Evidence That Bilayer Bending Rigidity Affects Membrane Protein Folding†

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ABSTRACT: The regeneration kinetics of the integral membrane protein bacteriorhodopsin have been investigated in a lipid-based refolding system. Previous studies on bacteriorhodopsin regeneration have involved detergent-based systems, and in particular mixed dimyristoylphosphatidylcholine (DMPC)/CHAPS micelles. Here, we show that the short chain lipid dihexanoylphosphatidylcholine (DHPC) can be substituted for the detergent CHAPS and that bacteriorhodopsin can be regenerated to high yield in mixed DMPC/DHPC micelles. Bacteriorhodopsin refolding kinetics are measured in the mixed DMPC/DHPC micelles. Rapid, stopped flow mixing is employed to initiate refolding of denatured bacterioopsin in SDS micelles with mixed DMPC/DHPC micelles and time-resolved fluorescence spectroscopy to follow changes in protein fluorescence during folding. Essentially identical refolding kinetics are observed for mixed DMPC/CHAPS and mixed DMPC/DHPC micelles. Only one second-order retinal/apoprotein reaction is identified, in which retinal binds to a partially folded apoprotein intermediate, and the free energy of this retinal binding reaction is found to be the same in both types of mixed micelles. Formation of the partially folded apoproptein intermediate is a rate-limiting step in protein folding and appears to be biexponential. Both apparent rate constants are found to be dependent on the relative proportion of DMPC present in the mixed DMPC/DHPC micelles as well as on the pH of the aqueous phase. Increasing the DMPC concentration should increase the bending rigidity of the amphiphilic bilayer, and this is found to slow the rate of formation of the partially folded apoprotein intermediate. Increasing the mole fraction of DMPC from 0.3 to 0.6 slows the two apparent rate constants associated with formation of this intermediate from 0.29 and 0.031 to 0.11 and 0.013 s^{-1}, respectively. Formation of the intermediate also slows with increasing pH, from 0.11 and 0.013 s^{-1} at pH 6 to 0.033 and 0.0053 s^{-1} at pH 8. Since this pH change has no known effect on the phase behavior of lecithins, this is more likely to represent a direct effect on the protein itself. Thus, it appears to be possible to control the rate-limiting process in bacterioopsin folding through both bilayer bending rigidity and pH.

The factors that drive the folding of integral membrane proteins are largely unknown. We examine a somewhat general hypothesis that the folding of transmembrane proteins is affected by the pressure exerted on them by the surrounding membrane lipids. This hypothesis is based on a previous suggestion by Gruner (1985) which has compelling support from a number of experimental observations (Lindblom et al., 1986; Keller et al., 1993).

The differential lateral pressures which exist in a fluid lipid monolayer are illustrated in Figure 1. In the chain region, there is a positive outward pressure due to chain collisions during bond rotational motion. Around the polar/apolar interface the pressure is negative, since the hydrophobic energy cost of exposing hydrocarbon to water causes the amphiphiles to crowd together and exclude areas of contact. In the head group region, steric hindrance, hydration, or electrostatic charge can lead to a repulsive, positive pressure, while direct hydrogen bonding between head groups can give rise to a negative pressure. Although the net lateral pressure must be zero (otherwise the monolayer will simply expand or contract), there will still be a general desire of the monolayer to bend. More formally, for most biologically relevant situations where the monolayer wishes to bend toward the water, the first moment of the lateral pressure [$\pi(z)$] must be non-zero, giving

$$\int \pi(z) z \, dz \geq 0 \qquad (1)$$

where z is the depth through the monolayer (Helfrich, 1973). Most bilayers are in a state of physical frustration as both monolayers wish to bend, but in the opposite sense. Increasing the desire of each monolayer to bend leads to a net increase in the magnitude of the differential lateral pressure. The hypothesis we investigate is that a protein embedded in the bilayer will experience these changes in pressure as the environment of the membrane is altered and this may change the way in which the embedded protein behaves and folds. Such effects have been observed for small molecules embedded in membranes (Cowsley et al., 1993; Castle, 1995). The rigidity of a single-component membrane to bending can be reduced by adding a lipid with a shorter chain

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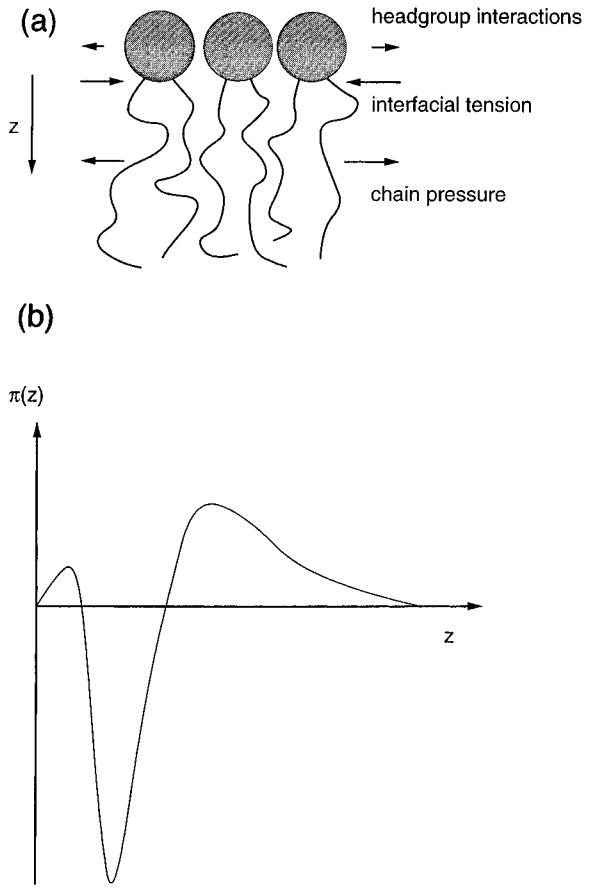

FIGURE 1: Differential lateral pressure profile and its molecular origins. Distinct local intermolecular forces are at play as a function of depth, *z*, through the monolayer (a). Such interactions will give rise to a lateral pressure, $\pi(z)$, profile having the qualitative form shown in panel b.

length but with an identical head group (Di Meglio et al., 1985; Ben-Shaul et al., 1987). This appears to be due to a sharp reduction in the pressure near the middle of the bilayer. We therefore anticipate that the refolding kinetics of a membrane-spanning protein would be affected by varying the bending rigidity in such a way. Changes in external pressure and temperature will also alter the lateral pressure profile of the lipid monolayer, as well as having a direct effect on the embedded or refolding protein. Thus, in order to change the micromechanics of the bilayer, we have altered the relative composition of the lipid membrane at constant temperature and pressure.

Bacteriorhodopsin is one of the few integral membrane proteins that can be refolded from a denatured to a native state *in vitro* (Huang et al., 1981; London & Khorana, 1982; Khorana, 1988), and methods have been developed to study the associated refolding kinetics (London & Khorana, 1982; Booth et al., 1995). It thus provides an ideal model system for this investigation. Bacteriorhodopsin is the only protein constituent of the purple membrane of *Halobacterium salinarium*, and functions as a light-driven proton pump (Oesterhelt & Stoeckenius, 1974; Stoeckenius & Bogomolni, 1982). The structure of bacteriorhodopsin has been determined to high resolution (Henderson et al., 1990), and the protein consists of a bundle of seven transmembrane α helices, together with a retinal chromophore which is covalently bound within the helix bundle via a Schiff base linkage to Lys 216.

Bacteriorhodopsin spontaneously refolds to a native state in mixed detergent/lipid micelles (Huang et al., 1981; London & Khorana, 1982), and the regeneration process has been characterized in mixed dimyristoylphosphatidylcholine (DMPC)[1] /CHAPS micelles (London & Khorana, 1982; Liao et al., 1983; Booth et al., 1995). The regeneration kinetics have been determined with millisecond time resolution using stopped flow mixing of denatured apoprotein in sodium dodecyl sulfate (SDS) micelles and mixed DMPC/CHAPS micelles to initiate folding and changes in protein fluorescence to follow protein folding (Booth et al., 1995, 1996). Several kinetic phases have been assigned to the regeneration process. A fast phase of about 250 s^{-1} accompanies mixing of the SDS and mixed DMPC/CHAPS micelles, and a phase of about 2−7 s^{-1} has been tentatively assigned to formation of an intermediate (I_1) on the regeneration pathway. A partially folded apoprotein intermediate has been identified (I_2), to which the retinal chromophore binds. This latter retinal binding reaction is the only second-order retinal/apoprotein reaction that has been identified in the regeneration process, and the product of this reaction is a retinal/apoprotein intermediate I_R, with retinal probably noncovalently bound within its binding pocket. The later stages in the regeneration process reflect covalent binding of retinal via its Schiff base linkage. The simplest sequential reaction scheme supported by the kinetic data is as follows (Booth et al., 1996)

$$\text{bO} \rightleftharpoons I_1 \rightleftharpoons I_2 \overset{R}{\rightleftharpoons} I_R \rightarrow I_3 \rightarrow \text{bR}$$

although a branched pathway, for example involving cis−trans isomerisations of proline residues, cannot be excluded. Formation of I_2 is essentially rate-limiting in protein folding and appears to be biexponential. The overall apparent rate of formation of I_2 is over 1 order of magnitude slower than the subsequent retinal binding (about 0.029 as opposed to 1.6 s^{-1}). This means the retinal binding reaction is difficult to resolve. However, by addition of retinal after I_2 has been allowed to form, it is possible to study this retinal binding reaction and estimate the associated free energy change (Booth et al., 1996).

The mixed DMPC/CHAPS systems used in these studies are thought to form mixed bilayer type micelles; however, the high proportion of the bulky and relatively rigid zwitterionic CHAPS detergent together with the differing types of head groups present in this mixed micelle system means that it is difficult to gain even a qualitative insight into the effect of CHAPS on the differential lateral pressures in the DMPC membrane. A possible alternative to CHAPS, where interpretation is simpler, is a short chain phosphatidylcholine lipid such as DHPC. This phosphatidylcholine lipid has a hydrocarbon chain of only seven carbon atoms which will reduce the bilayer-bending rigidity of the longer (fourteen carbon) chain DMPC. Mixtures of DMPC with DHPC are also thought to form bilayer discoidal mixed micelles, similar to those with CHAPS (Sanders & Schwonek, 1992), and mixed DMPC/DHPC systems have been shown to reconsti-

[1] Abbreviations: bO, bacterioopsin; CD, circular dichroism; CHAPS, 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate; DMPC, L-α-1,2-dimyristoylphosphatidylcholine; DHPC, L-α-1,2-dihexanoylphosphatidylcholine; FWHM, full width half-maximum; SDS, sodium dodecyl sulfate.

tute membrane proteins (Kessi et al., 1994; Sanders & Landis, 1995).

Here, we investigate the refolding of bacteriorhodopsin in mixed DMPC/DHPC micelles and compare the refolding kinetics to those in mixed DMPC/CHAPS micelles. The dependence of these refolding kinetics on the relative proportion of DMPC in the mixed DMPC/DHPC micelles is determined. However, since a process as complex as the refolding of bacteriorhodopsin is unlikely to depend solely on the micromechanical stresses in the membrane, we also present evidence on the effects of the pH of the aqueous phase on the folding kinetics. The results show that control of protein folding rates can be achieved by changes in both DMPC concentration and pH.

MATERIALS AND METHODS

Materials. 1,2-Dihexanoyl-*sn*-phosphatidylcholine (DHPC) and dimyristoylphosphatidylchoine (DMPC) were purchased from Avanti Phospholipids (Alabaster, AL), and SDS (electrophoresis grade) and *all-trans*-retinal were from Sigma (Dorset, U.K.). All other reagents and chemicals were of analytical grade.

Bacteriorhodopsin was isolated from *H. salinarium* (strain S9) according to the method of Oesterhelt and Stoeckenius (1974). Denatured bacterioopsin (bO) in SDS was prepared from purple membrane as described previously (Braiman et al., 1987; Booth et al., 1996).

Mixed DMPC/DHPC micelles were prepared by stirring DMPC and DHPC in buffer for 1 h followed by sonication in a bath sonicator for 30 min. The resulting clear micellar solution was either stored at room temperature and used within 24 h or stored for several days at 4 °C in 0.025% azide. Micelles stored for long periods in the absence of azide became cloudy. All mixed DMPC/DHPC micelle stock solutions contained 2% (w/v) total lipids, and the ratio of DMPC to DHPC was varied from 2.5:1 to 1:1.5 (weight ratio).

Measurements were made at three different pHs, using 10 mM phosphate buffer for pH 6 and 8 and 10 mM phosphate/citric acid buffer at pH 4.

Steady State Spectroscopy. Absorption spectra were recorded with a Varian Carey 1G UV/vis spectrophotometer with a 2 nm bandwidth and a 1 cm path length. Fluorescence spectra were measured with a Perkin-Elmer LS50 spectrometer with excitation at 290 nm and excitation and emission bandwidths of 2.5 nm. Fluorescence yields are the integrals of the fluorescence band from 300 to 500 nm. Bacteriorhodopsin was regenerated from bO as described previously (London & Khorana, 1982; Booth et al., 1996). Briefly, 8 μM bO in 0.2% SDS (w/v) was mixed with an equal volume of mixed DMPC/DHPC micelles, containing *all-trans*-retinal. Final concentrations were 4 μM protein, 0.1% SDS, 0.67% DMPC and 0.33% DHPC (for a 2:1 DMPC:DHPC weight ratio), 10 mM phosphate, and 0−8 μM retinal. Absorption and fluorescence spectra of regenerated bacteriorhodopsin were measured after overnight incubations. All incubations and measurements were performed in the dark or in dim light at 22 °C.

Bacteriorhodopsin regeneration yields were determined as described previously (Booth et al., 1996) using an extinction coefficient for bO in SDS of 66 000 cm^{-1} M^{-1} at 280 nm (Huang et al., 1981). The concentration of refolded bacteriorhodopsin was determined using an extinction coefficient of 49 000 cm^{-1} M^{-1} at 554 nm (see Results). This extinction coefficient was determined for mixed DMPC/DHPC micelles (2:1 DMPC:DHPC weight ratio) at pH 6. Regeneration yields were also determined by comparing the area of the chromophore absorption band for bacteriorhodopsin regenerated in mixed DMPC/DHPC micelles to that obtained in mixed DMPC/CHAPS micelles at the same protein concentration.

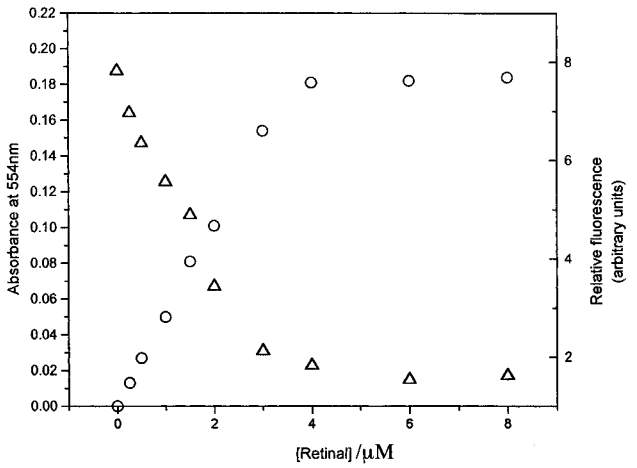

FIGURE 2: Changes in chromophore absorbance and protein fluorescence as a function of retinal concentration, on regeneration of bacteriorhodopsin in mixed DMPC/DHPC micelles (2:1 DMPC:DHPC weight ratio, pH 6): (○), changes in absorbance at 554 nm and (Δ) changes in protein fluorescence. Each data point represents measurements on separate bO samples (from the same bO preparation), to which retinal was added simultaneously. The bacteriorhodopsin concentration was 3.8 μM.

Time-Resolved Fluorescence Measurements. Experiments were performed using an Applied Photophysics stopped flow spectrofluorimeter as described previously (Huang et al., 1981; Booth et al., 1995, 1996). Experiments measuring protein refolding from bO were initiated by mixing equal volumes of 4 μM bO in 0.2% SDS (w/v) with DMPC/DHPC micelles (2% w/v total lipids). Final concentrations in the measuring cuvette were as for steady state measurements, except that the protein concentration was 2 μM. Retinal was included in the micelles where indicated, the final concentration being varied between 2 and 80 μM. For experiments involving addition of retinal to bO which had been allowed to equilibrate in DMPC/DHPC micelles for 1 h, bO in SDS was manually mixed with an equal volume of DMPC/DHPC micelles and left for 1 h. This pre-equilibrated bO was then mixed (in the stopped flow spectrometer) with an equal volume of mixed DMPC/DHPC/SDS micelles (1% w/v total lipids, 0.1% SDS) containing retinal (Booth et al., 1996).

Data Collection and Analysis. Experimentally determined rate constants were calculated from fluorescence data using the Marquardt−Levenberg algorithm, assuming multiexponential kinetics. Rate constants were determined from measurements taken on different time scales, 50 ms to 1000 s full scale with 2000 or 4000 data points per scale. Two, four, or eight transients were averaged per time scale.

All errors are quoted to one standard deviation.

RESULTS

Regeneration Yields. The retinal binding curve (Figure 2) for bacteriorhodopsin regenerated in mixed DMPC/DHPC

micelles (2:1 DMPC:DHPC ratio), was determined as described previously (Booth et al., 1996). Bacterioopsin in SDS was mixed with DMPC/DHPC micelles containing various concentrations of retinal. Recovery of the native-like chromophore absorbance was determined after overnight incubation in the dark at 22 °C. The absorption maximum of the dark-adapted regenerated chromophore was independent of retinal concentration and was about 554 nm, similar to that of 555 nm for bacteriorhodopsin regenerated in mixed DMPC/CHAPS micelles. Figure 2 shows the change in chromophore absorption at 554 nm as a function of added retinal. The chromophore absorbance increases linearly with retinal concentration until a maximum absorption is reached. Assuming a 1:1 retinal:bacterioopsin stoichiometry, an extinction coefficient at 554 nm was estimated from the initial gradient of the binding curve. A value of about 49 000 cm^{-1} M^{-1} (about 10% error between protein preparations) was obtained at 554 nm which is slightly lower than that of 55 000 cm^{-1} M^{-1} determined at 555 nm for DMPC/CHAPS micelles (Booth et al., 1996). The FWHM of the chromophore absorption band was the same in both types of mixed micelles (about 110 nm).

The yield of regenerated bacteriorhodopsin was determined from the relative concentrations of regenerated bacteriorhodopsin and the initial concentration of bO, as described previously (Booth et al., 1996). An average of four measurements on the same bO preparation gave a regeneration yield of 94 ± 5% (for a 2:1 DMPC:DHPC weight ratio, pH 6), which is in good agreement with that observed in DMPC/CHAPS micelles (98 ± 2%) (Booth et al., 1996). As previously reported for DMPC/CHAPS micelles, a larger variation in regeneration yield was found for different bO preparations (about ±13%). A comparison of the areas of the regenerated chromophore bands suggests that the oscillator strength of the chromophore absorption band in DMPC/DHPC micelles was the same as that in mixed DMPC/CHAPS micelles.

Regeneration yields in mixed DMPC/DHPC micelles at other DMPC and DHPC concentrations and pHs (see below) were determined both by a comparison of the relative areas of the regenerated chromophore absorption bands (at the same bO concentration), and by using the extinction coefficient at 554 nm of 49 000 cm^{-1} M^{-1}. The latter is likely to be a reasonable approximation, since the absorption maximum and FWHM of the native-like chromophore formed after regeneration under these conditions were similar to the data for Figure 2.

Figure 2 also shows the decrease in intrinsic protein fluorescence as a function of retinal concentration in mixed DMPC/DHPC micelles (2:1 DMPC:DHPC, pH 6). The change in protein fluorescence yield as a function of retinal appears to mirror the change observed in the chromophore absorbance, as was noted for DMPC/CHAPS (Booth et al., 1996).

Refolding Kinetics. Bacteriorhodopsin refolding kinetics were monitored using stopped flow fluorescence spectroscopy as described previously (Booth et al., 1996). Bacteriorhodopsin in SDS was rapidly mixed with DMPC/DHPC micelles (2:1 DMPC:DHPC, pH 6), and changes in protein fluorescence were time-resolved. The kinetic phases observed during the regeneration of bacteriorhodopsin in DMPC/DHPC micelles are very similar to those previously reported for DMPC/CHAPS micelles (Booth et al., 1996) (Figure 3).

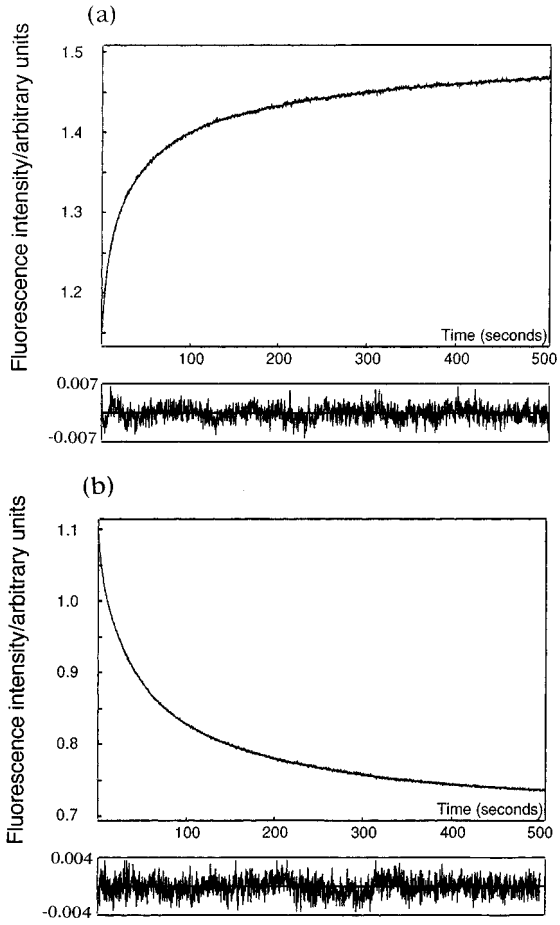

FIGURE 3: Changes in protein fluorescence with time on refolding bacteriorhodopsin in mixed DMPC/DHPC micelles (2:1 DMPC:DHPC weight ratio, pH 6) in (a) the absence of retinal and (b) the presence of retinal. Curves are the average of two transients. Values of 0.11 and 0.013 s^{-1} were resolved for $k_{obs}(1)$ and $k_{obs}(2)$, respectively, in the absence of retinal (a) and 0.096 and 0.012 s^{-1}, respectively, in the presence of retinal (b). Residuals to fits are shown below fluorescence curves.

Table 1 compares the rate constants determined for refolding bacterioopsin in mixed DMPC/DHPC and mixed DMPC/CHAPS micelles in the absence of retinal. The fast 200 s^{-1} phase is almost certainly due to mixing of the SDS and mixed DMPC/DHPC micelles, while the 2.4 s^{-1} phase corresponds to that previously observed in folding studies in mixed DMPC/CHAPS micelles, where it was tentatively assigned to a possible folding intermediate I_1. We have previously shown that, in the absence of retinal, bO forms a partially folded apoprotein intermediate I_2. This also appears to be the case in DMPC/DHPC micelles, and as previously reported for DMPC/CHAPS micelles, biexponential kinetics [$k_{obs}(1)$ and $k_{obs}(2)$] are associated with formation of I_2 (Table 1 and Figure 3a). Both $k_{obs}(1)$ and $k_{obs}(2)$ were found to be dependent on the concentration of DMPC in the mixed micelles (see below), and for a 1:1 weight ratio of DMPC to DHPC, the magnitudes and amplitudes of these rate constants are similar to those determined in DMPC/CHAPS micelles (1:1 DMPC:CHAPS weight ratio).

Essentially identical regeneration kinetics were observed in DMPC/DHPC micelles and DMPC/CHAPS micelles, when retinal was included at the start of refolding. Retinal quenches protein fluorescence which results in an overall

Table 1: Experimentally Determined Rate Constants for Refolding Bacterioopsin in Mixed DMPC/DHPC Micelles in the Absence of Retinal at pH 6

	experimentally determined rate constants (s^{-1})			
			formation of apoprotein intermediate I_2	
refolding system	micelle mixing	formation of I_1	$k_{obs}(1)$	$k_{obs}(2)$
DMPC/DHPC (2:1 weight ratio)	200	2.4	0.11[a]	0.013[a]
DMPC/CHAPS[b] (1:1 weight ratio)	250[b]	7.1[b]	0.13[b]	0.029[b]

[a] Values quoted are the average of three measurements, with an error of about ±20%. Relative amplitude of $k_{obs}(2)$ to $k_{obs}(1)$ ~ 2:1 (total amplitude is ~50% of that from denatured bO to regenerated bacteriorhodopsin, and the increase in the fluorescence amplitude during the 1.5 ms mixing dead time ~100%). [b] (Booth et al. (1996).

decay in fluorescence on folding (Figure 3b). The magnitudes of the rate constants $k_{obs}(1)$ and $k_{obs}(2)$ were similar to those in the absence of retinal; however, due to the presence of retinal, these two components are seen as a decay rather than a rise in fluorescence (compare panels a and b of Figure 3) (Booth et al., 1995). Longer components (~0.003 s^{-1}) were also observed when retinal was included in the refolding reaction which are associated with formation of the Schiff base (London & Khorana, 1982; Booth et al., 1995). As previously reported for DMPC/CHAPS micelles, the early kinetics (micelle mixing and formation of I_1) were independent of retinal.

In order to identify the step when retinal binds during bacteriorhodopsin regeneration, retinal was added at different times during regeneration and the dependence of each kinetic component on retinal concentration was determined (Booth et al., 1996). These experiments showed that, as for DMPC/CHAPS micelles, retinal binds to a partially folded apoprotein intermediate (I_2) to give a retinal/apoprotein intermediate (I_R). bO was incubated in mixed DMPC/DHPC micelles (2:1 DMPC:DHPC, pH 6) for 1 h in the absence of retinal, resulting in the establishment of an equilibrium between bO and I_2. Retinal was then added, allowing the retinal binding reaction

$$I_2 + R \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} I_R$$

to be clearly observed (Booth et al., 1996). This retinal binding reaction follows second-order kinetics and thus is not well approximated by a sum of exponentials (Booth et al., 1996). However, fitting the reaction to a single exponential gives a rough estimate of the rate. The apparent exponential rate constant, $k_{obs}(3)$, for this retinal/apoprotein reaction was about 3 s^{-1} (for a 2:1 mole ratio of retinal to protein), similar to that of 1.6 s^{-1} reported for DMPC/CHAPS micelles. When retinal is in excess, the retinal binding reaction will exhibit pseudo-first-order kinetics, according to the simplified rate law $k_{obs}(3) = k_{-3} + k_3[R]$ (Fersht, 1985; Booth et al., 1996), where k_{-3} is the first-order rate constant for retinal dissociation and k_3 is the second-order rate constant for retinal binding. Figure 4 shows this to be the case, and when retinal is in excess, $k_{obs}(3)$ increases linearly with retinal concentration. This was the only experimentally observed rate constant found to be dependent on retinal concentration, and hence, this is the only second-order reaction that could be detected between retinal and apoprotein. Values of 0.25 and 2.7 s^{-1} can be determined for k_{-3} and k_3, respectively, from Figure 4, using the equation $k_{obs}(3) = k_{-3} + k_3[R]$. Thus, an equilibrium constant of 0.094 μM^{-1} can be determined for this retinal

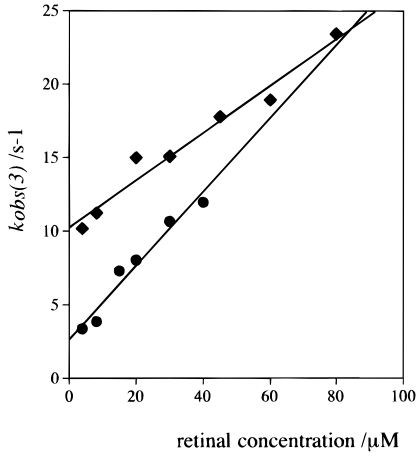

FIGURE 4: Dependence of the experimentally observed rate constant $k_{obs}(3)$ for the retinal binding reaction

$$I_2 + R \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} I_R$$

on retinal: retinal in excess over protein, protein to retinal mole ratios from 1:2 to 1:40, and protein concentration of 2 μM. Retinal was added after bacterioopsin had been allowed to equilibrate in mixed DMPC/DHPC micelles (2:1 DMPC:DHPC weight ratio) for 1 h: (●) pH 6 and (◆) pH 8. Linear fits to the data are shown.

binding reaction, giving a ΔG of −28 kJ mol^{-1} (pH 6, 295 K). This is in good agreement with that of −30 kJ mol^{-1} determined for the equivalent retinal binding reaction in mixed DMPC/CHAPS micelles (Booth et al., 1996).

Dependence of the Refolding Kinetics on DMPC Concentration. The regeneration kinetics of bacteriorhodopsin were determined both in the presence and in the absence of retinal at varying ratios of DMPC to DHPC (weight ratios from 2.5:1 to 1:1.5 DMPC:DHPC) at pH 6. The rate constants associated with micelle mixing and formation of I_1 were independent of DMPC concentration. The two experimentally determined rate constants associated with formation of I_2, [$k_{obs}(1)$ and $k_{obs}(2)$] decreased linearly with increasing DMPC concentration (Figure 5). The kinetics associated with Schiff base formation did not appear to depend on DMPC concentration; however, measurements on longer time scales are required to resolve these components accurately. The yield of regenerated bacteriorhodopsin did not vary with DMPC concentration over the concentration range studied.

Dependence of Bacteriorhodopsin Regeneration Kinetics on pH. Regeneration kinetics were also measured, for a 2:1 DMPC:DHPC ratio, at pH 4, 6, and 8, both in the presence and in the absence of retinal. The only constants found to be dependent on pH were $k_{obs}(1)$ and $k_{obs}(2)$ (Figure 5), both of which decreased with increasing pH. As previously reported for DMPC/CHAPS micelles, the regeneration yield was dependent on pH, with optimum regeneration of 94%

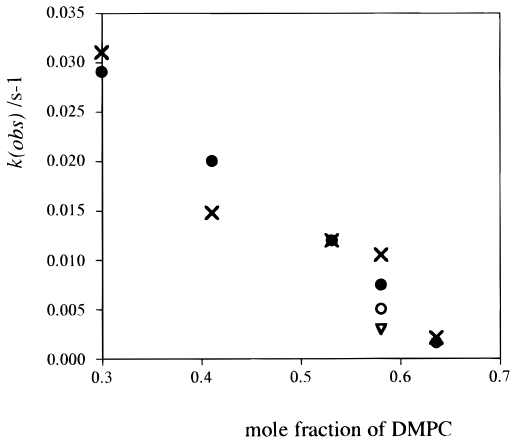

FIGURE 5: Dependence of the experimentally observed rate constants $k_{obs}(1)$ and $k_{obs}(2)$ on the concentration of DMPC for refolding of bacterioopsin in mixed DMPC/DHPC micelles. Values for $k_{obs}(1)$ have been divided by 10 for convenience: (●), $k_{obs}(1)$/10 and (×), $k_{obs}(2)$ at pH 6 and (▽), $k_{obs}(1)$/10 and (○), $k_{obs}(2)$ at pH 8. Values at pH 4 were 0.066 s^{-1} for $k_{obs}(1)$/10 and 0.1 s^{-1} for $k_{obs}(2)$, for a DMPC mole fraction of 0.58. DMPC mole fractions of 0.30 and 0.64 correspond to DMPC:DHPC weight ratios of 1:1.5 and 2.5:1, respectively.

occurring at pH 6. The regeneration yield at pH 8 was only slightly lower, being about 83%, while that at pH 4 was about 68%. The free energy associated with retinal binding (i.e. for the following reaction)

$$I_2 + R \underset{k_{-3}}{\overset{k_3}{\rightleftharpoons}} I_R$$

at pH 8 was found to be −24 kJ mol^{-1}, similar to that of −28 kJ mol^{-1} at pH 6 (see above and Figure 4).

DISCUSSION

We have developed a lipid-based refolding system for bacteriorhodopsin to replace the previously used detergent-based approach. Bacteriorhodopsin can be regenerated from a denatured state in mixed DMPC/DHPC micelles. Regeneration yields of about 94% can be achieved which are similar to those obtained with the previously used mixed DMPC/CHAPS refolding system (Huang et al., 1981; London & Khorana, 1982; Booth et al., 1995, 1996). The chromophore absorption band of the dark-adapted, regenerated bacteriorhodopsin in mixed DMPC/DHPC micelles has an absorption maximum at 554 nm, similar to that of 555 nm in mixed DMPC/CHAPS micelles. The oscillator strengths of the chromophore absorption bands in the two types of mixed micelles also agree to within 10%. Thus, the native chromophore is regenerated in mixed DMPC/DHPC micelles, reflecting recovery of native-like bacteriorhodopsin. The apparent rate constants associated with bacteriorhodopsin regeneration, determined by time-resolved fluorescence spectroscopy, are essentially identical in both types of mixed micelles, both in the presence and in the absence of retinal. Refolding and regeneration of bacteriorhodopsin are therefore likely to follow the same reaction scheme in both micelle systems. Retinal does not bind until late in the regeneration process, and there are several kinetic phases that are independent of retinal and assigned either to a change in the protein environment as a result of micelle mixing (200 s^{-1}) or to protein folding events (formation of I_1 and I_2) (Table 1). As previously reported for DMPC/CHAPS micelles, only one second-order retinal binding reaction is detected, when retinal binds to a partially folded apoprotein intermediate, I_2. The free energy of this retinal binding reaction is also the same (−28 kJ mol^{-1} or −30 kJ mol^{-1} at pH 6 and 295 K) in both refolding systems. Thus, the apparent kinetics and thermodynamics previously determined for bacteriorhodopsin regeneration are not artifacts of the use of CHAPS.

A key intermediate that has been identified in our studies of bacteriorhodopsin folding is the partially folded apoprotein intermediate, I_2, to which retinal binds. Omitting retinal from the refolding reaction facilitates study of the kinetics of formation of this intermediate. Formation of I_2 appears to be rate-limiting in protein folding. The two experimentally determined rate constants [$k_{obs}(1)$ and $k_{obs}(2)$] associated with I_2 formation depend on the relative proportion of DMPC present in the mixed DMPC/DHPC micelles, with both rates slowing as the proportion of DMPC is increased (Figure 5). The faster rates associated with micelle mixing and formation of the postulated intermediate I_1 are independent of DMPC concentration. An increase in the DMPC concentration will increase the rigidity of the mixed DMPC/DHPC bilayer type structures. We can estimate, from previous calculations of Ben-Shaul and co-workers on an amphiphile mixture of C_{12} and C_6 chain lengths (Ben-Shaul et al., 1987), that the bending rigidity of the DMPC/DHPC bilayer will increase approximately linearly by a factor of 2 as the DMPC mole fraction is increased from 0.3 to 0.7. This estimation assumes that the average cross sectional area per lipid remains constant with composition, which in our case is likely to be a good approximation since this is largely set by the head groups. It is therefore interesting to observe that, while there is a 2-fold increase in bending rigidity as the DMPC mole fraction is increased from 0.3 to 0.7, both $k_{obs}(1)$ and $k_{obs}(2)$ decrease 10-fold. Thus, the response of the protein to the changes in bending rigidity of the bilayer appears to be rather sensitive.

This effect of bilayer rigidity can be rationalized qualitatively as follows. An increase in the bending rigidity necessarily implies a net increase in the lateral pressure in the lipid hydrocarbon chain region, which in turn must enhance the stability of the final folded state of bacteriorhodopsin by increasing the compressive pressure on the seven helical bundle. Conversely, folding events which require the migration of protein moieties in the lipid matrix, such as the association of the transmembrane helices, will be slowed by the increase in lateral chain pressure. Thus, as we observe, we would expect to find the kinetics of the protein folding slowing as we increase the bilayer-bending rigidity. The effect of bending rigidity on the stability of the folded protein will be addressed in future studies.

It has been suggested that α helical membrane proteins fold in two stages (Popot & Engelman, 1990). The first involves the formation of independently stable transmembrane α helices and the second packing of these α helices to form native protein. We have previously suggested that in terms of this model the kinetics associated with formation of I_2 are most likely to relate to the second helix-packing stage [Booth et al., 1996; see Riley et al. (1997)]. Whether the transmembrane α helices form during micelle mixing or formation of I_1 remains to be established. (Neither of these latter two processes depends on DMPC concentration and therefore on bending rigidity.) The dominant driving force

for helix association to form native protein (in stage two of the proposed model) is thought to be a packing effect (helix−helix packing being favored over lipid−helix packing) (Lemmon & Engelman, 1994; Popot et al., 1994); however, achieving this final state will be hindered if the migration of the helices into contact is impeded by increased chain lateral pressure. Our assignment of I_2 formation to helix packing is therefore consistent with this idea, as the kinetics of formation of this intermediate slow as packing becomes more impeded. The fact that the apparent rate of helix packing is sensitive to the lateral pressure imposed by the lipids implies that the distance range of direct helix−helix interactions is small, in turn suggesting a small helix−helix interaction free energy.

Formation of I_2 also appears to depend on pH, with the two rate constants [$k_{obs}(1)$ and $k_{obs}(2)$] slowing as the pH is increased from 4 to 8. Changes in bulk pH of the aqueous phase could affect folding in two ways, through either a direct interaction with the protein or a lipid-mediated effect as a result of changes in lipid head group hydration. The latter seems less likely since only negligible changes on hydration of the phosphatidylcholine head group have been found over this pH range (Seddon et al., 1983). Thus, it seems that the folding of a membrane-bound protein can be affected by a direct effect of the aqueous bulk pH on the protein. In contrast to the bilayer rigidity effect, pH also affects the overall regeneration yield of bacteriorhodopsin. However, the effect of pH on regeneration yield does not correlate with the effect on the kinetics of I_2 formation; the latter is slowed by increasing the pH from 4 to 8, while a maximum is observed in the regeneration yield at pH 6.

DHPC has been used in several recent studies on membrane proteins. A study on the isolation of membrane proteins has shown that DHPC is superior to detergents such as CHAPS, and other nonionic detergents like octyl glucoside, for solubilizing many membrane proteins and preserving their biological activity (Kessi et al., 1994). In addition, it has been shown that it is possible to reconstitute some membrane proteins into mixed DMPC/DHPC micelles (Sanders & Landis, 1995), and it seems likely that these micelle systems will prove to be an effective model membrane system for NMR studies of membrane proteins (Sanders & Schwonek, 1992; Sanders, 1993). We have shown that such mixed DMPC/DHPC systems also provide a convenient model system for investigating the effects of the lipid bilayer on membrane protein folding.

The rate-limiting step in bacteriorhodopsin folding can be controlled both by the lateral pressure which the membrane lipids impose on the folding protein and by a direct pH effect on the protein itself. This work has not only given us an insight into how the lipid bilayer influences protein folding but also allowed us to control and slow folding. As a result, we have been able to apply circular dichroism spectroscopy in our studies of bacteriorhodopsin folding and obtain structural information on a transient folding intermediate (Riley et al., 1997). Thus, the ability to slow folding events, and effectively trap intermediates, opens up the study of membrane protein folding intermediates to a wide range of spectroscopic techniques.

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