Control of the Integral Membrane Proton Pump, Bacteriorhodopsin, by Purple Membrane Lipids of *Halobacterium halobium*

Anup K. Mukhopadhyay, Swetlana Dracheva, Salil Bose, and Richard W. Hendler*

Laboratory of Cell Biology, National Heart, Lung, and Blood Institute, National Institutes of Health, Bethesda, Maryland 20892

Received March 27, 1996; Revised Manuscript Received May 13, 1996[⊗]

ABSTRACT: Brief exposure of purple membrane (PM) to dilute Triton X-100 eliminates the actinic light effect on the relative amounts of fast M (M_f) and slow M (M_s) intermediates and alters the character and kinetics of the photocycle, without destroying the native BR trimers (Mukhopadhyay et al., 1994). Particular membrane lipids are removed during the Triton treatment, and adding back an extract of membrane lipids can repair most of the affected photocycle behavior (Dracheva et al., 1996). This paper defines conditions which are important in the reconstitution procedure, using a group of quantitative parameters which measure the extents of damage and repair. Circular dichroism in both the UV and visible ranges shows that Triton can disturb both the secondary structure of BR and its ability to polymerize into trimers. Whereas the damage to protein conformation could be reversed by lipids alone, the formation of trimers and recovery of normal photocycle behavior required both lipids and a high salt concentration.

Actinic light controls the nature and operation of the membrane proton pump, bacteriorhodopsin (BR),[1] in *Halobacteria* (Drachev et al., 1993; Komrakov & Kaulen, 1995; Shrager et al., 1995; and references therein). We obtained evidence that membrane lipids were intimately involved in this control process (Mukhopadhyay et al., 1994) by showing that 1−2 min exposure to 0.05% Triton X-100 removed the actinic light control and destroyed the M_f to O decay path while not affecting the native trimer structure of BR. That the membrane integrity was affected during the brief exposure to detergent was shown by a decrease in light scattering and a blue shift in BR absorbance. In recent studies (Dracheva et al., 1996), we showed that ∼25% of squalene and glycolipid sulfate and ∼7% of the phospholipid were lost from the membrane during a brief exposure of PM to 0.1% Triton X-100 and that the lost BR photocycle behavior can be recovered by treating Triton-exposed PM with isolated PM lipids.

In the present paper, we look more closely at the factors which are important in the reconstitution procedure. In order to define conditions which optimize the recovery of normal photocycle behavior, we have defined a group of parameters which quantify the extents of damage and recovery of BR photocyle activity. In addition, we have used circular dichroism to determine that Triton damages the secondary structure of BR and is capable of disrupting BR trimers. The damage to BR protein conformation could be reversed by adding back PM lipids alone, but the re-formation of lost BR trimers and recovery of normal photocycle behavior required both PM lipids and a high NaCl concentration.

[⊗] Abstract published in *Advance ACS Abstracts,* June 15, 1996.

[1] Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; PGP, diphytanylphosphatidyl glycerophosphate (methyl ester); PGS diphytanylphosphatidyl glycerosulfate; DPPC, dipalmitoylphosphatidylcholine; PG, diphytanylphosphatidylglycerol; PC, phosphatidylcholine; PA, phosphatidic acid; DPhPC, diphytanylphosphatidylcholine; DPhPS, diphytanylphosphatidylserine; GLS, glycolipid sulfate; SQ, squalenes.

EXPERIMENTAL PROCEDURES

Treatment of Purple Membrane (PM) with Triton X-100. Two hundred microliters of 1% aqueous Triton X-100 was added to a suspension of 100 μL of PM (5 mg BR/ml) and 1700 μL of 50 mM potassium phosphate (pH 7.2). The suspension was immediately centrifuged at 4 °C in a Beckman TL-100 ultracentrifuge using a TLA 100.3 rotor for 5 min at 200000*g*. Allowing for formation of vacuum, attaining speed, and stopping, the total time was 7 min. The pellet was washed by resuspending in 3 mL of H_2O and recentrifugation two times.

Reconstitution with PM Lipids. PM was isolated using procedures described by Oesterhelt and Stoeckenius (1974) as modified by Stuart et al. (in preparation). The modification was the replacement of the final sucrose layering step with 4−6 individual centrifugations of the PM in distilled water at 46200*g*. Using the ratio of absorbances at 280 and 570 nm as a measure of purity of BR, we compared the quality of BR preparations made by us, using the sucrose layering procedure, and from samples obtained from a group of major laboratories in BR research. The ratios for the layering procedure ranged from 1.5 to 2.0. The ratios for the simple centrifugation procedure were generally 1.6−1.8. Lipids were extracted from PM according to the method of Bligh and Dyer (1959) as described by Kates (1982) and suspended to a stock concentration of 4 mg/mL. Forty-two microliters was dried under N_2 at room temperature and suspended in 500 μL of 50 mM potassium phosphate in 4 M NaCl at pH 7.2, by sonication for 30 s at 0 °C. A Tekmar Model TM-50 (watt) high intensity ultrasonic processor was used with the output control set at 30 and the power monitor set at 25. The relative concentrations of BR and lipids were set at the levels normally present in PM (i.e., 1× level), unless otherwise indicated. Normally, BR accounts for 75% and lipids for 25% of the PM (Kates, 1986). Present in the reconstitution mixture were 500 and 168 μg, respectively, of BR (determined using ϵ_M (570 nm) = 63 000) and lipid. The mixture was held overnight in the dark at room

S0006-2960(96)00738-6

temperature. The PM was then washed by two centrifugations in 50 mM phosphate buffer (pH 7.2) at 65 000 rpm at 4 °C in a Beckman TLA 100.3 rotor for 6 min. The washing procedure removes essentially all of the Triton X-100 as indicated by the ratio of absorbances at 276 nm (A_{max} for Triton X-100) and 569 nm (A_{max} for BR). The ratio is ~1.65 for both the native BR and the washed pellet after Triton treatment, whereas the first supernatant fraction has a ratio of 69. In the NaCl studies, both lipid sonication and reconstitution were performed at the indicated NaCl concentration. In the pH studies, lipid sonication and reconstitution were performed at the indicated pH. After final washing, the PM pellet was suspended in 50 mM potassium phosphate (pH 7.2) for assay of the photocycle characteristics.

Reconstitution with Non-PM Lipids. DPPC, DphPC, DPhPS, PA, and PC were purchased from Avanti Polar Lipids, Inc., Alabaster, AL, and used as described above, in place of PM lipids where indicated.

Kinetic Analyses. Rapid sequential spectra were acquired following an actinic laser flash using a new form of spectrophotometer as previously described (Hendler et al., 1994; Mukhopadhyay et al., 1994). Spectral deconvolution and analysis were performed using singular value decomposition (SVD) (Hendler et al., 1994; Hendler & Shrager, 1994).

Circular Dichroism. CD spectra were recorded at room temperature using a JASCO J-710 spectropolarimeter and a cylindrical cell. UV spectra were collected over the range 180−240 nm with 0.1 nm resolution and 10 nm/min scan rate using a cell with 0.02 cm path length. Visible spectra were collected over the range of 400−700 nm with 1 nm resolution and 50 nm/min scan rate using a cell with 1 cm path length.

RESULTS

Figure 1 shows kinetics of changes at single wavelengths which correspond to the M (412 nm, top panel) and O (641 nm, middle panel) intermediates, and to the ground state (569 nm, bottom panel) in the BR photocycle. Shown in the figure are curves for the native (solid lines), Triton-treated (short dashed lines), and reconstituted (long dashed lines) PM preparations. The kinetics for M decay (top panel) in the native preparation consists of a fast (M_f, τ ~2 ms) and a slow (M_s, τ ~6 ms) component (Hendler et al., 1994). The fast component is absent in the Triton-treated PM, and the overall decay is markedly slower. Reconstitution restores the native decay characteristics. The kinetics for O decay (middle panel) in the native preparation shows a peak at ~6 ms. This reflects the early formation of O from the decay of M_f and the subsequent decay of O back to BR. The absorbance at 641 nm does not decay back to zero because the spectrum of BR cuts across this wavelength. The Triton-treated preparation shows no early peak, but instead a slow steady rise throughout the time course. This is consistent with the fact that Triton treatment abolishes the $M_f \rightarrow O$ decay pathway which is responsible for the early peak (Mukhopadhyay et al., 1994). Reconstitution reestablishes the native decay characteristics. The bottom panel shows the kinetics for recovery of the BR ground state. Native PM shows a fast and a slow component which correspond to the fast and slow components of the M intermediates. The Triton-treated preparation shows a loss of the fast component and an overall slowdown in the photocycle. Reconstitution reestablishes the presence of the fast component.

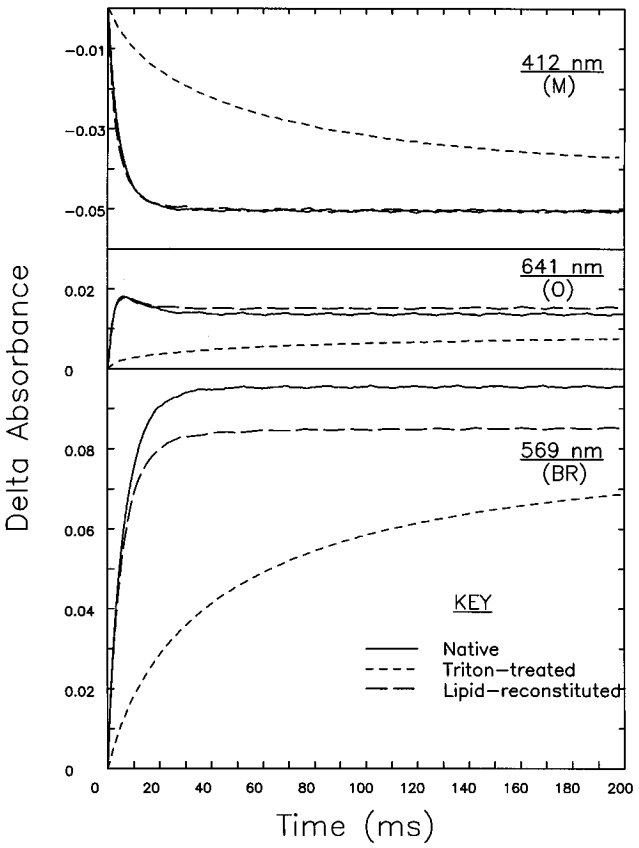

FIGURE 1: Time courses for changes in single wavelength absorbances obtained after an actinic laser pulse, obtained with native PM (solid lines), Triton X-100 treated PM (short dashed lines), and Triton-treated PM after reconstitution with PM lipids (long dashed lines). The top, middle, and bottom panels show data obtained at 412, 641, and 569 nm, respectively. These are near to the wavelengths of maximum absorbance for intermediates M, O, and native BR, respectively.

Quantitative Measurements of the Reconstitution of Functional Activity. We have identified 12 quantitative parameters to characterize the native BR photocycle(s), the degree of perturbation caused by brief exposure to Triton X-100, and the restoration of normal function obtained after reconstitution with lipids. These parameters are individually presented in Table 1 and discussed below. The actual magnitudes vary somewhat with the intensity of the laser light used to activate the photocycle. For uniformity, all quantitative comparisons were made from photocycles initiated with strong actinic light (80 pcp units; Hendler et al., 1994; Eisfeld et al., 1993). The first eight parameters are obtained in a singular value decomposition (SVD) deconvolution of the experimental data (Hendler & Shrager, 1994). Five are obtained directly from the raw data. One of these, the amount of M intermediate, was determined both from SVD analysis and from the absorbance of the raw data at 412 nm.

(1) Turnover of the M Intermediate. For native PM, containing 500 μg of BR, 54 mOD units of M intermediate turned over after a single high intensity actinic laser flash. In the Triton-treated case, there was about a 30% decrease in turnover, which was recovered after reconstitution.

(2) Average Time Constant (τ). As discussed above, native BR displays two forms of the M intermediate in 50 mM potassium phosphate at pH 7.2. The faster (M_f) has a τ near 2 ms, and the slower (M_s) a τ near 6 ms (Hendler et

Table 1: Triton Damage to, and Reconstitution of, BR Photocycles in Purple Membranes

	native	Triton-treated	reconstituted
1. ΔmOD M	54 ± 2 (9)	39 ± 2 (14)	54 ± 1 (11)
2. av τ (ms)	4.0 ± 0.1 (9)	61 ± 2 (14)	5.7 ± 0.7 (11)
3. M_f τ (ms)	2.5 ± 0.1 (9)	5.0 ± 0.8[a] (14)	2.2 ± 0.1 (11)
4. fraction M_f:			
H[c]	0.47 ± 0.01 (9)	0.09 ± 0.02[a] (14)	0.50 ± 0.01 (11)
L[c]	0.78 ± 0.02 (8)	xx[b] (14)	0.69 ± 0.02 (11)
5. ratio *L*/*H*	1.66 ± 0.04 (8)		1.36 ± 0.04 (11)
6. M_f[d] peak (nm)	643 ± 1 (9)	583 ± 3 (14)	640 ± 3 (11)
7. % A_{641} (cf. $A_{641} + A_{571}$)[e]	100 ± 0 (9)	30 ± 3 (14)	77 ± 2 (11)
8. reference peak (nm)	569 ± 0 (9)	562 ± 1 (14)	571 ± 0 (11)
9. ΔmOD BR	101 ± 3 (9)	66 ± 2 (14)	96 ± 3 (11)
10. ΔmOD M	53 ± 2 (9)	36 ± 2 (14)	53 ± 1 (11)
11. O_{max} (ms)	5.1 ± 0.2 (9)	200 ± 0 (14)	5.9 ± 0.4 (11)
12. $\Delta O_{max}/\Delta O_{end}$	1.45 ± 0.03 (9)	0.32 ± 0.02 (14)	1.22 ± 0.03 (11)
13. $\Delta O_{max}/\Delta M_t$	0.42 ± 0.01 (9)	0.08 ± 0.01 (14)	0.37 ± 0.01 (11)

[a] The fastest species of M present, which is not true M_f. [b] Very small signal; mole fraction <0.03; not true M_f. [c] *H* and *L* refer to high and low intensity laser flashes (see Experimental Procedures). [d] This is for the fastest species of M present, which may not actually be true M_f, as described in the text. [e] Percent positive absorbance at 641 nm compared to total at 641 and 571 nm.

al., 1994). The average τ is a weighted combination of these two species, obtained by multiplying each τ by the mole fraction of the species and summing. The average τ is near 4 ms for native PM. Treatment with Triton leads to the loss of M_f and the appearance of new much slower forms of M. The average τ, which is then computed as the sum of the products of τ and mole fraction for all of the species of M present, rises to ~60 ms after the brief exposure of PM to 0.1% Triton. Reconstitution returned the average τ to <6 ms.

(3) M_f τ (ms). Exposure of PM to Triton decreases the amount of true M_f (defined as having a τ near 2 ms and a direct decay path to the O intermediate) to zero or near zero. In the latter case, the fastest form of M remaining has a τ significantly above 2 ms. Reconstitution reestablishes true M_f.

(4) Mole Fraction of M_f. Following a high intensity actinic laser flash, the mole fraction of the species with τ near 2 ms compared to the total amounts of other forms of M is about 0.5 in native PM. Triton treatment either completely abolishes M_f or reduces its mole fraction to near zero. Reconstitution restores the native mole fraction of M_f.

(5) Ratio (L/H) of Mole Fraction of M_f at Low Intensity Laser Flash (L) Compared to High Intensity (H). The "cooperative" behavior of native PM (see Shrager et al., 1995) is manifest by the fact that, following a low intensity actinic laser flash (2.7 pcp units), the mole fraction of true M_f is appreciably greater than that following a high intensity flash (80 pcp units). For native PM, the ratio of mole fractions of M_f formed under the two conditions is near 1.7. After exposure to Triton, little or no true M_f is formed and the ratio decreases to 1 or less, or becomes meaningless (i.e., no true M_f is present). The mole fractions of the remaining, slower forms of M are not influenced by the intensity of the actinic laser flash. Reconstitution reinstates control of the mole fraction of M_f by the intensity of actinic light.

(6) Wavelength Position of Absorption Peak in Difference Spectrum for M_f. In native PM at neutral pH, M_f decays directly to the O intermediate with an absorption peak near 641 nm, whereas M_s bypasses O in returning to BR with an absorption peak near 571 nm (Hendler et al., 1994; Eisfeld et al., 1993). In Triton-treated PM, the decay route through the O intermediate is lost (Mukhopadhyay et al., 1994). Therefore, in native PM, the wavelength position of the positive peak in a difference spectrum showing the disappearance of true M_f is near 641 nm. In Triton-treated PM, there is very little true M_f (see above item 3), and the wavelength of the positive absorbance in the difference spectrum shifts downward toward 571 nm. In the briefest treatment with Triton, the maximum is seen near 585 nm. Reconstitution moves the positive absorption maximum back to ~641 nm.

(7) Percent of Absorbance at 641 nm Compared to the Sum of Absorbances at 571 and 641 nm. The decay of M_f through the O intermediate at neutral pH is an important characteristic of the native BR photocycle, and it is highly correlated with the control of the relative amounts of M_f and M_s generated by the energy of the actinic light. This parameter provides an additional quantitative measure of the M_f to O decay route. For native PM, near pH 7, the loss of absorbance at 412 nm (due to the decay of M_f) is compensated by a peak of absorbance near 641 nm (i.e., A_{max} for O intermediate). The ΔA at 571 nm is negative because of the disappearance of N intermediate whose turnover exhibits a τ near 2 ms (Hendler et al., 1994). Therefore, for native PM, the percent of A_{641} compared to the total positive A at 571 and 641 nm is 100. Upon exposure to Triton, little or no O is formed and the extent of the perturbation is quantitatively reflected by a decrease in the magnitude of this parameter to ~30%. Reconstitution increased the magnitude of this parameter to 77%.

(8) Wavelength Position for Light-Adapted BR in PM. In native PM, the maximum absorbance for BR is seen at 571 nm, whereas for Triton-treated PM it is blue-shifted to ~562 nm. This measurement is the same whether it is made directly with the PM or whether it is obtained by SVD in the deduced reference (i.e., background) spectrum. Reconstitution reestablished the wavelength position back to that of the native preparation.

(9) Turnover of BR (ΔmOD). As mentioned in item 1, the amount of M turnover is reduced by brief exposure to Triton X-100. This parameter, based on the magnitude of absorbance change at 569 nm, shows that there was a corresponding decrease in the overall turnover of BR. Reconstitution restored the native turnover level.

Table 2: Importance of NaCl and pH in Reconstitution

	NaCl concentration (M)[a]				pH		
	0	1	2	4	6	7	8
1. ΔmOD M	34	50	53	53	53	54	50
2. av τ (ms)	53	19	4.4	5.0	7.2	5.4	4.7
3. M_f τ (ms)	3.2	2.1	2.1	2.1	2.3	2.2	2.4
4. fraction M_f:							
H	0.08[b]	0.29	0.50	0.51	0.48	0.51	0.52
L	0.08[b]	0.28	0.63	0.71	0.59	0.63	0.65
5. ratio *L*/*H*	0.9	1.00	1.26	1.38	1.23	1.35	1.25
6. M_f peak (nm)	594	617	639	641	642	641	642
7. % A_{641} (cf. $A_{641} + A_{571}$)	40	67	73	81	78	76	83
8. reference peak (nm)	566	566	569	570	569	569	569
9. ΔmOD BR	60	93	102	97	95	96	91
10. ΔmOD M	32	51	57	52	53	53	51
11. O_{max} (ms)	200	200	5.8	5.9	6.0	5.3	6.5
12. $\Delta O_{max}/\Delta O_{end}$	0.31	0.88	1.20	1.28	1.21	1.24	1.29
13. $\Delta O_{max}/\Delta M_t$	0.08	0.25	0.39	0.38	0.36	0.37	0.39

[a] NaCl was added to the standard assay buffer (i.e., 50 mM phosphate, pH 7.2). [b] The fastest species of M present, which is not true M_f.

(10) Turnover of M (ΔmOD). This parameter is the same as item 1 above, but here it is obtained directly from the absorbance change at 412 nm. It provides a confirmation of the result obtained by SVD. The results are essentially the same as seen in item 1.

(11) Presence of a Transient Peak in Absorbance at 641 nm. M_f decays immediately to the O intermediate with a τ near 2 ms, whereas both M_s and O that was formed from M_f decay back to BR with τ's near 6 ms (Hendler et al., 1994). Therefore, it is to be expected, and is actually found (Figure 1), that the absorbance near 641 nm (for the O intermediate) shows a peak which separates its early rise due to M_f decay from its own subsequent decay to BR (Hendler et al., 1994). When the M_f to O decay pathway is lost, this peak does not occur, and instead, only a slow gradual rise in absorbance near 641 nm is seen throughout the whole time course. For native PM this peak is clearly seen at ~6 ms. In Triton-treated preparations, the peak is absent. Reconstitution restores the peak at ~6 ms.

(12) Ratio of Absorbances at 641 nm at ~6 ms and at 200 ms. When the native $M_f \rightarrow O$ pathway is intact, the magnitude of the peak (discussed in item 11) is reflected by the ratio of absorbances at the peak time (~6 ms) and at the end of the 200 ms time course. For native PM, this ratio is ~1.4, whereas for Triton-treated preparations it is ~0.3. Reconstitution restored the ratio to a value of ~1.2.

(13) Ratio of the Change in Absorbance at 641 nm (O Intermediate) from the Start of the Time Course to the Peak at ~6 ms, to the Total Change in Absorbance at 569 nm (M Intermediate). The relative amount of M decaying through the O intermediate is quantitatively reflected by the amount of O formed at ~6 ms compared to the total amount of M that was formed. This ratio is ~0.4 for native PM and ~0.08 for Triton-treated preparations. Reconstitution restored the ratio back to near 0.4.

Summary of Quantitative Changes after Triton Treatment and Reconstitution. Brief exposure of native PM to 0.1% Triton X-100 causes the following overall changes in native BR photocycle characteristics. There is a loss of true M_f (items 3, 4, and 13), a marked slowdown in overall M turnover (item 2), a loss of the $M_f \rightarrow O$ decay pathway (items 6, 7, and 11−13), a decreased turnover of BR (items 1, 9, and 10), a shift in the wavelength of maximum absorbance in the PM (item 8), and a loss of the ability of actinic laser light to modulate the mole fraction of M_f (item 5). The last column of Table 1 shows that all of the parameters are restored by reconstitution of the damaged PM with native PM lipids. Although, restoration was quantitatively substantial, it was not 100%. This is indicated in item 5 where the actinic light effect was restored to 1.36 compared to 1.66 in the control, and in items 7 and 12 where the relative amount of $M_f \rightarrow O$ pathway restored was somewhat less than in the native membranes.

On the Physical Reality of a Distinct M_f Membrane Lipid Entity. Table 1 shows the near total quantitative loss of the true M_f species (items 3 and 4). True M_f species is defined as the species with τ near 2 ms *which decays directly to the O intermediate*. The table also shows decreases in the amounts of BR and M turning over in the Triton-treated PM compared to both the native and reconstituted membranes (items 1, 9, and 10). Single-tail Student *T* tests show that the confidence levels for these differences are greater than 99% in all cases. The fact that reconstitution restored M turnover to the native level is supported by two-tail *T* tests which showed 72% and 96% confidence levels for items 1 and 10, respectively, that the same population was sampled.

The mole fraction of true M_f in native membranes, evoked at high laser intensity, was 0.47 (item 4). This represents 0.47×54 (item 1) = 25 mOD. After Triton treatment <0.09 of 39 mOD units, or ~3 mOD units, of M_f were formed. The actual amount of true M_f is less because the fastest form of M remaining after Triton treatment is used in this calculation, and this is not true M_f. The total loss of ~22 mOD units of true M_f represents ~40% of total M (i.e., 54 mOD) in the native membranes. There was a comparable loss in turnover of ~35% of the total BR in the native PM (item 9). It appears that fixed proportions of BR may be physically destined to turn over as either M_f or M_s. Removal of particular lipids from PM by brief treatment with Triton X-100 (Dracheva et al., 1996) prevents the turnover of that proportion of BR which was set to form M_f, and possibly some of the BR destined to form M_s (40% is greater than 35%). Adding back PM lipids restored the turnover of the affected BR population.

Critical Factors for Successful Reconstitution. Sodium chloride is required for the reconstitution of functional activity lost by exposure of PM to dilute Triton. Table 2

Table 3: Importance of BR Concentration on Triton Damage and Reconstitution[a]

	Triton-treated, μg of BR			reconstituted, μg of BR		
	250	500	1000	250	500	1000
1. ΔmOD M	16	34	77	30	59	102
2. av τ (ms)	75	66	54	5.8	6.6	6.5
3. M_f τ (ms)	8.0	3.8	3.2	2.4	2.5	2.4
4. fraction M_f:						
H	0.29[b]	0.02[b]	0.07[b]	0.49	0.52	0.51
L	xx[c]	xx[c]	xx[c]	0.70	0.68	0.65
5. ratio *H*/*L*				1.43	1.31	1.28
6. M_f peak (nm)	567	586	592	642	640	638
7. % A_{641} (cf. $A_{641} + A_{571}$)	12	40	44	81	80	69
8. reference peak (nm)	564	563	564	569	569	571
9. ΔmOD BR	27	62	137	56	105	182
10. ΔmOD M	14	32	75	30	56	103
11. O_{max} (ms)	200	200	200	6.0	8.3	6.7
12. $\Delta O_{max}/\Delta O_{end}$	0.35	0.32	0.40	1.27	1.17	1.20
13. $\Delta O_{max}/\Delta M_t$	0.08	0.08	0.08	0.38	0.36	0.36

[a] Triton concentration fixed at 0.1%. Lipid used for reconstitution fixed at 168 μg. [b] Not true M_f. [c] Signal too small to resolve M_f species.

Table 4: Importance of Added PM Lipid Concentration in Reconstitution

	relative lipid concn[a]					
	1/8×	1/4×	1/2×	1×	2×	3×
1. ΔmOD M	41	44	44	49	49	53
2. av τ (ms)	48	36	8.3	3.7	4.0	3.9
3. M_f τ (ms)	2.6	2.1	1.8	1.8	1.8	1.7
4. fraction M_f:						
H	0.09[b]	0.13	0.40	0.47	0.49	0.52
L	xx[c]	xx[c]	0.66	0.85	0.68	0.69
5. ratio *H*/*L*			1.65	1.80	1.39	1.33
6. M_f peak (nm)	589	601	642	642	642	641
7. % A_{641} (cf. $A_{641} + A_{571}$)	42	50	80	89	86	75
8. reference peak (nm)	564	566	569	569	569	568
9. ΔmOD BR	72	80	85	95	93	97
10. ΔmOD M	40	44	45	50	49	53
11. O_{max} (ms)	200	200	3.4	4.0	3.5	3.6
12. $\Delta O_{max}/\Delta O_{end}$	0.39	0.57	1.36	1.43	1.46	1.41
13. $\Delta O_{max}/\Delta M_t$	0.10	0.14	0.29	0.37	0.37	0.40

[a] The 1× level is the amount of lipid normally present in PM containing 500 μg of BR (i.e., 168 μg). [b] This is for the fastest M species present, which is not true M_f. [c] Signal too small to resolve M_f species.

Table 5: PM Lipid Added to Native PM

	concn added[a]			
	native	1/2×	1×	2×
1. ΔmOD M	61	62	53	62
2. av τ (ms)	3.9	3.8	3.8	3.9
3. M_f τ (ms)	2.5	2.3	2.3	2.4
4. fraction M_f:				
H	0.53	0.52	0.55	0.54
L	0.84	0.76	0.71	0.65
5. ratio *H*/*L*	1.58	1.46	1.29	1.20
6. M_f peak (nm)	645	645	645	645
7. % A_{641} (cf. $A_{641} + A_{571}$)	99	98	93	90
8. reference peak (nm)	570	571	571	571
9. ΔmOD BR	113	109	94	107
10. ΔmOD M	62	63	53	64
11. O_{max} (ms)	5.0	6.2	6.0	6.9
12. $\Delta O_{max}/\Delta O_{end}$	1.30	1.20	1.19	1.19
13. $\Delta O_{max}/\Delta M_t$	0.41	0.45	0.44	0.44

[a] The 1× level is the amount of lipid normally present in PM containing 500 μg of BR (i.e., 168 μg).

shows that lipids added to Triton-treated membranes, in the absence of NaCl, do not restore any of the lost native characteristics. Partial reconstitution is achieved at 1 M NaCl whereas nearly complete reconstitution is achieved at concentrations greater than 2 M. The same table shows a broad pH optimum in the range of pH 6−8.

Higher ratios of Triton to PM appear to cause more loss of native BR photocycle characteristics, as expected. In Table 3, this trend is seen in the slowing of M turnover (items 2 and 3), the decrease in amount of true M_f (item 4), and loss of the $M_f \rightarrow O$ decay path (items 6 and 7). Reconstitution of the Triton-damaged PM with the 1× level of PM lipids was successful in restoring function in all cases.

The essentiality of PM lipids in the reconstitution process and evidence of a stoichiometric relationship between the damaged membranes and added lipids are illustrated in Table 4. Examination of all of the quantitative parameters shows marked improvement as the lipid concentration was raised through the 1/8×, 1/4×, and 1/2× levels. Improvement in reconstitution between the 1/2× and 1× levels was less dramatic. The results with 2× and 3× levels indicate that, at higher lipid/PM ratios, some damage to the PM occurs. This was confirmed by adding PM lipids to native PM (Table 5). The ability of actinic light to modulate the relative levels of M_f and M_s (item 5) and the magnitude of the peak for O formation at ∼6 ms (item 12) appear to be diminished at the higher levels of added lipid.

Lipid Specificity for Successful Reconstitution. Table 6 shows that neither a mixture of phospatidylcholines from egg lecithin (PC), nor purified DPPC, nor phosphatidic acid (PA) was effective in supporting the reconstitution of normal photocycle behavior to Triton-treated PM. The structure of these lipids differs from that of PM lipids in (at least) two important ways: (1) the fatty acids are bound to glycerol in ester rather than ether linkages, and (2) the fatty acids are not phytanoic acid. On the other hand, diphytanyl PC, which although not a normal constituent of PM does have two phytanoic acids bound to glycerol through ether links, did support a partial reconstitution of the lost photocycle characteristics. However, the lost $M_f \rightarrow O$ pathway was not reestablished (parameter items 6, 7, 11, and 12). It is interesting that a related phospholipid, diphytanyl PS, was not at all effective in the reconstitution.

Triton-Induced Damage to Protein Conformation and Trimer Structure of BR and Recoveries by Addition of Lipids in the Presence and Absence of NaCl. Figure 2A shows damage to the protein conformational structure of native BR (solid line) exposed to 0.1% Triton X-100 for ∼2 min (short dashed line) and 30 min (long dashed line). Figure 2B reshows the native and ∼2 min Triton-treated preparations as references to gauge the extent of repair of damaged protein structure by addition of PM lipids in the presence of 4 M NaCl (long dashed curve superimposed on solid curve (native)) and in the absence of salt (series of unconnected points below curves for native and lipid + salt curves). These data demonstrate that a major fraction of the damaged secondary structure is recovered by addition of lipids in the absence of NaCl. In a separate study based on analysis by infrared spectroscopy, it was found that Triton induced a disruption of lipid head group hydrogen bonding and a loss

Table 6: Lipid Specificity

	lipid added						
	native	PC	DPPC	PA	DPhPC	DPhPS	total PM
1. ΔmOD M	57	35	33	34	39	31	45
2. av τ (ms)	3.0	50	46	45	9	48	5.6
3. M_f τ (ms)	2.0	2.9	2.7	1.8	1.5	3.2	1.7
4. fraction M_f:							
H	0.53	0.09	0.10	0.11	0.43	0.13	0.51
L	0.83	xx[a]	xx[a]	xx[a]	0.65	xx[a]	0.65
5. ratio *H*/*L*	1.57				1.51		1.27
6. M_f peak (nm)	645	578	584	586	581	577	641
7. % A_{641} (cf. $A_{641} + A_{571}$)	100	33	37	32	41	26	71
8. reference peak (nm)	569	562	563	562	568	562	568
9. ΔmOD BR	107	64	58	60	71	55	80
10. ΔmOD M	57	34	32	33	40	30	45
11. O_{max} (ms)	3.8	200	200	200	200	200	4.3
12. $\Delta O_{max}/\Delta O_{end}$	1.49	0.41	0.41	0.60	0.99	0.43	1.23
13. $\Delta O_{max}/\Delta M_t$	0.41	0.09	0.08	0.12	0.28	0.09	0.36

[a] Signal too small to resolve M_f species.

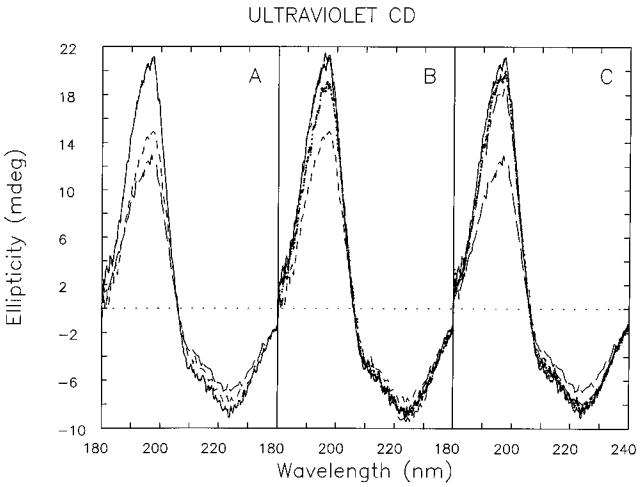

FIGURE 2: Ultraviolet CD spectra for native (containing 500 μg of BR), Triton-damaged, and lipid-reconstituted PM. The procedures for Triton damage and reconstitution are described in the Experimental Procedures section. (A) Native PM (solid line), Triton-treated for ~2 min (short dashed line), and for 30 min (long dashed line). (B) Recoveries of lost conformational structure after ~2 min exposure of PM to Triton. The solid and the short dashed lines shown here for reference are the same as shown in panel A. Reconstitution with lipids at the 1× level (i.e., 168 μg) (long-short dashed line superimposed on solid line for native BR). Reconstitution with 1× lipids in the absence of the 4 M NaCl normally present (discontinuous points just below the solid and long−short dashed lines). (C) Recoveries of lost conformational structure after 30 min exposure of PM to Triton. The solid and the long dashed lines shown here for reference are the same as shown in panel A. Reconstitution at the 1× lipid level (discontinuous points just below the solid line) and at the 3× level (long−short line below the 1× lipid level line.

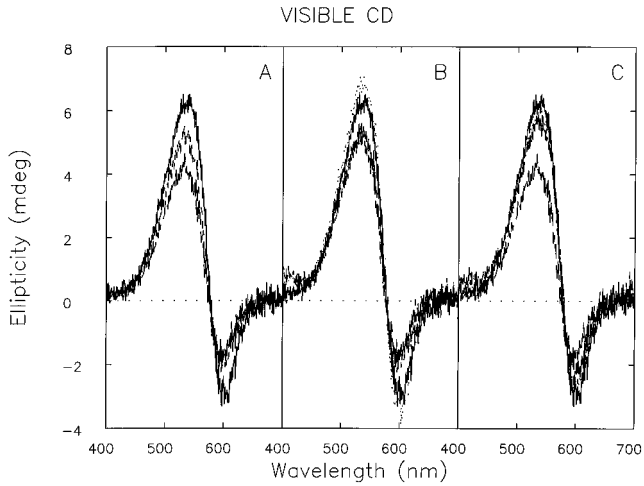

FIGURE 3: Visible CD spectra for native (containing 500 μg of BR), Triton-damaged, and lipid-reconstituted PM. The procedures for Triton damage and reconstitution are described in the Experimental Procedures section. (A) Native PM (solid line), Triton-treated for ~2 min (short dashed line), and for 30 min (long dashed line). (B) Recoveries of lost exciton signal after ~2 min exposure of PM to Triton. The solid and the short dashed lines shown here for reference are the same as shown in panel A. Reconstitution with lipids at the 1× level (i.e., 168 μg) (dotted line superimposed on solid line for native BR). Reconstitution with 1× lipids in the absence of the 4 M NaCl normally present (short−long dashed line very close to the Triton-treated case (short dashed line)). (C) Recoveries of lost exciton signal after 30 min exposure of PM to Triton. The solid and the long dashed lines shown here for reference are the same as shown in panel A. Reconstitutions at the 1× (dotted line) and at the 3× lipid levels (long−short line) are superimposed and located between the two reference lines (for control and Triton-treated preparations).

in β-turn and α_{II}-helical structures in BR (Barnett et al., 1996). Figure 2C shows that, in the presence of 4 M NaCl, nearly all of the native conformation lost during 30 min exposure to 0.1% Triton can be recovered. Figure 3A shows that, at ~2 min exposure of PM to 0.1% Triton X-100, there was a loss of ~20% of the exciton signal in the visible range (that has been correlated with the BR trimer structure (see Mukhopadhyay et al., 1994)). This amount of loss of exciton signal is greater than that which we reported earlier (Mukhopadhyay et al., 1994), but the ratio of Triton to BR in the current studies is 4 times greater than was shown to damage the normal photocycle in the earlier studies. Figure 3B shows that the recovery of the native exciton signal (dotted line compared to top solid line) requires that NaCl be present during reconstitution. Reconstitution in the absence of NaCl resulted in no re-formation of trimer structure (the Triton-treated and reconstituted (−NaCl) curves are superimposed). Figure 3C shows that the exciton signal lost after 30 min treatment of PM with Triton X-100 (bottom line compared to top line) is recovered equally well at both the 1× (dotted line) and 3× (middle solid line superimposed on dotted line) levels. This correlates with the observation that normal BR photocycle characteristics were recovered at both the 1× and 3× levels (Table 4). The reconstituted exciton signal is

∼90% of the native signal. This is really 100% recovery of the native signal because, at 30 min exposure to 0.1% Triton, 10% of the original BR was lost from the membrane and recovered in the supernatant obtained from pelleting the Triton-treated membranes. Comparing Figures 2B and 3B shows that salt is not required for recovery of lost protein conformational structure of the BR, but is required for recovery of lost trimer structure and lost functionality of the photocycle as discussed in this paper.

DISCUSSION

There is a relatively long history of studies in which damage to BR structure, properties, and/or function caused by treatment with detergents has been reported. In many of these studies, a regain of lost characteristics was accomplished by replenishing with either native or foreign lipids. It is important to distinguish the different kinds of earmarks of functionality which have been studied previously. One important activity is the ability to pump protons when the treated BR is incorporated into liposomes (Lind et al., 1981; Szundi & Stoeckenius, 1988). This functionality, however, requires neither the presence of native PM lipids nor the normal trimer structure of BR (Rigaud et al., 1988). Another criterion of BR integrity is the red shift in the absorption maximum caused by deionization, which is lost by delipidation. In addition, the delipidated membranes show a shift in the p*K* for the red to blue color transition from ∼4 to ∼1.4 (Szundi & Stoeckenius, 1988). Replenishment with foreign neutral lipids reestablished proton-pumping but did not raise the p*K* of the transition nor show the shift in color caused by deionization. Reconstitution with an excess of native acidic phospholipids did raise the p*K* of the transition to ∼4.5 (Lind et al., 1981).

It has been known for some time that monomers in the presence of native lipids, prepared by exposure of PM to Triton X-100, could partially re-form trimers and a hexagonal lattice after removal of the Triton by extensive (6 weeks) dialysis (Cherry et al., 1978). The fact that this could be accomplished in the absence of any additions demonstrated that other cellular components are not required for this self-assembly process. Another important step in defining conditions that favor re-formation of the 2D hexagonal array of BR trimers from monomers obtained by exposure of PM to Triton X-100 was that of Sternberg et al. (1992), who showed that endogenous polar PM lipids (PGP and PGS) and high NaCl play key roles in the re-formation of the hexagonal lattice from BR monomers. The studies reported in this paper involve a markedly different kind of disruption in BR photocycle behavior which can be repaired by reconstitution with native PM lipids. We have previously shown that profound changes occur upon brief exposure of PM to dilute 0.05% Triton X-100, even though the trimer structure of the BR remained intact (Mukhopadhyay et al., 1994). Specifically, the treatment removes the ability of high energy actinic light to alter the mole fraction of slow M intermediate (M_f) to total M, alters the decay path of M_f to BR, causes the formation of new species of M intermediates, and elicits a great delay in overall M turnover. The conclusion that the deleterious effects of the Triton treatment were most likely due to disruption of essential BR−lipid interactions was substantiated (Dracheva et al., 1996), where it was shown that particular lipids are removed from PM during exposure to Triton and that all lost functions of the Triton-treated PM could be restored by reconstitution of the treated PM with isolated PM lipids.

The goals of the present investigation were to explore the factors which are important in the reconstitution of normal photocycle behavior and to correlate the loss and recovery of photocycle characteristics with changes in BR protein conformation and state of aggregation. To accomplish these aims, we have identified twelve quantitative parameters to measure the extent of damage to the normal BR photocycle caused by brief exposure to 0.1% Triton X-100, and the extent of reversal of these defects by reconstitution with native PM lipids. It should be noted that the use of 0.1% Triton in the current studies caused about a 20% loss in trimer structure compared to ∼3% loss in the earlier studies where 0.05% Triton was used.

It was found that NaCl, in high concentration (above 2 M), was necessary in order to restore native BR photocycle characteristics to Triton-damaged membranes. This observation is consistent with the importance of high NaCl in the re-formation of the hexagonal array previously reported by Sternberg et al., described above. It was also established that the requirement for phospholipids is specific. In native PM there are ∼10 lipid molecules per BR monomer. These include approximately 5 PGP, $^1/_2$ each of PG and PGS, 3 GLS, 1 SQ, and ∼0.2 vitamin MK-8 (Kates, 1986; Dracheva et al., 1996). Phospholipids which lacked the ether-linked phytanoic fatty acids were ineffective. Although DPhPC (diphytanylphosphatidylcholine) was partially effective, DPhPS (diphytanylphosphatidylserine) was inneffective.

Whereas our earlier studies emphasized the importance of lipids in the normal turnover and control of the BR photocycle by actinic light, the present studies show that BR trimer−lipid interactions are essential for the normal photocycle. Specifically, we have found that lipids added in the absence of NaCl can restore a major fraction of protein conformational structure of BR lost by exposure of PM to Triton, but not trimer structure that was lost, nor normal photocycle characteristics. Although in our earlier studies more than 97% of the trimer structure remained intact after brief Triton treatment, NaCl was still required for reconstitution of the normal photocycle. This suggests that NaCl plays two roles. One is to allow added lipid to reach the critical BR site(s?) for reconstitution, and the other is to repair damage to both the secondary structure and integrity of the trimer state of BR.

In addition to providing answers to questions about the operation and control of the BR photocycle, the current studies are of interest in demonstrating an active role for membrane lipids in the normal function of an *in situ* integral membrane protein. Interest in the domain organization of lipids and proteins in biological membranes and their possible interactions has heightened as evidenced by a recent international conference on this subject at NIH (1994).[2] At the time of the conference there was no system presented which so clearly demonstrated the active role of membrane lipids in controlling integral protein function as the case presented in the current studies.

[2] Fogarty International Center Conference on Domain Organization in Biological Membranes, Lister Hill Auditorium, National Institutes of Health, Bethesda, MD, March 2−4, 1994. The proceedings of this conference are published in volume 12 of *Molecular Membrane Biology*, 1995.

In our continuing studies to define the critical features in the lipid control of BR function, we will try to identify which specific lipids are important for the recovery of particular native characteristics of the BR photocycle and whether certain lipid-dependent conformational states of the protein are critical for the normal operation of the BR photocycle.

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BI960738M