Membrane-Mediated Control of the Bacteriorhodopsin Photocycle

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ABSTRACT: The ability of actinic light to modify the proportion of fast and slow forms of the M intermediate (i.e., M_f and M_s) in the bacteriorhodopsin (BR) photocycle is lost by exposure of the purple membrane (PM) to 0.05% Triton for 1–2 min. The decay path of M_f through the O intermediate is also lost, and new, much slower kinetic forms of M appear. In this brief exposure, the trimer structure for BR, as measured by circular dichroism (CD) exciton coupling and sedimentability, is unaffected. The optical properties of the treated PM are affected within seconds of exposure to the detergent as indicated by an increase in transmittance and a blue shift in the wavelength of maximum absorbance for the ground state. Different concentrations of Triton cause reproducibly different changes in the kinetics of the system. These observations support the view that the BR trimer–membrane interaction is important in controlling the BR photocycle.

It has been known for about 15 years that increasing the intensity of the laser flash used to initiate the bacteriorhodopsin (BR) photocycle dramatically alters the proportion of fast and slow forms of M intermediate (i.e., M_f and M_s) that are present [see Hendler et al. (1994) for references]. This combined with the fact that BR is present in the purple membrane (PM) in the form of crystalline trimers (Henderson et al., 1990) has led to the idea that light may exert a photocooperative effect whereby a single photon impinging on the trimer target would lead to a photocycle involving M_f whereas two or more photons would lead to a photocycle involving M_s [see Hendler et al. (1994) for references]. It has been observed that treatment of purple membranes with Triton, which leads to the loss of the BR trimer structure, is accompanied by loss of the "photocooperative" [1] effect (Danshina et al., 1992). Nonetheless, the natural presence of the ordered trimer structure in PM does provide presumptive evidence for the possible existence of true photocooperativity.

In this work, we have investigated more thoroughly the process by which treatment of PM with Triton leads to the loss of "photocooperativity" as well as the trimer structure. The main finding is that "photocooperativity" can be lost with such mild Triton treatment that none of the trimer structure appears to be affected. Evidence is presented that the integrity of the membrane, as demonstrated by its light-scattering properties, *is* affected with the same apparent time constant as the loss of the "photocooperativity". The interaction of BR with the membrane, as indicated by the wavelength of maximum absorbance, is also affected on the same time scale. These observations show that if a true photocooperative process occurs, a membrane-BR structure is the functional unit rather than the trimer *per se*. It is also possible to account for the effect of actinic light to modify the BR photocycle without invoking photocooperativity (see footnote 1 and Discussion).

MATERIALS AND METHODS

Purple membranes (PM) were isolated from *Halobacterium salinarium* (a variant of S9P kindly provided by Jeffrey Stuart) using procedures described by Oesterhelt and Stoeckenius (1974) as modified by Stuart et al. (in preparation).

Rapid spectral kinetics were performed using a new type of ultrarapid-scan multichannel spectrometer built at the National Institutes of Health (Hendler et al., 1993, 1994; Cole et al., 1994). Spectral deconvolutions and kinetic analyses were performed using SVD (Hendler et al., 1994; Hendler & Shrager, 1994). The monitoring light was provided by a 75-Watt continuous xenon lamp (Model LPS-220, Photon Technology International, South Brunswick, NJ) and the actinic light by a Neodymium YAG laser with a 5-ns pulse at 532 nm (Hendler et al., 1993). The laser flash impinged on a 1-cm^2 area on the face of the cuvette at 90° relative to the monitoring beam. The "high"-intensity level of the laser in this work was 6.0 mJ, and the "low" level, 0.03 mJ. These levels are 80 and 4 PCP (photoconversion parameter) units, respectively, as defined by Eisfeld et al. (1993).

For Triton treatment of PM, a 10% (v/v) stock of Triton X-100 (Research Products International Corp., Elk Grove Village, IL) was prepared fresh on the day of experiment. Treatment was performed at room temperature in the dark with ~0.5 mg of PM/mL of 50 mM phosphate buffer, pH 7.2. Triton was removed from the treated PM suspension by Bio-Beads (SM-2, 20–50 mesh, Bio-Rad) with gentle stirring at room temperature in the dark. Nonsedimentability of BR at 200000*g* for 40 min in a Beckman TL-100 ultracentrifuge using TLA 100.3 rotor was used as a criterion for the depolymerization of BR trimers to monomers.

Delipidation of PM was performed using CHAPS and dodecyl maltoside (DM) as described by Seigneuret et al. (1991). Delipidated BR was solubilized with 0.5% Triton (w/v) and subsequently treated with Bio-Beads to remove Triton. Lipid-soluble phosphate was obtained by distributions between aqueous and organic phases as described by Bligh

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[1] In this paper, the observed ability of light to alter the distribution of M_f and M_s species will be described as "photocooperativity" with quotation marks to indicate that a true photocooperative effect has not been proved and that it is possible to explain the observations using noncooperative models. However, since photocooperativity may play a role in the BR energy transduction process, it must be considered until there is reason to definitely abandon the idea. The term "photocooperativity" is used for convenience and simplicity, to describe the dependence of the photocycle on light intensity. In a separate paper, we critically examine all of the evidence for and against photocooperativity (Shrager et al., in preparation).

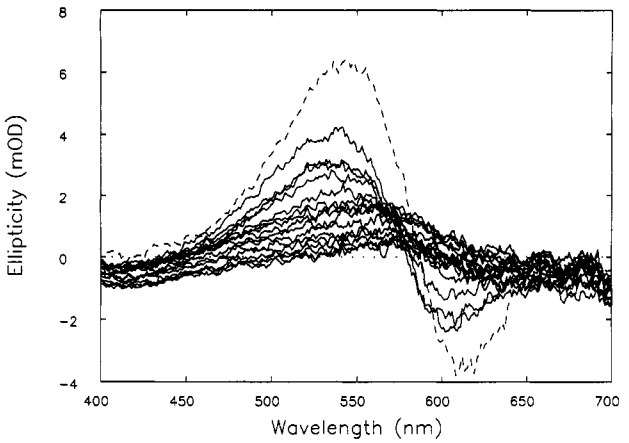

FIGURE 1: Circular dichroic spectrum of native purple membrane (dashed line) and the progressive decay in the spectrum during 5-h exposure to 0.2% Triton (solid lines). See text for further details.

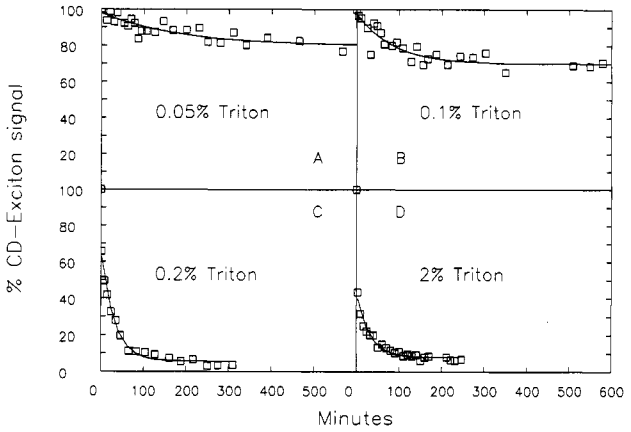

FIGURE 2: Kinetics of loss of the CD exciton signal in the presence of Triton. The magnitude of CD exciton signal was taken as the difference in ellipticity between the maximum value near 545 nm and the minimum value near 615 nm (see Figure 1). The signal was normalized to 100% before the addition of Triton at the indicated concentration. The solid lines are the results of fitting to a 1-exponential model. The τ values were 187, 84, 31, and 30 min, respectively, for 0.05%, 0.1%, 0.2%, and 2% Triton, respectively. In the cases of 0.2% and 2% Triton, the first time point was eliminated for the exponential fittings.

and Dyer (1959), with one major modification. During the several extractions and separation of aqueous and nonaqueous phases one is faced with two (undesirable) alternatives. Either try to collect the entire bottom (nonaqueous) phase each time and run the risk of including some upper (aqueous) phase, or sacrifice some of the bottom phase to avoid contamination of the inorganic phase and obtain a less than quantitative recovery of lipid-soluble phosphate. We have found that the addition of β-carotene as an internal standard avoids both problems. The carotene is extremely hydrophobic, it has a strong absorbance at 460 nm, and its presence does not interfere with the subsequent pyrolysis and determination of P_i. A known amount of carotene is added to the original sample. In the separations, some bottom phase is always sacrificed to avoid contamination by upper phase. At the end, the yield of lipid-soluble phosphate is corrected back to 100% on the basis of recovery of carotene. Lipid phosphorus was determined according to Rouser et al. (1966). We have compared the results obtained using the Bligh and Dyer extraction procedure to another, simpler, way to assay lipid phosphate and inorganic phosphate. If a sample has these two forms of phosphate, then the determination of phosphate after pyrolysis should yield the sum of the two species, whereas determination in the absence of pyrolysis should yield only P_i. The difference should be the amount of lipid phosphate. We find that this concept is true, and we obtain the same result on duplicate samples assayed both ways.

CD spectra were recorded at room temperature over the range 400–700 nm at 1-nm resolution and 50 nm/min scan rate in a JASCO J-600 spectropolarimeter using a cylindrical cell of 1-cm path length. An SLM-Aminco DW2000 spectrophotometer was used to record steady-state absorption spectra.

Stopped-flow experiments were performed in a KinTek Model SF-2001 system (KinTek Instruments, University Park, PA). The PM suspension at a concentration of 1 mg/mL was rapidly mixed with an equal volume of 2× the desired Triton concentration in buffer.

RESULTS

Kinetics of the Loss of BR Trimer Structure in the Presence of Triton X-100. Figure 1 (dashed line) shows a typical CD exciton spectrum of intact purple membrane. The coexistence of a positive lobe with a maximum ellipticity near 545 nm and a negative lobe with a minimum ellipticity near 610 nm is considered diagnostic of the native trimer structure of membrane-associated BR (Cassim, 1992). The solid lines show the progressive decay of the CD spectrum after treatment of the membrane with 0.2% Triton over a period of 5 h. The disappearance of the bilobed exciton spectrum and its replacement by a single diminished and broad positive ellipticity with a maximum near 550 nm coincide with the loss of sedimentability of BR at 200000*g* for 40 min. These well-known observations are interpreted as the release of BR as monomers from the original native membrane-associated trimer units. The time course and maximum extent of this process are influenced by the concentration of Triton (Figure 2). The difference in the magnitudes of the ellipticity between the maximum and minimum values was used as a measure of the strength of the exciton signal. With 0.05% Triton, ~20% of the signal was slowly lost in 10 h, whereas with 0.1% Triton, ~30% was lost in the same time period. Complete dissipation of the signal could be obtained in about 4 h in the presence of 0.2% and 2% Triton. At the higher Triton concentrations, there was an immediate large drop in the magnitude of the signal. As will be shown below, this is due to a sharp decrease in the light-scattering properties of the PM suspension and cannot be considered as part of the kinetics of loss of the structure responsible for the exciton spectrum. The steady decrease in exciton signal was fitted to a single-exponential decay equation which yielded τ values of 187, 84, 31, and 30 min, respectively, for 0.05%, 0.1%, 0.2%, and 2% Triton treatments.

It should be noticed that increasing Triton concentration from 0.2% to 2% did not decrease the single exponential τ for trimer breakdown. The kinetics of depolymerization were further studied by SVD. At 0.05% Triton, a single transition was seen with a τ of 195 min. The difference CD spectrum showed the loss of signal at near 545 nm and the rise near 610 nm (Figure 3A), which are the main changes associated with the dissipation of the exciton signal. At all higher Triton concentrations, a two-step process was seen during the degradation. The difference CD spectrum for the first step showed essentially the mirror image of the exciton spectrum (Figure 3A). This is the process in which the exciton spectrum disappears. The second difference spectrum showed a general decrease in ellipticity with a broad trough near 590 nm (Figure 3B). The τ values were 79, 25, and 28 min for the first

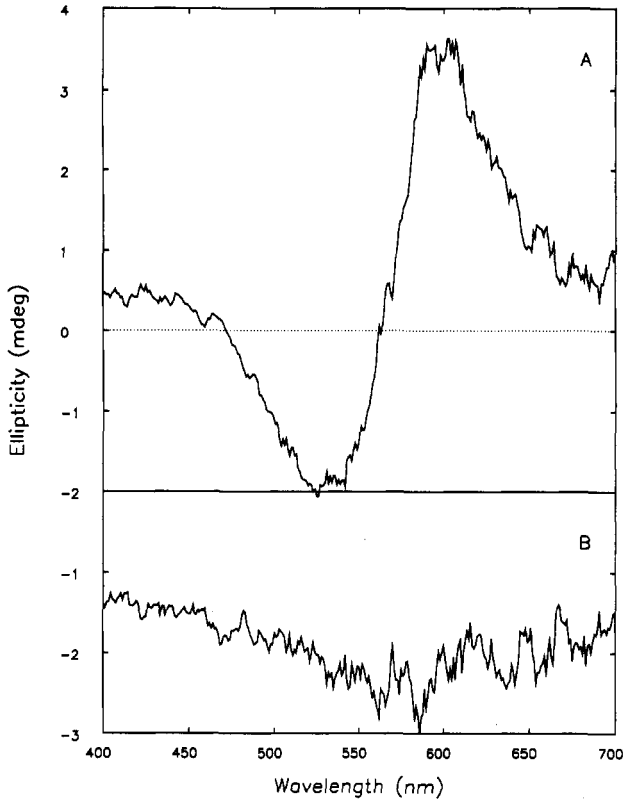

FIGURE 3: Difference CD spectra constructed from an SVD analysis of the changes occurring during a 5-h exposure of PM to 0.2% Triton. The fitted τ values were 25 min for the transition shown in panel A and 230 min for that shown in panel B.

transition, and 312, 229, and 51 min for the second, for the 0.1%, 0.2%, and 2% Triton treatments, respectively. The two-phase process seen by SVD may represent first the conversion of trimer to dimer, with loss of the exciton signal, followed by a slower breakdown of dimer to monomer. The second phase is more sensitive to Triton concentration than the first. This conclusion was further tested by first destroying the membrane with CHAPS, a detergent which has been shown to yield a sedimentable form of partially delipidated BR, as opposed to Triton which produces a nonsedimentable form of more extensively delipidated BR. The results are described at the end of this section after the effects of mild lipid disruption are presented.

Differences in Effects on the Photocycle Caused by Treatment with either 0.05% or 0.1% Triton. The photocycle of native PM shows two forms of the M intermediate, distinguished by their time constants (τ) as "fast" and "slow" (i.e., M_f and M_s). The percent of M_f relative to the total amount of M is high when very low laser intensity is used to activate the photocycle, and it diminishes as the laser intensity is increased. At pH ~7 it is seen that ~76% M_f at 0.03 mJ (low *I*) diminished to 56% at 6.0 mJ (high *I*) (Table 1 and Figure 4A). The τ's for M_f and M_s were ~2 and ~6–7 ms, respectively. Table 1 also shows that exposure of PM to 0.05% Triton for 2 min resulted in profound changes. The percent of M_f formed at high laser intensity increased from 56%, seen in the control, to 73%, and τ_s increased from ~6 to ~23 ms. There was a small but real decrease in τ_f. These abrupt changes at 2-min exposure to detergent were complete and remained essentially unchanged even after 2 h, although a small continuing increase in τ_s occurred. It is also seen that the ability of laser light to modulate the percent of M_f to M_{total} is greatly diminished. In fact, it appears that the system response, at least in terms of percent of M_f formed, is locked

Table 1: Treatment with 0.05% Triton

condition	% M_f	τ (ms) M_f	τ (ms) M_s	total M (mOD)
		Control		
high *I*	56	2.0	5.9	92
low *I*	76	2.2	7.8	8.7
		0.05% Triton		
2 min high *I*	73	1.9	23	78
5 min high *I*	73	1.9	24	78
10 min high *I*	76	1.8	25	74
15 min high *I*	76	1.8	25	74
20 min high *I*	77	1.8	26	73
24 min low *I*	79	1.7	31	7.2
53 min high *I*	75	2.0	27	69
60 min low *I*	77	1.6	30	6.7
88 min high *I*	74	1.8	27	62
93 min low *I*	80	1.7	31	5.7
122 min high *I*	73	1.9	28	63
150 min high *I*	75	1.7	23	75
160 min low *I*	80	1.6	29	7.6
		After Bio-Bead Treatment		
60 min high *I*	57	2.6	14	74
70 min low *I*	65	2.5	15	8.0
40 h high *I*	58	2.6	13	76
40 h low *I*	67	2.5	14	8.5

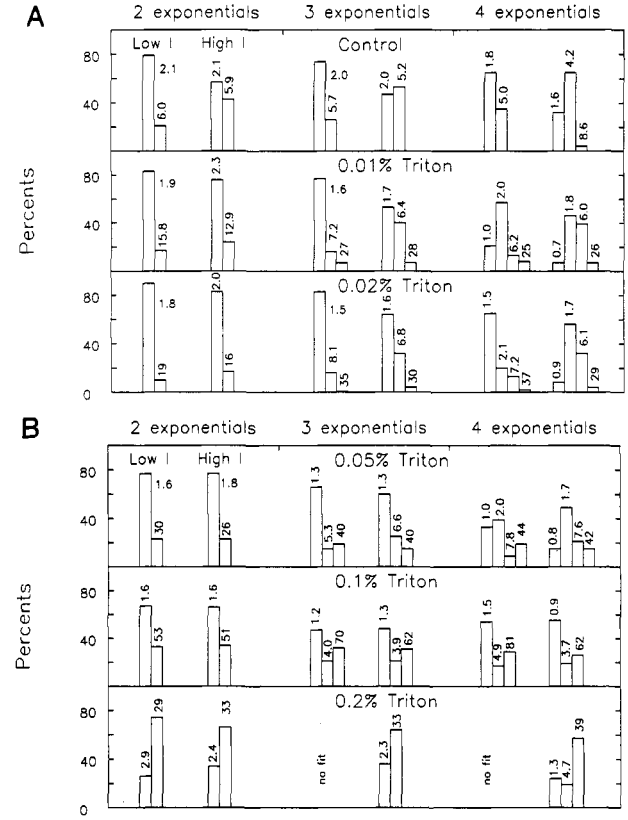

FIGURE 4: Distribution and time constants for M intermediates formed in photocycles at pH 7 initiated by low-intensity ("Low I"; 0.03 mJ) and high-intensity ("High I"; 6 mJ) laser flashes for PM in the presence of different concentrations of Triton. Each set of bars represents a complete time course involving hundreds of time points analyzed by SVD. The relative concentrations (heights of the bars) and τ values in ms (either vertically above or to the right of the bar) are shown. The graph shows the results of 2-, 3-, and 4-exponential fittings of the data. In each case, the left-hand set of bars represents the low-*I* laser flash, and the right-hand set, the high-*I* laser flash. See the text for further details.

in the low-level laser intensity response seen in the control (i.e., high percent M_f). The bottom part of Table 1 shows the results of an attempt to reverse the effects of the detergent,

Table 2: Treatment with 0.1% Triton

condition	% M_f	τ (ms) M_f	τ (ms) M_s	total M (mOD)
		0.1% Triton		
2 min high *I*	45	2.5	30	77
7 min high *I*	46	2.3	32	72
12 min high *I*	52	2.0	33	68
17 min high *I*	56	1.9	35	67
22 min low *I*	62	1.6	42	5.5
55 min high *I*	65	1.6	52	65
60 min low *I*	68	1.6	53	5.3
94 min high *I*	67	1.5	58	56
		After Bio-Bead Treatment		
60 min high *I*	57	1.8	11	57
70 min low *I*	60	2.0	12	6.0

by removing it with Bio-Beads. Some of the effects were reversed. The percent of M_f at high I was reduced from ~75% back to ~57%. τ_f was raised from ~1.9 to ~2.5 ms, and τ_s was reduced from ~30 to ~14 ms. The two most dramatic effects, however, were not reversed. The apparent "photocooperative" [1] effect was not restored, nor was the primary decay route of M_f through O. The decay of M_f through O is demonstrated by the SVD-derived difference spectra in which the loss of M_f is seen to be accompanied by the formation of O, not of BR (Hendler et al., 1994). The actual difference spectra are not reproduced here.

The effects of exposure of PM to 0.1% Triton are shown in Table 2. The results are similar to and at the same time different from those obtained by exposure to 0.05% Triton. The most striking difference is that whereas 0.05% Triton stabilized the system in the high percent M_f state characteristic of activation with the low laser intensity, the 0.1% Triton exposure put the system in the low percent M_f state characteristic of activation with high laser intensity. Other differences were the higher τ obtained for M_s, the slower diminution of τ for M_f from ~2 to ~1.5 ms, and the continued slow increase of percent M_f from 45% to 68%. The similarities are that the effects of exposure are evident within 2 min, M_f decays to BR rather than through O, and there is a virtual loss of control of the percent of M_f by the intensity of the laser flash. Treatment of the Triton-exposed preparation with Bio-Beads to remove the detergent resulted in a lowering of τ_s from ~60 to ~12 ms. There was, however less change in percent M_f and in τ_f as compared to the case after treating the 0.05% Triton-exposed PM with Bio-Beads.

SVD Analysis of Kinetic Effects of Treatment of PM with Different Concentrations of Triton. In the cases where the PM was exposed to Triton, SVD analyses showed that additional kinetic species were present. For a full description of the SVD analysis, the reader is referred to Hendler and Shrager (1994). For each condition analyzed, such as the control or a particular Triton concentration, SVD generates from 3 to 6 fundamental time course vectors. Fittings for a specified number of exponentials are performed simultaneously on all of the vectors. For example, if 5 vectors are being fitted for 2, 3, and 4 exponentials, at two different laser light intensities, $5 \times 3 \times 2 = 30$ fittings are performed. A summary of all of the fittings is presented in Figure 4. For these data, a total of 154 fittings were required. Therefore, it is impractical to show all of the residuals. The statistical reliability for the existence of the additional components comes from three criteria, the standard errors of the fitted values, the sum of the squared deviations, and the dependency values which can vary from unity to infinity. Dependency values are discussed in Hendler and Shrager (1994). Values below 50 indicate independent components. Higher values occur when more components are included in the fit than are justified by the data. For the data shown in Figure 4, the standard errors were extremely small, the dependency values were low, and the sum of the squares decreased with increasing the number of exponentials from 2 to 4. Therefore, the additional components seen after Triton treatment appear to be real. We have prepared a summary of all of the statistical data associated with the fittings in five tables, which will be provided upon request. The results of the SVD analyses are displayed in Figure 4, panels A and B, in the form of paired sets of bar graphs, one representing a low-energy laser actinic flash and the other a high-energy flash. The height of each bar represents the percent of an individual M intermediate with the time constant (in ms) shown either directly above or to the right of the bar. In the control case (Figure 4A, top), only two forms of M with τ's near 2 (M_f) and 6 ms (M_s) were seen. The 2-exponential fitting shows the high M_f ratio (i.e., M_f/M_s) seen at low-intensity laser flashes and the very much decreased ratio seen at high laser flashes. The same qualitative picture is seen in the 3- and 4-exponential fits, but the quantitative extent of the change in M_f ratio is much greater in the more highly resolved analyses. That fewer bars are sometimes present than the number of exponentials in the fit is explained by the fact that some of the resolved difference spectra did not include the M intermediate. The "cooperative" [1] light intensity effect on the M_f ratio seen in the control is lost in the 2-exponential fittings of all of the cases where Triton was present (Figure 4). Further confirmation of an observation made in discussing Tables 1 and 2 is also apparent, namely, that at Triton concentrations below 0.05%, the M_f ratio appears "frozen" at the high level characteristic of low laser intensity activation in the control, whereas at 0.1% Triton, the M_f ratio is "frozen" at a value more representative of high laser intensity activation. At 0.2% Triton, the M_f ratio is reversed even further than actually obtainable at the highest levels of laser intensity activation of the control.

In the case of 0.01% Triton, the 3-exponential fit shows the "cooperative" [1] light effect that was not seen in the 2-exponential fitting. In this case, τ for M_f was decreased while the M_s component was resolved into two species, one with a τ near 6 ms and the other near 28 ms. Further resolution of species is seen in the 4-exponential fittings, and the "cooperative" [1] light effect on the ratio of species with τ's near 2 and 6 ms is still evident. At 0.02% Triton, less of the modulation of M_f ratio is seen than in the 0.01% Triton case, and at all higher Triton concentrations the effect is gone. One other important finding in this series of studies is not shown in Tables 1 and 2 nor in Figure 4. We have previously shown that M_f decays directly to the O intermediate whereas M_s decays directly back to BR (Hendler et al., 1994). Even the briefest exposure (1 min) to the lowest Triton concentration (0.01%) alters this fact. In the 2-exponential fittings, the difference spectrum for the decay of M_f shows that the principal decay path of M_f is directly to BR rather than to O. The interesting finding, however, is that in the 4-exponential fittings of the 0.01% and 0.02% Triton exposures, the M component with a τ near 2 ms does decay directly to O. At all higher concentrations, this is not the case. Therefore, a correlation exists between the presence of the $M_f \rightarrow$ O path and the "cooperative" [1] effect of laser light intensity.

In summary, Triton treatment produced many dramatic effects. (1) The apparent "photocooperativity" [1] was greatly impaired or eliminated before the BR trimer structure was affected . (2) The percent M_f was fixed in the high range at

Table 3: Rapid Triton-Induced Optical Changes

	Triton concentration					
	0%	0.05%	0.1%	0.5%	1%	2%
			ΔA_{max} (%)			
1 s	0	−8	−9	−17	−20	−28
270 s	0	−28	−39	−58	−66	−88
			τ (s)			
1		1	0.6	0.9	0.6	1
2		96	12	18	14	10
3			388	333	258	170
4						262

Triton concentrations below 0.05% and in the low range at higher Triton concentrations. (3) The path of M_f decay through the O intermediate was diminished at 0.01% and 0.02% Triton and eliminated at higher Triton concentrations. (4) The ability of laser light to modulate the M_f ratio appears to be correlated with the presence of the $M_f \rightarrow O$ decay path. (5) The time constants are affected; τ_f is diminished and τ_s is increased. (6) Additional new kinetic species became evident.

Rapid Effects of Triton Treatment of the Purple Membrane. The dramatic effects of Triton treatment shown in Tables 1 and 2 and Figure 4 occur much more quickly than the loss of trimer structure as indicated by the CD exciton spectrum (Figure 2) and sedimentability of the PM. Of particular interest is the loss of the apparent "photocooperative" [1] effect of activation light on the relative amount of M_f formed in the photocycle. That Triton does affect the physical properties of the PM within the first seconds of exposure was shown by stopped-flow experiments, analyzed by SVD. A summary of these experiments is presented in Table 3. There was a dramatic lowering of the optical density of the suspension within the 1st second of exposure of PM to Triton, at all concentrations tested. The fitted τ for this kinetic phase is ~1 s. Subsequent kinetic phases exhibited τ's of ~10 s and ~4–6 min. The phases with $\tau > 1$ s involved a blue shift of the wavelength of maximal absorbance.

Kinetic Effects Caused by Delipidation of the Purple Membrane. Native purple membrane is made up of ~75% BR and ~25% lipid. The molar ratio of lipid to BR has been reported to be from ~5 (Stoeckenius et al., 1975) to ~13 (Blaurock & Stoeckenius, 1971). Other values between these extremes are listed by Kates et al. (1982). The reported molar ratio of phospholipid to BR varies from ~4 (Kates et al., 1982) to 10 (Bakker & Caplan, 1978). The variation may be due to the fact that different strains of *Halobacteria* were analyzed in different laboratories. We find ~10 phospholipids per BR. Having established that mild disruption of membrane integrity (treatment with 0.05% Triton for 2 min) causes profound changes in the nature and kinetics of BR photocycles, it was decided to look at the other extreme; that is, to remove as much of the membrane lipids as possible and to observe the nature of the BR photocycles in delipidated trimers and monomers. The data are presented in Table 4. The purple membrane control shows the presence of the two forms of M, M_f and M_s, and the ability of the actinic laser light intensity to regulate the relative proportion of these two species (i.e., "photocooperativity" [1]). CHAPS treatment, which removed 62% of the phospholipid, left 70% of the original exciton signal intact. Three, instead of two, M species were found, and all of these had larger τ's than the control. "Photocooperativity" [1] was lost. Dodecyl maltoside (DM) treatment of the CHAPS-treated preparation removed 40% of the residual phospholipid, but did not change the magnitude of the exciton signal. In both of these cases, the BR was sedimentable at 200000*g* in 40 min, indicating that depolymerization to the monomer stage had not occurred. Triton treatment of either the CHAPS-treated or the CHAPS- and DM-treated samples destroyed the exciton signal. These samples were no longer sedimentable, even at 500000*g*, verifying the complete monomerization of the BR. It has been shown by Huang et al. (1980) that the combined use of Triton with other detergents can remove 90–99% of endogenous lipids. An important distinction between the CHAPS-treated and the CHAPS/DM-treated samples is that subsequent removal of the Triton from the former with Bio-Beads reestablished more than half of the exciton signal that was lost in the presence of Triton, whereas in the latter case, the exciton signal was not restored. Either additional important lipids were removed by the DM treatment or DM itself remained at some crucial locus on the BR, preventing polymerization. At any rate, repolymerization of monomeric BR can occur in the absence of Triton. After Triton removal, there was a tendency for the BR to slowly aggregate. In all of the treated samples the "photocooperative" [1] effect was lost and three species of M intermediate were found, all of which had larger time constants than were present in the control. Table 4 also shows the kinetic analyses of Triton-treated samples embedded in acrylamide gel. This was done to prevent the aggregation and settling of these samples which otherwise would occur during measurement. There was very little difference, however, for the samples in the gel compared to those in suspension.

DISCUSSION

The main conclusion of this work is that the membrane exerts a profound influence on the nature and kinetics of the BR photocycle. Exposure of PM to low concentrations of Triton for only 2 min causes no change in the magnitude of the CD exciton spectrum nor in the sedimentability of BR, both of which are diagnostic of the membrane-associated trimer BR structure. Nonetheless, the following pronounced changes in the photocycle are elicited: (1) M_f which normally decays directly to the O intermediate, decays instead directly back to BR. (2) The ability of strong laser light to change the ratio of M_f/M_s is lost. (3) New and much slower forms of the M intermediate arise.

The fact that low concentrations of Triton affect the physical properties of the membrane on a time scale comparable to the effects listed above is shown in stopped-flow experiments monitored by SVD analyses of optical spectra. Within the first second of exposure there is a marked decrease in light scattering of the suspension. During the next several minutes there is a blue shift in the wavelength of the maximum absorbance.

Small differences in the Triton concentration cause different alterations in the photocycle. This suggests that distinct local membrane–BR associations are of importance. For example, concentrations of Triton <0.05% tend to favor the formation and stability of M_f species, whereas higher Triton concentrations favor the slower forms of M. Since there is only a single species of BR protein, the appearance of new species upon exposure to mild detergent conditions suggests that the interaction of the BR with membrane components is crucial in determining photocycle behavior. This also suggests that the presence in normal BR of two distinct types of photocycle, which are regulated by light levels (Hendler et al., 1994), also involves membrane lipids. Recently, we have obtained evidence by both infrared spectroscopy and lipid chemistry that specific lipid interactions with BR are perturbed by the

Table 4: Effects of Delipidation on Exciton Signal and Kinetic Species[a]

treatment	P-lipid [% (PL/BR)]	exciton [% (mdeg)]	I	kinetic species of M: τ (ms) (%)
		Control Purple Membrane		
none	100 (10)	100 (23)	H	2.9 (44); 6.9 (56)
			L	2.5 (81); 7.0 (19)
		In Suspension		
CHAPS	38 (4)	70 (16)	H	11 (18); 47 (45); 231 (37)
			L	9 (16); 48 (53); 274 (31)
CHAPS, Triton		6.5 (1.5)	H	13 (7); 60 (43); 245 (50)
			L	21 (16); 74 (42); 264 (42)
CHAPS, Triton, Bio-Beads		39 (9)	H	6 (14); 36 (8); 242 (52)
			L	2.4 (19); 24 (42); 237 (39)
		In Acrylamide Gel		
CHAPS, Triton, Bio-Beads		39 (9)	H	6 (17); 58 (30); 347 (53)
			L	4 (15); 37 (41); 290 (44)
CHAPS, DM, Triton, Bio-Beads		6.5 (1.5)	H	5 (24); 37 (32); 281 (44)
			L	4 (22); 32 (37); 252 (41)

[a] CHAPS and DM (dodecyl maltoside) were present at 5% (w/v), and Triton was present at 1%. The BR was sedimentable at 200000*g* in 40 min in all cases, except in the presence of Triton. Removal of Triton reestablished sedimentability. Phospholipid (P-lipid) is expressed both as percent and (in parentheses) as the molar ratio of phospholipid to Br, based on a ratio of 10 in the native purple membrane (see text). The exciton signal is expressed both as percent and (in parentheses) as the (+) peak to (−) trough difference in mdeg for 1 mg/mL in a 1-cm light path. The addition of DM to a CHAPS-treated sample removed 40% of the remaining P-lipid (i.e., 15% of the original P-lipid) but did not decrease the magnitude of the exciton signal. "I" represents light intensity of the laser flash which was 6 mJ for H (high) and 0.03 mJ for L (low). Each kinetic species of photocycle intermediate M found is presented with the time constnat (τ) in ms, followed by the percent of total M shown in parentheses, with individual species separated by semicolons.

same mild detergent treatment which alters the photocycles (unpublished).

The SVD analysis has shown that a correlation exists between the $M_f \rightarrow O$ decay pathway and the ability of laser flash intensity to regulate the M_f/M_s ratio. However, this relationship does not always apply. We have found that when PM is sonicated for 10 min in the presence of asolectin and NaCl, M_f still decays through the O intermediate, but there is no regulation of the M_f/M_s ratio by laser intensity (unpublished).

Extensive delipidation of BR using a combination of detergents leads to photocycles containing three forms of the M intermediate. The τ values for these range from 3 to 20 ms, from 30 to 60 ms, and from 230 to 350 ms, depending on the specific treatment. It is interesting that the most completely delipidated monomer preparation still shows three species of M intermediate. If a single BR protein is present, the heterogeneity is most likely due to either different isomeric forms of retinal and/or different kinds of tightly associated lipids. These possibilities will be explored in future studies. Milder et. al. (1991) studied the effects of six different detergents on the photocycle of purified (i.e., delipidated) BR. Where our two studies overlap, there are some direct parallelisms in the findings. In the presence of Triton X-100 at pH 5, they saw three kinetic species for M decay, with τ's of 3.7, 16, and 160 ms. They also found that the difference spectrum for M decay in the native membrane absorbed further to the red (~650 nm) compared to that for detergent-treated BR monomer (~610 nm). This is consistent with our finding that in the native membrane M_f decays through O, but that in detergent-treated preparations it does not.

The SVD analyses of the CD spectrum during exposure to different concentrations of Triton show a two-phase process, the first of which was associated with the loss of the exciton signal. Additional studies with CHAPS and dodecyl maltoside, which remove 60–75% of the membrane lipids and dissipate 30% of the CD exciton signal, suggest that the original intact membrane is needed to fully stabilize the trimer structure. Addition of Triton after CHAPS leads to the virtual loss of the exciton signal and to nonsedimentability of the BR. Subsequent removal of the Triton by Bio-Beads restores sedimentability and more than half of the exciton signal. These observations support the view that some lipids are needed to allow the monomers to interact and form dimers and trimers, that Triton prevents this interaction, and that the intact membrane stabilizes the trimer structure. As to the structural basis for the exciton signal, there are two views. The original view is that intratrimeric coupling of the individual BR monomers is the basis (Ebrey et al., 1977). More recently, Cassim (1992) has presented arguments in favor of intertrimeric coupling. In either case, the two-step process that we find in the loss of the exciton signal could be due to a degradation of the trimer through a dimer stage to monomers.

The fact that the apparent "photocooperative" [1] effect of laser light intensity on the photocycle has been dissociated from the integrity of the native trimer structure of BR does not eliminate the possible importance of photocooperativity nor of the essential role of the trimer structure in such a process. The results reported in this paper say that if the trimer structure is of importance in a photocooperative process, the membrane plays a crucial role. We are interested in the possibility that the control of the BR photocycle by laser light intensity may involve photocooperativity. Alternatively, the phenomenon may be explained without photocooperativity if heterogeneous populations of BR with different quantum efficiencies are present. There is another possible explanation based on a homogeneous population of BR. If two forms of the M intermediate are serially connected in the photocycle, each in reversible equilibrium with it's nearest neighbor [see Lanyi (1993)], light may affect one or more of the rate constants to produce and regulate phenomenological fast and slow forms of M. It should be noted that Lanyi does not propose such an effect of light on these constants. Furthermore, additional speculations are required to account for the fact that M_f decays through the O intermediate whereas M_s does not (Hendler et al., 1994; Eisfeld et al., 1993). All of these possibilities, as well as several others, are presented and quantitatively evaluated in a separate paper (Shrager et al., in preparation).

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