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Effects of Detergent Environments on the Photocycle of Purified Monomeric Bacteriorhodopsin[†]

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ABSTRACT: Time-resolved difference spectra have been obtained for the photocycle of delipidated bacteriorhodopsin monomers (d-BR) in six different detergent micelle environments that were prepared by two new detergent-exchange techniques. A global kinetic analysis of the photocycle spectra for d-BR in each detergent environment was performed. Comparison of these results with those obtained for the photocycle of bacteriorhodopsin in purple membrane (PM) shows that there is one fewer kinetically distinguishable process for monomeric BR between the decay of the K intermediate and the rise of the M intermediate. Assuming a sequential pathway occurs in the photocycle, it appears that the equilibrium between the L and M intermediates is reached much more rapidly in the detergent micelles. This is attributed to a more direct interaction between Asp-85 and the proton on the nitrogen of the Schiff base of retinal for BR in the detergents. Equilibrium concentrations of late photocycle intermediates are also altered in detergents. The later steps of the photocycle, including the decay of the M intermediate, are slowed in detergents with rings in their hydrocarbon region. This is attributed to effects on conformational changes occurring during the decay of M and/or other later photocycle intermediates. The lifetime of dark adaptation of light-adapted d-BR in different detergent environments increases in environments where the lifetime of the M intermediate increases. These results suggest that the high percentage of either unsaturated or methyl-branched lipids in PM and the membranes of other retinal proteins may be important for their effective functioning.

Bacteriorhodopsin (BR)[1] is the primary protein found in the cell membrane of *Halobacterium halobium*. Absorption of visible light by the chromophore of BR, a protonated Schiff base of retinal, initiates a photocycle whose function is to pump one or more protons across the cytoplasmic membrane (Ottolenghi, 1982; Stoeckenius & Bogomolni, 1982; Ottolenghi & Sheves, 1989). A large number of studies have been reported on how light absorption by BR eventually leads to the transfer of a proton across the membrane.

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[1] Abbreviations: BR, bacteriorhodopsin; $C_{12}E_8$, octylethylene glycol dodecyl ether; d-BR, delipidated BR monomers; d-BR/CHAPSO, d-BR in 16 mM 3-[(3-cholamidopropyl)dimethylammonio]-2-hydroxy-1-propanesulfonate; d-BR/NG, d-BR in 18 mM nonyl β-D-glucopyranoside; DDM, dodecyl β-D-maltoside; HTX, hydrogenated Triton X-100; NG, nonyl β-D-glucopyranoside; PM, purple membrane; TX, Triton X-100.

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Many studies have shown that after absorption of light BR goes through a number of intermediates that can, at least to some extent, be distinguished by their different spectral characteristics. Though there is no general agreement as to the details of the photocycle, there is general agreement that in the nanosecond to millisecond time regime the excited BR goes through at least five intermediates that are known by their distinguishing spectral characteristics; these intermediates are commonly called K, L, M, N, and O (Lozier et al., 1975; Beach & Fager, 1985; Hofrichter et al., 1989). It has been shown that at times earlier than 10 ns there are a number of intermediates that eventually lead to these later intermediates (Sharkov et al., 1985; Mathies et al., 1989; Ottolenghi & Sheves, 1989) and that the K intermediate seen on the nanosecond time scale is spectrally different from the K intermediate seen on the picosecond time scale (Shichida et al., 1983; Milder & Kliger, 1988).

Many authors have noted that both the rise and decay of the M intermediate are kinetically biphasic (Hanamoto et al., 1984; Dancshazy et al., 1986, Mauer et al., 1987a,b; Varo & Lanyi, 1990). This and other evidence have led to either of three explanations of the basic nature of the photocycle of BR: (1) there are two or more different conformations of light-adapted BR that are in thermal equilibrium and/or photoequilibrium and that may have somewhat different photocycle kinetics (Dancshazy et al., 1988; Diller & Stockburger, 1988; Birge et al., 1989; El-Sayed et al., 1989); (2) there is only one form of light-adapted BR, but it has branches in its photocycle that lead to two forms of the L and/or M intermediate that are not in equilibrium with each other (Kalisky et al., 1981; Kalisky & Ottolenghi, 1982; Renard & Delmelle, 1985; Mauer et al., 1987a,b); (3) there is only one form of light-adapted BR, and its photocycle occurs in a linear sequence (Lozier et al., 1975; Xie et al., 1987; Chernavskii et al., 1989; Hofrichter et al., 1989; Ames & Mathies, 1990; Varyo & Lanyi, 1990). Recent transient absorption and resonance Raman results indicate that the photocycle can be described by a linear sequence from a single light-adapted BR and that from nanoseconds to milliseconds the results are consistent with either of the mechanisms (Ames & Mathies, 1990; Varo & Lanyi, 1990) shown in Schemes I and II. Within these schemes other intermediates may be postulated to occur, but if they exist they would be too spectrally similar to those already observed to have been easily identified.

Scheme I

$$\mathrm{K} \rightarrow \mathrm{L} \rightleftarrows \mathrm{M_I} \rightarrow \mathrm{M_{II}} \rightleftarrows \mathrm{N} \rightleftarrows \mathrm{O} \rightarrow \mathrm{BR}$$

Scheme II

$$\mathrm{K} \rightarrow \mathrm{L} \rightleftarrows \mathrm{M} \rightleftarrows \mathrm{N} \rightarrow \mathrm{O} \rightarrow \mathrm{BR}$$

Almost all of the studies on the photocycle of BR have been of the protein in its native membrane (PM), where it is found in a two-dimensional crystalline array with each BR part of a local trimeric structure (Henderson & Unwin, 1975). Due to its hydrophobic character (Engelman & Zaccai, 1980), BR is not soluble in water without its native lipids or some other external solubilizing agent (Stoeckenius & Bogomolni, 1982; Miercke et al., 1989a). Thus, to study functional BR in conditions other than in PM, it is necessary to have either detergent, artificial lipids, or a mixture of both as a solubilizing agent.

It would clearly be interesting to study the photocycle of BR in different environments to see the effect the specific environment of PM has on the photocycle. Furthermore, since many of the studies of site-directed mutants of BR are done on monomeric BR (Khorana, 1988; Holz et al., 1989; Subramaniam et al., 1990), it is also important to know the effect that removing BR from PM has on its photocycle. Detergent solutions are often used in structural and functional studies of membrane proteins, yet there have been few systematic studies on the effect that different detergents have on membrane protein function (Helenius & Simons, 1975; Helenius et al., 1979; Fong et al., 1982). Since the photocycle of BR is both well studied and quite complex, it may be able to serve as a basis for describing the effect different detergents have on protein function [for some previous studies of BR in environments other than PM, see Dencher and Heyn (1978), Dencher et al. (1983), Lam and Packer (1983), and Iwasa et al. (1985)].

For BR and other membrane proteins [e.g., halorhodopsin (Duschl et al., 1988) and cytochrome *c* oxidase (Robinson et al., 1984)], the best detergent for gentle, efficient solubilization is different from the optimum detergent for purification; another, different detergent may then be best suited for experiments such as crystallization (Michel, 1983; Garavito et al., 1986; Kühlbrandt, 1988) and functional studies. Thus, it is useful to be able to vary the detergent that forms the micelles that solubilize the membrane protein. Herein we report the successful replacement of CHAPSO by five other detergents in d-BR micelles by using either dye–ligand affinity chromatography or multiple high-pressure filtration steps.

We have previously shown that it is possible to obtain stable and very pure BR in detergent micelle solutions that retain less than 4% of the original native lipids from PM (Miercke et al., 1989a,b). In the current work we use a number of different highly purified detergent micelle solutions to study the effect of micelle composition on the photocycle. In the detergent solutions we find there is one fewer kinetically distinguishable process in the time regime of the decay of the K intermediate and the rise of the M intermediate than in PM. We also find that the apparent decay of the M intermediate is significantly slower in detergents that have a rigid moiety in their hydrocarbon portion. A correlation between the apparent rate of the decay of the M intermediate and the rate of dark adaptation of the light-adapted BR is also seen for the different detergent solutions.

EXPERIMENTAL PROCEDURES

Preparation of Protein Samples. PM, obtained from *H. halobium* strain JW-3, was provided by Walther Stoeckenius at USCF. Absorption spectroscopy, quantitation of BR, and preparation of d-BR/CHAPSO in buffer A (100 mM NaCl and 20 mM acetate, pH 5) were done as previously described (Miercke et al., 1989b). Initial exchange of CHAPSO by NG (Sigma), along with removal of NaCl in preparation for chromatography, was done by multiple high-pressure filtration steps with a 12-mL stirred cell, YM-30 membrane (Amicon), and 20 psi at 4 °C; 1 mL containing 7 mg of protein was diluted to 10 mL in buffer A containing 18 mM NG without NaCl and was concentrated to 2 mL three times. This was followed by dye–ligand chromatography at 4 °C to remove any denatured protein that was in the preparation. Protein samples were applied to a 1 × 3 cm column of Red A (Amicon) that was prepared according to the manufacturer's specifications and equilibrated with 18 mM NG and 5 mM acetate, pH 5 (buffer B). Upon sample application, the matrix was washed with 4 bed volumes of buffer B followed by 2 bed volumes of buffer B containing 0.1 M NaCl, and the protein was then eluted with 0.5 M NaCl in buffer B. Protein eluted from the affinity column was concentrated to 3 mg of d-BR/mL and dialyzed (Spectra/Por 6, MWCO 25 000) against

100 mL of 18 mM NG in buffer A for 5 days at 4 °C in the dark. The d-BR/NG had a dark-adapted absorbance ratio ($A_{280\mathrm{nm}}/A_{548\mathrm{nm}}$) of 1.5, which represents pure monomeric BR protein (Miercke et al., 1991). Total recovery from the column was 90–100%. Prior to flash photolysis, d-BR/NG was diluted to 0.4 mg of d-BR/NG with fresh dialysis buffer and filtered (0.2 μm, Gelman Acrodisc).

Dye–ligand chromatography could not be used for the preparation of monomeric BR in other detergents used in this study; BR either did not adhere to the matrix or could not be eluted in conditions amenable for further use. In these cases, multiple high-pressure filtration steps were used. DDM (Calbiochem), $C_{12}E_8$ (Nikkol), TX (Pierce), and HTX (Pierce) replaced NG by multiple filtration steps as described above except for the following differences: (1) the initial sample was 2–3 mg; (2) dilutions were made with 1.5 mM $C_{12}E_8$, 0.06% TX, 0.15% HTX, or 4 mM DDM in buffer B; (3) final dialysis was for 7–10 days versus buffer A containing the appropriate amount of detergent at the concentration used for detergent exchange. This method does not denature BR (or bovine rhodopsin, unpublished results) since the $A_{280\mathrm{nm}}/A_{548\mathrm{nm}}$ ratio in DDM, HTX, and $C_{12}E_8$ was essentially unchanged. Due to the high absorbance by TX at 280 nm, an accurate absorbance ratio could not be obtained in this detergent. Dialysis for long periods of time followed by a 4–6 dilution in fresh dialysis buffer ensured insignificant levels of the detergent being replaced and gave the new detergent at the concentration desired. The final detergent concentrations used were the minimal amount required to prevent aggregation of the d-BR [for a more thorough discussion of detergent exchange by multiple filtration steps, see Miercke (1986)].

Each detergent solution was 20 mM in acetate and 100 mM in NaCl and was stored at pH 5 (4 °C), as we have found this to be the optimum pH for the stability of monomeric BR (Miercke et al., 1989a). As most studies were done at pH 5, no further adjustments of these solutions were carried out. For studies where the effect of pH on photocycle kinetics was studied, the solutions were also made 4 mM in phosphate and then an appropriate amount of either a NaOH or an HCl solution was slowly added to give the desired pH. Studies of BR in PM were carried out in solutions with 1 mM each of sodium phosphate, sodium acetate, and sodium borate and 10 mM NaCl at pH 7.

Time-Resolved Spectroscopy and Kinetic Analysis. The photocycle was studied by using a laser flash photolysis apparatus that utilized a 7-ns, 532-nm pulse from a Quanta-Ray DCR-2 Nd:YAG laser as the pump source and an EG & G Q3CP-2 xenon flash lamp ($\tau \simeq 5$ μs) as the probe source (Lewis et al., 1987). The excitation and probe beams entered the sample at right angles to each other, and the probe beam was polarized at the magic angle (54.7°) with respect to the pump beam polarization to eliminate the possibility of photoselection artifacts occurring in the observed kinetics. After passage through the sample, the probe beam was focused into a Jarrell-Ash 0.27-m polychromator equipped with a 250-μm entrance slit and a 150 groove/mm holographic grating. The spectrum was dispersed across a PAR 1420 optical multichannel analyzer (OMA) that was gated on for 10 ns by a PAR 1302 pulser. A Hoya UV26 filter was placed in front of the entrance slit of the polychromator to eliminate artifacts in the red part of the spectrum due to near-UV light emerging at second order from the polychromator. Transient difference spectra were obtained by taking the log of the ratio of the probe signal without and with laser excitation. Temporal information was obtained by changing the relative position in time between the pump laser pulse and OMA gate pulse. The position in time of the probe flash lamp pulse was changed along with the change in the OMA gate pulse to ensure that the same amount of light was collected at all data collection times.

Samples for the kinetic studies were placed in a 2 × 10 mm fluorescence cuvette that sat inside a thermostated cell holder (20.5 ± 0.5 °C) such that the probe beam traversed the long axis of the cuvette. Light adaptation of each sample was carried out with a Cole-Parmer 150-W focused quartz–halogen lamp that had a light flux of 0.2 W/cm^2 after infrared light was filtered with a heat filter and light of wavelengths shorter than 450 nm was filtered with a Corning 3-69 glass filter. In most cases 1 min of illumination was sufficient to achieve full light adaptation by the twin criteria of no further red shift in the absorption spectrum with further excitation and the lack of a red-absorbing transient species in the 10 μs–1 ms time regime after laser excitation. Such a red-absorbing species would be indicative of the C_{610} intermediate obtained from residual 13-cis chromophore undergoing its photocycle (Iwasa et al., 1981; Hofrichter et al., 1989). In the case of the detergents where the dark adaptation was relatively rapid (<20 min), a low light level (0.04 W/cm^2) was maintained on the sample during data collection to ensure that the BR remained in the all-trans form during data collection. For the detergents where the dark adaptation rate was slower, a low light level was not maintained, as in these cases the decay of the M intermediate is so slow (vide infra) that there would have been a significant buildup of M in the photostationary state during the data collection. For each sample condition (e.g., detergent, pH), transient difference spectra were taken at 30–40 different delay times after laser excitation, and at least three spectra were taken per decade in time. Care was taken to obtain a higher density of transient difference spectra in time regimes where there was an obvious large change in the difference spectrum with time. Typically, for each aliquot of sample solution, data were taken at either one or, in the detergents where BR is very photostable, two times. At each time, 12–24 transient difference spectra were taken and averaged.

The data were analyzed by a global spectral/kinetic fitting routine adapted for use on IBM/clone personal computers. In short, the data set to be analyzed is a series of transient difference spectra taken at different times following sample photolysis. Since these spectra have redundant spectral information, we used a singular value decomposition (SVD) approach (Matlab, The Math Works) to decompose them into basis spectra, with the first few representing the relevant information and the succeeding ones representing noise. The significant basis spectra were then used in a nonlinear least-squares routine (Matlab, The Math Works) to obtain the rate constants that couple the difference spectra. This nonlinear routine assumes that every kinetic process is first order. One chooses the number of first-order processes, and the resulting fit gives the rate constants and "b-spectra" that minimize the sum of the residuals between the resultant calculated spectra and the actual experimental spectra:

$$\Delta\mathrm{OD}(\lambda,t) = \sum_{i=0}^{n} b_i(\lambda)\,\exp(-k_i t) \qquad (1)$$

The criteria for goodness of fit involves the magnitude of the sum of the residuals and whether there is any systematic spectral structure in the residuals. In practice, one guesses the number of first-order processes that the data represent and obtains a fit. One then tries a fit with one more kinetic decay and observes if the residuals improve (decrease) significantly. The ultimate number of kinetic decays chosen is the number at which the addition of the last kinetic decay to the analysis significantly decreased the residuals, while the addition of one more kinetic decay does not significantly decrease the residuals. This is a somewhat subjective process, and it is discussed in detail by Hug et al. (1990), Nagle et al. (1982), and Xie et al. (1987).

The SVD/nonlinear least-squares fit only gives the kinetic rate constants and the intermediate transient difference spectra directly when the mechanism is that of purely parallel decays of all components starting from the experimental zero time. It is well-known that a purely parallel decay mechanism is not the case for the photocycle of BR and that the photocycle is primarily sequential. The results from the global fit can be applied to the description of the photocycle for a sequential photocycle if one knows the actual mechanism. Then it is possible to obtain the actual difference spectra as linear combinations of the b-spectra obtained in the fit. However, if one does not know the mechanism, it is necessary to postulate one and from this one can obtain putative spectra and rate constants. It is fairly straightforward to obtain the difference spectra if one assumes a purely sequential model with no back-reactions, and in this case the rate constants obtained in the fit are just the rates between successive intermediates. If one assumes equilibria are established between successive intermediates, the rate constants and difference spectra become linear combinations of those obtained in the fit, with the rate values and spectra depending on both the specific mechanism and the equilibrium constants between the intermediates. Whatever the mechanism chosen to calculate the difference spectra, the absorption spectrum of each intermediate will be the sum of the difference spectrum obtained for the intermediate and the percentage of the light-adapted BR spectrum that represents the fraction of the material undergoing the photocycle.

The analyses were carried out on either a 80386/80387 25-MHz computer with 4 MB of memory or a 80386/80287 20-MHz computer with 1 MB of memory. The difference spectra at each time had absorption values at 155 wavelengths evenly spaced between 350 and 720 nm. It was possible to include more than 50 time points in our analysis with the larger/faster machine, and a 5 exponential fit required about 5 min. Analysis using the smaller/slower machine was limited to a maximum of about 18 times. This necessitated our splitting the analysis into two time regimes. This was possible for all the samples in detergent, as there was always at least a 2 order of magnitude difference in time between the end of the rise of M and the beginning of the decay of M. The rates for the rise and decay of M are not as cleanly separated in time in PM, and in this case the spectra at all times had to be analyzed simultaneously.

The rate of dark adaptation of BR in detergent solutions was measured by determining the wavelength in the visible region with the largest absorption difference between the light- and dark-adapted forms (typically near 560 nm). The sample was then light adapted, as described above, for 1 min or more and quickly placed in an IBM 9420 spectrophotometer. The absorption versus time was recorded at the wavelength of greatest difference between light-adapted and dark-adapted forms, and the kinetic rate constant was determined by fitting the result to a single first-order process.

RESULTS AND DISCUSSION

Comparison of the BR Photocycle in PM and in NG. Spectral/kinetic data were obtained for both BR in the native purple membrane (PM) and delipidated BR monomers in NG micelles (d-BR/NG). Time-resolved spectra for these two conditions are displayed in Figure 1. Clearly, there are some differences in the photocycle of BR for the two solvent conditions. One difference between the two is that a rise in the absorption centered near 410 nm occurs concurrently with the decay in the absorption centered near 610 nm for d-BR/NG but not for PM. Also, during the time regime of the decay of the K intermediate and the rise of the M intermediate, there is a small rise followed by a decay in the absorption near 500 nm in the photocycle of PM that is not seen in d-BR/NG. Another difference in the two photocycles is that the red absorption that grows in and decays during the decay of the M intermediate absorbs further to the red in PM (≈650 nm) than in d-BR/NG (≈610 nm).

The b-spectra, obtained from a global fit of the data shown on Figure 1 to eq 1, are shown in Figure 2. Also shown in Figure 2 are the transient difference spectra for PM and d-BR/NG obtained from the b-spectra if a purely sequential photocycle without back-reactions is assumed. Shown in Figure 3 are the intermediate spectra that are obtained from these difference spectra by adding to each the appropriately scaled light-adapted BR spectrum to correct for the amount of BR photolyzed. The PM kinetics are best fit by a 5 exponential process, while the d-BR/NG kinetics are best fit by a 4 exponential process. A comparison of the difference spectra of the two shows that the second in d-BR/NG is similar to the third in PM. This suggests that there is one less photointermediate observed in the detergent solution because the first and second processes that occur in PM occur simultaneously in d-BR/NG.

As seen in Figure 3, some of the putative intermediate spectra for both PM and d-BR/NG do not have a single band in the visible region. This has been seen before for PM (Hofrichter et al., 1989) and probably indicates that a simple sequential pathway is not an accurate representation of the actual mechanism. Others have shown that equilibria probably exist between at least some of the intermediates (Chernavskii et al., 1989; Ames & Mathies, 1990; Varo & Lanyi, 1990), and two plausible mechanisms, as shown in Schemes I and II of the introduction, are currently supported by recently published spectral/kinetic results. Regardless of the details, both schemes postulate that an equilibrium is reached between the L and M intermediates. Thus, it appears that in PM the first observed kinetic process is the decay of K to L and the second is the establishment of an equilibrium between L and M. Since both the first and second decay processes observed in PM occur during the first process of d-BR/NG, it appears that in d-BR/NG, unlike in PM, the equilibrium between L and M is obtained concurrently with the decay of the K intermediate. Thus, a major difference between the photocycle kinetics of PM and d-BR/NG is that the equilibrium between L and M is established much faster in the detergent solution. Note in Figure 3 that for the purely sequential model the intermediate spectra for the third intermediate in PM and the second intermediate in d-BR/NG are quite similar, each showing an apparent equilibrium between L and M intermediates. The relative amounts of L and M do not appear to be greatly different in the two cases, yet the rate at which the equilibrium is established is much faster in the detergent, implying that both the forward and reverse rates between L and M are greatly increased in d-BR/NG compared to PM. Thus, detergent solubilization seems to decrease the activation barrier between L and M.

Since a major difference between L and M is the state of protonation of the Schiff base of retinal, the rate of protonation/deprotonation between these two states must be en-

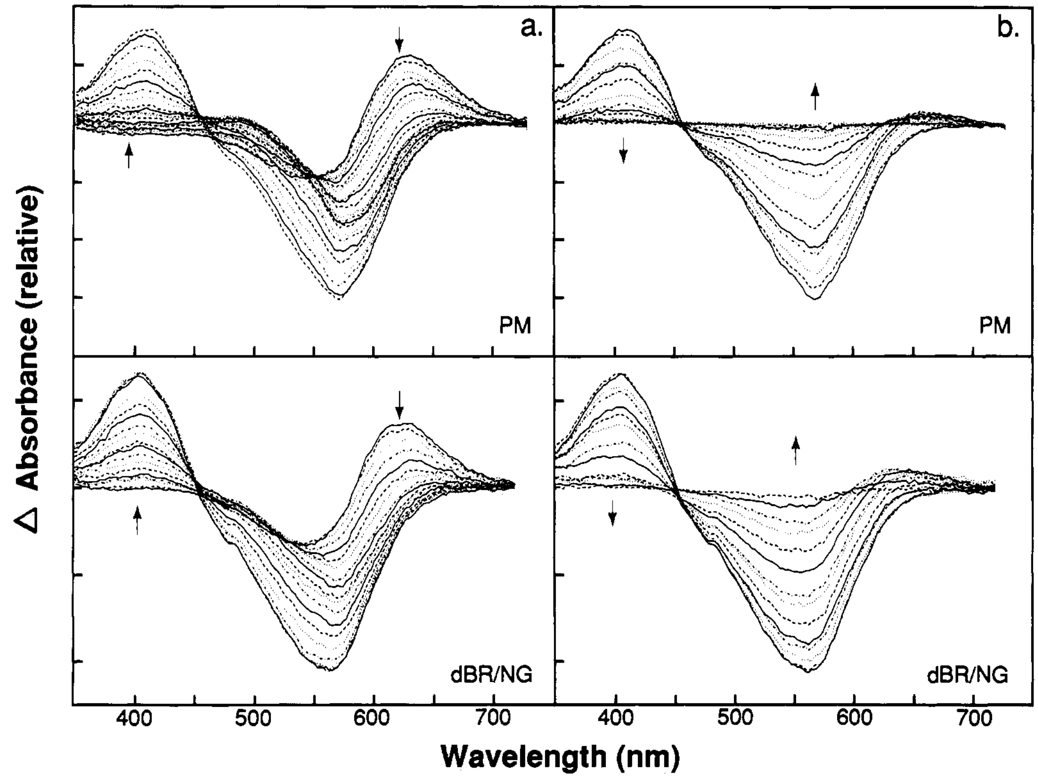

FIGURE 1: Transient difference spectra obtained for PM and d-BR/NG after excitation at 532 nm with a 2-mJ, 7-ns laser pulse. (a) Short-time data showing the rise of the M intermediate. The times after laser excitation for the spectra for PM (top) are 50 ns, 100 ns, 250 ns, 350 ns, 500 ns, 750 ns, 1 μs, 1.5 μs, 2 μs, 3 μs, 5 μs, 7.5 μs, 10 μs, 15 μs, 25 μs, 35 μs, 50 μs, 75 μs, 100 μs, 180 μs, 250 μs, and 500 μs; the times after laser excitation for the spectra for d-BR/NG (bottom) are 50 ns, 100 ns, 250 ns, 350 ns, 500 ns, 750 ns, 1 μs, 1.5 μs, 2 μs, 3 μs, 4 μs, 5 μs, 7.5 μs, 10 μs, 15 μs, 25 μs, 35 μs, 50 μs, and 100 μs. (b) Long-time data showing the decay of the M intermediate and the return of the initial light-adapted state. The times after laser excitation for PM (top) are 750 μs, 1 ms, 2 ms, 3 ms, 3.5 ms, 5 ms, 7.5 ms, 10 ms, 12 ms, 15 ms, 20 ms, 25 ms, 30 ms, 40 ms, and 50 ms; the times after laser excitation for the spectra for d-BR/NG (bottom) are 50 μs, 100 μs, 250 μs, 500 μs, 1 ms, 1.5 ms, 2 ms, 3 ms, 5 ms, 7.5 ms, 10 ms, 20 ms, and 40 ms. Arrows indicate the direction of change of the signal with increasing time after laser excitation.

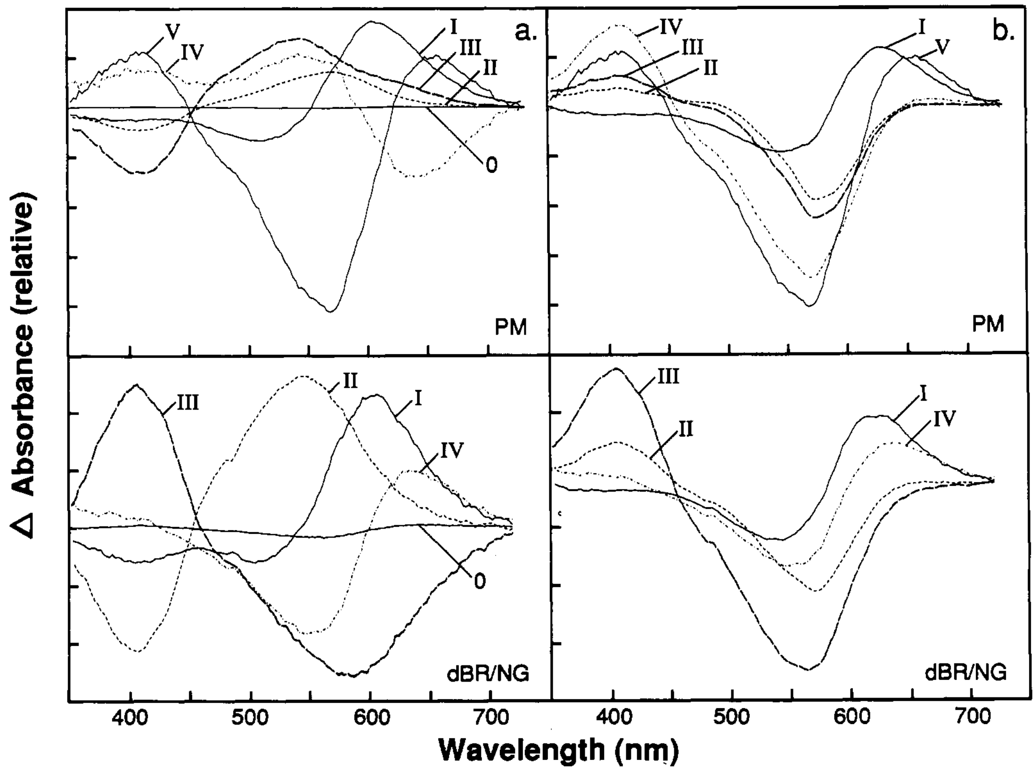

FIGURE 2: (a) B-Spectra obtained after the SVD/nonlinear least-squares kinetic fit of the data shown in Figure 1 for PM (top) and d-BR/NG (bottom). (b) Difference spectra for PM (top) and d-BR/NG (bottom) obtained from the lifetimes and b-spectra of the SVD/nonlinear least-squares fit by assuming that the photocycle mechanism is purely sequential, with no equilibria between intermediates. The Roman numerals indicate the sequence of the spectra in time.

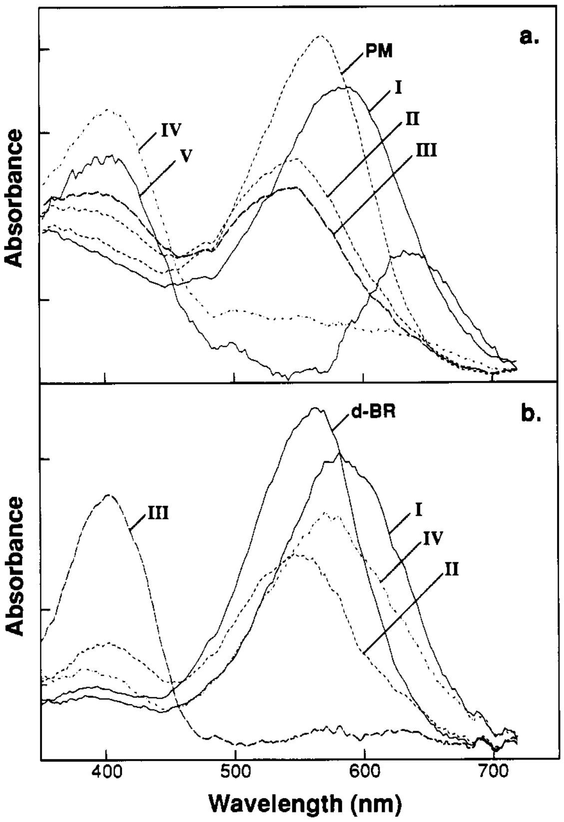

FIGURE 3: Spectra of the transient intermediates obtained by adding back a portion of the light-adapted spectrum of either PM or d-BR/NG to the difference spectra obtained by assuming a purely sequential decay mechanism (Figure 2b): (a) PM; (b) d-BR/NG. A more complex mechanism probably occurs in the photocycle of PM and d-BR/NG (see text). The Roman numerals indicate the sequence of the spectra in time.

Table I: Lifetimes of the Observed Kinetic Processes for BR in Various Detergent Micelles at pH 5[a,b]

	sequence of observed kinetic processes				
detergent[a]	1 (ns)	2 (μs)	3 (ms)	4 (ms)	5 (ms)
NG	690	9.3	2.0	9	
DDM	750	10.3	7.1	10	
$C_{12}E_8$	580	14.8	4.4	15	
TX	780	10.0	3.7	16	160
HTX	890	13.2	3.6	17	250
CHAPSO	860	10.2	7.4	70	670

[a] All solutions were buffered with 20 mM acetate and had 100 mM NaCl. [b] Typically, the lifetime of each kinetic process was reproducible to within ±15% when different sets of data, taken under identical experimental conditions, were analyzed.

hanced for BR in the detergent. This might imply that in d-BR/NG the counterion to the Schiff base nitrogen is in a position such that it can more readily interact with the nitrogen's proton. Interestingly, Varo and Lanyi (1990) have shown that for PM in high pH solutions there is some rise of the M intermediate concurrent with the decay of the K intermediate, implying that the equilibration of the L and M intermediates is also rapid under that solvent condition. The absorption maximum of BR is probably regulated by a complex series of interactions between the chromophore and the protein (Blatz et al., 1972; Kliger et al., 1977; Milder & Kliger, 1977; de Groot et al., 1989; Ottolenghi & Sheves, 1989). However, there is very strong evidence that the position of the counterion of the Schiff base nitrogen strongly influences the absorption maximum, with a closer counterion corresponding to a smaller red shift (Blatz et al., 1972; de Groot et al., 1989). The absorption maximum of light-adapted PM at neutral pH is 568 nm, while that of light-adapted d-BR/NG is 553 nm and that of light-adapted PM in high pH solutions is around 550 nm (Muccio & Cassim, 1979). Thus, light-adapted d-BR/NG and high pH PM have very similar absorption maxima that are blue-shifted from the absorption maximum of PM at neutral pH. This might be due to a smaller separation between the counterion and the Schiff base nitrogen in both d-BR/NG and high pH PM. This is also consistent with the more rapid deprotonation/protonation kinetics in the L to M transition for both d-BR/NG and high pH PM.

Mutagenesis studies of BR have strongly implicated Asp-85 as the primary counterion of the protonated Schiff base of retinal, as a strongly red-shifted BR is produced at all pHs when a neutral residue is substituted for it (Butt et al., 1989; Otto et al., 1989; Subramaniam et al., 1990). Mutagenesis studies have also revealed the crucial role that Asp-85 plays in the deprotonation of the Schiff base, as the deprotonated M form is not produced in the photocycle when Asp-85 is replaced by a neutral residue (unpublished results; Stern et al., 1989). Thus, the results from the current study imply that in d-BR/NG and in PM at high pHs there is a structural change in the BR that leads to placement of Asp-85 closer to the proton of the Schiff base. The exact nature and extent of how the structure has changed from the neutral PM form cannot be surmised from the current data, but it probably does not entail significant changes in the secondary structure of the protein, as far-UV circular dichroism studies of BR in either high pH PM (Muccio & Cassim, 1979) or detergent-solubilized BR (Reynolds & Stoeckenius, 1977; Dencher & Heyn, 1978; Jap et al., 1983; Lam & Packer, 1983; Mao & Wallace, 1984) have shown that these environments do not strongly perturb the secondary structure. Thus, it appears that changes in the external environment of the protein may subtly affect the tertiary structure of the protein, such as the relative positions of the helices.

In addition to the differences noted above between the photocycles of PM and d-BR/NG during the decay of the K intermediate and the rise of the M intermediate, differences between them were observed during the decay of the M intermediate. The spectra of the last intermediate observed before the return of light-adapted BR (intermediate spectrum IV for d-BR/NG and V for PM as shown in Figure 3) have different spectral characteristics. According to Scheme I, these spectra will reflect the concentrations of the species in the equilibrium between the M, N, and O intermediates. As M is the only intermediate that absorbs strongly near 410 nm, PM appears to have a higher amount of M than d-BR/NG at this point in the photocycle. The red band is centered near 575 nm in d-BR/NG while it is centered near 635 nm for PM, indicating that N is more prevalent than O in the equilibrium in d-BR/NG while O is more prevalent than N in the equilibrium in PM.

Comparison of the Photocycle of BR in Various Detergents. The photocycle kinetics of highly purified BR in detergent micelles were determined in five other detergents at pH 5: DDM, $C_{12}E_8$, TX, HTX, and CHAPSO. In all of the detergents, the decay kinetics of the K intermediate, the kinetics of the rise of the M intermediate, and the spectra obtained for the photocycle intermediates were similar to those reported above for d-BR/NG. Table I reports the rate constants obtained from the SVD/nonlinear least-squares fit of the photocycle kinetics for BR in all of the detergents.

The kinetics at the later times, consisting of the decay of the M intermediate and the return to light-adapted BR (processes 3 and 4 or 3, 4, and 5), did vary significantly in the

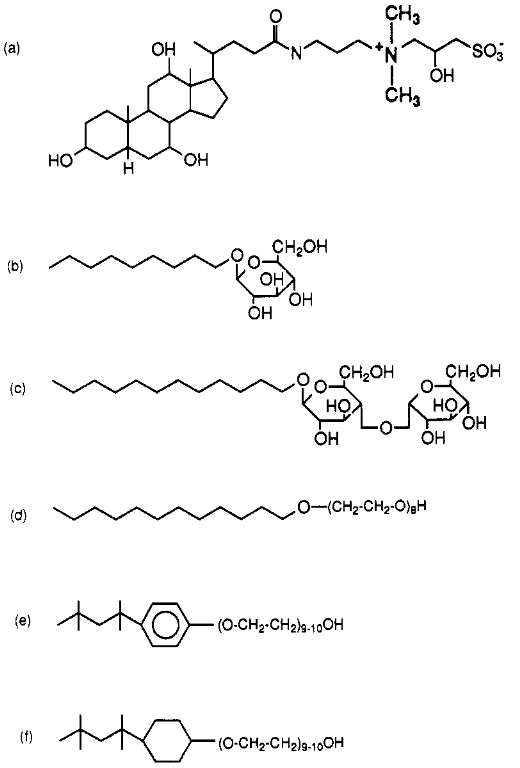

FIGURE 4: Structures of the detergents used to form micelles of d-BR: (a) CHAPSO; (b) NG; (c) DDM; (d) $C_{12}E_8$; (e) TX; (f) HTX.

detergents (Table I). The M decay kinetics of d-BR in the various detergents appear to involve either two or three distinguishable kinetic processes (thus a total of four or five distinguishable kinetic processes through the observed portion of the photocycle). Detergents with two kinetic decays for the M intermediate are NG, DDM, and $C_{12}E_8$, and for them the slowest decay of M and recovery of BR have a lifetime no longer than 15 ms. Detergents with three kinetic decays for the M intermediate are TX, HTX, and CHAPSO, and for them the slowest decay is at least an order of magnitude slower than for BR in the other class of detergents.

Figure 4 shows the chemical structures of the six detergents in whose micelles we studied the photocycle kinetics of BR. The most obvious difference between the detergents in the two classes is that those that have three observed rates for the decay of M and recovery of BR have at least one hydrocarbon ring in their hydrophobic region while the others do not have this feature. Previous work on fluorescence polarization/depolarization of aromatic hydrocarbons added to micelles in water suggests that rigidity, or increased microviscosity, may be a factor that defines the differences between the two classes of detergents. Addition of cholesterol to aqueous micelles of quaternary ammonium salts with aliphatic chains of 12, 14, 16, and 18 carbons leads to much greater polarization in the fluorescence of perylene in the micelles, implying that the rigid hydrocarbon structure of cholesterol increases the microviscosity of the micelle interior (Shinitzky et al., 1971). A fluorescence polarization study with 2-methylanthracene has shown that micelles of the cholesterol-like detergent sodium taurocholate have much greater microviscosity than micelles of the aliphatic detergent sodium lauryl sulfate (Chen et al., 1975). Additionally, HTX micelles induce nearly as high a fluorescence polarization in perylene as sodium taurocholate micelles and a much higher fluorescence polarization than sodium dodecyl sulfate micelles, indicating that movement of perylene is restricted within THX micelles (Nithipatikom & McGown, 1988). From these studies it can be seen that a detergent with a hydrocarbon ring can form micelles whose interior acts to restrict the movement of embedded species.

While the rigidity of the microenvironment surrounding the BR in the micelle may play a role in slowing down the later part of the photocycle, other factors probably contribute to the changes in the kinetics as well. The lipids of the native membrane of BR are negatively charged, and this leads to a much lower effective pH environment for BR in PM than the pH in the bulk solution (Szundi & Stoeckenius, 1989). Upon replacement of the native lipids by neutral detergents, BR will see a pH nearer to that of the bulk solution; thus, monomeric BR sees an effectively higher pH than BR in PM at the same solution pH. This may partially explain why rapid L to M equilibration occurs at a higher solution pH for PM than it does for d-BRs. However, rapid L to M equilibration and slow M decay occur under the same pH conditions in PM while they do not for d-BR/NG, implying that factors other than pH must be important in affecting the photocycle kinetics of d-BR.

The accessibility of water within the micelle may also influence the rate of decay of later photocycle intermediates. The kinetics of apparent M decay in dehydrated films of BR show similarities to the kinetics reported here for d-BR in micelles of detergents with hydrocarbon rings. Korenstein and Hess (1977) reported that the decay kinetics of the transient M absorption are triphasic and much slower than in fully hydrated films of PM. They showed that a diffusion rate limiting proton transfer is not consistent with their data and posited that the slowed decay of the 412-nm absorption could be due to an inhibition of conformational changes caused by the dehydration. Similarly, it has recently been shown (Fukuda et al., 1990) that BR has a slow M decay in heavily delipidated PM. The authors note that surface pH effects only partially account for the slowing of the apparent M decay and suggest that conformational changes are inhibited in their heavily delipidated BR preparations.

While later photocycle steps may be slowed by the higher viscosity of the microenvironment of detergents with rigid hydrocarbon moieties, specific detergent–protein interactions might actually be a more significant factor. Baldwin and Hubbell (1985a,b) have shown for bovine rhodopsin that the meta I to meta II transition is inhibited when the protein is embedded in bilayers of lipids with short acyl chains or with *n*-alkyl chains. They ascribed this to unfavorable lipid–protein interactions exerting an effective pressure on the protein that inhibits the expansion of the protein during the meta I to meta II step. Bilayers of lipids with methyl groups or double bonds allow meta II formation, presumably because these lipids can more readily undergo gauche kinks and thus specifically solvate the protein. In the case of d-BR, detergents with rigid hydrocarbon moieties may solvate the protein poorly and produce more unfavorable interactions between the protein and hydrocarbon than detergents with alkyl chains. This might cause the slowing down of steps in the photocycle that involve significant protein conformational changes.

To the extent that rigidity of the environment is an important factor in the inhibition of the apparent decay of the M intermediate in the micelles of detergents with a hydrocarbon ring, there is the implication either that in the decay of at least one of the later intermediates there is a transformation to an intermediate with a larger volume or that the activated pathway to at least one of them involves an increase in volume. This is consistent with a previous kinetic photoacoustic study that demonstrated a volume increase associated with the observed decay of the M intermediate in PM (Ort

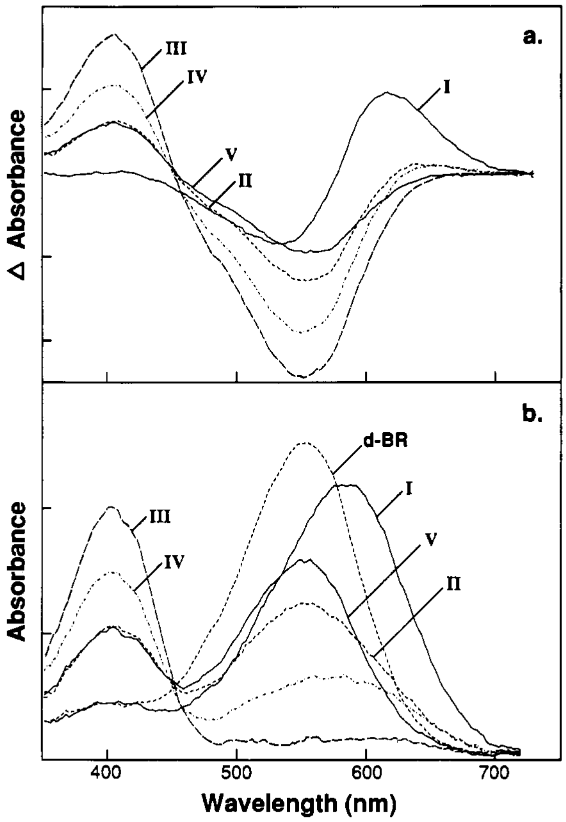

FIGURE 5: (a) Transient difference spectra obtained for the photocycle of d-BR/CHAPSO by assuming a purely sequential kinetic model. (b) Transient intermediate spectra obtained for d-BR/CHAPSO by adding a portion of the light-adapted spectrum to the difference spectra. The Roman numerals indicate the sequence of the spectra in time.

& Parson, 1978). The photoacoustic work also showed that the rate of volume change slowed as the pH was raised and that this change in rate had an apparent p*K* of about 8. This slowing of the rate with increasing pH is very similar to that associated with the slowing of the N to O kinetics that is also observed at higher pHs in PM (Kouyama et al., 1988; Varo & Lanyi, 1990), and it suggests that at least one effect that occurs in the detergents in which the apparent decay of M is slowed is that the micelle environment retards the decay of N to O. However, from the complexity of the kinetics and the intermediate spectra obtained, it is likely that the detergents affect more than just the decay of N to O in the long-time kinetics.

To try to understand the processes going on in the different detergents, we looked at the transient intermediate spectra obtained from the kinetic analysis of BR in the various micelles. Figure 5 shows both the transient difference and intermediate spectra obtained for d-BR/CHAPSO when a simple linear sequence of intermediates without back-reactions is assumed to occur. In the long times (>1 ms, processes 3, 4, and 5) the M intermediate, which absorbs near 410 nm, is a part of all of the intermediate spectra. Thus, if a sequential reaction is an appropriate model for the photocycle of d-BR/CHAPSO, at least two equilibria must be reached between M and later intermediates.

As mentioned above, the environments of the detergents tested produce a BR whose photocycle in the later time (>1 ms) kinetics can be described in either of two ways. Among the detergents with the very long lived kinetics (>20 ms), CHAPSO slows down the late part of the photocycle more than either TX or HTX. The cause of this behavior cannot be definitively established at this time, but it is consistent with rigidity of the hydrophobic portion of the detergent affecting the rate of conformational changes in the later steps of the photocycle. Thus one might expect CHAPSO, with its steroidal hydrophobic portion, to have a greater effect than the detergents that have only one ring in their hydrophobic portion.

Table II: Lifetimes of the Observed Kinetic Processes for BR in either NG or CHAPSO Micelles at Various pHs[a,b]

	sequence of observed kinetic processes				
pH	1 (ns)	2 (μs)	3 (ms)	4 (ms)	5 (ms)
NG					
4	600	10.3	2.9	8	
5	690	9.3	2.0	9	
6	590	9.9	1.4	12	
7	550	9.7	1.3	10	
CHAPSO					
4	880	12.2	23[c]	390[c]	
5	860	10.2	7.4	70	670
6	1090	10.3	10.1	150	1000

[a] Solutions were buffered with 20 mM acetate and 4 mM phosphate and had 100 mM NaCl, except at pH 5, where phosphate was not added. [b] Typically, the lifetime of each kinetic process was reproducible to within ±15% when different sets of data, taken under identical experimental conditions, were analyzed. [c] In CHAPSO at pH 4, the fourth process corresponds to the fifth in CHAPSO at pH 5 and 6, and the third process corresponds to the third and fourth processes in CHAPSO at pH 5 and 6.

The effect of the rigidity of the detergent on decay kinetics has also been noted for the meta I to meta II transition of bovine rhodopsin, a somewhat similar retinal protein (Litman et al., 1981; Konig et al., 1989). In the membranes of both PM and the rod disks of rhodopsin, the lipids have no rigid moieties in their hydrocarbon region. Rhodopsin has a high percentage of highly unsaturated lipids (Miljanich et al., 1979), while the lipids in PM have a number of methyl groups branched off their hydrophobic chains (Kates et al., 1982). The current work points to the possibility that these types of lipids make up the membranes of these retinal proteins because they need to have an environment that allows the later portions of their photocycle to occur rapidly enough for them to function effectively. This points to one possible similarity in the mechanics of retinal proteins, even though they can have very different end functions. It may be that the mechanism by which they translate the energy stored in the photoisomerization event to their function (i.e., energy or information transduction) is through a structure change that entails a transient volume increase. The degree to which solvation and rigidity effects, rather than surface charge or solvent accessibility effects, control late processes in the photocycle will require further study.

pH Effect on the Photocycle of BR in Detergents. As d-BR/NG and d-BR/CHAPSO were each typical of the two types of micelle environments, we studied how pH affects the photocycle of d-BR in each. The kinetic results, shown in Table II, show that in the pH range 4–7 there are much smaller pH effects for d-BR/NG than for d-BR/CHAPSO (data at pH 7 in CHAPSO were not obtained as the rate constant of the final decay was so slow it was difficult to study). For d-BR/NG there does not appear to be significant change in the short time ($\tau \simeq 600$ ns and 10 μs) kinetics, but there is a factor of 2 increase in the first long-time rate (process 3, presumably the rate of M going to equilibrium with N and O) and a small increase in the decay lifetime of the final equilibrated intermediates back to light-adapted BR (process 4) with increasing pH. For d-BR/CHAPSO, changing the pH does not significantly change the short-time kinetics, but there is clearly a lengthening of the longest decay time with increasing pH. Also, at pH 4 there are only two distinguishable kinetic processes on the millisecond time scale, while

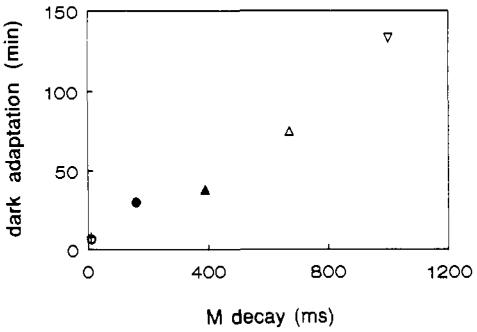

FIGURE 6: Plot of the observed lifetime of dark adaptation versus the lifetime of M decay for d-BR under different solvation conditions at 20 °C, pH 5 (unless otherwise noted): (+) d-BR/NG; (○) d-BR/DDM; (●) d-BR/TX; (▲) d-BR/CHAPSO (pH 4); (△) d-BR/CHAPSO; (▽) d-BR/CHAPSO (pH 6).

at pH 5 and 6 three kinetic processes are observed. This implies that back-reactions between intermediates in the sequence are sensitive to pH in the CHAPSO micelles and that the rate of one of the back-reactions becomes large enough to be important at pHs higher than 4. Since the rate of the slowest process decreases with increasing pH, one possible inference is that a protonation of a residue is involved in the rate-limiting step of this kinetic process.

We have noted above that both PM at high pH and BR in detergents appear to have similar structural changes that affect the rate at which L comes to equilibrium with M. High pH in PM slows down the N to O rate (Kouyama et al., 1988; Varo & Lanyi, 1990), and it is not surprising that a slowing down of the later components of the photocycle for BR is observed at higher pHs in detergents. Why a much smaller pH effect is seen for the more fluid detergent NG than for the more rigid detergent CHAPSO is not clear. One possibility is that the surface to bulk pH relationship is different in the two detergent micellar solutions. Both are uncharged detergents, but the zwitterionic nature of the polar headgroup in CHAPSO could give rise to differences in either the surface to bulk pH relationship or proton permeability properties. The smaller pH effect could also be due to differences in solvent accessibility in the different detergent micelles. Alternatively, it is possible for d-BR/CHAPSO that raising the pH of the solution leads to changes in the structure of the micelle that increase its microviscosity and/or change the protein–detergent interaction.

Dark-Adaptation Rates of BR in Detergent. While studying the photocycle of BR in the various detergents, we noted that the solutions varied in their rate of dark adaptation. We determined the dark-adaptation rates and found there to be a strong correlation between them and the rates of the later part of the photocycle, with the solutions with slower M decays having slower dark adaptations. Figure 6 shows the kinetic lifetime of the dark adaptation versus the kinetic lifetime of the final kinetic process both for detergents with and without hydrocarbon rings at pH 5 and for d-BR/CHAPSO at different pHs. These lifetimes are very highly correlated ($r = 0.982$, $m = 0.120$ min/ms, $b = 3.78$ min), with those conditions that increase the photocycle lifetime also increasing the lifetime of dark adaptation. If the slowing of the photocycle of BR in detergent micelles with rigid moieties can be primarily attributed to retardation of conformational changes, attribution of the slowed dark-adaptation rate to the same cause seems reasonable. It appears that the protein with the all-trans Schiff base of retinal has a smaller volume than the protein with the 13-cis Schiff base and/or that in the activated state between them the protein has an expanded volume. Interestingly, the correlation is also followed by d-BR/CHAPSO at various pHs (Figure 6).

CONCLUSIONS

We present the application of two new methods for changing the detergent environment of monomeric d-BR. Both methods are gentle and require relatively small amounts of new detergent. The general applicability of one of the techniques, dye–ligand affinity chromatography, may be limited as both efficient binding and subsequent removal of the protein are required. However, we found for d-BR that repeated filtration works very well, suggesting that this may be a generally useful technique for changing the environments of membrane proteins.

When compared to PM at neutral pHs, the equilibration of L and M in monomeric BR in purified detergent micelle solutions is much more rapid. This, along with the blue shift in the absorption maximum of light-adapted BR in the solutions studied (from 568 to 552–558 nm), suggests that the structure of the protein in detergent conditions has the Asp-85 residue closer to the proton of the protonated Schiff base of retinal than in PM at neutral pH. The similarity of the absorption maxima of light-adapted BR in detergents and in PM at high pHs, and their apparent similarities in the speed by which the L and M equilibration is achieved, suggests that a similar protein structural change occurs for PM at high pH, perhaps placing its Asp-85 nearer to the charge on the protonated Schiff base.

Differences were observed for the later time kinetics, starting with the decay of the M intermediate (>1 ms), between detergents that either did or did not have at least one ring in their hydrophobic region. For BR in detergents without a hydrocarbon ring, M was observed to decay in two steps and the lifetimes of the slower decay were relatively short (<15 ms). The detergents with one or more hydrocarbon rings had much slower long-time kinetics. The decreased rates are partially attributed to rigidity in the detergent micelles inhibiting a volume increase that must be necessary for the decay of the M and/or other later intermediates. From these and other studies one can conclude that at least one reason why BR and possibly rhodopsin and other retinal proteins have nonrigid native lipids is so that the external environment does not act to slow down the decay of later photointermediates in the photocycle. Differences in effective surface charge and solvent accessibility in different detergent micelles may also play a role in determining the rates of the later processes in the photocycle of BR.

A strong correlation was observed between the lifetime of the slowest photocycle decay and the lifetime of dark adaptation for the same sample. Rigid detergents and higher pHs in d-BR/CHAPSO gave slower dark adaptation, suggesting that a protein volume increase is involved in the isomerization process.

This work demonstrates the large influence the solvation environment has on the photocycle of BR. Upon detergent solubilization, early steps in the photocycle are affected by small structural changes induced in the protein. In later steps of the photocycle the external environment provided by the detergent micelle affects processes dependent on structural changes in the protein. This work points out the importance of studying a membrane protein in various environments, as the functions of many other membrane proteins are probably also affected by solvation environment.

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Ligand-Induced Interaction between α- and β-Type Platelet-Derived Growth Factor (PDGF) Receptors: Role of Receptor Heterodimers in Kinase Activation†

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ABSTRACT: Two types of PDGF receptors have been cloned and sequenced. Both receptors are transmembrane glycoproteins with a ligand-stimulatable tyrosine kinase site. We have shown earlier that ligand-induced activation of the β-type PDGF receptor is due to the conversion of the monomeric form of the receptor to the dimeric form [Bishayee et al. (1989) *J. Biol. Chem. 264*, 11699-11705]. In the present studies, we have established the ligand-binding specificity of two receptor types and extended it further to investigate the ligand-induced association state of the α-receptor and the role of α-receptor in the activation of β-receptor. These studies were conducted with cells that express one or the other type of PDGF receptor as well as with cells that express both types of receptors. Moreover, ligand-binding characteristics of the receptor were confirmed by immunoprecipitation of the receptor-^{125}I-PDGF covalent complex with type-specific anti-PDGF receptor antibodies. These studies revealed that all three isoforms of PDGF bind to α-receptor, and such binding leads to dimerization as well as activation of the receptor. In contrast, β-receptor can be activated only by PDGF BB and not by PDGF AB or PDGF AA. However, by using antipeptide antibodies that are specific for α- or β-type PDGF receptor, we demonstrated that in the presence of α-receptor, β-receptor kinase can be activated by PDGF AB. We present here direct evidence that strongly suggests that such PDGF AB induced activation of β-receptor is due to the formation of a noncovalently linked α-β receptor heterodimer.

Platelet-derived growth factor (PDGF)[1] is a mitogen for cells of connective tissue origin. It is a disulfide-bonded dimeric protein composed of two nonidentical but highly homologous chains, known as A and B chains [for reviews, see Ross et al. (1986) and Heldin and Westermark (1989)]. All three possible isoforms of PDGF, namely, PDGF AA, PDGF BB, and PDGF AB, have been identified and isolated from different sources including tumor cells (Antoniades et al., 1979; Deuel et al., 1981; Raines & Ross, 1982; Stroobant & Waterfield, 1984; Heldin et al., 1986; Hammacher et al., 1988). They are biologically active in inducing mitogenesis. The B chain of PDGF has more than 90% sequence homology with the cellular homologue of the v-sis oncogene protein of simian sarcoma virus (Waterfield et al., 1983; Doolittle et al., 1983). The mitogenic and transforming activities of PDGF are mediated through its interaction with a high-affinity cell-surface receptor. PDGF receptor, a transmembrane glycoprotein of 180 kDa, is a member of the tyrosine kinase family of receptors. The intrinsic tyrosine kinase activity of the receptor is obligatory for its mitogenic function [reviewed in Williams (1989)]. Two types of PDGF receptors (α and β) have been cloned and sequenced (Yarden et al., 1986; Gronwald et al., 1988; Claesson-Welsh et al., 1988; Matsui et al., 1989). α- and β-type PDGF receptors have identical structural features. However, at the amino acid level, there is only 43% overall sequence homology between the two receptor types (Matsui et al., 1989). The ligand-binding specificity of these two receptors also differs considerably (Heldin et al., 1988; Hart et al., 1988).

We and others have investigated changes in the structure and association state of the PDGF receptor that are induced by ligand binding (Bishayee et al., 1989; Heldin et al., 1989). Such studies conducted either with a homogeneous preparation of β-type receptor (Heldin et al., 1989) or with β-receptor-specific antibodies (Bishayee et al., 1989) revealed that one

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[1] Abbreviations: BS^3, 3,3′-bis(sulfosuccinimido)suberate; HEPES, 4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid; NP-40, Nonidet P-40; PDGF, platelet-derived growth factor; PMSF, phenylmethanesulfonyl fluoride; SDS, sodium dodecyl sulfate; Tris, tris(hydroxymethyl)aminomethane.

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