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Membrane Protein Conformational Change Dependent on the Hydrophobic Environment†

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ABSTRACT: Two conformational states of the coat protein of the filamentous bacteriophage M13 have been detected in detergent solution by using magnetic resonance techniques. When 3-fluorotyrosine is incorporated in place of the two tyrosine residues in the protein, four ^{19}F nuclear magnetic resonance signals are observed, two for each conformer of the protein. The equilibrium between the two forms can be modulated by pH, temperature, and detergent structure. The rate of interconversion of the isomers is rapid on the minutes time scale but is slow relative to the T_1 relaxation time of the fluorine resonances of ~50 ms. The conformational change between the conformers results in the perturbation of a basic residue in the protein such that this group has a pK_a of ~9.5 in one state which shifts to 10.5 or more in the other conformational state. The temperature dependence of the equilibrium suggests an enthalpy difference of about 10 kcal/mol which is offset by entropy to give nearly zero free energy difference between the states at pH 8.3 in deoxycholate solution at room temperature. This suggests a substantial reorganization of the noncovalent interactions defining the two conformational states. The conformational equilibrium is strongly dependent on detergent structure and the presence of phospholipid in the detergent micelle. The results are not consistent with a strong, specific lipid binding to the protein but appear to be consistent with a more general effect of the overall micelle structure on the conformational state of the protein.

The interactions of lipids and proteins have been examined by a number of physical and biochemical techniques. The classic electron spin resonance studies of Jost et al. (1973) using spin-labeled lipids detected a motionally restricted component of the lipids in the presence of membrane proteins and coined the term "boundary lipid" to describe this motionally restricted component. More recent deuterium magnetic resonance studies of labeled lipids in the presence of proteins suggest that the interaction between most lipids and membrane proteins is characterized by rapid exchange [on the nuclear magnetic resonance (NMR)[1] time scale of 10^{-5}–10^{-6} s] of lipid molecules between the boundary lipid environment and the bulk lipid environment (Oldfield et al., 1978; Paddy et al., 1981). These measurements suggest that this exchange rate is similar to that of lipid diffusing in the absence of protein. Such a result implies that the interactions between lipid and protein are likely to be weak and generally lack the binding specificity that one might associate with the presence of well-defined lipid binding sites along the protein surface. Thus, it appears that the bulk of lipid–protein interactions reflect a nonspecific solvation of the hydrophobic exterior region of membrane proteins by the hydrophobic lipid.

Detergents often mimic the effects of lipid in mediating activity of membrane proteins because they provide the hy-

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[1] Abbreviations: DOC, sodium deoxycholate; CD, circular dichroism; SDS, sodium dodecyl sulfate; DMPC, dimyristoylphosphatidylcholine; DOPC, dioleoylphosphatidylcholine; DPG, bovine diphosphatidylglycerol; NMR, nuclear magnetic resonance; MCI, micellar conformation I of M13 gene 8 protein; MCII, micellar conformation II of M13 gene 8 protein; TFA, trifluoroacetic acid; *L/P*, [lipid]/[protein] ratio; *L/P/D*, [lipid]/[protein]/[detergent] ratio; Tris-HCl, tris(hydroxymethyl)-aminomethane hydrochloride; EDTA, ethylenediaminetetraacetic acid.

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drophobic environment necessary for the protein. While the role of both lipid and detergent specificity for a particular enzyme activity can be assayed with relative ease, the structural interactions of lipids and/or detergents with membrane proteins are more difficult to probe. In this report, we monitor a conformational change in the hydrophobic gene 8 protein of the filamentous bacteriophage M13 (Dettman et al., 1982, 1984). This conformational change is coupled to the chemical nature of the hydrophobic environment.

Gene 8 protein exists as an integral membrane protein during one stage of the viral life cycle (Smilowitz et al., 1972; Denhardt et al., 1978). This small, 5240-dalton protein is the major coat protein of M13, and it differs from the related coat proteins of bacteriophages f1 and fd by only one amino acid (Asbeck et al., 1969; Nakashima & Konigsberg, 1974; Van Wezenbeek et al., 1980). The first 20 amino-terminus residues form an acidic domain, and the central 19 residues comprise the hydrophobic domain, with the remaining carboxy terminus forming a basic protein of 11 residues. The two tyrosines of the protein occur at positions 21 and 24, in the most hydrophobic region. However, their precise location in bilayers and detergent micelles is in dispute (Woolford & Webster, 1975; Chamberlain et al., 1978; Wickner, 1976).

In this paper, we present evidence that the nature of the hydrophobic environment around the filamentous bacteriophage coat protein can modulate a structural change in the protein. This conformational change is monitored by fluorine nuclear magnetic resonance using a coat protein preparation in which 3-fluorotyrosine has been incorporated in place of the tyrosines. In deoxycholate micelles, four resonances are observed, two each for the two conformers of the protein. Addition of phospholipid changes the equilibrium between these conformers. The data suggest that this may not be the result of specific binding to the protein but may result from an overall structural change of the detergent–phospholipid micelle which favors one conformer over the other. This suggests that general features of the structure of the lipid environment, such as bilayer thickness, surface charge, etc., may modulate protein structure and activity in a natural membrane.

Experimental Procedures

Materials. *Escherichia coli* AT2471 (Hfr, *thi*, *relA*, *tyrA*, λ^-) was obtained from Peter von Hippel, Institute of Molecular Biology, University of Oregon, Eugene, OR. *m*-Fluoro-DL-tyrosine was from either Sigma (St. Louis, MO) or Aldrich Chemical Co. (Milwaukee, WI). Bio-Gel A-0.5m was obtained from Bio-Rad Laboratories (Richmond, CA). Sodium deoxycholate, DMPC, DOPC, and beef heart DPG were purchased from Sigma. Octyl glucoside was from Calbiochem (San Diego, CA). Dialysis tubing for protein concentration was size 8, from VWR Scientific Inc. (San Francisco, CA).

Growth of Fluorotyrosyl M13 Phage. M13 Fluorotyrosyl phage were grown as described elsewhere (Hagen et al., 1978) with the following modifications. The media were supplemented with 0.2% glucose and 20 μg/mL methionine. Additional tyrosine was found necessary for maintaining growth. Phage were precipitated several times with 4% poly(ethylene glycol) and banded in CsCl (18 h, 15 °C, 40000 rpm in a Beckman VTi50 rotor). The phage bands were dialyzed against 20 mM Tris-HCl (pH 9.0), 20 mM NaCl, and 1 mM EDTA buffer and lyophilyzed.

Purification of Gene 8 Protein. Phage disruption and gene 8 protein isolation were as described by Woolford & Webster (1975) with the following changes: solubilization of phage solutions up to 20 mg/mL was done in 48 mM DOC, 10 mM Tris-HCl, and 1 mM EDTA, pH 8.5. After addition of 1–3 drops of $CHCl_3$, the solutions were incubated 1 h at 37 °C, applied to a Bio-Gel A-0.5m column (2.5 × 90 cm) equilibrated with 30 mM DOC, 10 mM Tris, 1 mM EDTA, and 0.2% NaN_3, pH 8.5, and eluted with the equilibration buffer. The protein fractions were concentrated into the desired buffer by vacuum dialysis and never lyophilized. Protein concentrations were assayed by $E_{280} = 1.65$ cm^2 mg^{-1}.

Determination of Fluorotyrosine Incorporation. Samples of fluorotyrosyl gene 8 protein were dialyzed into 0.05 M NH_4HCO_3, and aliquots of 3 nmol were hydrolyzed with constant-boiling HCl at 110 °C for 20 h. The hydrolysate was precolumn derivatized with *o*-phthalaldehyde, and the amino acids were separated on an Altex ultrasphere ODS Column using reverse-phase high-performance liquid chromatography on a Beckman 322 liquid chromatograph. The method is described in detail elsewhere (Kehry et al., 1983; Turnell & Cooper, 1982). Detection was by fluorescence spectroscopy on a Gilson Spectra-Glo fluorometer fitted with 330–380-nm excitation and 430–600-nm emission filters. Derivatized 3-fluorotyrosine eluted 2.5 min after tyrosine. The degree of percent incorporation was calculated from the ratio of the standardized intensities of 3-fluorotyrosine and tyrosine and was found to be 76% for the material used in this study.

NMR Methods. All ^{19}F NMR spectra were obtained at 339 MHz on a Nicolet NT-360 spectrometer equipped with an Oxford instruments superconducting solenoid. The spectrometer was operated in the Fourier transform mode with a sweep width of ±2500 Hz, a pulse width of 27 μs (90°), an acquisition time of 200 μs, and a 1-s delay between transients. For spin–lattice relaxation measurements (T_1), the inversion–recovery sequence (180–τ–90) was used, and the recycle delay was increased to 1.7 s. The selective saturation experiment employed a long 180° selective pulse of 12 ms generated by a gated, heteronuclear decoupler. The nonselective 90° observed pulse was 27 μs, and the recycle delay was 1.0 s. Temperature control was achieved with a computer-controlled Nicolet variable-temperature regulator. Integrated intensities for equilibrium constant determinations were derived from deconvoluted spectra by using Nicolet software. All chemical shifts are reported relative to internal trifluoroacetic acid (TFA) at pH 8.7, and negative shifts are upfield from TFA.

CD Methods. Circular dichroism measurements were performed on a Jasco J-500C spectrometer equipped with a Lauda K-2/R (Brinkmann Instruments) for temperature regulation. The instrument was calibrated with *d*-10-camphorsulfonic acid. All spectra at a given temperature were taken in triplicate and corrected for buffer contributions at the same temperature. Protein concentrations were determined on a Cary 15 UV spectrophotometer.

Other Methods. Detergents were exchanged by extensive dialysis against the desired buffer. For phospholipid-containing samples, the desired amount of lipid was added as a solid to a solution of gene 8 protein in the appropriate detergent containing buffer. All pH values are direct meter readings from a Radiometer pH Meter 26.

Results

Demonstration of Two Protein Conformations in Detergent Micelles. Figure 1A shows the ^{19}F NMR spectrum of gene 8 protein with 3-fluorotyrosine incorporated into the protein to a level of 76% in place of tyrosine. The protein has been solubilized in deoxycholate. The spectrum shows the presence of four resonances, a relatively intense pair of downfield lines at −61.1 and −61.5 ppm and a less intense upfield pair of lines

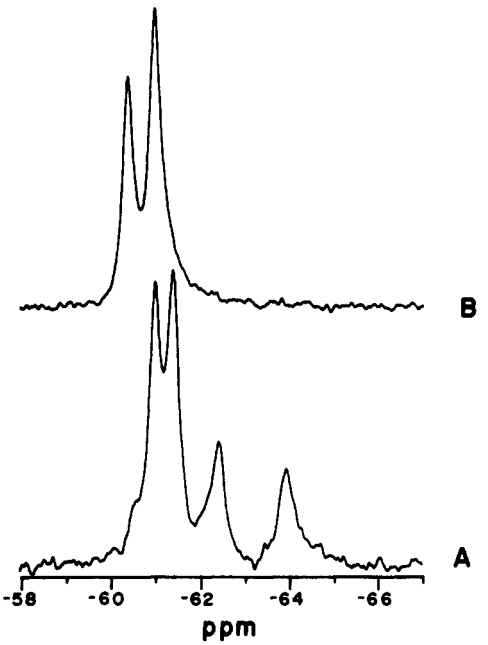


FIGURE 1: ^{19}F NMR spectra at 339 MHz of M13 3-fluorotyrosyl gene 8 protein at 14 °C, pH 8.3, in deoxycholate or octyl glucoside micelles. The conditions for (A) were 2.0 mg/mL (0.39 mM) protein, 30 mM DOC, and 20 mM $NaHCO_3$ diluted with 14% D_2O and for (B) 2.2 mg/mL (0.42 mM) protein, 30 mM octyl glucoside, and 20 mM $NaHCO_3$ diluted with 28% D_2O. Both spectra were 1500 transients with a line broadening of 25 Hz. Shifts are relative to trifluoroacetate as an internal standard.

at −62.5 and −64.0 ppm. Figure 1B shows the spectrum obtained when the same preparation of gene 8 protein was solubilized in the detergent octyl glucoside. Only the two downfield resonances are observed at −60.4 and −61.0 ppm. Thus, it appears that the two tyrosine residues of gene 8 protein each give rise to single resolved resonances in octyl glucoside.

However, in deoxycholate, there appear to be two resonances for each tyrosine. The arguments presented below strongly suggest a second conformational state of the protein in the presence of this detergent. In addition, these two conformations interconvert in a reversible fashion.

Under all conditions in deoxycholate, we find the integrated intensities of each resonance of the downfield pair to be equal. Similarly, the integrated intensities of each resonance in the upfield pair of lines are always equal whenever these lines are sufficiently intense to quantitate. However, the integrated intensity of the downfield pair is not simply related to that of the upfield pair. These observations are most consistent with the view that the protein exists in two distinct conformational states, one represented by the downfield pair of resonances and the other represented by the upfield pair. For the purposes of further discussion, we will refer to the form represented by the downfield pair as micellar conformation I (MCI) and that represented by the upfield pair as micellar conformation II (MCII).

The spectrum shown in Figure 1A was found to be independent of protein concentration over about a 25-fold range of protein concentration (0.2–5 mg/mL) in the same buffer and detergent and at the same temperature (data not shown). This appears to rule out any association–dissociation phenomenon to account for the two conformations seen in Figure 1A.

The reversibility of the interconversion between MCI and MCII was illustrated in Figure 2. This shows a series of spectra in which the temperature is varied from 4 to 54 °C in deoxycholate with bicarbonate buffer (pH 8.7 at 25 °C).

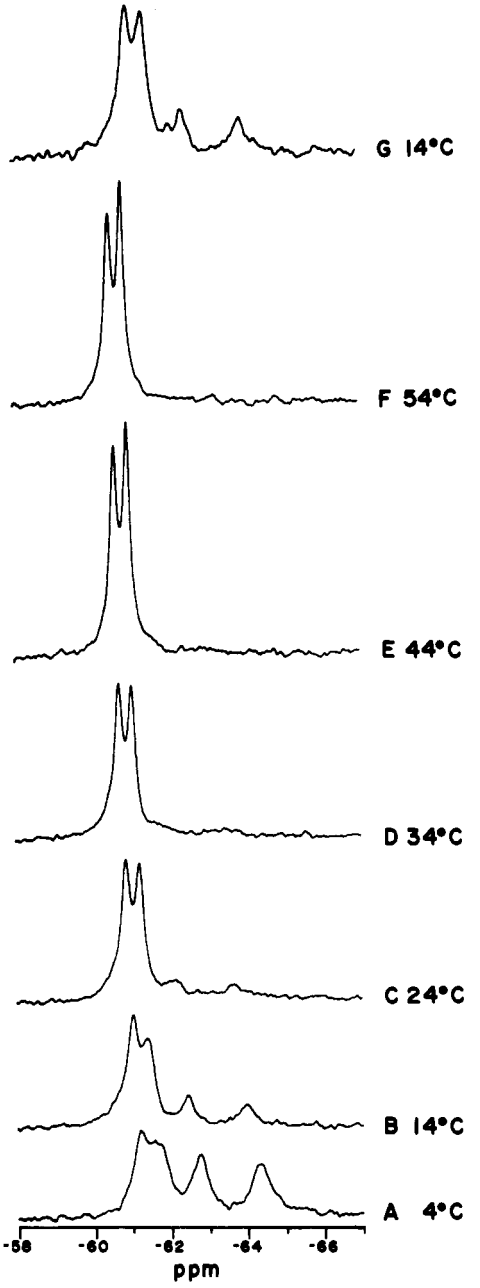


FIGURE 2: Temperature dependence of the interconversion between MCI and MCII of M13 3-fluorotyrosyl gene 8 protein. The spectra were 2.7 mg/mL gene 8 protein in 30 mM DOC and 20 mM $NaHCO_3$, pH 8.7, diluted with 33% D_2O at (A) 4, (B) 14, (C) 24, (D) 34, (E) 44, (F) 54, and (G) 14 °C. Spectrum G was recorded after acquiring spectra A–F in random order and illustrates the reversibility of interconversion. All spectra were 2000 transients with a line broadening of 25 Hz.

MCI is seen to increase from 54% of the total area at 4 °C to nearly 100% above 44 °C. Even after several hours at 54 °C (or below), any previous equilibrium can be reestablished on return to the original temperature. The spectrum in Figure 2G illustrates this reversibility. It was recoeded after the series of spectra (A–F) were accumulated in which the temperature was varied from 4 to 54 °C before returning to 14 °C. The spectra obtained before and after heating are essentially indistinguishable.

When spectra were recorded at 64 °C, the resonances were observed to broaden substantially. Cooling of the sample to a lower temperature did not regenerate a low-temperature spectrum. We interpret this as an essentially irreversible aggregation of the protein and did not explore temperatures

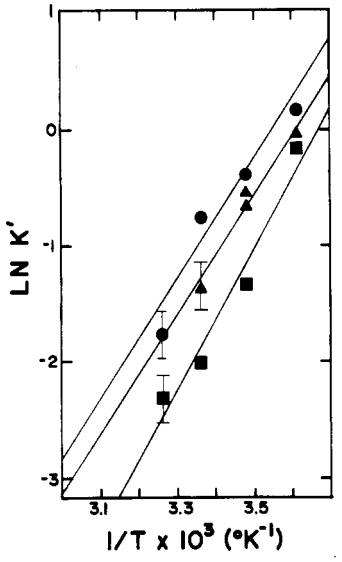


FIGURE 3: van't Hoff plot of the interconversion between MCI and MCII. Apparent equilibrium constants were obtained from experiments similar to Figure 2 and calculated as described under Experimental Procedures. Exact experimental conditons were the following: 2.7 mg/mL protein, 30 mM DOC, and 20 mM $NaHCO_3$, pH 8.7, diluted with 14% D_2O; 2000 transients, 25-Hz line broadening; (●) 2.0 mg/mL protein, 2.0 mg/mL DMPC, 30 mM DOC, and 20 mM $NaHCO_3$, pH 8.3, diluted with 14% D_2O; 1500 transients, 50-Hz line broadening; and (▲) 1.8 mg/mL protein, 1.8 mg/mL DOPC, 30 mM DOC, and 20 mM $NaHCO_3$, pH 8.8, diluted with 33% D_2O; 3000 transients, 25-Hz line broadening. The lines were calculated by a linear least-squares program, and the derived enthalpies are (■) −12.2, (●) −10.0, and (▲) −10.2 kcal/mol.

above 54 °C for equilibration studies.

Under reversible conditions, we write the equilibration of the two conformers as

$$\text{MCI} \rightleftharpoons \text{MCII} \tag{1}$$

and define an apparent equilibrium constant

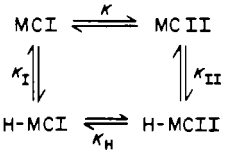
$$K' = [\text{MCII}]/[\text{MCI}] \tag{2}$$

The relative concentrations of each species can be calculated from the relative areas of each of the pairs of resonances. A van't Hoff plot of these data (Figure 3) yields an enthalpy for the interconversion of the conformers of −12 kcal/mol at pH 8.7. The entropy change is −42 entropy units.

The spin–lattice relaxation rate, $1/T_1$, was determined for each of the four resonances seen in Figure 1A by standard inversion recovery techniques. In deoxycholate–bicarbonate buffer at pH 8.3, and 14 °C, these values are similar, ranging from 20 to 27 s^{-1}. From these data, it is possible that the rate of chemical equilibration of MCI and MCII could cause averaging of the $1/T_1$ values for the pairs of lines connected by the exchange process. This possibility was ruled out, however, by using a selective inversion technique (Dahlquist et al., 1975). In the data shown in Figure 4, the most upfield line has been selectively inverted by a long, weak pulse. After a time interval, a nonselective observation pulse was employed to monitor relaxation and/or exchange in the system. The data are presented as difference spectra showing the position of the excess magnetization introduced by the selective pulse at the various times indicated. As seen in the figure, no magnetization was transferred to another chemical environment during the times used. Only the decay of the excess magnetization originally produced by the selective irradiation is observed. This decay was found to be exponential with a rate of 31 s^{-1}, in reasonable agreement with the value of 27 s^{-1} determined by the nonselective method. Thus, chemical exchange must be slow relative to these T_1 relaxation rates but has reached equilibrium in the time necessary to adjust the temperature and begin spectral accumulation (about 0.5 h).

Investigation of the pH dependence of MCI and MCII was undertaken to explore the possibility that a simple protonation–deprotonation of the phenol moiety of the tyrosine rings or some other ionizable group accounted for the two micellar conformations. The deoxycholate–gene 8 protein micelles were titrated over a pH range of 7.5–12.0 at 4 °C where the equilibrium between the conformers was easily determined and chemical shifts accurately measured. Increasing pH shifts the equilibrium toward MCI with essentially 100% MCI above pH 10.5 (Figure 5).

The data were quantified by using the simple scheme shown below in which MCI and MCII have a proton binding residue whose proton dissociation constants are K_I and K_{II}, respectively. The unprotonated and protonated forms of MCI and MCII equilibrate with equilibrium constants K and K_H. Using



NMR, we measure an apparent equilibrium constant K' which represents the ratio of all forms of MCII to MCI.

$$K' = \frac{\Sigma[\text{MCII}]}{\Sigma[\text{MCI}]} = \frac{[\text{MCII}] + [\text{H–MCII}]}{[\text{MCI}] + [\text{H–MCI}]} \tag{3}$$

If K_H equals the pH-independent microscopic constant:

$$K_H = \frac{[\text{H–MCII}]}{[\text{H–MCI}]} \tag{4}$$

then the measured constant, K', can be related to K_H and the ionization constants by the relationship

$$K' = K_H \frac{[\text{H}] + K_{II}}{[\text{H}] + K_I} \tag{5}$$

The value of K' will be pH independent and equal to K_H at low pH. As pH increases and [H] becomes comparable to K_I, the value of K' will decrease until [H] is lower than K_{II}, when again the value of K' will be pH independent. A plot of the experimentally determined value of K' vs. pH is shown in Figure 6. The value of K' is essentially independent of pH from pH 7.5 to 8.5 and then decreases to an immeasurably small value at values greater than 10.5. The solid line in Figure 6 has been calculated by using eq 5 with K_H equal to 1.1 and pK_I equal to 9.5. The ionization constant of the ionizable group in MCII, K_{II}, is too small to be measured. This suggests that pK_{II} must be 10.5 or larger.

Figure 7 shows the pH dependence of the chemical shift of the downfield line of MCI as a function of pH as taken from Figure 5. The solid line is a theoretical single titration curve for a group of $pK_a = 10.4$. This pK_a represents a group in MCI which changes the environment of the fluorotyrosine residue. It does not appear to be the same group which modulates the conformational equilibrium since its pK_a is 1 unit different from pK_I.

Protein–Detergent–Lipid Micelle Conformations. Addition of phospholipids to gene 8 protein in deoxycholate micelles modulates the equilibrium between the protein conformations. Figure 8 presents the spectra obtained in the presence of various amounts of the lipid (DMPC) and deoxycholate at constant protein concentration, pH, and temperature. Comparison of Figure 8A (without DMPC) and Figure 8B (with DMPC at 2 mg/mL) clearly shows the differential effects of the addition of phospholipid to the solution. The most upfield

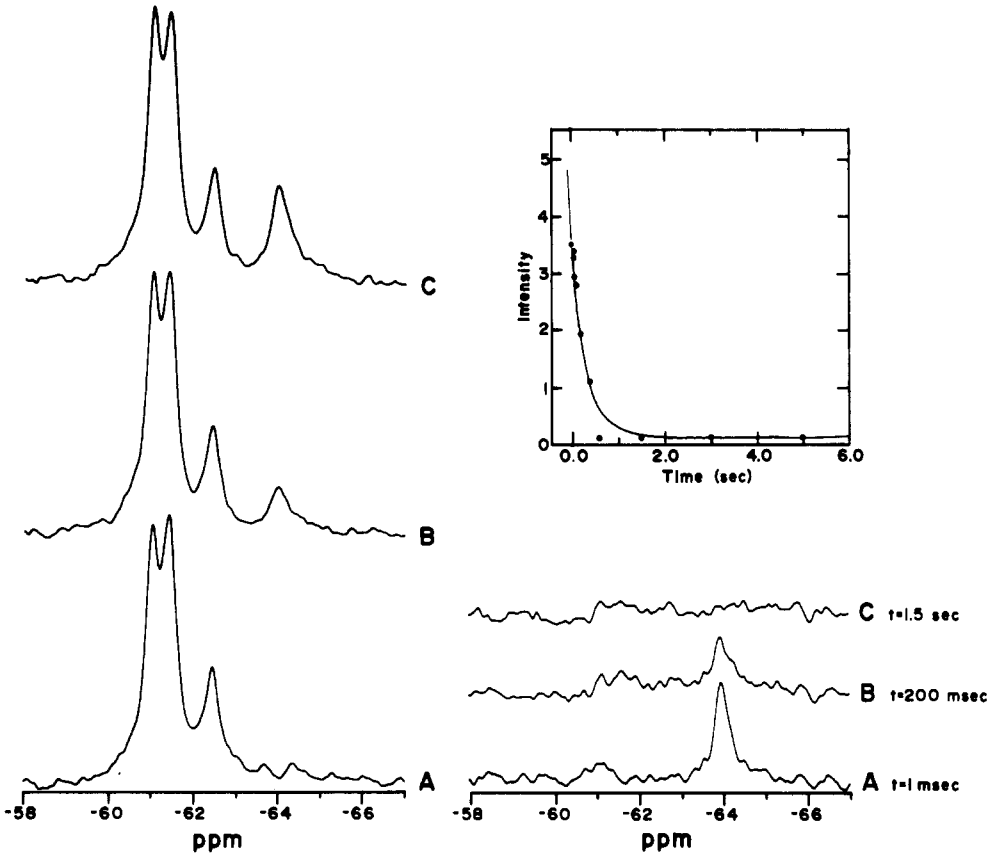


FIGURE 4: Selective saturation of the most upfield resonance of fluorotyrosyl gene 8 protein in DOC micelles. The left panel shows representative spectra obtained by waiting t seconds before application of the observed pulse. The excess magnetization is shown in the right panel where the same spectra are displayed as difference spectra from the final spectrum of the series in which t was much greater than $5T_1$ (t = 5 s). The inset shows the decay of excess magnetization plotted against t. The rate of decay is 31 s^{-1}. Conditions were the same as in Figure 1A except 1200 transients were collected, and the line broadening was 50 Hz.

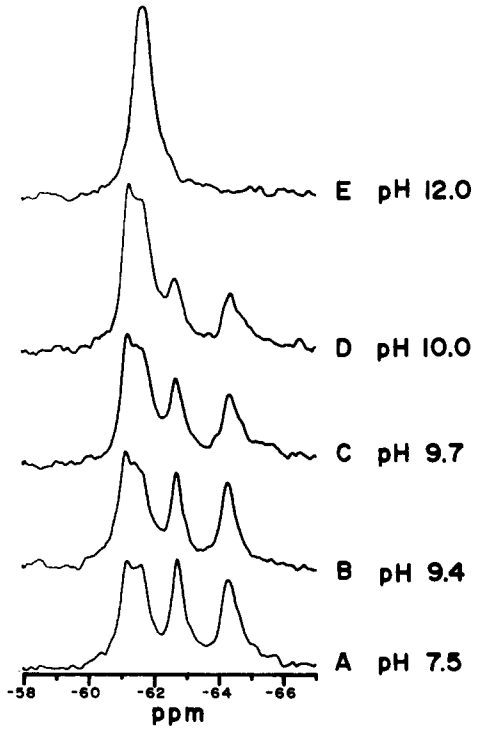


FIGURE 5: pH dependence of interconversion between MCI and MCII of gene 8 protein in DOC micelles at 4 °C. These spectra are typical of the extrema and midpoint of the titration and were taken at pH values of (A) 7.5, (B) 9.4, (C) 9.7, (D) 10.0, and (E) 12.0. Conditions were 1.5 mg/mL gene 8 protein in 30 mM DOC and 20 mM $NaHCO_3$ diluted with 33% D_2O. All spectra have 50-Hz line broadening and either 2000 (A) or 3000 (B–E) transients.

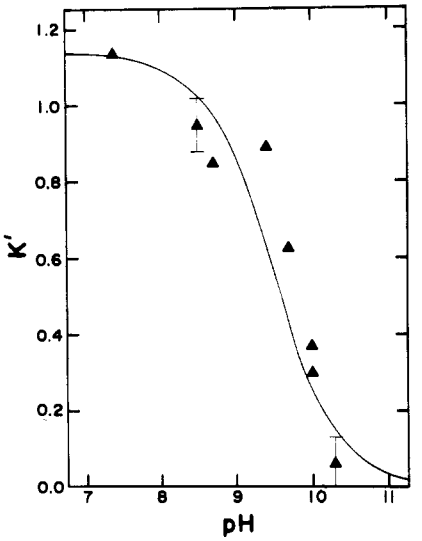


FIGURE 6: pH titration curve of MCI–MCII equilibrium. The solid line is the theoretical curve derived from eq 5 (see Results) with pK_I = 9.5 and K' = 1.14. The pK_I and K values were derived from the linear least-squares best-fit break point and intercept, respectively, of a ln K' vs. pH plot of the data from Figure 5 (not shown). The solid triangles are experimentally determined apparent equilibrium constants from Figure 5 by measuring the ratio of the integrated intensities of the resonances corresponding to MCII and MCI.

resonance shifts 1 ppm downfield while the most downfield resonance is substantially broadened. Simultaneously, there is an increase in the relative amount of MCII observed. As more lipid is added, the equilibrium shifts back to favor MCI (Figure 8C). There are at least two explanations for this

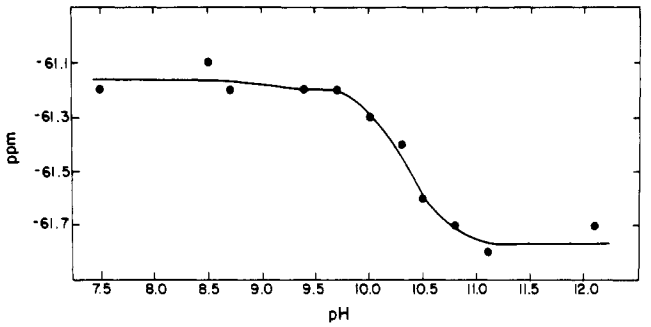


FIGURE 7: pH titration curve of fluorotyrosine of gene 8 protein represented by the most downfield resonance in Figure 5. Chemical shifts were taken from the spectra shown in Figure 5 and are relative to internal TFA. The solid line represents a theoretical single titration curve of $pK_a = 10.4$.

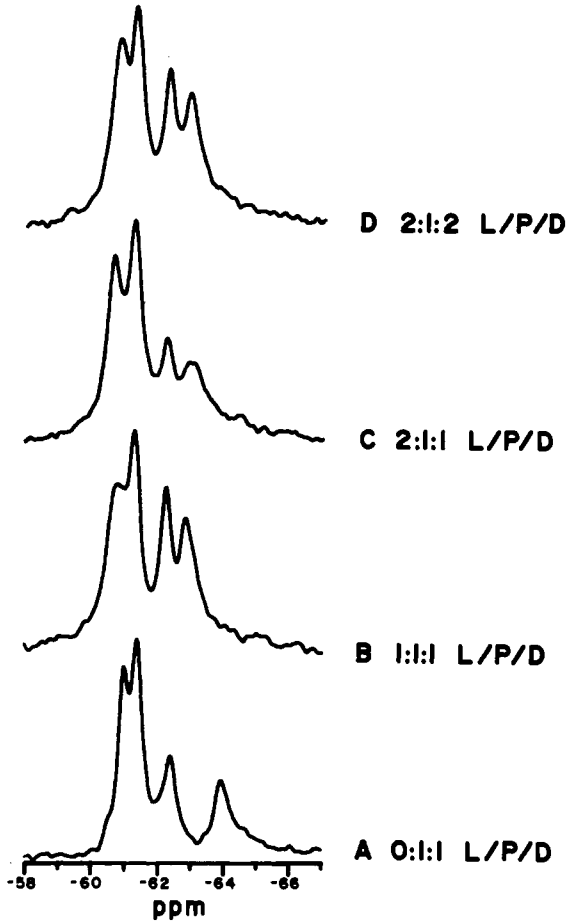


FIGURE 8: Reversible phospholipid titration of 3-fluorotyrosyl gene 8 protein in deoxycholate micelles at 14 °C. Successive additions of solid DMPC were made to solutions of 2.0 mg/mL protein, 30 mM DOC, and 20 mM $NaHCO_3$, pH 8.3, diluted with 33% D_2O. (A) No DMPC; (B) 2.0 mg/mL DMPC; (C) 4.0 mg/mL DMPC; and (D) no further DMPC but solid DOC added to double the DOC concentration (40 mM). The spectra were 1500 transients with a line broadening of 50 Hz.

phenomenon. In one case, the lipid is binding to this protein, perhaps in a distinct site, which results in a change in the distribution of conformations. Alternatively, the structure of the protein–lipid–detergent micelle varies with lipid concentration, causing the change in the protein conformational equilibrium. Figure 8D shows the spectrum which results when the detergent is increased to generate the same lipid to detergent ratio but twice the ratio of lipid to protein as employed for the spectrum shown in Figure 8B. The spectra observed in Figure 8B and Figure 8D are nearly identical. This result rules out a strong stoichiometric binding of the lipid to the protein. The results are consistent either with weak binding to a distinct site(s) or with the structure of the detergent–lipid micelle being responsible for the alteration in the conformational equilibrium. This latter view is consistent with the observation that the addition of the DPG or DOPC gave results very similar to DMPC (data not shown), suggesting little lipid specificity in the interaction. It is also possible that distinct but nonspecific binding sites exist.

Experiments to determine the temperature and pH dependence of the micellar conformations in the presence of lipid were qualitatively consistent with the results obtained in deoxycholate micelles alone. Enthalpies of interconversion were determined to be −10 kcal/mol for DMPC at pH 8.3 and −10.1 kcal/mol for DOPC at pH 8.9 (Figure 3).

Circular Dichroism. Previous circular dichroism (CD) and Raman studies [see Nozaki et al. (1978) and Fodor et al. (1981) and references cited therein] have shown that the secondary structure of M13 (fl, fd) gene 8 protein depends on history of treatment and environment of the protein. The two micellar conformations we observe could reflect an equilibrium between the various forms previously detected by circular dichroism. Thus, we performed several CD experiments to test this possibility.

The CD spectra of fluorotyrosyl gene 8 protein in Figure 9A illustrate the temperature dependence of gene 8 protein in deoxycholate micelles. As temperature increases, there is a slight overall decrease in the ellipticity. At 4 °C, the molar ellipticity value at 208 nm is −20 100 deg cm^2 $dmol^{-1}$, and the corresponding value at 44 °C is −18 300 deg cm^2 $dmol^{-1}$. Assuming an ellipticity value of +500 for a random coil (Nozaki et al., 1978), we calculate only a 10% change in total helicity of gene 8 protein for this transition. The CD spectrum of gene 8 protein in octyl glucoside micelles shows a similar temperature dependence (Figure 9B). Under these conditions at 4 °C, the molar ellipticity value at 208 nm is −15 500 deg cm^2 $dmol^{-1}$, and the corresponding value at 44 °C is −14 100 deg cm^2 $dmol^{-1}$. This transition also reflects a 10% change in total helicity. Finally, the temperature-dependent CD spectra of gene 8 protein in deoxycholate–lipid (DMPC) mixed micelles are shown in Figure 9C. The molar ellipticity at 208 nm ranges from −20 600 to −18 800 deg cm^2 $dmol^{-1}$ and reflects a 9% change in overall helical content. These data suggest that the helical contents of MCI and MCII are nearly equal. Comparison of the molar ellipticity values suggests that MCI and MCII are conformational substates of the "b" form described by the studies of Nozaki et al. (1978) and Fodor et al. (1981). This point is further considered under Discussion.

Discussion

The fluorine NMR studies described here have detected two spectroscopically distinct forms of the bacteriophage coat protein encoded by gene 8 of the filamentous bacteriophage M13 when fluorotyrosine is substituted for tyrosine. The fact that the ratio between the two forms, MCI and MCII, varies reversibly and that this reversible ratio depends on the chemical nature of the detergent solvent, temperature, and pH rules out a number of trivial explanations for the observations. For example, it is not possible that the two forms are simply different in covalent structure as a result of proteolysis, processing, or partial incorporation of the 3-fluorotyrosine used to replace the two tyrosines of the protein.

Sedimentation and electrophoresis studies in the anionic detergents SDS and deoxycholate demonstrated that gene 8 protein can exist as a dimer (Cavalier et al., 1976; Makino et al., 1975). To exclude that an equilibrium of protein monomers and dimers could explain the two conformers, we determined the protein concentration dependence of the MCI

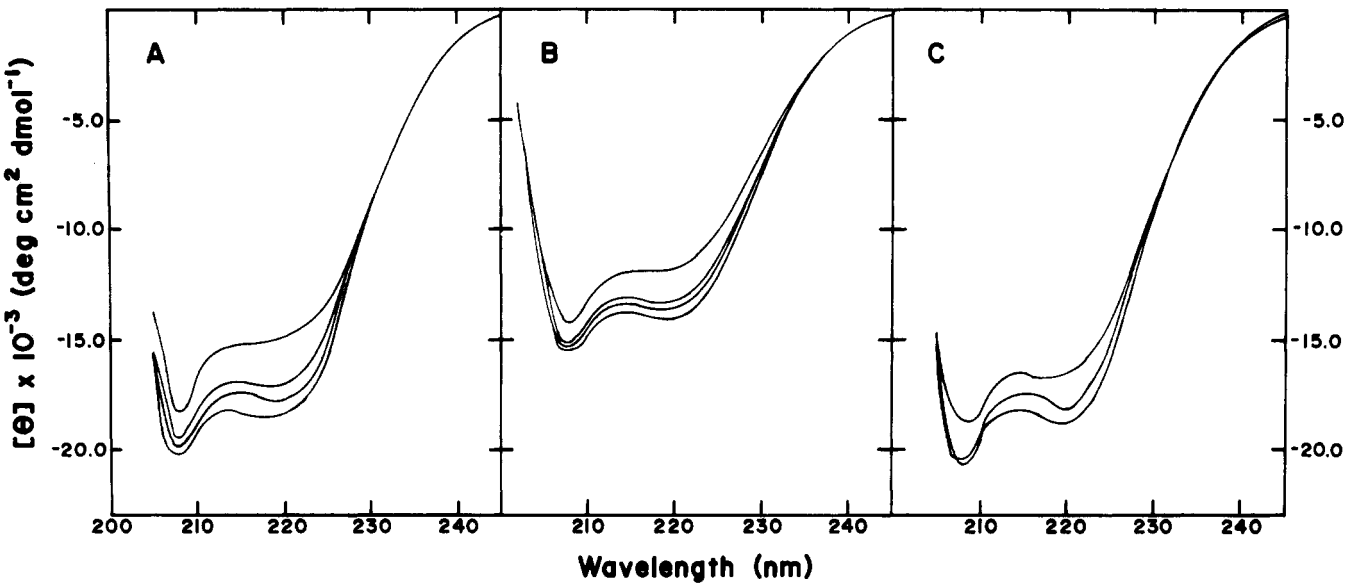


FIGURE 9: CD spectra of 3-fluorotyrosyl gene 8 protein shown as a function of temperature in deoxycholate micelles, octyl glucoside micelles, and deoxycholate–lipid mixed micelles. Note that the ellipticity decreases as the temperature increases. (A) 0.2 mg/mL protein in 30 mM DOC and 20 mM $NaHCO_3$, pH 8.9, at 4, 14, 24, and 44 °C. (B) 0.2 mg/mL protein in 30 mM octyl glucoside and 20 mM $NaHCO_3$, pH 9.3, at 4, 14, 24, and 44 °C. (C) 0.2 mg/mL protein and 0.2 mg/mL DMPC in 30 mM DOC and 20 mM $NaHCO_3$, pH 8.9, 4, 14, and 44 °C.

and MCII equilibrium. We observed no change in equilibrium over a 25-fold range of protein concentration. Therefore, we conclude that MCI and MCII do not arise from association or dissociation of protein dimers or other multimer interactions.

The two conformations appear to represent more global structural differences rather than localized differences in the immediate region around the substituted tyrosine residues. If the structural differences were the result of strictly local changes, such as aromatic ring "flipping", one would expect the detailed energy differences between the two substructures to be independent and dissimilar at the two tyrosine residues. This would argue for different equilibrium constants at each tyrosine residue in these local structures. However, we observe the same equilibrium constant for the tyrosines. Thus, we conclude either that the factors which control changes in two different microenvironments are exactly identical or, more likely, that the two tyrosines are coupled to a structural transition in the protein which changes the environment of both tyrosine residues simultaneously.

The observed enthalpy difference of 10 kcal/mol between the two states also argues for more extensive conformational alterations than a simple aromatic ring flip. If ring flipping were responsible, then the equilibrium energy difference between MCI and MCII would only result for each tyrosine residue having a different interaction at the 3-position fluorine and the 5-position hydrogen with the same microenvironment. It is not expected that the energy difference could be as large as 10 kcal/mol since the aromatic fluorine is nearly the same size as a proton and its electrons are interacting with the aromatic system. Again, independent partitioning of each tyrosine residue into its two conformations would be expected, and this behavior is clearly not observed.

The observed slow rate of interconversion between these conformers further supports the notion of a fairly extensive structural change involved in the equilibrium between MCI and MCII. The equilibration of conformers is complete in the 0.5 h necessary between successive ^{19}F NMR spectra. A careful examination of the data presented in Figure 5 suggests that some magnetization may be transferred from the most upfield to the most downfield resonance in a time comparable to T_1. The data are only suggestive, but it is possible that interconversion occurs at a rate closer to 10 s^{-1} than to the 10^{-3} s^{-1} value needed to complete equilibration in 0.5 h. Sykes and co-workers measured rotational rates for 3-fluorotyrosine-substituted gene 8 protein in small phospholipid vesicles (~310-Å diameter) and found rates faster than 10^6 s^{-1} (Hagen et al., 1978). Their data support our conclusion of more extensive structural changes occurring between MCI and MCII.

The conformational equilibrium appears to be modulated by an ionizable group in MCI whose pK_a is 9.5. The pK_a of this residue shifts to a value greater than 10.5 in MCII and accounts for the equilibrium pH dependence. While this pK_a value is suggestive of the tyrosine phenol moiety, careful analysis of the spectra tends to rule out any major role for it in a modulating MCI–MCII equilibrium. Free 3-fluorotyrosine has a pH-dependent chemical shift of ~0.5 ppm centered about a pK_a of 8.0 as the phenol moiety ionizes (Sykes & Weiner, 1980; Lu et al., 1976). Of the two downfield resonances corresponding to MCI, only the most downfield one shifts with pH whereas the other is invariant in position (Figure 5). In contrast, all four resonances exhibit temperature-dependent chemical shifts over the same equilibrium constant range (Figure 2). Quantitation of chemical shift vs. pH yields a pK_a of 10.4 for the phenol moiety of the 3-fluorotyrosine represented by the most downfield resonance (Figure 7). This pK_a is unexpectedly high but consistent with the high pK_a value of 12.4 for the tyrosine residues of gene 8 protein in SDS micelles at 45 °C as measured by Cross & Opella (1981).

It appears that the ionization state of at least one fluorotyrosine residue (represented by the upfield resonance of MCI, Figure 5A) is completely independent of the protein conformational equilibrium in deoxycholate micelles since by chemical shift criterion, it does not change ionization state over a pH range of 7.5–12. The ionization of the other tyrosine residue (the downfield resonance of MCI, Figure 5A) is also distinct from the ionizable group coupled to the protein conformational equilibrium, since its pK_a differs by about 1 pH unit from the value controlling the conformational equilibrium. We conclude that the pK_a reflects the ionization of a group(s) other than the two tyrosines. Possible candidates for this moiety include one (or more) of the five lysine residues in gene 8 protein. There are no arginine or histidine residues

in gene 8 protein (Van Wezenbeek et al., 1980). Alternatively, the conformational equilibrium could be mediated by changes in deoxycholate micelle structure coupled to pH. It seems unlikely that a pK_a of 9–10 could be due to the ionization of the carboxyl group of deoxycholate.

Conformational changes in the protein have been observed by using circular dichroism and Raman spectroscopy (Nozaki et al., 1978; Fodor et al., 1981). In deoxycholate and SDS micelles, the protein is a dimer with a CD spectrum characteristic of the "b" form observed in deteregent solution using CD. This form is approximately 50% α helix and is also present in lipid vesicles formed from unsaturated lipids. The "a" form is seen in the intact virion and is nearly 100% helical while the "c" form is observed in reconstituted membrane preparations with saturated lipids and has a CD spectrum consistent with about 50% β structure.

Our measurement of the small helicity changes associated with the MCI and MCII equilibrium in detergent suggests that these are more subtle than a to b or b to c transitions. The b to c form transition requires nearly complete exchange of detergent solvent for saturated phospholipids and results in about a 5000 deg cm^2 $dmol^{-1}$ decrease in ellipticity at 208 nm (Fodor et al., 1981). In contrast, the average ellipticity decrease for the MCII to MCI conversion is 1700 deg cm^2 $dmol^{-1}$ at 208 nm. This change is independent of the chemical nature of the solvent and thus is observed under conditions when only MCI is present. Furthermore, the presence of small amounts of phospholipids does not alter the ellipticity change. In contrast, the same amount of phospholipid significantly alters the NMR measured equilibrium between MCI and MCII. We conclude that both MCI and MCII are b-form conformations. It is important to recognize that all our NMR work involves the fluorine-substituted protein. It is possible that MCI and MCII are specifically associated with only the fluorine-substituted protein.

The pH-dependent alteration of viral coat proteins is not without precedence. Tomato bushy stunt virus (TBSV), bromegrass mosaic virus, and other related icosahedral viruses undergo a reversible, pH-dependent expansion (Incardona & Kaesberg, 1964; Robinson & Harrison, 1982). Recently, Robinson and Harrison demonstrated that the structural basis of capsid expansion in TBSV is a localized protein subunit conformational change which allows accommodation of different packing environments of the folded domains. Although the physiological significance is unknown for TBSV, this adaptability to environment may be important in virion assembly, disassembly, or some other process. Since MCI and MCII have only been observed under nonphysiological conditions, it is difficult to incorporate tham into a model of virion assembly. However, conversion to MCII releases 10–12 kcal/mol. This enthalpy may be available to drive virion assembly providing the entropy change is appropriate. Thus, protein flexibility may provide both environmental adaptation and energy storage mechanisms.

Our observation that the addition of lipid could change the MCI–MCII equilibrium is tantalizing. It is suggestive of phospholipid-induced protein structural transitions one might hope to observe in other membrane proteins. However, some care must be exercise in this interpretation. It seems clear that the effects of phospholipid are not a result of strong stoichiometric binding to a specific site on the protein. The results are most consistent with the view that the effect is really on the micelle structure which acts to solvate the protein. There is ample evidence that incorporation of phospholipid into bile salt micelles (Mazer et al., 1980; Müller, 1981) changes the micelle structure. This apparent effect on general micellar structure is further supported by the observation that octyl glucoside strongly favors MCI while deoxycholate allows both MCI and MCII to exist in nearly equivalent amounts.

The conclusion that the hydrophobic environment of the lipid and/or detergent can cause protein conformational change without necessarily binding to a discrete site on the protein may be important for our understanding of lipid–protein interactions in bilayer systems. Recent spectroscopic studies of such systems do not suggest the existence of strong, specific interactions between certain lipids and proteins. Rather, the data are more consistent with a picture of relatively nonspecific lipid solvation of membrane proteins (Paddy et al., 1981). In this sense, the gene 8 protein–detergent system may represent a good model for these interactions. If this is the case, it seems clear that the structure of the lipid environment could easily regulate protein conformational equilibria. By providing certain important membrane proteins with conformational equilibria coupled to membrane structure, the activities of these proteins could be regulated by variations in fatty acid chain length of head group. These factors in turn could be controlled by the cell in response to environmental signals.

While such arguments are not new to this work, the clear demonstration of a protein conformational equilibrium which is coupled to the structure of its hydrophobic solvent gives these arguments somewhat more credence and may offer an experimental system to test these ideas.

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Conformational Flexibility of Neurophysin As Investigated by Local Motions of Fluorophores. Relationships with Neurohypophyseal Hormone Binding†

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ABSTRACT: Flexibility of various structural domains of neurophysin and neurophysin–neurohypophyseal hormone complexes has been investigated through the fast rotational motion of fluorophores in highly viscous medium. Despite seven intrachain disulfide links, it is shown that some domains of neurophysin remain highly flexible. Dimerization of neurophysin does not affect the structural integrity of the individual subunits, each subdomain being conformationally equivalent within each protomer of the unliganded dimer. The absence of heterogeneous fluorescence anisotropy precludes the existence of a dimer tautomerization equilibrium. Binding of the hormonal ligands to neurophysin dimer promotes a large conformational change over the whole protein structure as assessed by differential alterations of the flexibility–rigidity and intrasegmental interaction properties of domains that do not participate directly to the dimerization/binding areas. The order of free-energy coupling between ligand binding and protein subunit association has been evaluated. Data are consistent with a model in which the first mole of bound ligand stabilizes the dimer by increasing the intersubunit contacts while the second mole of ligand induces most of the described conformational change. Accordingly, the positive cooperativity between the two dimeric binding sites is linked mainly to the binding of the second ligand. The induced structural change is perceived differently by each subunit as assessed by opposite local motions of Tyr^{49} in each liganded protomer and leads to the formation of a dimeric complex with a global pseudospherical symmetry although containing domains of local asymmetry.

The posterior pituitary contains a class of highly disulfide-linked proteins, neurophysins, associated with both biosynthesis and transport of the neurohypophyseal hormones, ocytocin and vasopressin, along the hypothalamo neurohypophyseal tract [for recent reviews, see Cohen et al. (1983), Chaiken et al. (1983), Richter (1983), Breslow (1984), and Pickering & Swann (1984)]. Within the neurosecretory granule two distinct neurophysins are found in noncovalent association with a different hormone although each neurophysin is able to bind vasopressin or ocytocin with similar affinities in vitro.

Studies of the polymerization process of neurophysins demonstrated that these proteins self-associate in a form of

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