Backbone Dynamics of a Model Membrane Protein: ^{13}C NMR Spectroscopy of Alanine Methyl Groups in Detergent-Solubilized M13 Coat Protein†

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ABSTRACT: The filamentous coliphage M13 possesses multiple copies of a 50-residue coat protein which is inserted into the inner membrane of *Escherichia coli* during infection. ^{13}C nuclear magnetic resonance (NMR) spectroscopy has been used to probe the structure and dynamics of M13 coat protein solubilized in detergent micelles. A comparison of backbone dynamics within the hydrophobic core region and the hydrophilic terminal domains was obtained by biosynthetic incorporation of [3-^{13}C]alanine. Alanine is distributed throughout the protein and accounts for 10 residues (i.e., 20% of the total). Similar ^{13}C NMR spectra of the protein have been obtained in two anionic detergents, sodium deoxycholate and sodium dodecyl sulfate, although the structures and physical properties of these solubilizing agents are quite different. The N-terminal alanine residues, assigned by pH titration, and the penultimate residue, assigned by carboxypeptidase A digestion, give rise to analogous peaks in both detergent systems. The pK_a of Ala-1 ($\sim$8.8) and the relaxation parameters of individual carbon atoms (T_1, T_2, and the nuclear Overhauser enhancement) are also generally similar, suggesting a similarity in the overall protein structure. Relaxation data have been analyzed according to the model-free approach of Lipari and Szabo [Lipari, G., & Szabo, A. (1982) *J. Am. Chem. Soc. 104*, 4546-4559]. The overall correlation times were obtained by fitting the three experimental relaxation values for a given well-resolved single carbon atom to obtain a unique value for the generalized order parameter, $\mathcal{S}^2$, and the effective correlation time, τ_e. The former parameter reflects the spatial restriction of motion, and the latter, the rate. Assuming symmetric rotation of the methyl group, $\mathcal{S}^2$ effectively reports the reorientation of the C2-C3 axis, i.e., protein backbone motions. The polypeptide backbone is thus shown to approach rigidity in the hydrophobic core and parts of the hydrophilic regions. Substantial mobility is apparent only at the extreme ends of the polypeptide chain (Ala-1 and Ala-49).

The conformational dynamics of macromolecules is currently a topic of great interest (Karplus & McCammon, 1981; Wüthrich & Wagner, 1984). ^{13}C nuclear magnetic resonance (NMR)[1] spectroscopy is a particularly useful technique in this regard and has been used to investigate the dynamics of the coat protein from bacteriophage M13 when solubilized in detergent micelles.

M13 is a male-specific, nonlytic coliphage [for a review, see Denhardt (1975)]. It is a simple nucleoprotein particle comprising a circular single-stranded DNA molecule surrounded by 2700 copies of a major coat protein (gene 8 protein) in a regular array (Marvin & Wachtel, 1975). During the reproductive cycle of M13, the coat protein is deposited as an integral protein in the inner membrane of *Escherichia coli* as phage DNA enters the cell (Smilowitz et al., 1972). Large quantities of new coat protein, synthesized via a procoat precursor (Sugimoto et al., 1977; Chang et al., 1978), become similarly located prior to extrusion of progeny phage [Smilowitz, 1974; for a recent review, see Wickner (1983)]. Mature coat protein consists of only 50 amino acid residues. The sequence resembles that of a typical integral membrane protein: a 19-residue highly hydrophobic core is flanked by an acidic N-terminal and a basic C-terminal region (Asbeck et al., 1969, Nakashima & Konigsberg, 1974). M13 coat protein, like many other amphiphilic proteins, aggregates in aqueous solutions in the absence of detergents (Cavalieri et al., 1978). In the experiments described here, sodium deoxycholate and sodium dodecyl sulfate were chosen as solubilizing agents, since their protein-containing micelles are known to be discrete particles of uniform size. Both detergents possess a single negative charge. Under the conditions of ionic strength and pH employed in these experiments, the coat protein exists as a dimer which binds either 16 molecules of DOC or about 60 molecules of SDS (Makino et al., 1975). Monomeric coat protein is difficult to obtain; Nozaki et al. (1978), in an extensive characterization, suggest the monomer to be the dominant species only in very high concentrations of guanidinium chloride. A number of NMR studies using a wide variety of nuclei have involved M13 or the closely related phage fd (Dettman et al., 1982, 1984; Cross & Opella, 1979, 1980, 1981; Gall et al., 1982).

To obtain an overall dynamic picture of the molecule, [3-^{13}C]alanine was chosen as a biosynthetic label. Alanine contributes 10 residues (20% of the total), and these are distributed between the hydrophilic and hydrophobic domains (Figure 1). Since the methyl group (C3) is only one bond removed from the α-carbon (C2), it effectively reflects

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[1] Abbreviations: NMR, nuclear magnetic resonance; T_1, spin-lattice relaxation time; T_2, spin-spin relaxation time; NOE, nuclear Overhauser enhancement; $\Delta\nu_{1/2}$, line width at half-height; SDS, sodium dodecyl sulfate; DOC, sodium deoxycholate; Tris, tris(hydroxymethyl)aminomethane; ppm, parts per million; CD, circular dichroism; DFP, diisopropyl fluorophosphate; TLCK, N^{α}-*p*-tosyl-L-lysine chloromethyl ketone; TPCK, N^{α}-*p*-tosyl-L-phenylalanine chloromethyl ketone; Me_4Si, tetramethylsilane.

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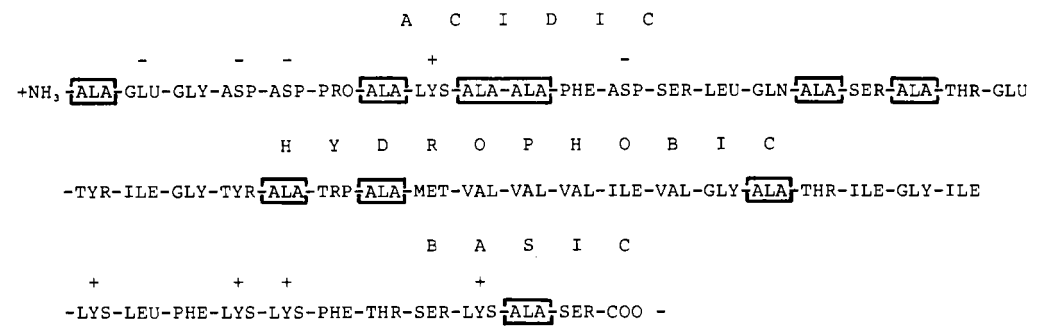

FIGURE 1: Sequence of M13 coat protein (Asbeck et al., 1969; Nakashima & Konigsberg, 1974) showing the distribution of the 10 alanine resonances.

backbone motions. The alanine methyl spectra of coat protein in SDS and DOC are directly comparable. All 10 alanine resonances are either assigned specifically to individual residues or restricted to local clusters by pH titration (Ala-1) or by limited proteolysis. Relaxation data (T_1, T_2, and NOE measurements) were analyzed according to the model-free approach of Lipari & Szabo (1982a), in which the rate of motion is expressed as an "effective" correlation time, τ_e, and the spatial restriction as a generalized order parameter, $\mathcal{S}^2$. It is thus shown that the hydrophobic core (which is buried within the micelle) and parts of the hydrophilic domains are essentially rigid in both detergents, i.e., tumbling only as the micelle tumbles. In contrast, the extreme ends of the protein (as reflected by Ala-1 and Ala-49) appear to be moving more rapidly.

Theory

Relaxation of a protonated carbon atom may be assumed to take place almost enirely by dipolar interactions with attached protons (Allerhand et al., 1971). The equations for the spin–lattice relaxation time, T_1, the spin–spin relaxation time, T_2, and the nuclear Overhauser enhancement (NOE) are as follows (Abragam, 1961) in an isotropically tumbling rigid molecule:

$$\frac{1}{NT_1} = \frac{1}{20}\frac{\gamma_H^2\gamma_C^2\hbar^2}{r^6}[J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_H + \omega_C)] \quad (1)$$

$$\frac{1}{NT_2} = \frac{1}{40}\frac{\gamma_H^2\gamma_C^2\hbar^2}{r^6}[4J(0) + J(\omega_H - \omega_C) + 3J(\omega_C) + 6J(\omega_H) + 6J(\omega_H + \omega_C)] \quad (2)$$

$$\eta = \frac{I_{irrad} - I_0}{I_0} = \frac{\gamma_H}{\gamma_C}\left[\frac{6J(\omega_H + \omega_C) - J(\omega_H - \omega_C)}{J(\omega_C - \omega_H) + 3J(\omega_C) + 6J(\omega_C + \omega_H)}\right] \quad (3)$$

where N is the number of attached protons, γ_C and γ_H are the gyromagnetic ratios of carbon and protons, respectively, r is the length of the C–H vector, $\hbar = h/2\pi$, η is the NOE, I_{irrad} and I_0 are the intensities in the presence and absence of proton irradiation, respectively, and $J(\omega)$ is the spectral density function (which defines the frequencies available for relaxation).

The motions of individual C–H vectors (which give rise to NMR relaxation times) are necessarily complex. The interpretation of relaxation data is thus dependent on a suitable choice of correlation function to describe the motion, such that an appropriate spectral density (its Fourier transform) may be obtained and used in eq 1–3. One approach is thus to choose a physically reasonable motional model and derive the correlation function; however, this may lead to overinterpretation of the data and further suffers from the possibility that the original model may not provide the only adequate fit to the data. Lipari & Szabo (1982a) define a correlation function which does not rely on preconceived models and fully extracts the unique information from a relaxation data set providing the internal motions are sufficiently fast. This is termed the "model-free approach" and has been used here for the analysis of alanine C3 relaxation times. The model-free approach reduces data to a measure of spatial restriction (the generalized order parameter, $\mathcal{S}^2$) and a measure of the rate of motion (the effecive corrrelation time, τ_e). The spectral density function is given as

$$J(\omega) = 2\left[\frac{\mathcal{S}^2\tau_c}{1 + (\omega\tau_c)^2} + \frac{(1 - \mathcal{S}^2)\tau}{1 + (\omega\tau)^2}\right] \quad (4)$$

where $\tau^{-1} = \tau_c^{-1} + \tau_e^{-1}$. The generalized order parameter $\mathcal{S}^2$ may vary between 1 (complete order), in which the second term in eq 4 vanishes and the equation reduces to the rigid isotropic case [$J(\omega) = 2\tau_c/[1 + (\omega\tau_c)^2]$, and 0, in which case relaxation is completely determined by the internal motions. These equations are valid if $\tau_c >> \tau_e$ and if overall motion is isotropic (Lipari & Szabo, 1982a). Once the $\mathcal{S}^2$ and τ_c values have been extracted, they may be interpreted within the framework of an appropriate model.

For an alanine methyl group, if symmetric rotation occurs about the methyl group axis (i.e., the C2–C3 bond), then $\mathcal{S}^2$ is given by

$$\mathcal{S}^2 = \mathcal{S}_{axis}^2(3\cos^2\beta - 1) = 0.111\mathcal{S}_{axis}^2$$

where β is the angle between the axis of the methyl group and the C–H bond (Lipari & Szabo, 1982b). The angle β is taken to be 70.5°, assuming perfect tetrahedral geometry of bonds about C3. The maximum value of $\mathcal{S}^2$ is thus 0.111 if the above assumptions hold. $\mathcal{S}_{axis}^2$, on the other hand, varies between 0 and 1 and is the order parameter with respect to the motions of the C2–C3 bond. It is often more convenient to think in terms of $\mathcal{S}_{axis}^2$ rather $\mathcal{S}^2$, since the former property relates directly to protein backbone motions.

Experimental Procedures

Materials

E. coli KA197 (CGSC 5243, Hfr, thi$^-$, pheA27, relA1, λ^-) was obtained from Dr. B. Bachmann, Coli Genetic Stock Centre, Yale University School of Medicine, DL-[3-^{13}C]Alanine (93.5%) was from MSD Isotopes (Pointe Claire, Dorval, Québec). The proteases carboxypeptidase A (DFP treated), α-chymotrypsin (TLCK treated), and proteinase K were from

Sigma Chemical Co. (St. Louis, MO). Worthington trypsin (TPCK treated) was obtained from Cooper Biomedical Ltd. (Mississauga, Ontario). Sodium dodecyl sulfate was from Bio-Rad (Richmond, CA), Sephacryl S200 Superfine was obtained from Pharmacia (Canada) Ltd. (Dorval, Québec), and sodium deoxycholate was from Sigma. M13 was from laboratory stocks.

Methods

Growth of Labeled Phage. [3-^{13}C]Alanine-labeled phage was prepared by growing *E. coli* KA197 on M63 minimal medium (Miller, 1972) supplemented with thiamin hydrochloride (0.005 g L^{-1}), L-phenylalanine (0.04 g L^{-1}), and DL-[3-^{13}C]alanine (0.08 g L^{-1}). One-liter cultures were generally used; these were inoculated with 20 cm^3 cells grown to mid-log phase on L broth (Miller, 1972). M13 was added to a multiplicity of infection of 20 when the optical density at 600 nm reached 0.1. Growth was continued until the optical density became constant (1.0–1.2). Cells were then removed by centrifugation, and M13 phage was harvested as described by Wickner (1975). Phage was stored as a freeze-dried powder, desiccated at 0 °C.

Preparation of Detergent Micelles. Deoxycholate micelles were prepared as described by Woolford & Webster (1975), except that 25 mM sodium borate, pH 9.0, was used in place of sodium bicarbonate (to avoid carbamylation of primary amino groups). Typically, 30 mg of labeled phage was dissolved in 2 cm^3 of Tris-HCl, pH 7.5, and an equal volume of buffer containing 0.1 M ammonium bicarbonate and 70 mM sodium deoxycholate, pH 9.0, was added. The mixture was shaken vigorously at 37 °C in the presence of 0.4 cm^3 of chloroform until all the chloroform had evaporated (2–3 h). The coat protein containing micelles were then separated from phage DNA by gel filtration on Sephacryl S200 SF equilibrated with 25 mM sodium borate, pH 9.0, and 8 mM sodium deoxycholate, and protein-containing fractions were concentrated on an Amicon ultrafiltration apparatus using a YM10 membrane. SDS micelles were prepared similarly by using 10 mM SDS in place of DOC. Samples were prepared for NMR by concentrating to half the required volume and adding an equal amount of borate/detergent buffer in ^2H_2O. Concentrations were measured by the absorbance at 280 nm using a molar absorbance of 8290 M^{-1} cm^{-1} (Dettman, 1984).

NMR Spectroscopy. ^{13}C spectra were recorded at 75 MHz on a Nicolet NT-300WB NMR spectrometer; 3.5 cm^3 of sample was contained in a 12-mm tube at concentrations typically between 0.7 and 1.0 mM protein in 50% ^2H_2O at 23 °C, pH 9.0. Protons were decoupled by using the MLEV16 decoupling sequence (Levitt & Freeman, 1981; Levitt et al., 1982) except during T_1, T_2, and NOE measurements, where broad-band noise decoupling was used. The pulse width was 15 μs (54°), and the minimum delay of 500 μs was left between pulses except during relaxation experiments. Chemical shifts are quoted relative to Me_4Si and measured with respect to internal dioxane at 67.37 ppm (Shindo et al., 1978). pH values are quoted as direct meter readings, uncorrected for isotope effects. pK_a values were obtained from a nonlinear least-squares fit of the chemical shift as a function of pH to the Henderson–Hasselbalch equation using a computer program written by Dr. Jean-Robert Brisson.

T_1 measurements were made by using the inversion recovery sequence (180°–τ–90°) with a recycle delay of 4 s. T_2 values were determined by using the Hahn spin-echo sequence (90°–τ–180°–τ) with a recycle delay of 3 s. At least six τ values were used in T_1 and T_2 experiments. Averaging 4000–5000 scans under the above conditions gave good signal to noise ratios. The NOE was measured in a gated decoupling experiment, collecting alternate scans (with and without NOE) into adjacent blocks of memory. All relaxation data were collected by using a sweep width of ±1000 Hz and 4K data points. The carrier frequency was placed at 25 ppm. Spectral parameters for other experiments are recorded in the appropriate figure legend.

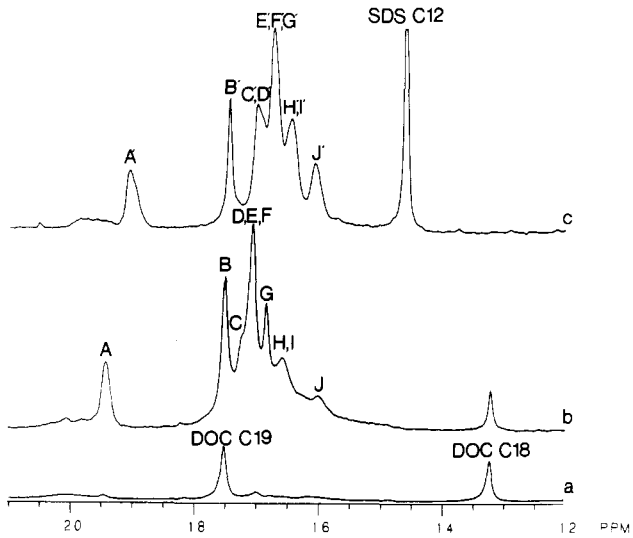

FIGURE 2: Methyl region of the proton-decoupled ^{13}C NMR spectrum of detergent-solubilized M13 coat protein at 75 MHz. 32K data points were collected by using a sweep width of ±8064 Hz with the carrier frequency placed at 98 ppm. (a) Natural-abundance spectrum in DOC micelles (2.5 mM protein, pH 9.0, 100 000 scans, 2-Hz line broadening). (b) [3-^{13}C]Alanine-labeled protein in DOC micelles (1.0 mM protein, pH 9.0, 20 000 scans, 2-Hz line broadening). The 10 alanine resonances are labeled A–J. (c) [3-^{13}C]Alanine-labeled protein in SDS micelles (1.0 mM protein, pH 8.9, 30 000 scans, 2-Hz line broadening). The 10 alanine resonances are labeled A′–J′. Detergent resonances in all spectra are indicated.

Proteolytic Digestion Procedures. All proteolytic digestion procedures were designed to give complete digestion in 10–20 h at room temperature. Carboxypeptidase A digestions were performed at a ratio of 1:100 mol:mol enzyme to protein at pH 8.0 in DOC. In SDS, a molar ratio of 1:15 was used, and digestion was never complete due to denaturation of the enzyme. Trypsin was used at 1:20 000 on a weight basis, at pH 9.0. Coat protein was prepared for trypsin digestion by prior treatment with carboxypeptidase A to remove alanine-49 and serine-50. Carboxypeptidase (in a molar ratio of about 1:20 with protein) was added to protein in DOC micelles before the gel filtration step, which separates protein-containing micelles from phage DNA. The mixture was left for 2 h at 37 °C to allow digestion to proceed to completion before continuing with the preparation as described earlier. Proteinase K digestion was carried out with approximately 1:5000 enzyme:protein (w:w), also at pH 9.0.

RESULTS

Assignments. The natural-abundance ^{13}C NMR spectrum of M13 coat protein is overwhelmingly dominated by detergent resonances arising from the 24 deoxycholate or 12 SDS carbon atoms. The methyl region of unlabeled protein solubilized in deoxycholate is given in Figure 2a. Comparison with the assigned spectrum of cholic acid (Barnes & Geckle, 1982) indicates the peak at 13.24 ppm to be C18 and that at 17.52 ppm to be C19, i.e., the ring-associated methyl groups. Protein methyl resonances can barely be detected above the base line. [3-^{13}C]Alanine-labeled protein, by contrast, shows a series of intense peaks (Figure 2b) in the methyl carbon region. No

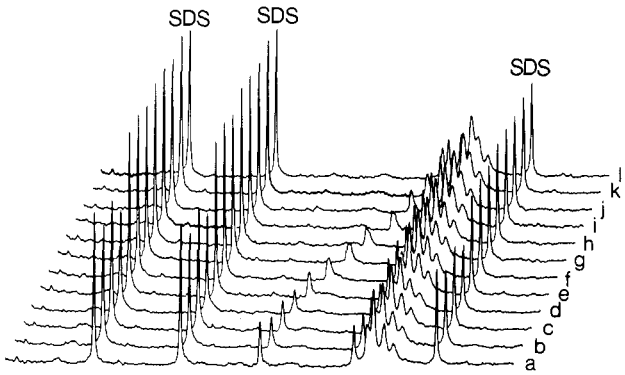

FIGURE 3: pH titration of [3-^{13}C]alanine-labeled coat protein in SDS showing the assignment of peak A′ to Ala-1. Spectra are from 12 to 38 ppm. pH values are (a) 10.15, (b) 9.97, (c) 9.67, (d) 9.50, (e) 9.20, (f) 8.95, (g) 8.70, (h) 8.40, (i) 8.21, (j) 7.94, (k) 7.75, and (l) 7.40. Protein concentration was 1.0 mM, 4000 transients were averaged, and a line broadening of 2 Hz was used.

major differences were observed elsewhere in the spectrum. When the labeled protein was hydrolyzed to its constituent amino acids, very little label was found anywhere except in the alanine methyl group. The extent of incorporation of the labeled amino acid was not determined. The methyl carbons shown in Figure 2b thus arise entirely from the 10 alanine methyl groups, together with 2 of the 3 deoxycholate methyls. One of these (C19) lies directly under one of the protein peaks. The extent of this contribution can be gauged from the upfield deoxycholate resonance (C18) at 13.24 ppm. The alanine resonances are distributed over a range of approximately 3.5 ppm at pH 9.0.

Figure 2b shows that seven resonances can be resolved. Curve fitting of a fully relaxed spectrum obtained in the absence of an NOE (in a gated decoupling experiment) indicates the large peak at 17.09 ppm to arise from three residues and the peak at 16.59 ppm from two residues. All 10 alanine residues are thus accounted for and have been labeled A–J. Peak J actually appears to be composed of at least three overlapping peaks, i.e., exchanging between several similar sites.

A comparable spectrum obtained in SDS micelles is given in Figure 2c. In view of the large differences in structure of the two detergents, the alanine methyl spectra seem remarkably similar. Curve fitting in this case showed the largest peak (16.71 ppm) to arise from 3 carbons and the peak at 16.41 ppm from 2 carbons, again accounting for 10 alanine methyl groups. The alanine methyl carbons in SDS are labeled A′–J′. In this case, the detergent resonances do not overlap with the protein spectrum nor do any of the protein peaks indicate any obvious conformational heterogeneity to be present.

Assignment of Alanine-1 by pH Titration. The N-terminal alanine resonance is easily assigned since the chemical shift of the methyl group is sensitive to the protonation state of the α-amino group (Horsley & Sternlicht, 1968). This proved to be the most downfield resonance (A and A′) in both detergents. Figure 3 shows a pH titration of [3-^{13}C]alanine-labeled coat protein in SDS micelles; a similar set of spectra was obtained in deoxycholate (data not shown). The pK_a values of the α-amino group were determined to be 8.8 in DOC and 8.7 in SDS by a nonlinear least-squares fit to the Henderson–Hasselbalch equation. The rest of the spectrum is insensitive to pH within the limits 8.05–10.25 (DOC) and 7.40–10.60 (SDS). The value of 8.05 was taken for the lower limit in the deoxycholate experiment because of the large changes in micellar structure which occur if the pH approaches the pK_a of the DOC carboxyl (Small, 1971). All spectral changes observed within these ranges were fully reversible.

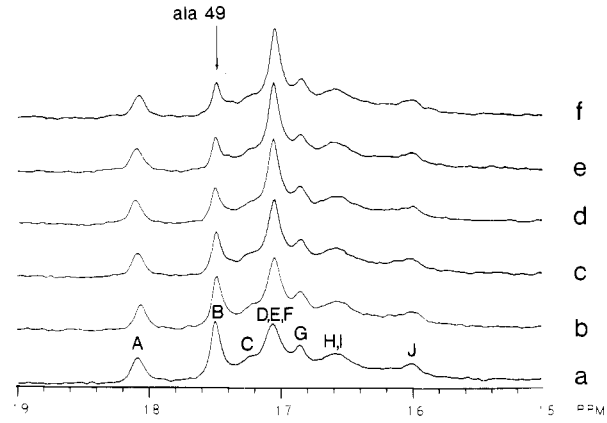

FIGURE 4: Time course of the carboxypeptidase A digestion of coat protein in DOC micelles, illustrating the assignment of Ala-49. Only the spectral region showing labeled methyl carbons is given (0.7 mM coat protein, pH 8.0, 1:100 enzyme:protein mol:mol ratio, 23 °C, 5000 scans/spectrum, 2-Hz line broadening). (a) Zero time; (b) 1.41 h; (c) 2.83 h; (d) 4.25 h; (e) 5.66 h; (f) 7.08 h. Ala-49 (peak B) superimposes a DOC methyl group. Free alanine superimposes peak D, E, F.

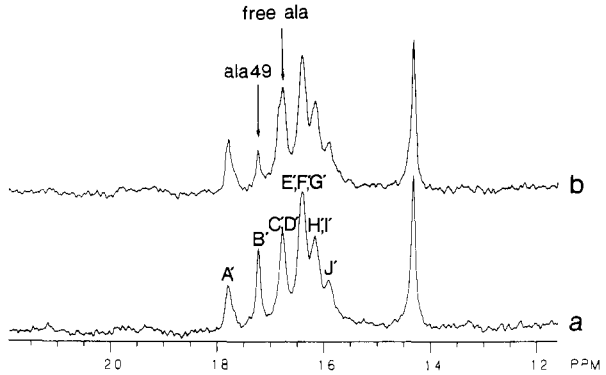

FIGURE 5: Assignment of alanine-49 to peak B′ in SDS micelles (1 mM protein pH 7.8, 22 °C, 1000 scans) by carboxypeptidase A digestion. (a) Before carboxypeptidase treatment; (b) after addition of carboxypeptidase at a 1:15 enzyme:coat protein molar ratio. No further digestion was apparent in succeeding spectra.

Proteolytic Digestion Studies. (*i*) *Carboxypeptidase A Digestion.* Carboxypeptidase A progressively removes C-terminal residues from a protein until it encounters a basic residue. Serine-50 and alanine-49 will thus be cleaved from M13 coat protein, leaving lysine-48 exposed as the new C-terminus. Figure 4 shows a typical time course of the digestion of [3-^{13}C]alanine-labeled coat protein at pH 8.0; peak B (17.52 ppm) disappears, leaving only the underlying DOC methyl resonance. The free alanine resonance superimposes peak D, E, F (17.08 ppm) at pH 8.0. Its chemical shift is pH sensitive, and it can be removed completely by gel filtration on Sephadex G25. Proteolytic digestion studies in SDS are more difficult since the protease rapidly loses activity when exposed to SDS solutions. Nevertheless, large amounts of carboxypeptidase A significantly reduce the intensity of peak B′ (17.42 ppm) (Figure 5). The analogous resonance in both spectra was thus shown to arise from the penultimate residue, Ala-49.

(*ii*) *Trypsin Digestion.* M13 coat protein in SDS micelles (pH 8.0) is unaffected by trypsin (protein:trypsin 10:1 w:w ratio) over a time period of 24 h; however, coat protein in deoxycholate is readily digestible (Woolford & Webster, 1974). Digestion products were monitored by high-voltage paper electrophoresis at pH 6.5. A small amount of trypsin (1:20 000 w:w at 25 °C, pH 9.0) removes two peptides approximately simultaneously over a time period of about 20 h.

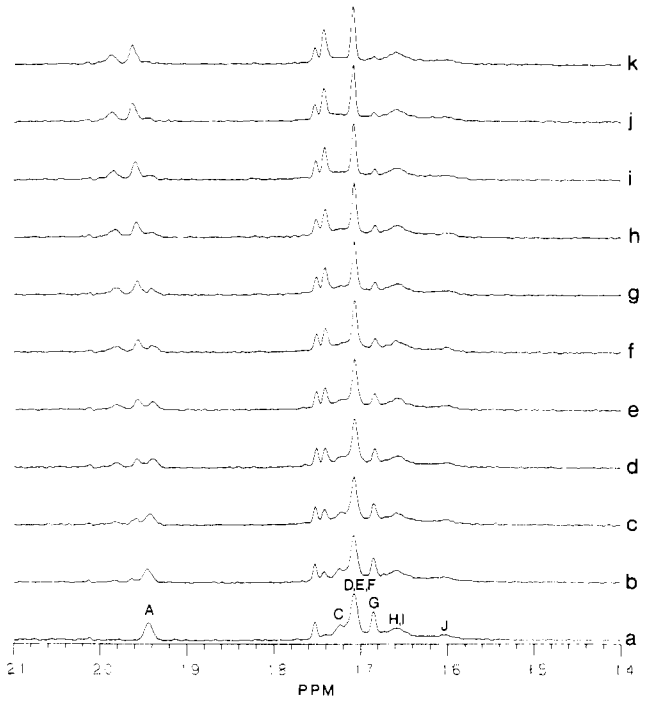

FIGURE 6: Time course of trypsin digestion of carboxypeptidase A treated M13 coat protein in DOC micelles. Ala-49 and Ser-50 were removed prior to digestion under conditions described in the text. [0.7 mM protein, 1:20000 trypsin:protein (w:w) ratio, 23 °C, 5000 scans (1.4 h)]. Resolution was enhanced by weighting the free induction decay by multiplication with a double exponential function. (a) Zero time; (b) 2.81 h; (c) 5.61 h; (d) 8.41 h; (e) 11.22 h; (f) 14.02 h; (g) 16.83 h; (h) 19.64 h; (i) 22.44 h; (j) 25.24 h; (k) 28.05 h. Alanines-7, -9, and -10 were assigned to peaks C and G and a contributor to peak D, E, F.

These peptides were isolated and identified by amino acid analysis as residues 1–8 (acidic) and 49–50 (neutral). Much larger amounts of trypsin (about 1:100 w:w) are required to cleave the other lysine residues at a comparable rate. The enzyme can thus be made selective for two bonds only: Lys-8–Ala-9 and Lys-48–Ala-49. The assignment experiment was simplified further by treatment with carboxypeptidase A prior to separation of the protein-containing micelles from phage DNA. Free alanine was removed from the protein during the Sephacryl S200 gel filtration stage (see Methods) and tryptic cleavage restricted to one site: Lys-8–Ala-9.

There are three alanine residues clustered about lysine-8 (Ala-7, Ala-9, and Ala-10; Figure 1). These should all experience significant chemical shift changes upon cleavage of the Lys-8–Ala-9 bond, especially Ala-9 which becomes the new N-terminal amino acid of the protein. A time course of the limited tryptic digestion of carboxypeptidase A treated coat protein in DOC micelles is shown in Figure 6. Changes occur in only four resonances, indicating that the protein structure in general is not greatly influenced by removal of the terminal eight residues. Gel filtration on Sephadex G25 of the completed digest (not shown) removes intensity from peak D, E, F and completely eliminates the peak at 19.64 ppm, showing these to correspond to the peptide 1–8. It is concluded that alanine residues 7, 9, and 10 give rise to peaks C and G and contribute to peak D, E, F.

(iii) Proteinase K Digestion: Assignment of Hydrophobic Residues. The hydrophobic segment of M13 coat protein in deoxycholate micelles is protected against the action of proteases by the binding of detergent. The hydrophilic ends, by contrast, are accessible to enzymes and are presumably more exposed (Woolford & Webster, 1975). Those alanine residues

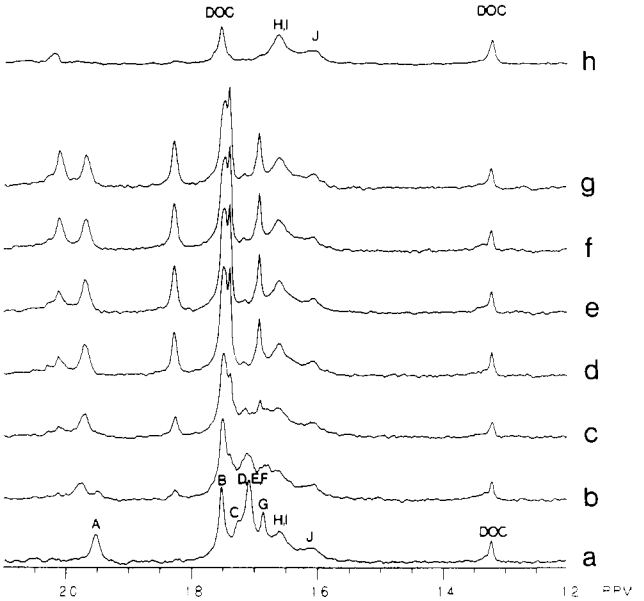

FIGURE 7: Proteinase K digestion of coat protein in DOC micelles. (a) Zero time; (b) 1.13 h; (c) 2.26 h; (d) 3.39 h; (e) 4.52 h; (f) 5.65 h; (g) 6.78 h; (h) sample after gel filtration on G25 to remove small peptides. Peaks H, I, and J are the protected hydrophobic residues. Conditions: 4000 scans/spectrum, 0.7 mM coat protein, 2-Hz line broadening.

Table I: Summary of Resonance Assignments of [3-^{13}C]Alanine-Labeled Coat Protein in Sodium Deoxycholate and Sodium Dodecyl Sulfate Micelles

peak	δ_{DOC}	Deoxycholate assignment	comments
A(1)[a]	19.44 (pH 9)	Ala-1	pH titration
B(1)	17.52	Ala-49	carboxypeptidase A digestion
C(1)	17.25	Ala-7, -9, or -10	trypsin, chymotrypsin
D, E, F(3)	17.08	Ala-7, -9, or -10; Ala-16 and -18	trypsin, chymotrypsin
G(1)	16.85	Ala-7, -9, or -10	trypsin, chymotrypsin
H, I(2)	16.59	Ala-25, -27, or -35	proteinase K
J(1)	16.02	Ala-25, -27, or -35	proteinase K

peak	δ_{SDS}	SDS assignment	comments
A′(1)[a]	19.04 (pH 8.9)	Ala-1	pH titration
B′(1)	17.42	Ala-49	carboxypeptidase A digestion
C′(1)	16.98	unknown	
D′(1)	16.89	unknown	
E′, F′, G′(3)	16.71	unknown	
H′, I′(2)	16.41	Ala-25, -27, or -35	proteinase K
J′(1)	16.05	Ala-25, -27, or -35	proteinase K

[a] Numbers in parentheses are relative integrated peak intensities in the absence of the NOE.

contained within the hydrophobic core have been assigned by treatment with proteinase K, a protease of broad specificity which cleaves the hydrophilic ends of M13 coat protein leaving the hydrophobic core intact. A time course of proteinase K digestion is given in Figure 7a–g, showing the gradual release of small alanine-containing peptides. When no further spectral changes were observed, the small peptides were removed by gel filtration on Sephadex G25, and the micelle-bound residual protein was reconcentrated (Figure 7h). It is thus shown that the two upfield peaks (peak H, I and peak J), of relative intensity 2:1, correspond to the three hydrophobic alanine residues. Alanines-16 and -18 must therefore contribute to peak D, E, F. A similar experiment in SDS (data not shown)

Table II: T_1, T_2, and NOE Values of [3-^{13}C]Alanine-Labeled Coat Protein in DOC and SDS Micelles

peak	δ	T_1 (s)	NOE	T_2 (ms)	$\Delta\nu_{1/2}$(from T_2) (Hz)	$\Delta\nu_{1/2}$(obsd)[a] (Hz)
			Deoxycholate			
A (Ala-1)[b]	19.44	0.62	1.5	119	2.7	13
B (Ala-49)	17.52	0.45	1.3	98	3.2	10
C	17.25	0.34	1.2	62	5.1	11
D, E, F	17.09	0.31	1.2	72	4.4	13
G	16.85	0.30	1.3	80	4.0	9
H, I	16.59	0.25	1.4	20	11.2	20
J	16.02	0.28	1.4	29	16.1	33
			SDS			
A′ (Ala-1)[b]	19.04	0.74	1.5	133	2.4	
B′ (Ala-49)	17.42	0.45	1.5	108	2.9	4
C′	16.98	0.32	1.2	63	5.0	9
D′	16.89	0.32	1.2	67	4.7	10
E′, F′, G′	16.71	0.32	1.3	69	4.6	11
H′, I′	16.41	0.29	1.5	52	6.0	13
J′	16.05	0.25	1.65	40	5.8	13

[a] $\Delta\nu_{1/2}$(obsd) is the line width measured directly by using curve-fitting procedures. [b] pH 9.0 in DOC, pH 8.9 in SDS.

yielded a comparable result; i.e., peak H′, I′ and peak J′ were protected from proteolytic cleavage. All assignments are summarized in Table I.

Analysis of Relaxation Parameters. T_1, T_2, and NOE measurements were made on each resolvable peak in the spectrum of both SDS- and DOC-solubilized coat protein. Sometimes this involved measurements on more than one carbon atom simultaneously (e.g., peak D, E, F in DOC which comprises three individual resonances), and the recorded value thus reflects an average. Relaxation parameters are summarized in Table II. Comparison of the data obtained in DOC with that in SDS revealed no major differences for peaks of similar chemical shift, suggesting no major differences in structure and dynamics in the region of the alanine probe and that the overall correlation times (τ_c) are similar.

To extract dynamic information from data obtained at a single frequency in the absence of knowledge of the overall correlation time, all three relaxation parameters are required. T_2 values were determined directly since contributions to the line width from sources other than T_2 are substantial and cannot be ignored (see Table II). Further, and of potentially wider significance, the suggestion of conformational heterogeneity of the DOC hydrophobic peak (peak J) was reinforced by the observation that both peak J and peak H, I in DOC are very much broader than suggested by the T_2 values. Such heterogeneity of structure, in terms of very broad or asymmetric peaks, has been observed for a number of carbonyl carbon resonances in DOC (G. D. Henry, J. H. Weiner, and B. D. Sykes, unpublished results).

From eq 3, the maximum possible value of η is 1.988, and the minimum is 0.153. The upper limit is expected only for a protonated carbon (i.e., one undergoing dipolar relaxation) and if the extreme narrowing condition is satisfied (Kuhlman et al., 1970). The relatively large NOEs (η = 1.2–1.6) observed for M13 coat protein–detergent complexes suggest that substantial internal motions are occurring within the molecule.

Relaxation data obtained for the alanine methyl groups have been interpreted in terms of the model-free approach (Lipari & Szabo, 1982a) assuming overall motion to be isotropic (see Theory). For a methyl group, the significant motions are rotation about the C2–C3 (α–β) axis and movement of the C2–C3 (α–β) relative to the tumbling of the micelle as a whole. The unknowns at this stage are τ_c (the overall correlation time), τ_e (the effective correlation time of the internal motions), and $\mathcal{S}^2$ (the generalized order parameter). Assuming relaxation to be dipolar, eq 1–3 were used to describe relaxation,

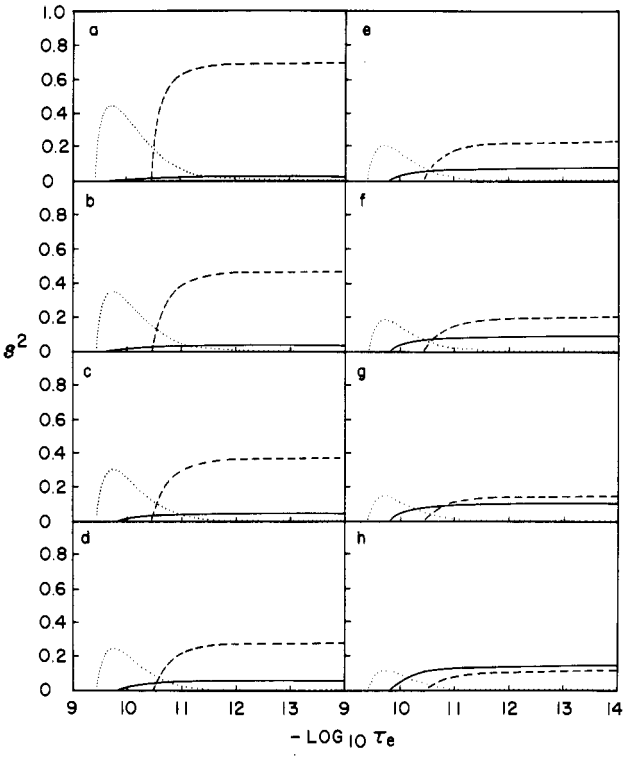

FIGURE 8: Computer-generated curves illustrating the determination of τ_c for Ala-49 in SDS micelles. Possible values of $\mathcal{S}^2$ over all physically reasonable values of τ_e are defined T_1 (---), T_2 (—), and NOE (···) values. τ_c is varied as follows: (a) 30 ns, (b) 20 ns, (c) 16 ns, (d) 12 ns, (e) 9.5 ns, (f) 8 ns, (g) 6 ns, (h) 4 ns. The lines intersect at the "correct" τ_c value, in this example, at 9.5 ns (e). The corresponding $\mathcal{S}^2$ value is 0.06, well below the theoretical maximum of 0.111 for a symmetrically rotating methyl group.

employing eq 4 as the spectral density function. For a methyl group, N = 3 and the C–H bond length was taken as 1.09 Å. Owing to the $1/r^6$ dependence of the relaxation rate on internuclear distances, protons other than those directly bonded to the carbon atom in question do not contribute significantly to its relaxation (Oldfield et al., 1975). A computer program was written to generate values of $\mathcal{S}^2$ while varying τ_e over a physically reasonable range for each of the experimentally determined T_1, T_2, and NOE values. A plot of $\mathcal{S}^2$ vs. $-\log \tau_e$ thus yields three curves corresponding to each parameter. This is illustrated in Figure 8 for Ala-49 (peak B′) in SDS. These curves are of significance only in the region $0 \leq \mathcal{S}^2 \leq 1$ since

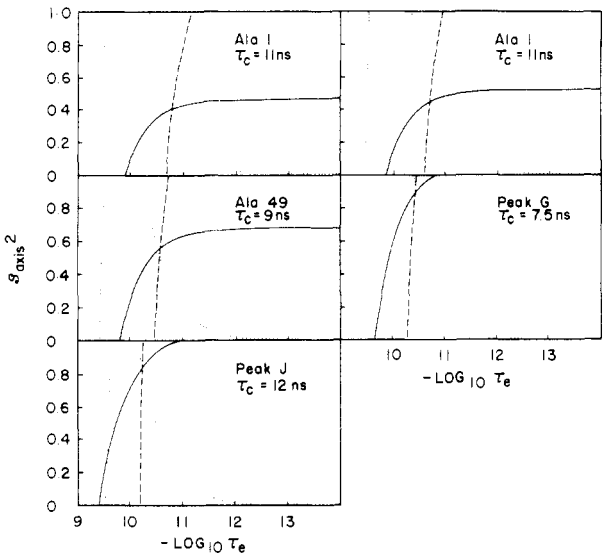

FIGURE 9: Determination of τ_c values for various alanine methyl resonances. Computer-generated curves as in Figure 9 except using $\mathcal{S}_{axis}^2$ (the order parameter with respect to protein backbone motions) in place of $\mathcal{S}^2$. ($\mathcal{S}_{axis}^2 = \mathcal{S}^2/0.111$; $0 \leq \mathcal{S}_{axis}^2 \leq 1$ for a symmetrically rotating methyl group.) (Left panels) Selected well-resolved protein resonances in SDS micelles. (Right panels) Selected well-resolved resonances in DOC micelles. T_1 (- - -); T_2 (—); NOE (···). Average values of these correlation times were used to generate the numbers given in Table III.

the model-free approach endows physical meaning only to numbers within these limits.

The overall correlation time, τ_c, was fitted as follows: the T_1 curves shown in Figure 8 represent the possible combinations of $\mathcal{S}^2$ and τ_e which could give rise to the experimentally determined number, assuming a value for τ_c. The same is true for the T_2 and NOE curves. Since only one value for each of $\mathcal{S}^2$ and τ_e can exist for any individual carbon atom, it follows that the three curves should intersect at a single point if τ_c is chosen correctly. This is also shown in Figure 9; an acceptable fit is achieved by using a correlation time of 9.5 ns (which is a physically reasonable number for the protein–detergent complex). The τ_e value is 27 ps, and $\mathcal{S}^2$ is 0.06.

$\mathcal{S}^2$, the order parameter, relates to the spatial restriction of motion, encompassing both rotation about the methyl group axis and movement of that axis. If the methyl group is assumed to be rotating freely, or jumping between three equivalent sites, this number is necessarily small ($\leq$0.111). The value of 0.06 obtained above is thus well within the possible range. As described under Theory, it is possible to remove the methyl group rotation from the order parameter by considering $\mathcal{S}_{axis}^2$ ($\mathcal{S}_{axis}^2 = \mathcal{S}^2/0.111$). $\mathcal{S}_{axis}^2$, which is the order parameter with respect to the methyl group symmetry axis, varies between 0 and 1. Plots which are exactly analogous to those of Figure 8 may be drawn with $\mathcal{S}_{axis}^2$ replacing $\mathcal{S}^2$ (see Figure 9); practically, these represent a 9-fold expansion of the ordinate. τ_e is unaffected. However, plots of $\mathcal{S}_{axis}^2$ vs. τ_e are more informative than $\mathcal{S}^2$ plots because they relate to the backbone motions of the polypeptide chain, since the C2–C3 bond is rigidly attached to the protein backbone. Thus, in the example above, $\mathcal{S}_{axis}^2$ for alanine-49 in SDS is 0.5, indicating a significant amount of mobility at the C-terminus of the polypeptide chain.

τ_c values were obtained in this way for the most well-resolved single carbon resonances. Figure 9 shows the $\mathcal{S}_{axis}^2$ vs. –log τ_e plots from which the correlation times were derived for both DOC and SDS. Values of 11, 9, and 12 ns were obtained for peak A′ (Ala-1), peak B′ (Ala-49), and peak J, respectively, in SDS. In DOC, peaks A (Ala-1) and G yielded values of 11 and 7.5 ns.

Table III: $\mathcal{S}^2$, $\mathcal{S}_{axis}^2$, and τ_e Values for Labeled Alanine Methyl Groups in DOC- and SDS-Solubilized M13 Coat Protein

peak	assignment	$\mathcal{S}^2$	$\mathcal{S}_{axis}^2$	$\tau_e \times 10^{11}$ (s)
	DOC			
A	Ala-1	0.05	0.5	2.1
B	Ala-49[a]	0.08	0.7	2.2
C	Ala-7, -9, or -10	0.12	1.1	3.1
D, E, F	Ala-7, -9, or -10; Ala-16 and -18	0.10	1.0	3.1
G	Ala-7, -9, or -10	0.11	1.0	3.5
H, I	hydrophobic alanines	0.12	1.1	4.8
J	hydrophobic alanine	0.11	1.0	4.3
	SDS			
A′	Ala-1	0.04	0.4	1.6
B′	Ala-49	0.06	0.5	2.7
C′/D′	unassigned	0.11	1.0	3.1
E′, F′, G′	unassigned	0.10	0.9	3.1
H′, I′	hydrophobic alanines	0.10	0.9	3.1
J′	hydrophobic alanine	0.09	0.8	6.3

[a] Contains contribution from DOC methyl group.

The agreement between values obtained for different peaks in the spectrum is within acceptable limits. The correlation time of a protein may also be estimated from the Stokes–Einstein equation [see Marshall (1978) and Brauer & Sykes (1984)]. Accounting for the apparent partial specific molar volume of the bound detergent, τ_c was estimated to be approximately 6 ns in DOC (Dettman, 1984) and 9 ns in SDS. In fact, the order parameter ($\mathcal{S}^2$ or $\mathcal{S}_{axis}^2$) is not critically sensitive to the choice of correlation time. Taking the example of alanine-49 in SDS micelles, it can be shown that $\mathcal{S}_{axis}^2$ does not change significantly as τ_c is increased from 7 to 13 ns. τ_e, on the other hand, is more sensitive to the choice of τ_c, varying from 22 to 30 ps. We can thus feel confident that our choice of τ_c is reasonable. An average τ_c value (9 ns in DOC, 11 ns in SDS) was used to extract $\mathcal{S}_{axis}^2$ and τ_e from the relaxation parameters measured for all peaks in the spectrum.

With a knowledge of τ_c in hand, it is no longer necessary to use all three relaxation parameters, although the figures given in Table III are actually derived from the T_1, T_2, and NOE data unless indicated. The exceptions are peak H, I and peak J (the hydrophobic residues) in DOC where T_1 and NOE values alone were used to calculate $\mathcal{S}^2$ and τ_e.

The use of an average correlation time generally leads to three intersections of two lines (i.e., a "triangle" of points as shown in Figure 8) rather than a single triple-intersection point. The numbers given in Table III therefore reflect an average of three values; however, the experimental relaxation times generally gave good approximations to a single crossover point. The worst fits were peak D, E, F in DOC and peak E′, F′, G′ in SDS, which both arise from three carbon atoms.

All the recorded values of $\mathcal{S}_{axis}^2$ are less than or equal to 1 within experimental error, as predicted under Theory. The $\mathcal{S}_{axis}^2$ and τ_e values are very similar for assigned and analogous peaks in the spectra of M13 coat protein in DOC and SDS micelles. This suggests that the protein adopts a similar conformation in both detergents. Further, the $\mathcal{S}_{axis}^2$ values clearly fall into two groups: those from the extreme terminal regions of the protein (Ala-1 and Ala-49) which are significantly less than 1 and those from the rest of the protein which approach or equal 1. Large amplitude motions of the polypeptide backbone occur only at the very ends of the protein.

Discussion

Apart from its intrinsic interest as a viral coat protein and an integral membrane protein, the coat protein of bacterio-

phage M13 is a useful system spectroscopically, since it is small and easily purified and labeled amino acids may be incorporated biosynthetically. Alanine methyl groups were chosen as a label because this residue is well distributed between the hydrophobic and hydrophilic domains. Alanine therefore provides comparable information about contrasting sites. This residue in general shows little preference for being in or out of an aqueous environment in a protein (Tanford, 1973). Although an alanine auxotroph (which would ensure almost 100% labeling) is unavailable, alanine was readily taken up by the bacterial cells and used in protein synthesis. Metabolic scrambling of the label was barely detectable in spite of the potential for deamination to pyruvic acid and metabolism through the tricarboxylic acid cycle. Very small amounts of label appeared in certain α-carbon atoms, indicating that this pathway is not entirely absent.

The large number of alanine residues in this protein (10), amounting to 20% of the total, has allowed a good general picture of the molecule to be obtained. Two residues, Ala-1 and the penultimate residue, Ala-49, were assigned very easily in both detergents by pH titration and carboxypeptidase A digestion, respectively. The remainder of the alanine methyl resonances in DOC micelles were successfully assigned to local clusters within the molecule. The lack of detectable tryptic (or chymotryptic) activity in SDS solutions was a hindrance to resonance assignment, although tentative conclusions can be drawn by analogy with DOC. It seems likely that these proteases were rapidly denatured by exposure to SDS solutions, since the Lys-48–Ala-49 bond which is potentially cleavable by trypsin, is accessible to carboxypeptidase A. Furthermore, the N-terminal region is very readily removed in SDS by proteinase K.

An advantage of the alanine methyl group label is that it can be directly related to the motions of the polypeptide backbone at the C2 (α) carbon to which the methyl group is attached. The relevant order parameter is $\mathcal{S}_{axis}^2$. $\mathcal{S}_{axis}^2$ can be interpreted in terms of a model, if required; for example, the diffusion in a cone model (in which the methyl group symmetry axis is allowed to diffuse within a cone of semiangle θ) is physically acceptable. With the use of eq 16b of Lipari & Szabo (1982b), an $\mathcal{S}_{axis}^2$ value of 0.4 (that of the N-terminal alanine in SDS) relates to an angle θ of 58°. Similarly, an angle of 52° is obtained for Ala-1 in DOC and Ala-49 in SDS and 38° for Ala-49 in DOC. As $\mathcal{S}_{axis}^2$ approaches 1, of course, θ approaches zero.

The values of τ_e are more difficult to interpret than $\mathcal{S}^2$ or $\mathcal{S}_{axis}^2$. τ_e contains contributions from both the rate of methyl rotation and the rate of backbone motions. The methyl rotations, however, are very rapid and account for the small values of τ_e (12–60 ps). These fast motions are probably responsible for the large NOE values (up to about 80% of the maximum value). The model-free approach applies specifically to the analysis of fast internal motions. Lipari & Szabo (1982b) provide a set of empirical criteria which determine the applicability of their approach to practical situations. According to their rules, the data presented here should be accurate to within a few percent.

Although some problems still exist in relating these results to possible structures for the coat protein (due to irresolvable or unassignable resonances), two points stand out clearly. First, as far as can be determined by the alanine probe, the nature of the detergent does not greatly affect the dynamics of the protein. Second, the greatest mobility occurs at the extreme ends of the molecule. Considering these observations in order, it is perhaps surprising that detergents with such differing structures and properties as SDS and DOC should have such little effect on the protein structure. Sodium deoxycholate is a bile salt which typically binds only to hydrophobic regions of proteins (Makino et al., 1973). It is generally considered to be a mild reagent and is often used in the isolation of membrane-bound enzymes (Helenius et al., 1979). SDS, on the other hand, is widely considered to be a denaturant. It does not generally discriminate between hydrophilic and hydrophobic regions (Robinson & Tanford, 1975); it binds to a large number of proteins in a strict weight:weight ratio (Reynolds & Tanford, 1970). SDS does not denature proteins in a classical sense but tends to induce secondary structure through hydrophobic interactions (Wu et al., 1981). The induced structure, of course, may be quite different from the native conformation; thus, enzymes are typically inactive in SDS. Nevertheless, for M13 coat protein, the pK_a of the terminal amino group is the same in both SDS and DOC (about 8.8), the assigned residues have similar chemical shifts, τ_c is similar for both detergent–protein complexes, and the T_1, T_2, and NOE values are comparable for peaks across the spectrum. Circular dichrosim spectra (Nozaki et al., 1976) are very similar for DOC- and SDS-bound coat protein, suggesting no major differences in secondary structure. Furthermore, this conformation was maintained in phospholipids, which are presumed to represent the native environment of the protein. It seems, then, that the use of SDS as a solubilizing agent is not unreasonable in the case of M13 coat protein, for which it may provide a membrane-mimetic environment. It should also be noted that the protein remains a dimer even at high SDS:protein ratios (Knippers & Hofmann-Berling, 1966). Possibly the negative charge at the micelle surface is instrumental in maintaining the similar conformations observed in the two detergents.

Our picture of M13 coat protein in either SDS or DOC is currently one in which only the extreme ends of the protein are capable of undergoing large amplitude motions. The rest of the molecule (the hydrophobic core and most of the N-terminal hydrophilic region) is rigid with respect to the protein backbone, and tumbling as the micelle tumbles. It should be pointed out that motions on a slow time scale may well occur but that the NMR experiment is insensitive to correlation functions with decay times longer than τ_c. Natural-abundance ^{13}C NMR experiments in both SDS (Cross & Opella, 1981; Opella et al., 1981) and DOC (G. D. Henry, J. H. Weiner, and B. D. Sykes, unpublished results) have shown approximately eight exceptionally sharp resonances in the α-carbon region, against a fairly broad background, also indicaing that certain regions of the molecule are significantly more mobile than others. It seems likely from our data that these resonances arise from both ends of the molecule, perhaps four from each terminus. This is in agreement with the prediction of Cross & Opella (1980).

This view of the molecule fits in quite well with other available data. CD spectra suggest the protein to be 50% α-helix and 30% β-sheet in both detergents (Nozaki et al., 1976, 1978; Cavalieri, 1976). Laser Raman experiments (Williams et al., 1977) in SDS imply more helix and less β-sheet; nevertheless, the concensus is that about 20% of the protein (10 residues) is unstructured. Furthermore, N–H exchange rates have been shown to be fastest for residues near the ends of the polypeptide chain (Henry et al., 1986).

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Registry No. SDS, 151-21-3; DOC, 83-44-3.

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