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High-Resolution Nuclear Magnetic Resonance Studies of the *Lac* Repressor. 3. Unfolding of the *Lac* Repressor Headpiece†

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ABSTRACT: At temperatures below 20 °C, the *lac* repressor headpiece (N-terminal amino acids 1–51) has a well-defined structure which is independent of ionic strength. Its unfolding with increasing temperature proceeds gradually with a characteristic transition temperature which depends on ionic strength. Unfolding has been studied by using NMR and CD. Shifts of several methyl and all of the tyrosyl resonances can be followed, allowing a detailed analysis of the temperature denaturation. At high ionic strength (1 M), the unfolding is complete at 85 °C, while at low ionic strength (0.01 M), it is complete by 65 °C. Native and partially unfolded structures are in rapid exchange during the unfolding, and the process appears completely reversible at all ionic strengths.

The isolated "headpiece" (HP) of the *lac* repressor, containing the 51 N-terminal residues (Geisler & Weber, 1977), has been shown to possess extensive structure and considerable flexibility (Wade-Jardetzky et al., 1979; Ribeiro et al., 1981). This small single-chain polypeptide has no disulfide bridges, making it particularly interesting for the study of protein folding. Changes of the structure with temperature were followed by CD and ^{1}H NMR. Unlike most globular proteins, this protein fragment unfolds in a gradual and continuous manner and in a completely reversible fashion. The temperature at which unfolding is complete is quite dependent upon ionic strength of the solution. Assignments of some of the resonances in the ^{1}H NMR spectrum of this protein to particular residues allow us to develop a model of this transition.

Materials and Methods

Preparations of HP were carried out as described previously (Ribeiro et al., 1981). The standard phosphate buffer at ionic strength ($\Gamma/2$) = 1 contained 260 mM K_2HPO_4, 40 mM KH_2PO_4, 200 mM KCl, 10^{-4} M dithiothreitol, and 10^{-4} M ethylenedinitriloacetic acid. Ionic strength was adjusted by dilution from $\Gamma/2 = 1$ to $\Gamma/2 = 0.01$. NMR experiments were performed on the 360-MHz spectrometer at the Stanford Magnetic Resonance Laboratory. CD measurements were made on a Jasco J-40 auto recording spectropolarimeter at Stanford. Temperature was measured and held to ±1 °C.

Results

The overall unfolding can be monitored by following the CD and NMR spectra as a function of temperature. As secondary and tertiary structures are disrupted, the ellipticity in the CD spectrum is reduced. This is shown for HP at high ionic strength in a D_2O solution in Figure 1. The CD data are consistent with an initial structure which is primarily a helix converting to a random coil. Figure 2 shows the 360-MHz ^{1}H NMR spectra of the HP in 0.3 M phosphate buffer at three temperatures (20, 60, and 80 °C). The low-temperature spectrum represents the native structure and the high-temperature spectrum the completely unfolded form, which is well approximated by a sum of the spectra of the constituent amino acids (Roberts & Jardetzky, 1970). The intermediate spectrum, taken at a temperature near the midpoint of the transition, does not show a mixture of peaks for the folded and unfolded forms but rather peaks with positions intermediate to them. This indicates that the folded and unfolded forms and possibly several partially folded forms are in rapid exchange throughout the temperature range. This is in sharp contrast to the characteristic finding of slow exchange between folded and unfolded forms for many globular proteins near the transition point (Baldwin, 1975).

There is nothing unusual about the transition as monitored by CD. However, NMR provides a method for distinguishing between slow and rapid exchange between folded and unfolded forms and for a more detailed study of the unfolding process. When lines which are assigned to particular residues in the molecule are followed, local and global changes can be distinguished. Large shifts of peaks with temperature are particularly obvious in three spectral regions: (a) 6.0–7.0 ppm containing the aromatic resonances (tyrosine and histidine),

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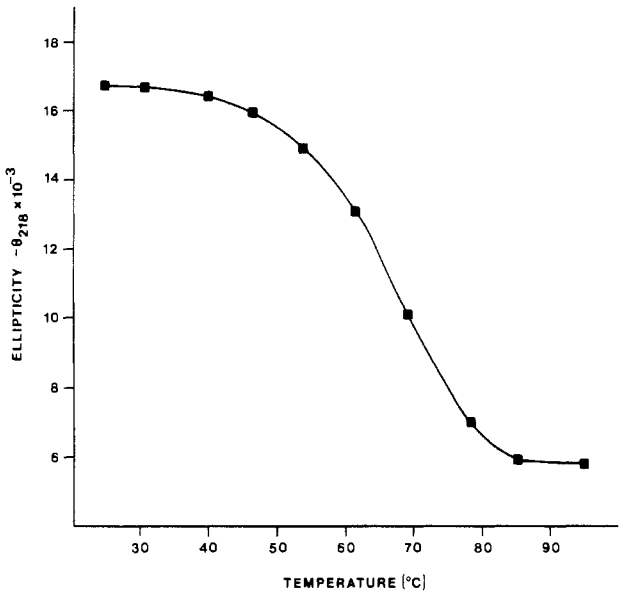

FIGURE 1: Change in circular dichroism of HP with temperature and plotted as the ellipticity remaining at temperature T (phosphate buffer $\Gamma/2 = 1$ in D_2O).

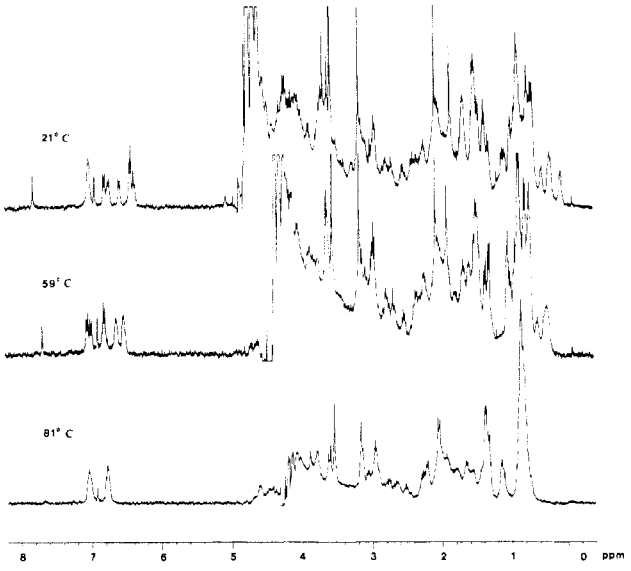

FIGURE 2: ^1H NMR spectra (360 MHz) of HP in phosphate buffer $\Gamma/2 = 1$ at 20, 60, and 80 °C at pD 7.8. At intermediate temperatures, no coexistence of folded and unfolded forms occurs.

(b) 3.9–5.0 ppm containing α-CH resonances, and (c) 0.0–2.0 ppm containing various methyl resonances.

The changes in the α-CH region are most difficult to follow because of the often complex structure of the resonances and interference of the HOD line. The chemical shifts for α-protons should be 4.2–5.0 ppm for a random coil and 3.8–4.5 ppm for a helical conformation. In HP, a substantial number of α-CH peak appear in the 3.8–4.3-ppm region, suggesting that at least part of the backbone is helical (Ribeiro et al., 1981; Chandrasekaran et al., 1979). With an increase in temperature, a downfield shift of a considerable number of resonances is observed. While the details cannot be followed in this region, the pattern is consistent with a helix–coil transition, with the two forms in rapid exchange (Markley et al., 1967).

Many changes can be observed in the methyl and tyrosyl regions which parallel each other. Almost all of these resonances show an initial gradual shift with temperature, followed by a much more rapid shift above about 55 °C. The universality of this pattern for resonances which come from widely separated regions of the molecule probably reflects cooperativity in the unfolding on a global scale, at least in the later stages.

The detailed temperature dependence of lines in the aromatic region is shown in Figure 3. The assignment of all lines to particular residues has been discussed elsewhere (Ribeiro et al., 1981). The initial gradual shift observed for the resonances of Tyr-17, Tyr-7, and the downfield half of Tyr-12 may represent a change in a local interaction, as with a neighboring charged group. This view is strengthened by the observation that these resonances shift with pH in a fashion consistent with titration of a nearby Asp or Glu (unpublished results). Above 55 °C, the resonances shift strongly with temperature, which reflects the overall unfolding. It seems most reasonable to assume that the large upfield shifts observed for Tyr-7, -12, and -17 come from stacking of these aromatic rings. As the tyrosines unstack with an increase in temperature, these shifts are removed.

Many of the lines show a significant broadening above 55 °C. This broadening is somewhat greater and occurs earlier at lower ionic strength. The cause for this broadening is not clear; it seems unlikely that the exchange rate between folded and partially unfolded forms would decrease with an increase in temperature to bring resonances into the intermediate-exchange time scale. It is possible that temperature gradients across the sample are responsible since only shifting resonances are significantly broadened. We also note that the temperature dependence provides a convenient method for assignment of the 2,6 vs. 3,5 resonances of all tyrosines since the assignments are unambiguous in the denatured form.

The temperature dependence of some lines in the methyl region is shown in Figure 4. Clearly, the general pattern of shifts is similar to those seen in the tyrosine region. Since assignments of most of these lines to particular residues are not yet available, it is difficult to draw detailed conclusions. NOE measurements have demonstrated that the highest field methyls are in proximity to tyrosines (Ribeiro et al., 1981). As unfolding occurs, the amount of time spent in the proximity of a tyrosine is reduced and the lines shift proportionately.

The resonances with initial shifts of 1.4 and 1.0 ppm, which converge at 1.15 to 1.10 ppm in the denatured spectrum, are identified as Thr-5, -19, and -34. Individual assignments to particular residues are not yet known. It seems likely that the single upfield shifting resonance is involved in hydrogen bonding, while the two downfield shifting resonances are in proximity to aromatic rings.

The two very sharp singlets at 2.05 and 1.85 ppm have been assigned as Met-1 and Met-42, respectively. It has been shown that Met-1 is not critical for maintaining structure and is apparently quite free to move (Ribeiro et al., 1981). This results in a very sharp line, with almost no temperature-dependent shift. Met-42, however, is significantly upfield shifted in the native protein, possibly from interaction with Tyr-47. The temperature dependence is analogous to the other peaks observed.

At lower ionic strength, the temperature required for complete denaturation is significantly reduced. Figure 5 compares spectra of the aromatic region for various salt concentrations and temperatures. Lines begin to shift strongly at lower temperature and are broadened more strongly at intermediate temperatures. Since overall unfolding occurs sooner at low salt, some of the initial "local" changes are not observed, for example, the separation of the downfield doublets of Tyr-7 and Tyr-47. The exact effects of salt are not known, but at high

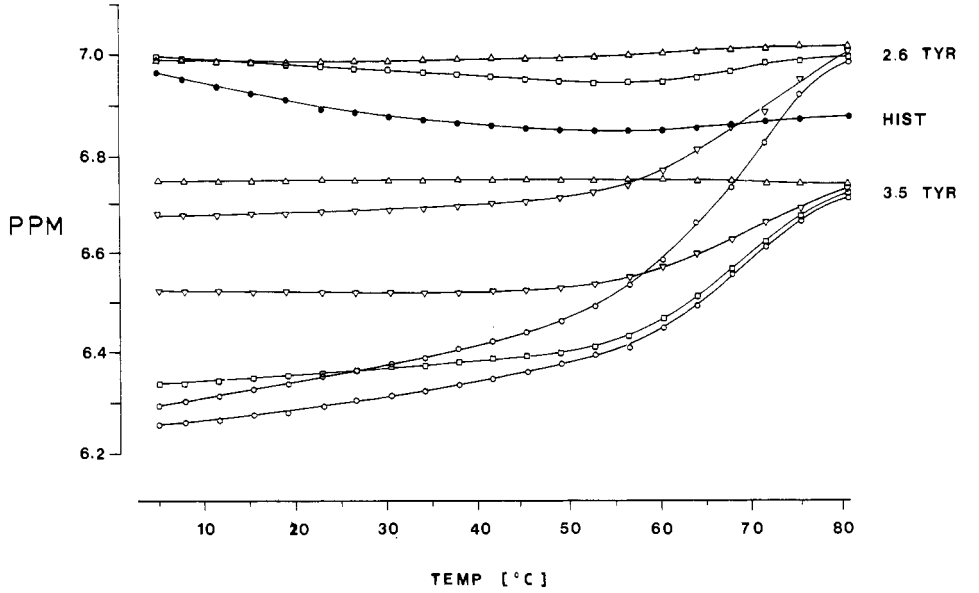

FIGURE 3: Temperature dependence of the chemical shifts in the aromatic region of HP (phosphate buffer $\Gamma/2 = 1$, pD 7.8). (●) Histidine C-2, (□) Tyr-7, (▽) Tyr-12, (○) Tyr-17, and (△) Tyr-47.

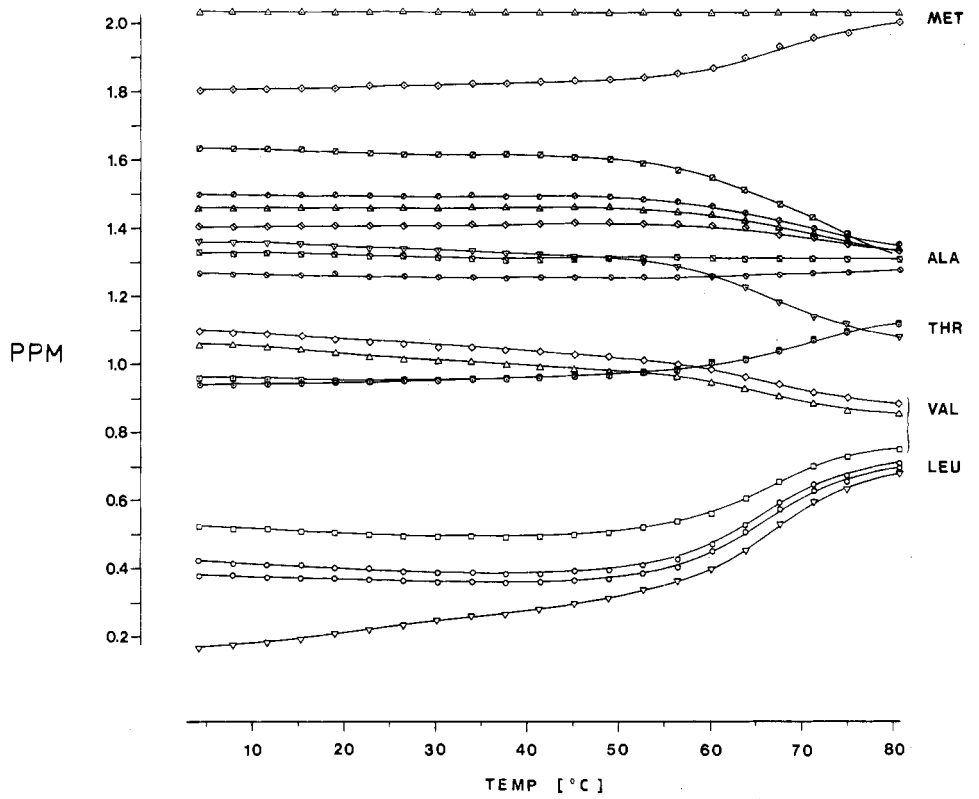

FIGURE 4: Temperature dependence of chemical shifts in the methyl region of HP (phosphate buffer $\Gamma/2 = 1$, pD 7.8).

ionic strength, repulsive interactions between charged side chains are reduced and the "hydrophobic" clustering of nonpolar groups enhanced (Kauzmann, 1954).

Discussion

Several types of spectroscopic behavior may be seen when protein unfolding is followed by NMR in an equilibrium experiment: (a) The chemical shift of some resonances remains constant with temperature until unfolding occurs, at which point the resonance corresponding to the folded form vanishes and a resonance from the denatured form appears. In this case, the folded and unfolded forms are always in slow exchange. This behavior is typical of "stable" parts of a protein structure. (b) The chemical shift of some resonances may drift continuously over a small temperature range before undergoing a discrete jump upon complete unfolding. Care must be taken in interpreting such findings to exclude effects of a neighboring titrating group whose pK may change with temperature or concentration of the denaturing agent. (c) Shifts may change continuously from their native form value to that of the unfolded form. Depending on shift differences and exchange rates, the lines, in this case, may or may not be exchange broadened.

Most of the well-studied small proteins—bovine pancreatic trypsin inhibitor (BPTI) (Wuthrich, 1976), ribonuclease (Benz & Roberts, 1975a,b), lysozyme (Cohen & Jardetzky, 1968;

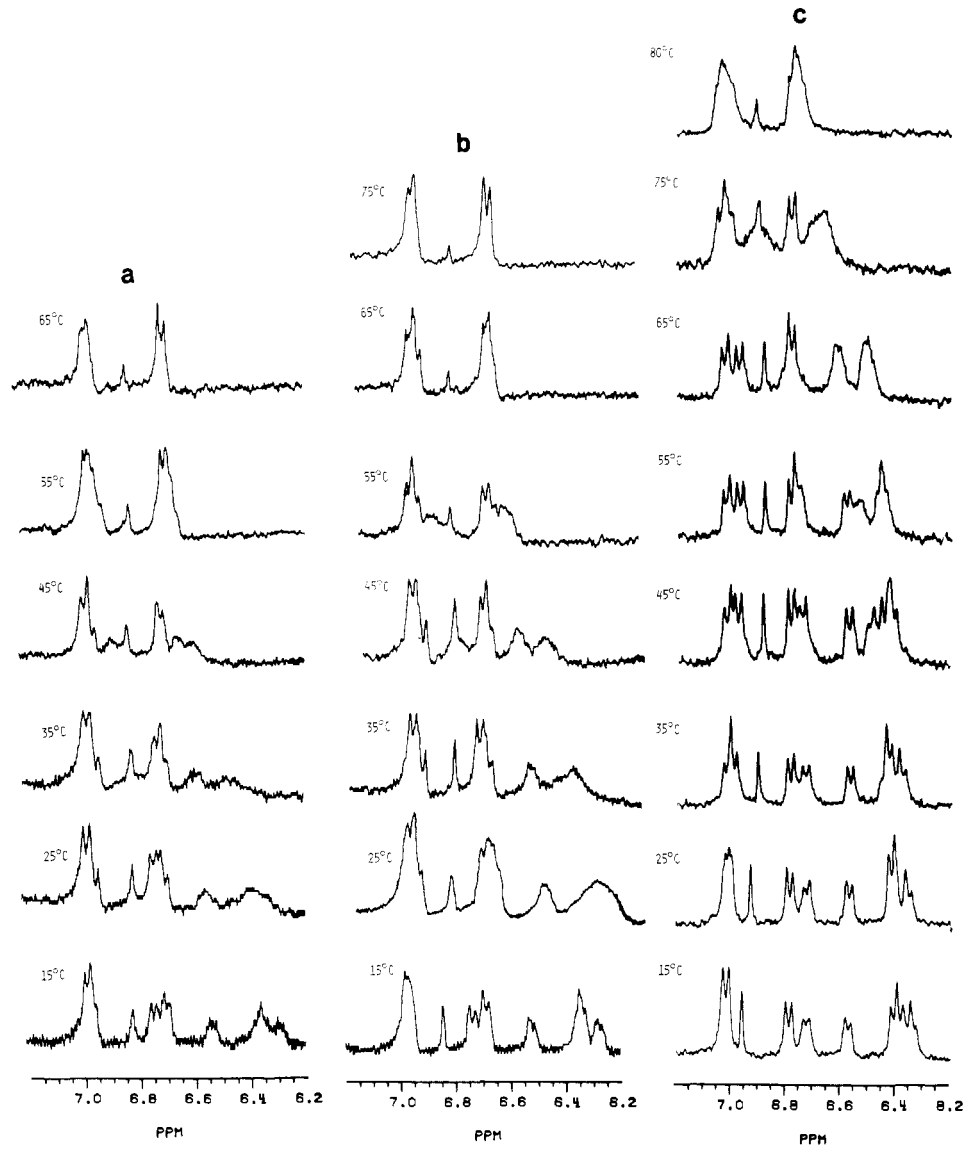

FIGURE 5: ^{1}H NMR spectra (360 MHz) of the aromatic region of HP as a function of temperature at pD 7.8 and (a) $\Gamma/2 = 0.01$, (b) $\Gamma/2 = 0.1$, and (c) $\Gamma/2 = 1.0$ phosphate buffer.

McDonald & Phillips, 1969), and parvalbumin (Cave et al., 1979)—show behavior of the (a) and (b) type. Clearly the HP of *lac* repressor falls in the (c) class.

These experiments demonstrate that there exists a large variation in the degree of cooperativity in protein folding. The unfolding of some proteins is totally cooperative to the point that it approaches a phase transition (BPTI, ribonuclease, lysozyme, parvalbumin), others are highly cooperative in some situations but not others (staphylococcal nuclease; Epstein et al., 1971; Jardetzky et al., 1971; Anfinsen & Scheraga, 1975), and some (HP) show very weak cooperativity. It is probably significant that disulfide cross-linked structures (BPTI, ribonuclease, lysozyme) show the most cooperative behavior. The transition is abrupt, exchange between folded forms and unfolded forms is slow, and hysteresis is commonly observed. Single-chain non-crosss-linked proteins (HP and staphylococcal nuclease), however, may unfold gradually, and *all* of the partially unfolded intermediates are in rapid exchange on the NMR time scale. However, they may also show a high degree of cooperativity (parvalbumin). When exchange is rapid, it is, of course, impossible to say anything about the number of intermediate unfolded species and their structures or any of the exchange rates. Yet, the existence of a folding transition involving rapid exchange is in itselt of considerable interest for our understanding of the folding process.

Further experiments investigating pH effects and denaturation by chemical means are currently under way. As further assignments of particular resonances become available, a more detailed picture of local interactions and how they are disrupted with temperature will emerge.

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Phospholipid Vesicle Formation and Transmembrane Protein Incorporation Using Octyl Glucoside†

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ABSTRACT: Removal of detergent from mixed micelles of egg yolk phosphatidylcholine and octyl glucoside leads to formation of unilamellar phospholipid vesicles with a diameter of about 230 nm. The same procedure applied to mixed micelles containing the transmembrane protein glycophorin A, in addition to lipid and detergent, produces vesicles of the same size with glycophorin incorporated into the bilayer. The pure lipid vesicles are highly impermeable to both anions and cations, and incorporation of up to 220 molecules of glycophorin per vesicle has little effect on permeability.

To acquire a detailed understanding of integral membrane proteins, one must be able to incorporate them in a highly purified state into unilamellar phospholipid vesicles where functional properties such as ion transport can be studied under conditions approximating the native state. Since membrane proteins are generally purified and characterized in detergent-solubilized form (Tanford & Reynolds, 1976), it is desirable to be able to form protein-containing vesicles directly from protein-containing detergent micelles. The purpose of this paper is to summarize the principles that apply quite generally to this problem and to present initial results for a simple model system, egg yolk phosphatidylcholine/octyl glucoside/human erythrocyte glycophorin. The vesicles formed in this system have been characterized with respect to size distribution, protein orientation in the membrane, and passive permeability of the membrane. The last characteristic is particularly important, for if incorporation of a transmembrane protein significantly increases the nonspecific permeability of the membrane, it would interfere with the ability to use the vesicles for specific transport studies. Glycophorin has no known transport function and was chosen for these studies because it is readily prepared in large quantity and in a pure state. It is reasonable to hope that it will be typical of transmembrane proteins, in general, in its effect on passive membrane permeability. It should be pointed out, however, that this protein has limited but finite solubility in aqueous solution in the nonliganded state (it is generally aggregated under these conditions) and that this is not a general property of transmembrane proteins.

To form complexes containing only lipid and protein from a solution of detergent-rich mixed micelles requires removal of detergent from the solution. This will decrease the solubility of the lipid and protein moieties, and the detergent-depleted micelles will aggregate to form larger particles (as one can observe visually since the solution becomes cloudy). However, these larger particles will not necessarily take the form of protein-containing lipid vesicles, and whether they do so must depend on two kinds of events that can occur during aggregation: (1) changes in local composition within a particle, both as a result of detergent removal and as a result of lipid and protein exchange between particles, and (2) molecular rearrangement in response to the compositional change.

The first process requires the movement of molecules through the aqueous solution, and the rate at which a given constituent can participate in producing a compositional change therefore depends on its solubility in an unassociated state in the aqueous medium. This solubility is very small for phospholipids and for most intrinsic membrane proteins. The attainable concentration for unassociated detergent depends on the critical micelle concentration but will always be much higher than that of lipid or protein. Detergent removal and aggregation of the remaining lipid and protein are therefore expected to occur without much change in the *local* lipid/protein ratio. It is clearly advantageous to fix this ratio at the ultimately desired value before the detergent is removed by starting with mixed micelles containing protein and lipid in the same particle.

Since purification of a membrane protein requires separation from other membrane-associated proteins originally present, it may be necessary to use such a high detergent concentration

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