Structure and Photodynamics of Bacteriorhodopsin in a Delipidated Contracted Lattice Form of Purple Membrane

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ABSTRACT: The influence of membrane supramolecular structure on the conformation and photodynamics of bacteriorhodopsin (bR) was studied by utilization of comparative absorption and circular dichroic (CD) spectroscopy of the native and a delipidated contracted lattice form of the purple membrane. The contracted form had similar absorption spectra in both the light-adapted, bR_{568}, and the photocycle intermediate M_{412} states of the bacteriorhodopsin as compared to the native lattice form. The excitonic contribution to the visible CD spectrum was slightly greater in the contracted form than in the native lattice form. However, in both forms excitonic coupling, which is indicative of membrane order, was lost during the M_{412} photoconversion. Furthermore, as deduced from CD spectra, the conformation as well as the global structural changes associated with the bR_{568} to M_{412} transformation was essentially unaffected by the lattice supramolecular structure. In contrast, the light–dark-adaptation and the M_{412} decay rates were greatly decreased in the contracted form as compared to those in the native form. Also, the contracted form, and more importantly any purple membrane system with a slowed photocycle, was bleached much more rapidly than native purple membrane in the presence of light and hydroxylamine. These data can be related to protein structural changes consistent with a model for the reversible tilting of the α-helical segments of the bacteriorhodopsin away from the membrane normal during the photocycle.

Bacteriorhodopsin (bR)[1] is a retinal-containing protein that functions as a light-activated proton pump in the purple membrane (PM) of *Halobacterium halobium*. This protein accounts for 75% of the dry weight of the PM, the remainder being lipids. This protein is localized as trimeric clusters in an extended hexagonal lattice with unit cell dimensions of 62.4 Å (Henderson, 1975). The effect of the lattice structure on proton translocation efficiency is still a matter of controversy. Klausner et al. (1982) have claimed that the presence of the lattice enhances the generation of the proton gradient while Cadadio and Dencher (1981) have suggested that the monomeric bR is as efficient a proton pump as the bR in the native supramolecular structure of the PM.

A number of techniques have been outlined by Henderson et al. (1982) which may be used to alter the purple membrane supramolecular structure by causing a contraction of the lattice unit cell dimensions from 62.4 to 59 Å without significantly changing the trimeric bR arrangements. These techniques involve either detergent-mediated delipidation of the membrane or neutralization of membrane negative charges (e.g., by lowering of the pH to <0.5 or the addition of transition metal halides). It was predicted that delipidation involved removal of at least 10–12 negatively charged lipids per unit cell (Henderson et al., 1982). In a similar vein, Glaeser et al. (1985) have reported that deoxycholate delipidation and lattice contraction led to the loss of at least 70% of the total PM lipids without significantly changing the basic trimeric unit.

This study was undertaken to assess whether the alteration of the hexagonal lattice due to delipidation may affect the photochemistry and the protein structural changes in bR occurring during the transition to the critical M_{412} photocycle intermediate. This spectroscopic intermediate is thought to represent the proton pumping step of the photocycle [see Stoeckenius (1985) for review and references]. The properties of the contracted lattice form of PM (cPM), generated by delipidation, were also compared to those of the native lattice form (nPM) by use of absorption and circular dichroic (CD) spectroscopy.

Analysis of the spectra indicated that although there are no significant differences in the structure of bR due to the modification of nPM to cPM, there are very drastic differences in the photodynamics of the bR. The dark-adaptation and the M_{412} decay rates are decreased while the light-dependent hydroxylamine bleaching rate is increased. It is shown that the retinal-regulated α-helical segment tilting paradigm proposed for the bR function by Cassim and co-workers (Muccio & Cassim, 1979a; Papadopoulos & Cassim, 1981; Draheim & Cassim, 1985a) is consistent with these results.

MATERIALS AND METHODS

The PM was isolated essentially by the method of Becher and Cassim (1975). The contracted lattice form was produced as described by Henderson et al. (1982) according to the Triton X-100 delipidation technique. The delipidated PM was washed extensively with double-distilled H_2O and resuspended at pH 6.6. The cPM generated by Triton X-100 treatment was similar to the deoxycholate-treated form (Glaeser et al., 1985) except that the Triton-treated preparation had a significantly smaller spectral blue shift as compared to nPM at the absorbance maximum (2 nm as compared to 5–7 nm). This may indicate a smaller perturbation of the chromophoric environment when the Triton X-100 method is used. The lipid phosphorus content was reduced by 62 ± 5% as judged by a total phosphorus assay (Bartlett, 1959). There was no re-

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[1] Abbreviations: nPM, native purple membrane; cPM, contracted lattice purple membrane; CD, circular dichroism; bR, bacteriorhodopsin.

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versible bleaching of the bR during this procedure since the addition of *all-trans*-retinal produced no change in the absorbance at 568 nm. Oriented films were made according to the procedures described by Muccio and Cassim (1979a), except a brief sonication followed by repeated Millipor filtration (2-μm pore size) was necessary to remove aggregates so that suitable thin films (OD_{570} of 0.01–0.04) for spectral studies could be formed.

Absorption spectra were recorded on a Cary 118C spectrophotometer with a far-UV modification and a scattered transmission accessory, and CD spectra were recorded on a Cary 60 spectropolarimeter with a 6003 CD attachment (Varian Associates, Inc., Instrument Group, Palo Alto, CA) or on a Jasco J-500A spectropolarimeter (JASCO, Inc., Easton, MD). In order to perform the low-temperature studies, the PM was suspended in 67% glycerol. The pH was adjusted to 9.3 in order to stabilize the M_{412} intermediate. The temperature was maintained at –70 °C in a glass Dewar flask with a dry ice/ethanol bath. A 500-W projector lamp was used to convert samples to the M_{412} intermediate during cooling. The projector lamp beam was filtered through a Corning 500-nm high-band-pass filter and 2 cm of 1 M aqueous $CuSO_4$ solution. To prevent backconversion artifacts in the CD measurements, the low-temperature spectra were measured point by point in the visible region as previously described by Draheim and Cassim (1985a). Under these conditions, nearly 100% M_{412} conversion was achieved.

M_{412} transients were recorded on a custom-designed flash photolysis unit built around a Nicolet Explorer Model 206 transient digitizer. Samples were flashed with an unfiltered xenon pulse (10 J total, 0.5-μs pulse width) from three flash lamps arranged coaxially around the cell inside a diffusely reflecting chamber. This setup produced approximately 15% photolysis.

Light-dependent hydroxylamine bleaching and dark-adaptation studies were carried out by the procedures previously given by Becher and Cassim (1976, 1977).

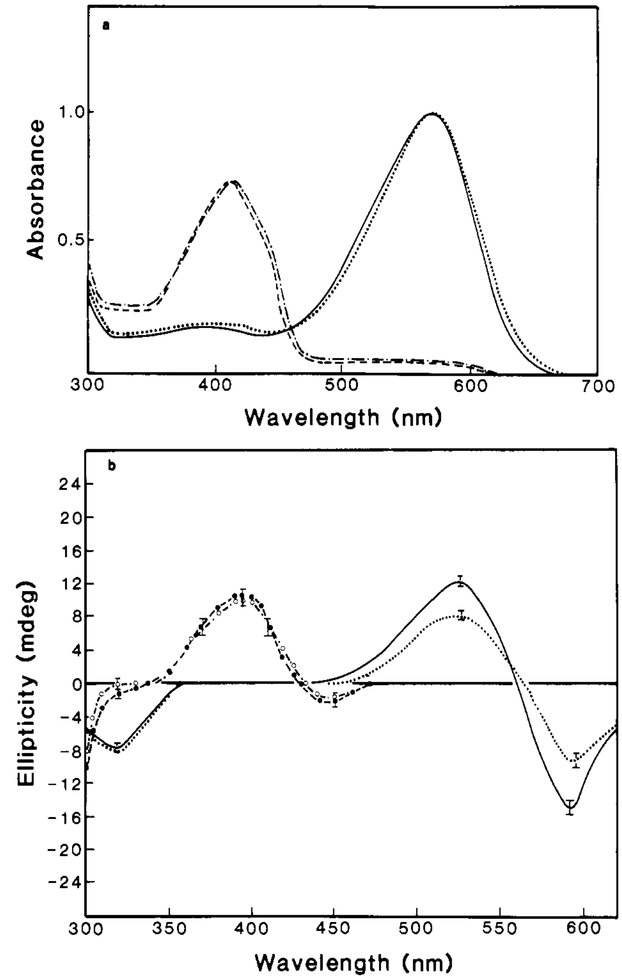

FIGURE 1: (a) Visible absorption spectra of the native, nPM, and the contracted, cPM, lattice forms of the purple membrane in the light-adapted, bR_{568}, and the photocycle intermediate, M_{412}, states. Samples were suspended in 67% aqueous glycerol, pH 9.3, and 79 μM bR in a 2-mm path-length cell at –70 °C. bR_{568} (···) and M_{412} (–·–) of nPM; bR_{568} (—) and M_{412} (– –) of cPM. The major difference between the two forms is a slight blue shift (2 nm) of the spectra of cPM relative to that of the nPM. (b) Visible CD spectra of the same samples. The M_{412} spectra were obtained point by point to prevent photoconversion artifacts from the measuring beam. In contrast to the absorption spectra, a significant intensity difference between the light-adapted forms is seen.

RESULTS AND DISCUSSION

Structural Studies

The visible absorption spectra of nPM and cPM are compared in Figure 1a. Upon contraction there was only a slight blue shift in the absorption maximum indicating a very small change in the local environment of the retinyl chromophore. The visible CD spectrum of cPM, however, exhibits an enhancement of the integrated intensity of the bands with biphasic waveform, which has been attributed to excitonic interactions among the retinals in the membrane lattice (Muccio & Cassim, 1979a), by about 40% relative to nPM as shown in Figure 1b. On the other hand, the 317-nm band, which has been attributed to static perturbation of a weakly allowed retinyl transition (Muccio & Cassim, 1979a), was almost unchanged upon contraction. This indicates that the perturbation of the retinyl chromophoric environment caused by lattice contraction is relatively small compared to that caused by polyhydric alcohols, low pH, or dark adaptation (Becher & Cassim, 1976; Muccio & Cassim, 1979b; Draheim & Cassim, 1985b). Also shown in Figure 1b are the visible CD spectra of the cPM and nPM photoconverted to the M_{412} photocycle intermediate. The M_{412} visible CD spectra are nearly identical for the cPM and nPM forms. Both lattice forms exhibit the same conversion of the spectrum from a biphasic to monophasic waveform indicating the loss of the excitonic contributions to the CD (Muccio & Cassim, 1979a). In a more detailed study of this photoconversion in the nPM, employing oriented film CD and linear dichroism as well, it was concluded that the interpretation most consistent with all the data and CD–exciton formalism is that the retinyl positions in the membrane lattice must have become disordered during this conversion (Draheim & Cassim, 1985a). It is also clear from Figure 1b that the biphasic to monophasic waveform conversion is identically, not precisely complete in both lattice forms. The very weak long-wavelength negative band was shown in the previous publication not to be excitonic in origin since it is also present in the oriented film spectrum of M_{412} of nPM in which excitonic contributions to the CD must vanish according to symmetry considerations (Draheim & Cassim, 1985a). It was suggested that this band may be attributed to a possible low-energy n–π^* transition of the chromophoric retinal resulting from the nonbonding orbitals of the Schiff-base nitrogen of the unprotonated Schiff-base linkage of the retinal lysine in the M_{412}.

In addition, these visible CD measurements suggest that the excitonic coupling is enhanced in the cPM form. In view of the fact that the trimeric bR unit is essentially the same in nPM and cPM (Henderson et al., 1982), the enhanced cou-

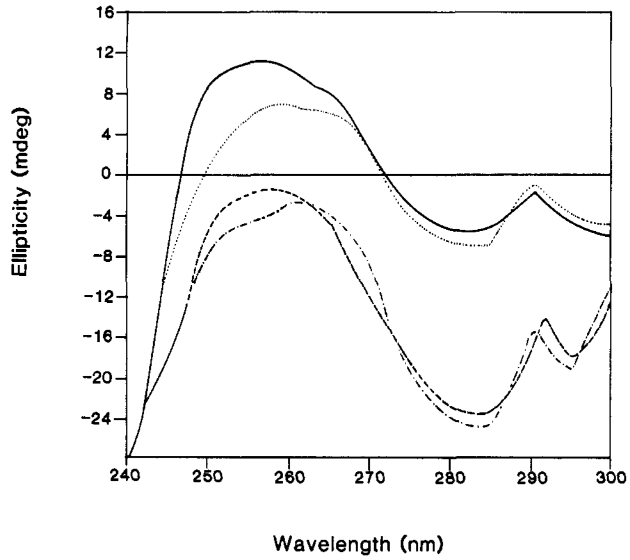

FIGURE 2: Near-UV CD spectra of the samples from Figure 1. All experimental conditions were the same as those in Figure 1.

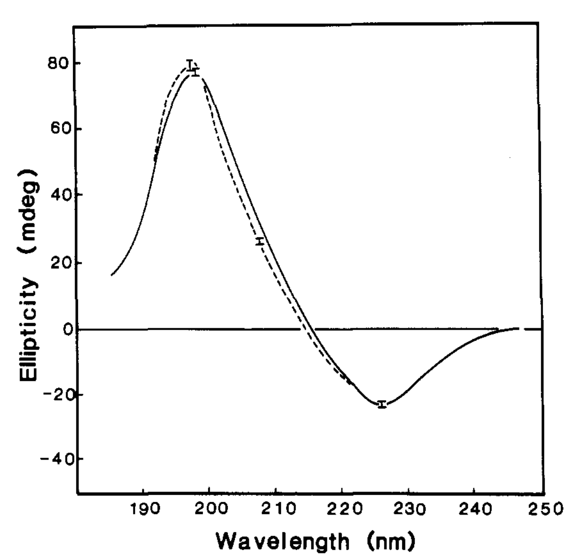

FIGURE 3: Far-UV CD spectra of cPM in the M_{412} (--) and the bR_{568} (—) states. Oriented film samples were at 15–20% relative humidity at 22 ± 1 °C and normalized to an OD_{568} of 0.1.

pling may point to the importance of interactions between chromophores in different trimers related by the three distinct C_3 axes. Also, since the intertrimeric distances are smaller in the cPM, the interaction potentials should be greater in proportion to the inverse cube of the distances between chromophores. The slight rotation of the trimeric units may also change these relative distances (Henderson et al., 1982). Significant intertrimeric coupling has been proposed for a weak coupling crystalline excitonic model of the PM where the Born–Oppenheimer approximation is not applied (Muccio & Cassim, 1981). Furthermore, the enhanced coupling may indicate an attenuation of damping effects on the exciton due to lattice vibrations.

The near-UV absorption spectra of the cPM and nPM forms (not shown) demonstrate that lattice contraction leads only to a small 7% increase in extinction between 270 and 285 nm. This change is reflected by an enhanced positive ellipticity in the 245–270-nm region of the near-UV CD spectra shown in Figure 2 similar to that observed in the presence of low glycerol concentrations (Draheim & Cassim, 1985b). There was little change in the CD spectra in the 285–295-nm region usually associated with transitions due to tryptophan residues. The decrease in absorbance in the near UV upon formation of the M_{412} species was nearly the same for both the cPM and nPM forms (not shown). Large changes in the near-UV CD spectra, which have been previously interpreted as global tertiary structural changes, were observed and were nearly identical. This suggests that the change in lattice structure of the PM leads to only minor local changes of the bR structure. The diffraction results of Henderson et al. (1982) and Glaeser et al. (1985) also demonstrate little tertiary structural changes of the bR associated with lattice contraction.

Previously, Cassim and co-workers (Muccio & Cassim, 1979a; Draheim & Cassim, 1985a) have shown that neither the M_{412} formation nor the light-dependent hydroxylamine bleaching causes any significant change in the solution far-UV CD spectra of the PM, indicating that net secondary structure changes of the bR must be negligible during these conversions. Similar measurements show that the cPM also undergoes no significant net secondary structural changes during photoconversion and does not differ significantly from nPM in secondary structure (data not shown).

Of greater interest are the far-UV CD spectral changes of oriented films of cPM during photoconversion. Previously, analysis of the oriented far-UV CD spectra of nPM, based on the CD–exciton formalism of the α-helical polypeptide, has suggested that the α-helical segments of the bR undergo a tilting transition during photoconversion to the M_{412} state (Draheim & Cassim, 1985a). It has been shown that nPM films under conditions of low humidity and high pH may have extraordinarily long M_{412} lifetimes (on the order of 30 s to several minutes) at ambient temperatures (Muccio & Cassim, 1979b; Kalisky et al., 1981). These conditions were reproduced for oriented cPM films, and M_{412} half-lives of as long as 30 min were observed. After a 500-W projector lamp illumination for 15 min, 70–80% M_{412} conversion could be achieved as estimated by the absorbance loss at 568 nm. Far-UV CD spectra could be obtained for these preparations at ambient temperatures with only about 5–7% decay of the M_{412} during the course of these measurements (Figure 3). These spectra were remarkably similar to those published previously for nPM at -70 °C (Draheim & Cassim, 1985a). The small shift of the crossover point to shorter wavelengths in the spectrum upon photoconversion indicates an increased contribution of the parallel polarized rotational strengths of the α-helix which are localized as a negative band at ca. 206 nm in the CD spectrum. This is consistent with a net tilting (ca. 5–15°) of the bR α-helical segments away from the membrane normal during this photoconversion (Muccio & Cassim, 1979a; Papadopoulos & Cassim, 1981; Draheim & Cassim, 1985a). This interpretation of the oriented α-helix CD spectra has also been experimentally supported by rhodopsin and cytochrome oxidase studies (Rothschild et al., 1980; Bazzi & Woody, 1985). More importantly, it is apparent from the spectral changes observed in Figures 1–3 that the pronounced structural changes of the PM associated with the photoconversion are not affected by the lattice supramolecular structure of the PM despite the large differences observed in the photodynamics of the bR discussed below. Furthermore, it is now clear that the tilting phenomenon observed previously in the M_{412} of the nPM cannot be a temperature-induced artifact.

Photodynamic Studies

Light–Dark-Adaptation Kinetics. A drastic difference between the nPM and cPM was seen in the light–dark-adaptation kinetics shown in Figure 4. Under identical conditions, the rate constant of the nPM dark adaptation was more than 3 times greater; that is, the contracted form dark adapted

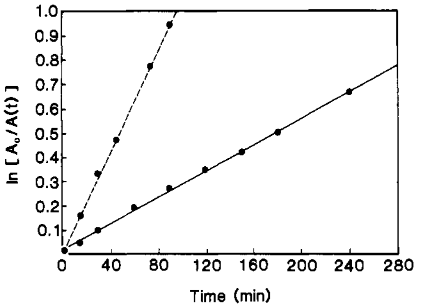

FIGURE 4: Light to dark adaptation kinetics of nPM (- -) and cPM (—). bR concentration was 15.8 μM in a 1-cm cell, pH 6.6, 23 ± 0.5 °C. The first-order rate constants for these processes are 0.0104 min^{-1} for nPM and 0.0027 min^{-1} for cPM. A is the difference absorption maximum at 586 nm during light to dark adaptation.

Table I: Decay Rates of the M_{412} Photointermediate in cPM, nPM, and Selected Perturbed nPM[a]

sample	rate constant, k (ms^{-1})	$t_{1/2}$ (ms)
cPM		
pH 7.0	0.0146 (0.18) 0.00229 (0.82)	302
pH 9.3	0.00074 (1.0)	940
nPM		
pH 7.0	0.529 (0.44) 0.179 (0.56)	2.6
pH 9.3	0.0293 (0.32) 0.1204 (0.68)	7.2
selected perturbed nPM		
25% NaCl–ether saturated		326
80% glycerol		247
8 M guanidine hydrochloride		157
50% glycerol		35
50% PEG-300		22

[a] Decay of cPM M_{412} could be adequately fitted by a single exponential at pH 9.3; at pH 7.0 the curve was better fitted with the addition of a second low-amplitude exponential. The nPM required a two-exponential fit at both pH values. Relative amplitudes of exponentials are given in parentheses. Conditions: xenon flash duration, 50 μs; $A_{570} = 0.5$; $T = 20$ °C. $t_{1/2}$ values for the selected perturbed nPM samples used in hydroxylamine bleaching experiments in Figure 5 were measured under the same conditions.

much more slowly than nPM. This indicates that the supramolecular differences between nPM and cPM are reflected in the light–dark-adaptation kinetics which have previously been shown to be critically dependent on the lattice state and the membrane lipid composition in vesicle reconstitution studies (Dencher et al., 1983).

M_{412} Decay Kinetics. From the spectral results, conformational changes accompanying the M_{412} state do not appear to be altered by lattice differences. However, the M_{412} species of the cPM, while apparently identical spectroscopically, is extraordinarily long lived compared to nPM under identical conditions. The half-time of decay at pH 7.0 and 20 °C was 302 ms and at pH 9.3 was 940 ms (Table I). The nPM suspensions under identical photolysis conditions have M_{412} half-lives more than 2 orders of magnitude shorter. Clearly, this dramatic difference in the decay rate must be due to the effects of the different supramolecular arrangement of the bR molecules since spectral studies have precluded significant secondary and tertiary structure differences. In a similar way, the orthorhombic supramolecular arrangement has been shown to slow the photocycle by an order of magnitude (Korenstein et al., 1985). The rate of M_{412} decay may be influenced by a number of factors involving the lipid microenvironment of the protein and in some cases is independent of bR aggregation state (Dencher & Wilms, 1975; Dencher et al., 1983). This suggests that altered protein–protein contacts per se are not the only factors affecting M_{412} turnover rate. Some recent studies on the effects of bulk solvent and state parameters on the photocycle may suggest a mechanism compatible with the observations of significant structural rearrangements of the photocycling bR. It has been demonstrated that charge motion (Rayfield, 1986) associated with the M_{412} state as well as the spectroscopic changes may be altered by changing bulk–solvent viscosity (Beece et al., 1981; Ahl & Cone, 1984) or temperature and pressure (Marque & Eisenstein, 1984). In many of these studies it has been observed that early photocycle intermediates thought to involve only localized changes (i.e., K_{590} and L_{550}) were essentially unaltered kinetically while the M_{412} was strongly affected. Furthermore, it is notable that during the L_{550} intermediate formation the excitonic interaction between the retinyl chromophores is retained while during the M_{412} formation it is lost (Draheim & Cassim, 1985a; Zimanyi et al., 1987). The explanation which fits all of these data most consistently is one involving displacement of the protein segments during the M_{412} formation and decay as previously proposed by Draheim and Cassim (1985a). Specifically, an increase in the rigidity of the bR environment in cPM would be expected to have a more perturbing effect on such delocalized structural changes than on small localized ones. In fact, Jost et al. (1978) have demonstrated with a spin probe method that the delipidated PM is even more rigid than the native PM. In addition, such delocalized changes, which can transform the bR molecule to a more open conformation, may be instrumental in decoupling the excitonic interactions as well as encourage the formation of transient aqueous channels. A transient solvent accessibility of the protein interior during the M_{412} has also been proposed on the basis of fluorescence measurements in various solvents (Plotkin & Sherman, 1984; Palmer & Sherman, 1985).

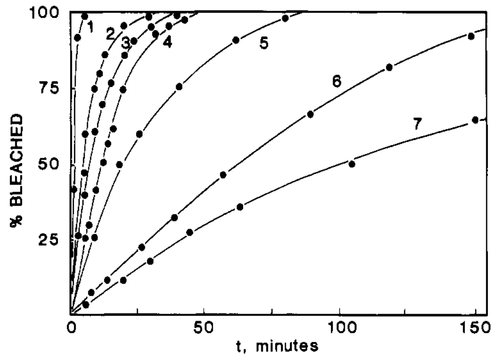

FIGURE 5: Light-mediated hydroxylamine bleaching of bR samples under various conditions which are known to retard the photocycle. Concentrations of bR were adjusted to 3.4 μM. Curves: (1) 25% NaCl/diethyl ether saturation, pH 7.0; (2) 80% glycerol; (3) cPM; (4) 8 M guanidine hydrochloride, pH 8.0; (5) 50% glycerol; (6) 50% PEG-300; (7) nPM.

Light-Dependent Hydroxylamine Bleaching Kinetics. Light-dependent hydroxylamine bleaching was carried out on both lattice forms (Figure 5). Under identical conditions the cPM was completely bleached after 55 min while the nPM preparation showed only about 75% bleaching after 250 min. Neither of the two forms showed any dark bleaching after 300 min.

In order to test the hypothesis that increased aqueous exposure time of the chromophore might be responsible for the enhanced bleaching rate, some other perturbations of nPM which are known to slow the M_{412} decay were also employed in the studies demonstrated in Figure 5 (Oesterhelt & Hess, 1975; Dencher & Wilms, 1975; Korenstein et al., 1979). The M_{412} decay half-lives for these perturbed nPMs, measured under the exact same experimental conditions used for the cPM and the unperturbed nPM, are also given in Table I.

These perturbations of the nPM range from a relatively mild one with glycerol to a rather severe one with diethyl ether saturation in the presence of 35% NaCl. Only the ether saturation perturbation led to any significant dark bleaching. However, this dark bleaching rate was more than 2 orders of magnitude slower than that in the presence of light illumination and hence did not significantly alter the results. It is conceivable that a solvent such as glycerol could penetrate the membrane bilayer and directly affect the bleaching reaction. However, the nonpermeant high molecular weight polyhydric alcohol PEG-300 also enhances the bleaching reaction relative to nPM which suggests that the solvent effect may be a surface-mediated phenomenon (Draheim & Cassim, 1985b). Nevertheless, in all these cases, the bleaching rate was markedly enhanced as compared to that in nPM. The effect of the various perturbation methods employed on the bleaching rate should depend on a number of complex factors. Therefore, the correlation between M_{412} lifetime and bleaching rate is not expected to be directly proportional, and as seen in Table I, it is not. However, a qualitative correlation seems to be present in that any perturbation which slows the M_{412} intermediate decay also seems to enhance the bleaching rate.

One essential factor affecting the bleaching rate should be increased aqueous accessibility during the photocycle. The dynamic-structure model of the bR based on the segmental-tilting hypothesis proposed by Cassim and co-workers (Muccio & Cassim, 1979a; Papadopoulos & Cassim, 1981; Draheim & Cassim, 1985a) can provide such accessibility. It is quite possible that there are other means of achieving accessibility. However, the point is that the qualitative correlation between M_{412} lifetime and bleaching rate is consistent with this proposed model.

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