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MINI-REVIEW

Importance of Lipids for Bacteriorhodopsin Structure, Photocycle, and Function

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Abstract—This review begins with a brief history of early studies on the involvement of lipids in certain bacteriorhodopsin (BR) properties. Such properties include the regulation of the p*K* for the purple to blue transition caused by deionization, and the reformation of trimers from monomers after exposure of the purple membrane to Triton X-100. Most of the review is devoted to newer studies which indicate an important role for the neutral lipid squalene in the functional stability of the fast-decaying M-intermediate, for its decay through a pathway involving the O-intermediate, and for the regulation of the relative amounts of slow-decaying and fast-decaying forms of M. Participation of a peripheral acidic amino acid in the overall expression of fast-decaying M is also discussed. Initial studies suggest that the acidic amino acid may be Asp36 and/or Asp38.

Key words: bacteriorhodopsin, lipids, photocycle, Triton X-100, purple membranes, reconstitution

There is a relatively long history of studies in which damage to bacteriorhodopsin (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 indications of functionality employed in these earlier studies. One important criterion is the ability to pump protons when the treated BR is incorporated into liposomes [1, 2]. This functionality, however, requires neither the integrity of normal membrane-lipid structure nor the trimer structure of BR [3, 4]. Another criterion of BR behavior is the red shift of 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 purple to blue color transition from ~2.7 to 1.4 [2]. It has been shown that the purple to blue transition is mediated by the protonation of Asp85 [5, 6]. Replenishment with foreign neutral lipids reestablished proton-pumping but did not raise the p*K* of the transition or show the color transition upon deionization. Reconstitution with an excess of native acidic phospholipids did raise the p*K* of the transition to ~4.5 [1].

It has been known for some time that BR-monomers prepared by exposure of purple membrane (PM) to Triton X-100 could partially reform trimers and a hexagonal lattice in the presence of native lipids, after removal of the Triton by extensive (6 weeks) dialysis [7]. 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.

Steinberg et al. [8] observed other important conditions that favor reformation of the 2D hexagonal array of BR trimers from monomers after exposure of PM to Triton X-100. Their studies showed that key roles in the reformation are played by endogenous polar lipids (phosphatidyl glycerol (PG) and phosphatidyl glycerosulfate (PGS)), and by a high NaCl concentration. The studies from our laboratory, described below, involve markedly different perturbations of the BR photocycle that include kinetics, transition pathways, and the regulation of photocycles by ambient light levels.

One of the fascinating aspects of the BR-photocycle is that it is regulated by the intensity of incident light. This phenomenon was first observed by Ohno et al. [9] and has been extensively confirmed and intensely studied (Hendler et al. [10] and references therein). An indicator of this effect is the relative amounts of fast-decaying (M_f)

Abbreviations: BR) bacteriorhodopsin; PM) purple membranes; GLS) glycolipid sulfate; PG) phosphatidyl glycerol; PGP-Me) phosphatidyl glycerophosphate (methyl ester); PGS) phosphatidyl glycerosulfate; SQ) squalene.

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and slow-decaying (M_s) forms of the M-intermediate present in a single turnover. The designation M_f describes a species of M which decays with a time constant near 2 msec at neutral pH in the wild type, and in which the decay has been shown to be accompanied by the formation of intermediate O [10-12]. The designation M_s describes a species of M which decays with a time constant near 6 msec, whose decay is accompanied by the formation of BR and not O [10-12]. Newer kinetic analysis based on a discrete parallel photocycle model (described below) shows that the M_f to O transition is a two step process which involves the intermediate N. At neutral pH, the mole fraction of M_f can vary from 0.8 at low light intensity to less than 0.5 at high intensity [10]. Several different explanations for the effect of light intensity on the relative amounts of M_f and M_s have been considered. 1) There is a photocooperative effect where a single photon hit on the trimer induces decay through an M_f and O pathway, whereas multiple photons induce decay by a direct $M_s \rightarrow$BR pathway. 2) All of the intermediates are linked by reversible reactions and light intensity can alter individual kinetic micro constants in such a way as to account for the overall observations. 3) There are separate photocycles. One contains both M_f and O whereas the other contains M_s and not O. The M_f/O cycle has a higher quantum efficiency and a smaller saturation level than the M_s cycle. The first two possibilities involve a single homogeneous photocycle, whereas the third involves separate photocycles.

Shrager et al. [13], evaluated published evidence available in 1995 to see if convincing support could be obtained for either the homogeneous cooperative or the purely heterogeneous model. It was concluded that neither of these two models by itself was sufficient to explain all of the reported observations, but that a mixed model based on both heterogeneity and cooperativity seems to be required. At that time, a specific model based on light-controlled micro constants had not been advanced. Subsequently, Varo et al. [14] proposed a model based on a single homogeneous photocycle containing two species of N and of M, where light is able to modify micro constants linking N and M intermediates in such a way as to influence the relative amounts of apparent fast and slow M-decays.

The trimer structure for BR presents an ideal target for photocooperativity. A single photon hit on one of the monomers can be envisioned to lead to a photocycle involving M_f and O, whereas multiple hits on the trimer would invoke a photocycle involving only M_s. The idea of heterogeneity of BR photocycles is not so straightforward. There is only a single form of the protein, which contains 248 amino acids in known sequence [15, 16]. Therefore, heterogeneity might be based on interactions with other membrane (i.e., lipid) components. To test the idea that lipid–BR interactions are important for a normal photocycle, it was desired to define conditions where these interactions could be disrupted while leaving the BR-trimer structure largely intact. The neutral detergent Triton X-100 was used in concentrations from 0.01 to 0.2% for exposure times 1-2 min [17]. The integrity of the trimer structure was assayed using circular dichroism (CD) exciton coupling in the visible range, and sedimentability. It was found that 0.05% Triton X-100 eliminated both M_f and O from the photocycle, destroyed the ability of actinic light to modulate the system, and induced the formation of new and much slower kinetic forms of M to appear. In addition, the light-scattering of the suspension was decreased and the wavelength of maximum absorbance for the ground state was blue-shifted. The trimer structure, however, appeared to have remained intact under the conditions of treatment. These observations supported the view that membrane–BR interactions are important for a normal BR-photocycle.

The purple membrane is 75% BR and 25% lipid by weight [18]. There are approximately 10 lipid molecules per BR-monomer. One of these, retinal, is covalently linked to BR. In terms of individual membrane-lipid molecules per BR-monomer, there are 1 squalene (SQ), 2 glycolipid sulfate (GLS), 5 phosphatidyl glycerophosphate (methyl ester) (PGP-Me), 0.5 phosphatidyl glycerol (PG), and 0.5 phosphatidyl glycerosulfate (PGS) [18, 19]. Recently, two novel lipids were found in a genetically engineered strain of *H. salinarum*, one a phosphosulfoglycolipid and the other a diphytanylglycerol analog of bis-phosphatidyl glycerol [20]. The former was equimolar to retinal while the latter was about 0.12 relative to retinal. The presence of these lipids in naturally occurring strains of *Halobacterium* has not been established. It was of interest to determine which of the naturally occurring lipids are most easy to remove by Triton X-100. For this purpose, treatment with 0.1% Triton X-100 was used on a time course where samples were removed and separated into soluble and pellet fractions by centrifugation at 200,000*g* [19]. It was found that within 2 min exposure to detergent, 25% of SQ and 20% of GLS were removed as well as 6% of the total phospholipid. This brief exposure to Triton X-100 produced serious perturbations in photocycle behavior. The central role of the lipid environment was shown by restoring normal photocycle behavior through the addition of PM lipids in the presence of 4 M NaCl [19]. The lipid-induced reconstitution was studied in much more detail by Mukhopadhyay et al. [21] who used 13 quantitative standards to measure the extent of disruption and reconstitution of the BR-photocycle. Reconstitution re-established the presence of M_f and its decay through O, as well as the regulation of the relative amounts of M_f and M_s by the intensity of actinic light. The reconstitution also removed the aberrant slow forms of M that were formed after Triton treatment. The vital role of high salt concentration for successful reconstitution with lipids was confirmed by titration of the extent of reconstitution as a function of NaCl

concentration. Lipid-specificity in the reconstitution process was shown by a strong preference for the unique phospholipid structure of the purple membrane as contrasted to that in egg lecithin and purified phospholipids isolated from non-archaeobacterial sources. In addition to extraction of lipids and disruption of their interactions with BR, the brief exposure to 0.1% Triton X-100 caused extensive damage to the secondary structure of BR as revealed by CD in the UV and about 20% depolymerization of trimers as revealed by visible CD. In a separate study employing infrared spectroscopy [22], it was shown that the treated protein suffered a loss of lipid head group hydrogen bonding, and in β-turn and α-helical structures. Addition of PM lipids in physiological salt concentrations repaired a major part of the damage to protein secondary structure but did not restore normal photocycle behavior. The work of Mukhopadhyay et al. [21] showed that addition of lipids in the presence of 4 M NaCl restored nearly all of the native protein structure. Lost trimer structure could not be repaired by addition of lipids in the absence of high NaCl concentration, but was repaired upon addition of lipid in the presence of 4 M NaCl. Subsequent studies with the individual isolated lipids of PM and various combinations of these showed that full reconstitution could be obtained with just two membrane lipids, SQ and PGP-Me, which functioned synergistically [23]. PGP-Me alone was quite effective in restoring lost M_s activity. Addition of SQ to such preparations resulted in recovery of the M_f and O pathway. It was somewhat surprising that, although GLS was nearly as susceptible to removal by Triton X-100 as SQ, and is in close structural association with tryptophan and tyrosine of BR [24], GLS was not essential for restoring normal structure and function to Triton-treated membranes.

The requirement for high salt concentrations during reconstitution suggests a need for charge neutralization during the process. PM is known to have a high negative surface charge. Charge-screening as described by the Gouy–Chapmann equation should be more effectively realized by cations with higher valency. That is, trivalent cations are much more effective than divalent, which in turn are much more effective than univalent. Furthermore, all cations of equal valency should be equally effective. It was found that consistent with the predictions of Gouy–Chapmann, higher valence cations were much more effective than lower ones, but cation specificity was also evident [25]. This finding is consistent with many other studies showing specific binding sites for individual cations of similar valence (see references in Bose et al. [25]). The most striking finding for specific binding of cations of the same valency was that protons were about five orders of magnitude more effective than Na^+ for facilitating reconstitution [25]. A plot of the extent of reconstitution vs. pH produced a Henderson–Hasselbach relationship with an apparent pK near 5. Thus, it appears that one of the charges requiring screening may be a peripheral acidic amino acid of BR. This suggests that to achieve reconstitution, negatively charged lipid must be brought very close to this site. From the observations described above that SQ appears to be important for the M_f and O pathway and that SQ and PGP-Me work synergistically, it is possible that the negatively charged lipid might be a complex of SQ and PGP-Me.

We have been pursuing the idea that an association between a neutral lipid and a peripheral acidic amino acid is crucial to the existence and functioning of M_f. Three dimensional mappings of BR in purple membrane show that two acidic amino acids in close contact with membrane lipids are Asp36 and Asp38 (structure 1CWQ [26]). Brown et al., using single exponential fittings, showed a threefold increase in the time constant for M decay in a D38N mutant [27]. Riesle et al., using five and six exponential fittings showed that M_f was completely absent in a D38R mutant and that M_s with a time constant of 5.3 msec in the wild type was replaced with two slower forms of M, one with a time constant of 57 msec (16%) and the other of 91 msec (84%) [28]. In our own work, using multichannel data analyzed by SVD-based least squares, we also find a drastic reduction of M_f (from 45 to 8%) and the appearance of two slower forms of M_s in a D38N mutant (unpublished). Similar results were obtained with a D36N mutant. We have recently shown that the kinetics of the BR photocycle in wild type PM at pH 7.2 are explainable by the operation of two parallel unidirectional photocycles, one fast and the other slow [29]. The fast cycle is represented by $\rightarrow L_f \rightarrow M_f \rightarrow N \rightarrow O \rightarrow BR$, and the slow by $\rightarrow L_s \rightarrow M_s \rightarrow BR$. When the kinetics of the D36N and D38N mutants are examined on the basis of this kinetic model, the fast cycle, involving M_f is drastically reduced and a third cycle with a slower form of M becomes predominant (unpublished). These kinetic results, which resemble the effects produced by brief exposure to dilute Triton X-100, support the notion that the functional stability of the fast form of M depends on a structural association that requires D36 and/or D38.

The importance of SQ, the only neutral lipid present in PM, for the functionality of M_f suggests that an electrically neutral environment around an acidic amino acid is a critical requirement. This idea is supported by the finding that the addition of decane to PM from wild-type Halobacteria, in the range of 0.01-0.2%, results in the conversion of M_s to M_f (unpublished). These observations could explain earlier unexpected findings that very small amounts of the neutral detergent Triton X-100 (i.e., of 0.01-0.02%) increased the relative amount of M_f present in wild type PM [17]. It is important to note, however, that the conclusion stated above on the positioning of SQ near Asp36 and Asp38 is not supported by recent X-ray diffraction data which places SQ away from these acidic amino acids in the hydrophobic groove

formed by the side chains of Leu19, Leu22, Val210, Va1213, Val217, Leu221, and Ser214 [30]. There seem to be two ways in which this apparent conflict may be resolved. One possibility is that the neutral lipid does reside at the hydrophobic site indicated by the X-ray data, but that in order for this site to be available, PGP-Me must be in place near Asp36/Asp38. The other is that as indicated by Brown in a recent review [31], there is the possibility that in the exposure of BR to detergents and crystallization in the presence of non-PM lipids, serious perturbations in native lipid–BR arrangements may occur. In view of our observations that a two minute exposure of PM to 0.1% Triton X-100 extracts lipids and completely disrupts the normal photocycle, we are receptive to Brown's caveat. More work is needed to resolve this question.

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