-helical conformation as found in the virus particle (Spruijt & Hemminga, 1991).

Sodium cholate is able to preserve the coat protein in a predominantly α-helical conformation. Sodium cholate is a weak detergent and has been used extensively as an intermediate hydrophobic environment for protein labeling and subsequent reconstitution in the phospholipid bilayers (Woolford et al., 1974; Makino et al., 1975; Stopar et al., 1996). The smallest protein aggregate in sodium cholate is assumed to be an α-helical coat protein dimer (Knippers & Hoffmann-Berling, 1966; Makino et al., 1975). Therefore, coat protein oligomerization in sodium cholate is thought to be a process in which coat protein dimers are joined together (Spruijt et al., 1989). Although dimers appear to be stable in sodium cholate, it was demonstrated that in the strong detergent sodium dodecyl sulfate (SDS)[1] , they dissociate into monomers (McDonnell et al., 1993). This suggests that the coat protein dimers in sodium cholate are stabilized primarily by noncovalent interactions.

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On the other hand, the *in situ* aggregational state of the coat protein reconstituted in phospholipid bilayers is very difficult to determine. Size-exclusion chromatography, HPSEC, has been employed previously (Makino et al., 1975; Spruijt et al., 1989; Spruijt & Hemminga, 1991). However, in the absence of detergents, only the size distribution of the entire lipid−protein complex can be determined, but no information about the size of the protein aggregate can be obtained (Tanford & Reynolds, 1976). In contrast, HPSEC in the presence of detergents disrupts the lipid−protein complexes and extracts the coat protein, thereby probably changing the protein−protein interactions and the original aggregational state of the coat protein in the bilayer (Spruijt et al., 1989). The same applies for SDS−PAGE gel electrophoresis, because it disrupts all the noncovalent interactions between proteins. The oligomeric state of the coat protein in lipid bilayers, as determined by sodium cholate−HPSEC, is characterized by weak protein−protein interactions that can be readily disrupted, because SDS−HPSEC elution profiles indicate the presence of only monomers (Spruijt & Hemminga, 1991).

Analysis of the dynamics of the spin-labeled proteins was used earlier to identify sites of interaction between neighboring protein units in the lipid bilayers (Bittman et al., 1984; Hubbell & Altenbach, 1994). When the spin-labeled part of the protein makes contacts with other structures in the protein, or between neighboring proteins, its motion is highly constrained. The steric restrictions sensed by the spin-label can thus be used to evaluate the local protein conformation (Wolkers et al., 1997), or the extent of protein−protein interactions at any residue (Hubbell & Altenbach, 1994). In addition, fluorescent labels covalently attached to the protein have been a valuable tool in determining information about the polarity of the environment. For example, the wavelength emission maximum gives information about the polarity of the fluorophore environment in different aggregational states of the protein (Spruijt et al., 1996).

In this paper, we describe the *in situ* aggregational behavior of the major coat protein isolated in sodium cholate and reconstituted in lipid bilayers. For this purpose, cysteine mutants of the coat protein were labeled with either a spin-label or a fluorescence label. The labeled coat protein mutants were examined under different conditions of protein association (by variation of ionic strength, temperature, and pH). This has enabled us to obtain detailed information about the protein−protein interactions in a membrane environment.

MATERIALS AND METHODS

Coat Protein Isolation and Spin-Labeling. The major coat protein mutants A49C and T36C were grown and purified from the phage as described previously (Spruijt et al., 1996). The major coat protein mutants were checked for their conformation and aggregational properties with HPSEC and CD as described previously (Stopar et al., 1996). The phage was disrupted in a mixture of 2.5% (v/v) chloroform, 100 mM sodium cholate, 150 mM NaCl, and 10 mM Tris-HCl at 37 °C, pH 7.0, with subsequent spin-labeling of the major coat protein mutants as described previously (Stopar et al., 1996). The spin-label 3-maleimidoproxyl was obtained from Aldrich Chemical Co. To separate the spin-labeled protein from the free spin-label and DNA, the mixture was applied to a Superose 12 prepgrad HR 25/60 column (Pharmacia) and eluted with 25 mM sodium cholate, 150 mM NaCl, and 10 mM Tris-HCl equilibrated to pH 7.0, pH 8.0, or pH 10.0. Fractions with an A_{280}/A_{260} absorbance ratio higher than 1.5 were collected and concentrated by Amicon filtration. The aggregational state of the protein at each pH value was checked by SDS−HPSEC and sodium cholate−HPSEC as described previously (Spruijt et al., 1989).

Coat Protein Reconstitution in Lipid Vesicles. The spin-labeled protein mutants were reconstituted in DOPC and DOPC/DOPG vesicles (80/20 mol/mol) as described earlier (Stopar et al., 1996) with the following modifications. Lipid vesicles were prepared by evaporating chloroform from the desired amount of DOPC or DOPC/DOPG mixture. The residual traces of chloroform were removed under vacuum overnight. The lipids were solubilized in 50 mM sodium cholate buffer (150 mM NaCl, 10 mM Tris-HCl) at the desired pH: pH 7.0, 8.0, or 10.0 by brief sonication (Branson B15 cell disrupter) in ice-cold water until a clear opalescent solution was obtained. The desired amount of spin-labeled mutant major coat protein isolated in 50 mM sodium cholate at pH 7.0, 8.0, and/or 10.0 was added to give a molar lipid/protein ratio of 200 (L/P 200). Dialysis was performed at room temperature against a 100-fold excess of 50 mM NaCl, 10 mM Tris-HCl buffer at pH 7.0 for the protein isolated in sodium cholate at pH 7.0; pH 8.0 for the protein isolated in sodium cholate at pH 8.0; or pH 10.0 for the protein isolated in sodium cholate at pH 10.0. The dialysis buffer was changed 4 times every 12 h. After dialysis, the proteoliposome suspension of the spin-labeled coat protein reconstituted at either pH 7.0, 8.0, or 10.0 in DOPC was divided into five aliquots and adjusted to the desired pH by a second dialysis against a 100-fold excess of 150 mM NaCl, 10 mM Tris-HCl buffer to obtain a final proteoliposome suspension at pH 6.0, 7.0, 8.0, 9.0, and 10.0. The dialysis buffer for the second dialysis was also changed 4 times every 12 h.

For ESR measurements, the samples were freeze-dried, resuspended in a volume of distilled water equal to the original volume, and then concentrated by centrifugation (Beckman XL-90 ultracentrifuge, 45 000 rpm, 1 h, 10 °C). During the second dialysis, samples with high and low salt concentration were prepared by dialysis against a 100-fold excess of 1000 mM NaCl (high salt), 10 mM Tris-HCl buffer, or 25 mM NaCl (low salt), 10 mM Tris-HCl buffer at the desired pH value. The aggregational state of the protein was checked by SDS−HPSEC and sodium cholate−HPSEC as described previously (Spruijt et al., 1989).

Chemical Cross-Linking Experiments. Spin-labeled major coat protein isolated in sodium cholate at pH 7.0 or pH 10 was cross-linked with 100 mM 1-ethyl-3-[3-(dimethylamino)-propyl]carbodiimide (EDC), at room temperature for 2 min. The cross-linking reaction was stopped by adding an excess of concentrated Tris-HCl buffer up to 0.8 M. The aggregational state of the sample was analyzed with Tricine/sodium dodecyl sulfate−polyacrylamide gel electrophoresis

[1] Abbreviations: CD, circular dichroism; DOPC, 1,2-dioleoyl-*sn*-glycero-3-phosphocholine; DOPG, 1,2-dioleoyl-*sn*-glycero-3-phosphoglycerol; ESR, electron spin resonance; HPSEC, high-performance size-exclusion chromatography; IAEDANS, *N*-[[(iodoacetyl)amino]ethyl]-1-sulfonaphthylamine; L/P, lipid to protein molar ratio; SDS, sodium dodecyl sulfate; EDC, 1-ethyl-3-[3-(dimethylamino)propyl]carbodiimide hydrochloride.

(Schagger & von Jagow, 1987). Molecular mass markers from horse heart globin were obtained from Pharmacia in the range from 2.5 to 16.9 kDa.

ESR Spectroscopy. Samples containing spin-labeled coat protein isolated in sodium cholate or reconstituted into DOPC or DOPC/DOPG bilayers were filled up to 5 mm in 100 μL glass capillaries. These capillaries were accommodated within standard 4 mm diameter quartz tubes. ESR spectra were recorded on a Bruker ESP 300E ESR spectrometer equipped with a 108TMH/9103 microwave cavity and with a nitrogen gas flow temperature regulation system. The ESR settings were 6.38 mW microwave power, 0.1 mT modulation amplitude, 40 ms time constant, 80 s scan time, 10 mT scan width, and 338.9 mT center field. Up to 20 spectra were accumulated to improve the signal to noise ratio. The first and second integrals of the ESR spectra were determined after base line correction. The effective rotational correlation times were determined from the line height ratios as described previously (Marsh, 1981).

Evaluation of the Immobilized Component. To estimate the fraction of the immobilized component in ESR spectra, only spectra recorded at low temperature (5 $\pm$ 0.5 °C) were used. In these spectra, the mobile and immobile components were clearly separated on the composed spectrum. To obtain the percentage of immobilized component, a pairwise subtraction method was used (Devaux & Seigneuret, 1985). The basic assumption of this method is that, to a first approximation, over a limited range of temperature only the ratio of the two components varies substantially, while the line shape of the components is affected only to a small extent (Andersen et al., 1981). Typically, spectra of the spin-labeled protein isolated in sodium cholate at pH 10.0 and reconstituted in DOPC bilayers at pH 10.0 were subtracted from the ESR spectra until an undistorted line shape, with no spikes, was generated. Double integration of the original spectra and the generated immobilized component spectra gives the relative amount of the immobilized spin-labels.

CD Spectroscopy. Circular dichroism measurements were performed on a Jasco J-715 spectrometer in the wavelength range from 190 to 290 nm at room temperature. Samples were prepared as described previously by Sanders et al. (1993).

Fluorescence Spectroscopy. For the purpose of steady-state fluorescence experiments, the A49C coat protein mutant was labeled with IAEDANS (Molecular Probes) directly after phage disruption, as described previously (Spruijt et al., 1996). Fluorescence-labeled protein was reconstituted into DOPC bilayers, similar to as described above for the spin-labeled coat protein, but with dialysis carried out in the dark to prevent photodegradation of the AEDANS. Steady-state fluorescence was performed on a Perkin-Elmer LS-5 luminescence spectrophotometer at room temperature. The absorbance of the samples was kept below 0.1 at the excitation wavelength. The excitation wavelength was 340 nm, and emission scans were recorded from 400 to 640 nm with an excitation and an emission bandwidth of 5 nm. Steady-state quenching studies of AEDANS-labeled A49C coat protein mutant were performed by addition of the same amount of spin-labeled A49C coat protein mutant (mol/mol) and acrylamide in the concentration range from 0 to 125 mM at room temperature.

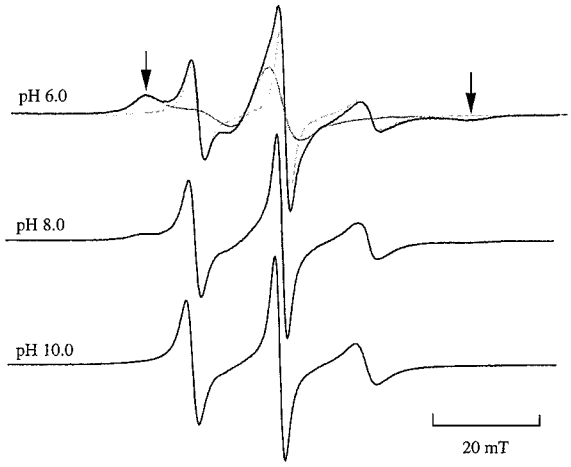

FIGURE 1: Conventional ESR spectra of the spin-labeled A49C major coat protein mutant in DOPC bilayers at a lipid/protein ratio of 200 (mol/mol). Samples were isolated in 25 mM sodium cholate at pH 7.0 and reconstituted into DOPC at the desired pH; from top to bottom pH 6.0, 8.0, and 10.0, respectively. Spectra are normalized to the same central line height. The temperature of the samples was 5 °C. Total scan width is 10 mT. The outermost peaks of the immobilized component in the ESR spectra are indicated with arrows in the pH 6.0 spectrum. The two component fit to the experimental spectrum is indicated in the pH 6.0 spectrum.

RESULTS

The ESR spectra of the spin-labeled A49C coat protein mutant isolated in 25 mM sodium cholate at pH 7.0 and subsequently reconstituted at high lipid to protein ratio (L/P 200) in DOPC bilayers at pH 6.0, 8.0, and 10.0 are given in Figure 1. The spectra display two well-resolved components: a sharp, three-line component corresponding to spin-labels undergoing fast isotropic motion, and a second broad component resolved in the outer wings of the spectrum, representing spin-labels undergoing restricted motion. The fraction of the immobile component dominates in the composite spectrum at pH 6.0, but it is dramatically reduced in the ESR spectrum at pH 10.0.

The fraction of motionally restricted labels was quantitated by spectral subtraction of the mobile component. (Devaux & Seigneuret, 1985). This enabled us to obtain a line shape of the immobilized component. This procedure works best at low temperature (5 °C), where the two components are well resolved. The mobile component used in the spectral subtraction is taken from the spin-labeled A49C coat protein isolated in 25 mM sodium cholate at pH 10.0 and subsequently reconstituted at L/P 200 in DOPC bilayers at pH 10.0. This component is characteristic for a single component with a fast isotropic motion with a rotational correlation time, τ_c, of 0.59 ns, and an isotropic splitting of 1.59 mT. The immobilized component obtained from spectral subtraction is characteristic for a slow mobility with an outer splitting of $2A_{max} = 5.93$ mT at 5 °C. The choice of the mobile component for the spectral subtractions is justified by the observation that several non-base-line isoclinic points are observed in the spectra in Figure 1, normalized to the same double integral (data not shown). These non-base-line isoclinic points indicate that the ESR spectra are a linear combination of the mobile and immobile spectra (Marriott & Griffith, 1974).The subtraction procedure leads to a reliable quantitation (the estimated error is $\pm$5%) of the fraction of the immobilized component.

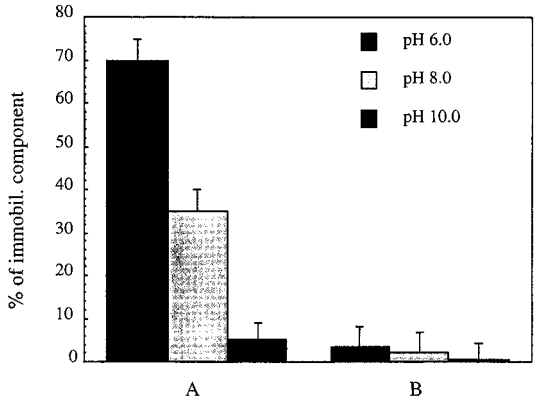

FIGURE 2: Fraction of the immobilized component of the spin-labeled A49C coat protein mutant in DOPC bilayers. The fraction of the immobilized component was estimated as described under Materials and Methods. The temperature of the samples was 5 °C. (A) The spin-labeled A49C coat protein mutant was isolated in 25 mM sodium cholate at pH 7.0 and reconstituted in DOPC at the desired pH; pH 6.0 (left bar), pH 8.0 (middle bar), and pH 10.0 (right bar). (B) The spin-labeled A49C coat protein mutant was isolated in 25 mM sodium cholate at pH 10.0 and reconstituted in DOPC at the desired pH: pH 6.0 (left bar), pH 8.0 (middle bar), and pH 10.0 (dark gray bar).

The fraction of motionally restricted component of the spin-labeled A49C coat protein mutant reconstituted in DOPC bilayers at L/P 200 is given in Figure 2. The fraction of the immobilized component of the coat protein isolated in 25 mM sodium cholate at pH 7.0 and subsequently reconstituted in DOPC bilayers at pH 6.0 is 70%. This fraction decreases dramatically to 5% if the protein was reconstituted in DOPC bilayers at pH 10.0 (Figure 2A). In contrast, the fraction of the immobilized component for the protein isolated in 25 mM sodium cholate at pH 10 and subsequently reconstituted in DOPC bilayers at pH 10.0 is low (<5%), and is not significantly influenced by different pH values (Figure 2B). It should be noted, that the fraction of the immobile component was strongly dependent on pH during protein isolation in sodium cholate. The disappearance of the immobile component is completely irreversible.

The fraction of the immobilized component was also dependent on the concentration of sodium cholate during protein isolation. When the concentration of sodium cholate at pH 7.0 isolation was increased from 25 to 100 mM, the fraction of the immobilized component decreased from 63 to 49%. If both the concentration of sodium cholate and the pH were increased, the immobilized component was completely removed from the ESR spectra. There was no immobilized component and no sodium cholate concentration dependence for the protein isolated in 25 mM sodium cholate at pH 10.0. If the pH of such samples was lowered at either high or low sodium cholate concentration, there was no increase in the fraction of the immobilized component, which indicates that the process is irreversible. Experiments with addition of wild-type protein to the spin-labeled protein isolated at either pH 7.0 or pH 10.0 were also performed. The fraction of the immobilized component and the spectral line shape were not changed upon titration of spin-labeled mutant with wild-type protein up to 10 times mol/mol excess.

The effect on the fraction of the immobilized component to high salt and low salt concentrations and to negatively charged lipids for the protein isolated in 25 mM sodium cholate at pH 8.0 and reconstituted in DOPC or DOPC/DOPG bilayers (L/P 200) at pH 6.0, 8.0, and 10.0 is given in Table 1. The fraction of the immobilized component was pH dependent in all systems studied, but was generally lower as compared to the protein isolated in sodium cholate at pH 7.0 and subsequently reconstituted in DOPC bilayers. There was again no significant immobilized component at pH 10.0. The fraction of the immobilized component at a given pH was not significantly dependent on different model systems, except for the case of high salt concentration, which shows a slight increase at pH 6.0.

Table 1: Fraction of Immobilized Component of Spin-Labeled A49C Coat Protein Mutant Isolated in 25 mM Sodium Cholate, pH 8.0, and Reconstituted into DOPC Bilayers, Lipid/Protein Ratio 200 (mol/mol), at the Desired pH[a]

pH	DOPC[a]	DOPC, low salt[b]	DOPC, high salt[c]	DOPC/DOPG (80/20 mol/mol)
6.0	18	19	24	20
8.0	6	7	10	7
10.0	1	2	3	2

[a] DOPC: 150 mM NaCl, 10 mM Tris-HCl. [b] DOPC, low salt: 25 mM NaCl, 10 mM Tris-HCl. [c] DOPC, high salt: 1000 mM NaCl, 10 mM Tris-HCl. [d] DOPC/DOPG (80/20 mol/mol): 150 mM NaCl, 10 mM Tris-HCl. The estimated errors are ±5%.

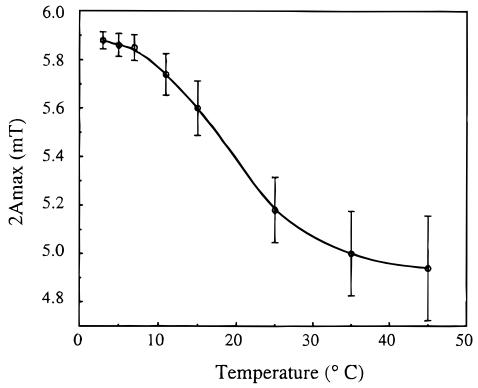

FIGURE 3: Outer hyperfine splitting, $2A_{max}$, of the immobilized component in the spin-labeled A49C coat protein mutant isolated in 25 mM sodium cholate and reconstituted in DOPC L/P 200 at pH 6.0, as a function of the temperature.

The temperature dependence of the immobilized component of the spin-labeled A49C coat protein mutant isolated in 25 mM sodium cholate at pH 7.0 and reconstituted in DOPC bilayers (L/P 200) at pH 6.0 is represented by the outer hyperfine splitting $2A_{max}$, as given by Figure 3. The line shape of the two components was temperature dependent. This makes it very difficult to determine the fraction of the immobilized component at higher temperatures. However, the outer hyperfine splitting $2A_{max}$, which could be identified up to about 45 °C, was used instead in Figure 3 to characterize the motional freedom of the motionally restricted spin probes. The temperature effect was fully reversible upon decreasing the temperature, indicating that no irreversible conformational change occurred during the temperature scan.

In all cases, the secondary structure of the coat protein in micellar samples was determined with CD. Within experimental error, no change in the spectral intensity or in the line shape was found between samples containing the coat protein isolated in sodium cholate at pH 7.0 and 10.0. The aggregational state of the protein was checked by sodium cholate−HPSEC. The protein−holate complex at 100 mM

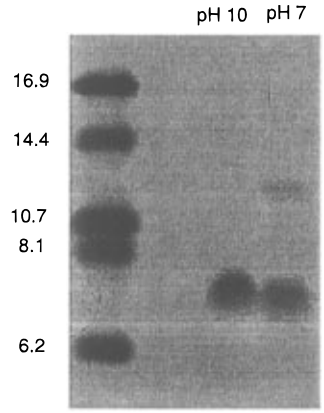

FIGURE 4: EDC-dependent cross-linking of the spin-labeled A49C major coat protein mutant isolated in 25 mM sodium cholate at pH 7.0 and 10.0 at room temperature. The aggregational state was analyzed by Tricine-SDS−PAGE.

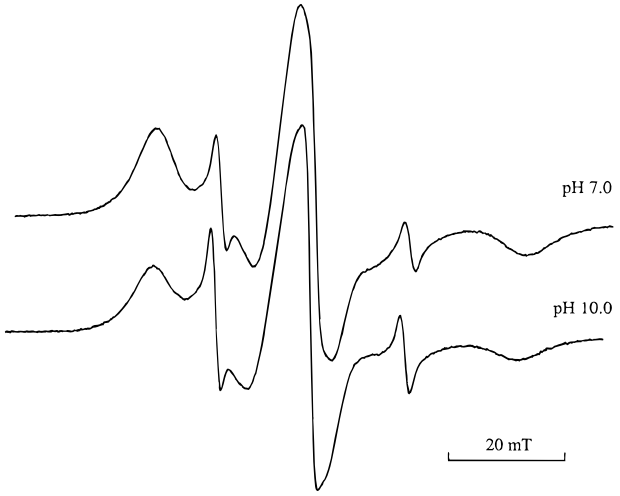

FIGURE 5: ESR spectra of spin-labeled T36C major coat protein isolated in 25 mM sodium cholate at pH 7.0 and 10.0. Spectra were recorded at room temperature and normalized to the same central line height.

sodium cholate was eluted from the column as a protein dimer peak for both pH 7.0 and pH 10.0 samples (data not shown). The aggregational states of the EDC cross-linked major coat protein isolated in sodium cholate at pH 7.0 and 10.0 and analyzed with SDS−PAGE are given in Figure 4. After EDC cross-linking, the major coat protein isolated at pH 7.0 was a mixture of monomers and dimers; at pH 10.0, it was monomeric.

Additional experiments were carried out with spin-labeled T36C coat protein mutant isolated in 25 mM sodium cholate at pH 7.0 and 10.0. The spin-label at position 36 is located in the transmembrane α-helical domain of the protein, and has a powder-like appearance with little segmental motion, as discussed previously (Stopar et al., 1996). The spectra of spin-labeled T36C mutant isolated in 25 mM sodium cholate at pH 7 and 10 are given in Figure 5. The high- and low-field peaks in the spectrum of the spin-labeled T36C mutant isolated at pH 10 are slightly line-broadened as compared to the spectrum at pH 7. To characterize the mobility of the spin-label, the outer hyperfine splitting $2A_{max}$ was measured from the ESR spectra. The motion of the spin-labeled coat protein was slightly reduced when the protein was isolated in sodium cholate at pH 7.0, giving a value for $2A_{max}$ of 6.30 mT, as compared to 6.12 mT for the protein isolated at pH 10.0 (data not shown).

Table 2: Wavelength Emission Maximum of AEDANS-Labeled A49C Coat Protein Mutant Isolated in 50 mM Sodium Cholate at pH 7.0 and/or pH 10.0, and Subsequently Reconstituted in DOPC Bilayers (L/P 200) at pH 6.0 and/or pH 10.0

coat protein isolation	wavelength max (nm)		
	sodium cholate	DOPC, pH 6.0	DOPC, pH 10.0
pH 7.0	484	489	492
pH 10.0	497	491	491

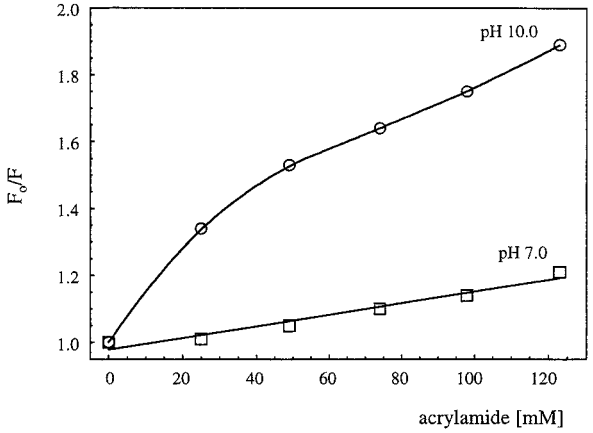

FIGURE 6: Quenching of the AEDANS-labeled A49C coat protein mutant with acrylamide in 50 mM sodium cholate at pH 7.0 and 10.0.

To further characterize the properties of the coat protein isolated in sodium cholate at pH 7.0 and 10.0, the A49C coat protein mutant was labeled with the fluorescence label IAEDANS. The fluorescence wavelength emission maximum of IAEDANS is indicative for the environment of the fluorophore (i.e., hydrophobic vs aqueous) as discussed previously for M13 coat protein mutants (Spruijt et al., 1996). The wavelengths of emission maximum for the coat protein isolated in 50 mM sodium cholate at pH 7.0 and 10.0 are given in Table 2.

The accessibility of the AEDANS-labeled coat protein to a quencher depends on the polarity of the fluorophore environment as well as steric effects (Mandal & Chakrabarti, 1988). To assess the steric effects imposed on the AEDANS-labeled coat protein isolated in sodium cholate at pH 7.0 (more hydrophobic environment) and at pH 10.0 (more hydrophilic environment), quenching studies were performed with acrylamide, which is a neutral quencher molecule. The accessibilities of fluorescence-labeled coat protein at both pH values are given in the Stern−Volmer plot shown in Figure 6. The quenching of the AEDANS-labeled coat protein at pH 7 by acrylamide was very low which is consistent with AEDANS labels being substantially protected from the solvent. In contrast, the quenching by acrylamide at pH 10.0 indicates that AEDANS labels are more accessible for the acrylamide, providing additional evidence for a change in the environment of the fluorescence label at increased pH.

In addition, ADEANS-labeled coat protein isolated in 50 mM sodium cholate at pH 7.0 and 10.0 was also quenched with spin-labeled A49C coat protein mutant isolated in sodium cholate at pH 7.0 or 10.0. The fluorescence intensity after mixing at a molar ratio of spin-labeled to fluorescence-labeled coat protein of 1/1 (mol/mol) was reduced by 12% for the coat protein isolated in sodium cholate at pH 7.0. In contrast, the fluorescence intensity was reduced by 5% in

the case coat protein was isolated in sodium cholate at pH 10.0 (data not shown).

DISCUSSION

The aggregational state of the major coat protein in micellar model systems has been investigated previously (Makino et al., 1975; Nozaki et al., 1976; Spruijt et al., 1989; Henry & Sykes, 1990; Sanders et al., 1991; Spruijt & Hemminga, 1991). It was also shown that mutant M13 coat proteins can exist in a range of conformational and aggregational states depending uniquely upon mutation type and locus [for a review, see Li et al. (1993) and Williams et al. (1995)]. In this paper, we report the *in situ* aggregational states of labeled mutant coat protein, as a model for wild-type coat protein, solubilized in sodium cholate and reconstituted in lipid bilayers. This requires that the mutation and labeling do not significantly change the properties of the protein. Previously we have demonstrated that, concerning the conformational and aggregational properties, the mutant coat proteins are indistinguishable from the wild-type M13 coat protein (Stopar et al., 1996). This means that labeled mutant coat protein can thus be used as a model system to characterize the aggregational behaviour of the coat protein in a membrane environment.

The ESR spectra of the spin-labeled A49C major coat protein mutant in DOPC bilayers isolated in 25 mM sodium cholate at pH 7.0 and reconstituted into DOPC at various pH values are shown in Figure 1. The two components in the ESR spectra that are seen at pH values below about 8.0 are typical for a mixture of immobilized and mobile spin-labels. The two spectral components are clearly separated, and the line shape of the immobile component indicates that there is no fast exchange between the components at a time scale of about 10^{-7} s (Horváth et al., 1988). Further evidence for this observation follows from the multiple non-base-line isoclinic points in the ESR spectra. These points indicate that the spectra are composed of a pH-dependent linear combination of the two components (Marriott & Griffith, 1974). Although the spin-labels in the immobilized component are significantly restricted in motion, they still have some anisotropic motion on the ESR time scale. This is deduced from the value of the outer hyperfine splitting $2A_{max}$ of 5.93 mT, which is significantly less than the rigid-limit value of nitroxide radicals, which is about 6.7 mT in an aqueous environment. This relatively fast anisotropic motion probably arises from the local motion of the spin-label and the cysteine side-chain on which it is attached, whereas the segmental mobility of the C-terminal polypeptide chain is restricted. The mobile, isotropic component in the spectrum suggests a large segmental flexibility of the C-terminal part of the coat protein mutant, which will additionally lead to an increased local side-chain motion of the spin-labeled cysteine.

Similar effects are found for the spin-labeled A49C coat protein mutant solubilized in sodium cholate: on increasing the pH from 6.0 to 10.0, the immobile component completely disappears. A very striking observation is that the disappearance of the immobile component in bilayers as well as in sodium cholate is completely irreversible: on decreasing the pH from 10.0 to 6.0, the immobile component does not reappear. In contrast, the temperature dependence of the immobilized component of the spin-labeled A49C coat protein mutant at pH 6.0, as represented by the outer hyperfine splitting $2A_{max}$ (see Figure 3), is fully reversible.

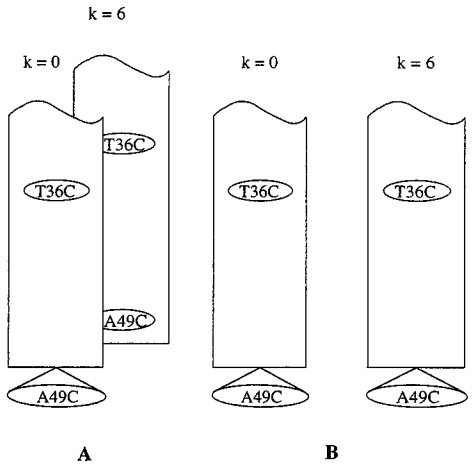

FIGURE 7: Schematic representation of a part of the transmembrane helix and C-terminus of the coat protein. (A) "Structural dimer" in sodium cholate micelles at pH 7.0. The two proteins are shifted relative to each other about 1.6 nm, similar to that found in the phage. (B) Disrupted structural dimer in sodium cholate micelles at pH 10.0. In this case, the two proteins are symmetric and in line with each other. The residues that are immobilized in the ESR spectra are indicated by numbered ellipses. The residues that are mobile are indicated by cones. The index *k* refers to the coat protein numbering in the phage as given by Marvin et al. (1994).

The following questions now arise: what limits the motion of the spin-label in samples at low pH, and what causes this irreversible pH effect? To solve these questions, it is most helpful to consider first the situation of the coat protein solubilized in sodium cholate. According to HPSEC experiments in 100 mM sodium cholate at pH 7.0, it is found that the protein at most has a dimeric state (Spruijt et al., 1989). Cross-linking experiments for the protein isolated at pH 7.0 (see Figure 4) further indicate that the protein has more tendency to cross-link, due to a closer proximity of the two proteins in the sodium cholate micelle. From the spin-label experiments, it follows that under these conditions the spin-label senses two different states of motion. This indicates that the two protein monomers in the dimer are asymmetric with respect to each other, and have different states of the C-termini: one monomer has a C-terminal part with a large amplitude of motion, whereas the C-terminus of the other monomer is strongly reduced in motion. This reduced motion roughly reflects the overall motion of a sodium cholate micelle containing two protein molecules (the rotational correlation time τ_c is estimated to be about 10 ns in pure water). These two states are consistent with a model where the proteins are dissolved from the phage particle as parallel dimers slide with respect to each other for about 1.6 nm. This dimer will be labeled as "structural dimer". In this state, the C-terminus of one monomer is sticking out in the aqueous phase and reflects a large motion, whereas the C-terminus of the other protein must be strongly limited in motion by very specific interactions with the neighboring protein molecule. This protein−protein interaction will limit the segmental flexibility of the C-terminus and result in the immobile component in the ESR spectrum. This model is depicted in Figure 7A. The distance between axes of nearest neighbors in the phage particle, in the index notation used by Marvin et al. (1994), is similar in both the 0 to 6 and 0 to 11 directions. However, in the 0 to 11 direction, the

α-helices make an angle with respect to each other (−18°), whereas in the 0 to 6 direction the α-helices are nearly parallel (−5 °). Also in the 0 to 6 direction strong interlocking of the apolar side chains of the coat protein molecules occurs. Therefore, it is hypothesized that during the sodium cholate solubilization at pH 7.0, the coat protein dissolves from the phage particle as 0−6 dimers with the same orientation as in the phage. This also explains the unusually strong association properties of the "structural dimer" in sodium cholate. This furthermore indicates that at pH 7.0 the solubilization power of sodium cholate is not strong enough to disrupt the 0−6 dimer interactions in the phage.

The situation described above at pH 7.0 reflects the state of the coat protein at high sodium cholate concentration (100 mM). From the ESR results, it is found that the fraction of the immobilized component increases at lower cholate concentrations. This can be explained by the formation of tetramers of the coat protein in the micelle as shown previously (Spruijt et al., 1989). The increased fluorescence quenching with spin-labeled coat protein at pH 7.0 is consistent with such an increased protein aggregation.

At pH 10, the immobile component is absent from the spectra. This is consistent with the absence of the specific dimer protein−protein interaction in cross-linked samples at pH 10.0 (see Figure 4). Nevertheless, sodium cholate micelles still contain two protein monomers, as deduced from HPSEC elution profiles of non-cross-linked samples. In this case, the dissociated dimer exists as two protein molecules within the same micelle, and the two spin-labels in the monomers must experience an identical environment, because there is no second component present. However, there are many orientations of the two proteins in the micelle, which would satisfy this condition. One of the possible orientations, which may be of relevance for the lipid bilayer situation, is depicted in Figure 7B.

Although the two proteins in Figure 7B are relatively close to each other, the spectra of spin-labeled A49C coat protein isolated in sodium cholate at pH 10.0 do not indicate extensive spin−spin broadening. This is consistent with a high mobility of the spin-labeled A49C protein, which effectively averages out the dipolar spin−spin interactions. In addition, different possible orientations of the two proteins in the micelle would further reduce spin−spin exchange by reducing the spin collision frequency. The outer splitting of the spin-labeled T36C protein isolated in sodium cholate at pH 10.0 has decreased as compared to the protein isolated in sodium cholate at pH 7.0 (see Figure 5). In addition, the outer lines in the spectrum at pH 10.0 are slightly broadened. The reduction of the outer splitting and the broadening effect may arise from an incomplete averaging of the powder components in the line shape, due to an increase of molecular motion after disruption of the "structural dimer". Part of the observed broadening may also arise from spin−spin interactions, but in the absence of any effect of dilution with wild-type protein on the ESR line shape, this effect is probably small.

The mobility and hydrophilic environment of the spin-labeled and fluorescence-labeled A49C coat protein mutant isolated in sodium cholate at pH 10.0 are consistent with an aqueous environment. In sharp contrast, the reduced C-terminal flexibility, the increased hydrophobicity of the AEDANS-labeled A49C coat protein mutant, and AEDANS protection from acrylamide quencher of the coat protein isolated in sodium cholate at pH 7.0 indicate that a significant number of the C-termini are in a different environment (see Table 2). Such an environment can easily be found in the structural protein dimer as discussed above.

The coat protein solubilized in sodium cholate and subsequently reconstituted into lipid bilayers has qualitatively the same aggregational tendency, suggesting that the aggregational states of the protein in two model systems are comparable. Moreover, structural dimers reconstituted into lipid bilayers at low pH show a tendency to form tetramers (increased immobilized component), and can be solubilized by the surrounding lipids only when the pH is increased. In the absence of titratable groups of DOPC in the experimental pH range, this indicates that the solubilization process is induced primarily by changing titratable groups of the major coat protein. It is a remarkable finding that the lipid membrane can accommodate the structural dimers, as shown in Figure 7A. This indicates that the coat protein in the structural dimer has the unique ability to change its conformation and topology to adapt to the water−membrane interface, and to cope with the hydrophobic forces within the membrane. In the case of the coat protein solubilized in sodium cholate micelles at pH 10.0, the structural dimers have been disrupted, and two identical protein monomers are observed, that stay together in the micelles (Figure 7B), probably because of the relatively weak solubilizing properties of sodium cholate. When the coat protein is reconstituted in lipid bilayers at pH 10.0, these disrupted dimers no longer exist, and the protein behaves as separate monomers, as has been found in previous work (Spruijt & Hemminga, 1991).

From the experiments shown in Table 1, it is found that the fraction of the immobilized component of the spin-labeled A49C coat protein mutant is not strongly dependent upon the ionic strength and the presence of DOPG in mixed DOPC/DOPG bilayers. This result further indicates that the reduction of spin-label motion is not caused by a specific lipid effect. At pH 7.0, the coat protein has a net charge of +1. By increasing the pH, the net charge of the protein will eventually become negative, for example, by deprotonating the lysine residues ($pK_a \approx 10.0$). This will give an increase in the net electrostatic repulsion between the protein molecules in the dimers, in competition with the hydrophobic interactions. At pH 10.0, this explains the complete dissociation of the structural protein dimer, in favor of protein−amphiphile interactions. However, the molecular details of this process are still missing.

The results in this paper illustrate the sequence of events during phage disruption with sodium cholate. Sodium cholate is a flat hydrophobic molecule with a weak solubilizing power. It can disrupt the phage particle only when the rigid phage structure is "loosened" with chloroform (Makino et al., 1975). Upon disruption of the loosened phage particle, it may be expected that sodium cholate disrupts the weak hydrophobic forces between the coat proteins by removing patches of parallel oriented coat protein, which turn up as "structural dimers" in sodium cholate at a low pH value. It may be expected that the "structural dimers" can reversibly aggregate, depending upon the sodium cholate concentration (Spruijt et al., 1989). At high sodium cholate concentration, however, the "structural dimer" is the smallest possible protein aggregate. The last step in coat protein solubilization is a disruption of the "structural dimer". In

sodium cholate, this process only takes place at an increased pH. This also implies that the pH should be increased to at least pH 10.0 during sodium cholate protein isolation or in lipid bilayer reconstitution, if one wants to study the monomeric form of the major coat protein in lipid bilayers.

Our data suggest that during disruption of the phage particle in sodium cholate at pH 7.0, the protein is solubilized as a "structural dimer", and that after increasing the pH to 10.0 this dimer is further disrupted. Clearly, at pH 7.0 phage disruption is not complete. There are, however, no experimental data available about the conformational state of the protein, or local pH values in the cytoplasmic membrane during phage disassembly. Apparently, *in vivo* complete dissociation of the coat protein can be achieved, because parental coat proteins can be reutilized in the assembly process (Smilowitz, 1974). This is believed to be a monomeric process (Russel, 1991). Furthermore, prior to assembly of the coat protein in the virus particle, neighboring α-helix units cannot have the same side-chain interlocking as in the assembled phage, because the formation of the original "structural dimer" in micelles and lipid bilayers turns out to be impossible. As suggested by Russel (1993), the interaction with the gene I product is likely to play a key role in bringing the major coat protein to the phage assembly site. At the assembly site, coat proteins are extruded from the membrane lipids in a controlled fashion by the virus assembly machinery, preventing aspecific protein aggregation in the membrane. Such a lipid-free monomer could then interact with the protein coat previously added to the elongating phage particle. To obtain the final side-chain interlocking in the virus particle, extrusion of the new phage through the membrane and addition of the coat protein must be correlated in such a way that the extruding phage moves 1.6 nm out of the plane of the membrane before the next coat protein is added.

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