Chemical and functional studies on the importance of purple membrane lipids in bacteriorhodopsin photocycle behavior

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Abstract In native purple membrane (PM), there are approximately 1 squalene, 2 glycolipid sulfate (GLS), and 6 phospholipid (PL) molecules per bacteriorhodopsin (BR) monomer [10]. Brief (~2 min) exposure to 0.1% Triton X-100 removes about 25%, 20%, and 6% of squalenes, GLS, and PL, respectively (this paper) while causing profound changes in the BR photocycle, including the loss of 'photocooperativity' [1]. The BR photocycle in Triton-treated PM can be restored to near normal behavior by reconstitution with native PM lipids. Isolated squalenes are not effective whereas PL alone partially restores normal photocycle characteristics.

Key words: Bacteriorhodopsin photocycle; Membrane lipid; Membrane protein; Photocycle regulation

1. Introduction

It has been known for some time that there are (at least) two forms of the M intermediate in the bacteriorhodopsin (BR) photocycle, one (M_f) with faster kinetics than the other (M_s), and that the level of actinic light alters the fractions of these two species (see [2–4] and references therein). More recently, it has been shown that M_f decays to the ground state through the O intermediate whereas M_s does not [4,5]. A recent study showed that very mild treatment of purple membranes (PM) with Triton X-100 destroyed the ability of actinic light to influence the photocycle and altered the decay pathway of M_f without disrupting the trimer BR structure [1]. It was not directly established, however, that membrane lipids were actually removed from the membrane by the Triton treatment. In this paper three specific questions are asked. (1) Does the brief exposure to mild Triton actually remove lipids? (2) Which particular lipid classes are most susceptible to perturbation by the Triton exposure? (3) Is it possible to restore normal function, including the ability of actinic light to control the BR photocycle, by adding back endogenous lipids?

2. Material and methods

2.1. General

Purple membranes (PM) were isolated from *Halobacterium halobium* ET1001 according to the method of Oesterhelt and Stoeckenius [6] as modified by Jeffrey Stuart at al. (in preparation). For Triton treatment of PM, a 1% stock solution of Triton X-100 (Res. Products Int. Corp., Elk Grove Village, IL) was prepared. Treatment for each time point was performed at room temperature with 50 mg BR in a volume of 10 ml using 0.1% Triton, followed by centrifugation at 200000×*g* in a Beckman TL-100 ultracentrifuge at 4°C using a TLA 100.3 rotor. For most purposes, centrifugation was for 20 min. The '0' time point sample was immediately centrifuged for the minimum possible time which, allowing for formation of a vacuum, attaining speed, and stopping, was 7 min. Optical measurements at 570 nm showed that no liberation of monomer occurred during the treatment, and previous work showed negligible loss of the trimer structure [1]. Lipid extraction of the pellets and supernatant fractions was performed using the method of Bligh and Dyer [7] as described by Kates et al. [8].

2.2. Quantitative analyses

Phosphate was determined on pyrolysed samples [9]. Glycolipid was assayed using the phenol/sulfuric acid procedure [10]. Sulfolipid determination was based on the barium chloranilate procedure [10]. Phosphate produces 18.6% as much color per μg in this assay as does sulfate. Therefore, corrections were made for the known amount of phosphate present. Because very little phosphate was present in the extracts from the early time points of exposure to 0.1% Triton, little or no correction was required for these times. The iodine value was determined essentially as described by Kates [8] with the following changes to increase sensitivity and to conserve lipid. Sample size was 0.25 ml (instead of 5.0 ml), the potassium iodide and Dam's reagent were diluted to 1/2 strength, and the thiosulfate concentration was decreased from 0.02 to 0.005 N.

2.3. Thin layer and column chromatography

TLC was performed according to Kates [10]. The identities of all BR lipids were based on their Rf position in comparison with those reported by Kates [10].

The separation of polar lipids from nonpolar was done by column chromatography according to Kates [10].

2.4. Reconstitution with endogenous lipids

The reconstitution procedure is described in detail elsewhere (Mukhopadhyay et al., in preparation). Briefly, dried lipids (either 325 μg total PM lipids, 15 μg squalenes, or 40 μg combined PL) were sonicated in 500 μl of 50 mM potassium phosphate (pH 7.1) in 4 M NaCl for 30 s at 0°C using a Tekmar model TM-50 sonicator. A Triton-treated, centrifuged PM pellet (500 μg BR) was suspended in the sonicated lipid solution and incubated overnight in the dark. The PM was then washed by two centrifugations in 50 mM phosphate (pH 7.2) and suspended to 3 ml in the same buffer for assay.

3. Results

3.1. Release of membrane lipids by 0.1% Triton X-100

Table 1 presents all of the raw data obtained from the native PM lipids and from the lipids extracted from the supernatant and pelleted fractions of PM after exposure to 0.1% Triton X-100 at room temperature for different amounts of

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Abbreviations: BR, bacteriorhodopsin; PM, purple membrane; PL, phospholipid; PG, phosphatidyl glycerol; PGS, phosphatidyl glycerosulfate; PGP, phosphatidyl glycerophosphate; GLS, glycolipid sulfate; TGD, triglycosyl diether.

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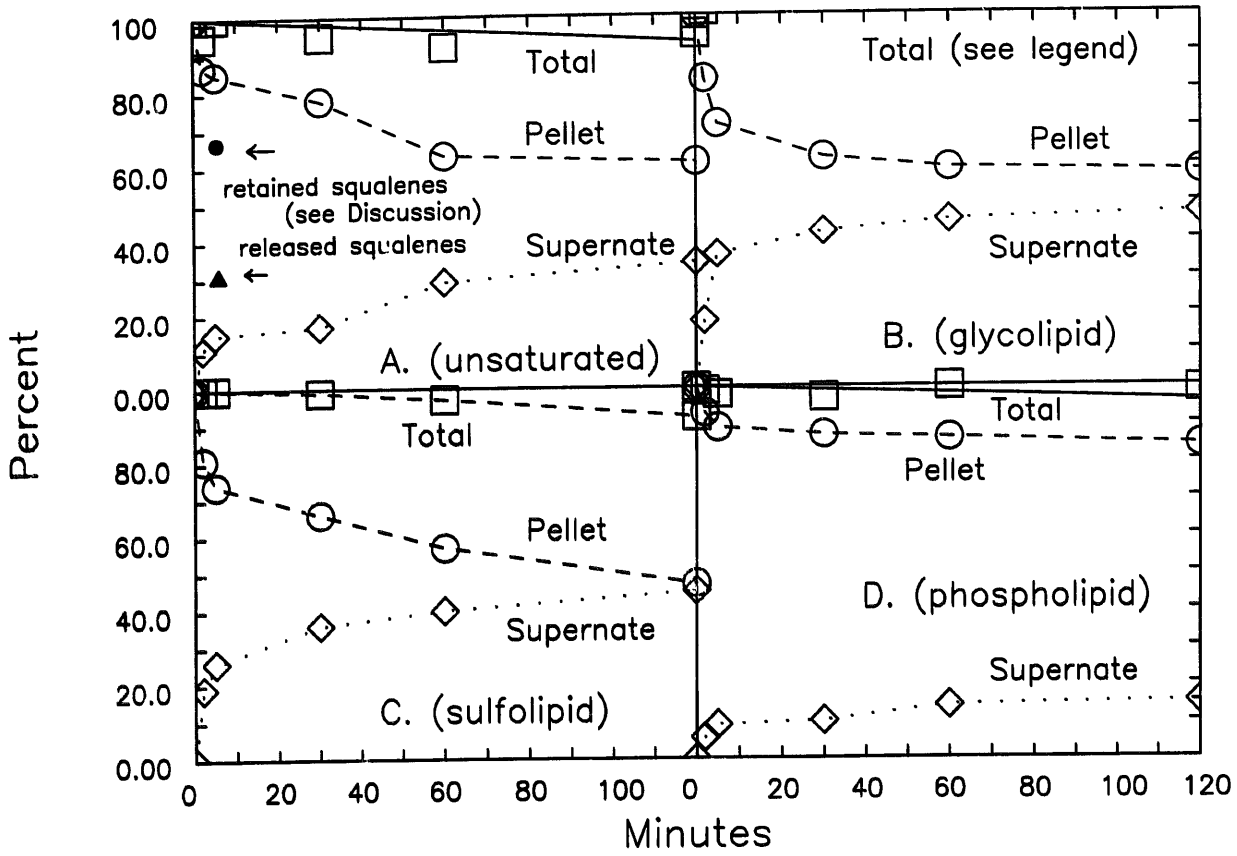

Fig. 1. Time courses showing percent release of unsaturated lipids (panel A), glycolipids (panel B), sulfolipids (panel C), and phospholipids (panel D) from PM exposed to 0.1% Triton X-100. The samples were separated into pellet and supernatant fractions by centrifugation after incubation at room temperature for the times shown on the abscissa. The earliest time point sample, shown at 2 min, was centrifuged at 4°C immediately after addition of Triton. Unsaturated lipids were assayed by their ability to bind I_2 [10], glycolipids lipids by the phenol/sulfuric acid procedure [10], lipid sulfate by the barium chloranilate procedure [10] and PL by the phosphomolybdate assay [9]. The solid symbols in panel A show the amounts of squalenes, as distinguished from retinal (see text). The individual points for total glycolipid (panel B) which were 100, 101, 107, 105, 104, and 105% respectively at 0, 2, 5, 30, 60 and 120 min, are not seen, because they are off scale.

time. Fig. 1 depicts the time courses of release of individual lipid classes during exposure of PM to detergent. Identities of released and retained lipids were verified by TLC. In order to save space, the results of TLC are described without showing photographs of the plates. The effects of the treatment on individual lipid classes are presented separately below.

(1) Unsaturated (neutral) lipids: Fig. 1A shows that ~11% of the total unsaturated lipids were liberated at the earliest time point. The unsaturated lipid fraction from purple membrane is made up of the squalenes and retinal in approximately equal amounts in terms of weight percent and degree of unsaturation [10]. Because retinal is not released by 0.1% Triton during the first 30 min [1], the initial release of ~11% and the release of ~15% of total unsaturated lipids in 5 min represents 20–30% of the squalenes. TLC showed that all classes of squalene (squalene, dihydrosqualene and tetrahydrosqualene) were liberated.

(2) Glycolipids: Fig. 1B shows that ~18% of the total glycolipids was quickly released and about one third was released in 5 min. Continued incubation out to 120 min liberated an additional 10%, while 60% remained associated with the membranes. TLC showed that the glycolipid present was almost entirely in a single spot which we identify as GLS.

(3) Sulfolipids: Fig. 1C shows that about 19% of the sulfate was immediately released, ~26% in 5 min, and ~45% in 2 h. The initial sulfate release parallels the initial glycolipid release.

(4) Phospholipids: Fig. 1D shows that approximately 6% of the total was quickly released, but extended incubation out to 120 min released a total of ~15% of the phospholipids. The extracted lipids were similar to the native PM lipid in the distribution of the three classes of phospholipids (i.e. there was no selective release of an individual PL class).

3.2. Reconstitution

Fig. 2 shows time courses for the decay of M (412 nm), the transient appearance of O (641 nm), and the restoration of BR (569 nm). Comparison of Triton-treated membranes (dotted lines) to native PM (solid lines) shows the loss of fast M (M_F), the transient peak for O at ~6 ms, and the initial fast recovery of BR. All of the lost properties are recovered by reconstitution with isolated PM lipids (long dashed lines). Initial reconstitution attempts with individual lipid fractions separated by silica gel chromatography are also shown (squalenes-short dashed lines; phospholipids-long-short dashed lines). With native PM, the ratio of mol fraction of fast M to total M with weak actinic (0.1 mJ) light, compared to strong (13 mJ) was 1.6. Triton-treatment destroyed fast M. The ratio was 0.83, 1.16, and 1.39 for the squalene,

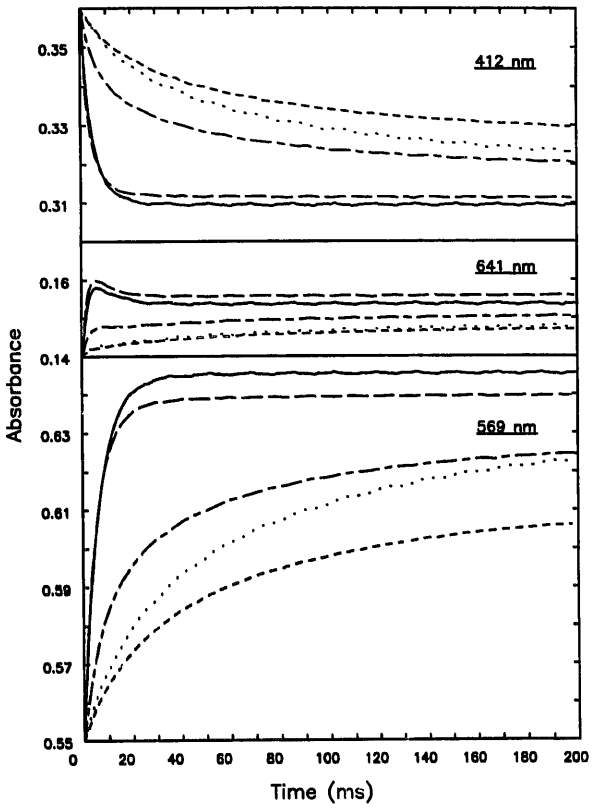

Fig. 2. Time courses for changes in single wavelength absorbances obtained after an actinic laser pulse, obtained with native PM (500 μg BR) (solid lines), Triton X-100 treated PM (dotted lines), and Triton-treated PM after reconstitution with 325 μg total PM lipids (long dashed lines), 15 μg squalenes alone (short dashed lines), or 40 μg phospholipids alone (long-short 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. See text for further details.
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PL, and total lipid reconstitutions, respectively. Triton removed, and reconstitution restored, the photocooperativity effect [11].

4. Discussion

Answers to the three specific questions listed in Section 1 were obtained. (1) Brief exposure of PM to Triton X-100, which dramatically alters the photocycle of BR, does extract lipids. (2) The lipid classes most susceptible to removal are squalenes and GLS, whereas PL are least susceptible. These results suggest important functional roles for squalenes and GLS, but do not rule out the possible importance of PL. (3) Normal photocycle behavior, including the ability of actinic light to modulate the relative amounts of fast and slow M intermediates, is restored to Triton-treated PM by reconstitution with extracted PM lipids. Differential effectiveness of li-

Table 1
Raw lipid analytical data from native PM and Triton-extracted samples

	I_2 value	Glycolipid	Sulfolipid		Phospholipid
			Raw	Corrected	
PM '0'	516 ± 8 (8)	0.244 ± 0.005 (10)	0.059 ± 0.002 (11)	0.047	0.305 ± 0.003 (12)
Sup.	58 ± 2 (4)	0.043 ± 0.003 (12)	0.009 ± 0.001 (4)	0.009	0.017 ± 0.002 (12)
Pel.	450 ± 2 (3)	0.202 ± 0.008 (6)	0.049 ± 0.001	0.038	0.283 ± 0.006 (9)
5 min					
Sup.	77 ± 9 (14)	0.087 ± 0.006 (5)	0.014 ± 0.002 (5)	0.012	0.028 ± 0.003 (4)
Pel.	441 ± 7 (14)	0.173 ± 0.007 (5)	0.046 ± 0.001 (5)	0.035	0.271 ± 0.007 (4)
30 min					
Sup.	87 ± 10 (6)	0.105 ± 0.007 (5)	0.018 ± 0.003 (5)	0.017	0.032 ± 0.004 (4)
Pel.	402 ± 4 (6)	0.151 ± 0.003 (5)	0.042 ± 0.001 (5)	0.031	0.264 ± 0.006 (4)
60 min					
Sup.	150 ± 4 (4)	0.109 ± 0.011 (4)	0.019 ± 0.003 (5)	0.019	0.042 ± 0.001 (3)
Pel.	325 ± 6 (4)	0.144 ± 0.006 (4)	0.039 ± 0.002 (5)	0.027	0.262 ± 0.007 (4)
120 min					
Sup.	173 ± 9 (3)	0.114 ± 0.007 (5)	0.024 ± 0.002 (3)	0.021	0.047 ± 0.001 (4)
Pel.	313 ± 8 (4)	0.142 ± 0.004 (5)	0.034 ± 0.003 (5)	0.022	0.255 ± 0.007 (4)

The data are presented as averages ± standard error of the mean for the number of determinations shown in parentheses. Determinations were made on the total starting PM lipids (PM) and on the supernatant and pellet fractions obtained from samples incubated at room temperature for 0, 5, 30, 60, and 120 min in the presence of 0.1% (w/v) Triton X-100, as described in the text. The zero time point is shown in quotes because although the preparation was centrifuged immediately after addition of Triton, some exposure to the detergent was experienced in the centrifuge.

Iodine value: The numbers represent μl 0.005 N $Na_2S_2O_3$. They are the differences in amounts between the titration of a blank sample (950 μl) and the lipid samples. For example, 516 μl is equivalent to 2.58 μatoms of I, or 1.29 μmol double bonds. The 100% (starting) amount of BR lipid in the assay was 1 mg.

Glycolipid: The numbers represent optical absorbance at 490 nm for the color produced in the assay for carbohydrates. The amount of absorbance in the starting 160 μg of BR lipid corresponded to the amount of color obtained from 31 μg glucose.

Sulfolipid: The numbers represent optical absorbance at 530 nm for the chloranilate anion produced in the reaction with sulfate, liberated from the lipids by pyrolysis. The corrected values were obtained by subtracting the amount of color due to phosphate which produces 18.6% of the chromophore on a per unit weight basis compared to sulfate. The amount of sulfate in the starting 0.8 mg of BR lipid used in the assay was 85 μg.

Phospholipid: The numbers represent optical absorbance at 820 nm produced in the phosphomolybdate assay for phosphate liberated from the lipid samples by pyrolysis. The amount of phosphate in the starting 6.67 μg of lipid used in the assay was 1.00 μg.

pid fractions in restoring function suggest specific roles played by individual lipids. A complete description of the roles played by each lipid class in the reconstitution of function requires testing all isolated lipids individually and in mixtures. These studies are currently under way.

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