PURPLE-TO-BLUE TRANSITION OF BACTERIORHODOPSIN IN A NEUTRAL LIPID ENVIRONMENT

ISTVAN SZUNDI AND WALTHER STOECKENIUS
Department of Biochemistry and Biophysics and Cardiovascular Research Institute, University of California, San Francisco, California 94143-0130

ABSTRACT The red shift in the absorption maximum of native purple membrane suspensions caused by deionization is missing in lipid-depleted purple membrane, and the pK of the acid-induced transition is down-shifted to pH ~ 1.4 and has become independent of cation concentration (Szundi, I., and W. Stoeckenius. 1987. *Proc. Natl. Acad. Sci. USA.* 84:3681–3684). However, the proton pumping function cannot be demonstrated in these membranes. When native acidic lipids of purple membrane are exchanged for egg phosphatidylcholine or digalactosyldiglyceride, bacteriorhodopsin is functionally active in the modified membrane. It shows spectral shifts upon light-dark adaptation, a photocycle with M-intermediate and complex decay kinetics; when reconstituted into vesicles with the same neutral lipids, it pumps protons. Unlike native purple membrane, lipid-substituted modified membranes do not show a shift of the absorption maximum to longer wavelength upon deionization. A partial shift can be induced by titration with HCl; it has a pK near 1.5 and no significant salt dependence. Titration with HNO_3 and H_2SO_4, which causes a complete transition in the lipid-depleted membranes, i.e., it changes their colors from purple to blue, does not cause the complete transition in the lipid-substituted preparations. These results show that the purple color of bacteriorhodopsin is independent of cations and their role in the purple-to-blue transition of native membranes is indirect. The purple and blue colors of bacteriorhodopsin are interpreted as two conformational states of the protein, rather than different protonation states of a counterion to the protonated Schiff base.

INTRODUCTION

Bacteriorhodopsin (bR), the only protein in purple membrane (pm), functions as a light-driven proton pump (1, 2; for recent reviews see references 3–5). Besides bR, which forms a two-dimensional lattice, the pm contains 25% by weight diether lipids, of which at least 80% are acidic; 70% of these are phospholipids, mainly the diether analogues of phosphatidylglycerophosphate, and the other 30% are glycosulfolipids (6–11). The membrane thus maintains a high negative surface charge density, even at low pH.

The absorption maximum of bR in pm changes from 568 to 605 nm upon titration with acids or removal of cations, and this transition is reversible (1, 12–18). The acid and deionized blue forms of bR have identical absorption and resonance Raman spectra (19). Further acidification with HCl turns the blue form into an "acid purple" form, which by absorption and resonance Raman spectroscopy is indistinguishable from the native purple form (17, 19).

The ability of cations to restore the original purple color to deionized blue membrane has generally been interpreted as a requirement for bound cations to maintain the native structure of bR. Scatchard plots indicated the presence of three to five high- to medium-affinity cation-binding sites and up to 10 lower affinity sites (15, 16, 20–22), and binding of the first cation had no effect on bR color (21). Some authors also considered the possibility that, due to the high negative surface charge density of pm, binding in a diffuse counterion layer might be involved (15).

We have recently shown (23) that reducing the high surface charge density by removal of acidic lipids from pm has a drastic effect on the characteristics of the color change. In the lipid-depleted membrane (LD-pm), the blue form of bR cannot be induced by deionization, cations are not required to maintain the purple color and the purple-to-blue transition is controlled entirely by proton concentration. The LD-pm is functionally defective; its light-dark adaptation and photocycle are significantly different from native membrane, and proton translocation cannot be demonstrated. Here we describe the purple-to-blue transition of bR in membranes where most of the native lipids have been substituted by neutral or zwitterionic lipids. These lipid-exchanged membranes light-adapt, have an only moderately altered photocycle and transport protons, thus allowing us to study the role of cations and lipids in proton-induced color changes of bR under more physiological conditions.

MATERIALS AND METHODS

Purple membrane was purified from *Halobacterium halobium* strain JW-3 according to the standard procedure (24). Lipids were extracted

from pm with CHAPS as described (23). The native lipids were exchanged for egg phosphatidylcholine (EPC-pm) or digalactosyldiglyceride (DGDG-pm) by incubating the delipidated pm (LD-pm) with the appropriate lipid in the presence of detergent. 5 mg of pm were delipidated three times with 5 ml of 20 mM CHAPS and then resuspended in 5 ml of the incubation medium which contained 20 mg lipid, 20 mM CHAPS and 20 mM buffer (acetate pH 5.2 or MES pH 6.2). After overnight incubation, a purple pellet was separated from the equally colored supernatant by centrifugation at 45,000 *g* for 20 min, and then washed several times with distilled water. These lipid-exchanged membrane fragments were embedded in polyacrylamide gels, deionized, and titrated as described (23).

The colored supernatant was used to obtain bR liposomes. It was dialyzed against distilled water four times and when run on a linear sucrose gradient (0–40%) it formed a single colored band near the top. For more complete removal of detergent, and/or a reduction of the dialysis time, the suspensions were passed through an Amberlite XAD (BDH Chemicals Ltd., Poole, UK) column. This reduced the extent of foaming when N_2 was bubbled through the suspension, but had no other observable effect on the preparations.

We confirmed by electron microscopy that the lipid-restored membranes from the pellet remained sheets; they were smaller and of more irregular shape than the native membranes and partly aggregated. The dialyzed supernatant contained mostly unilamellar vesicles of ~110 nm diameter.

Lipid phosphorus was measured by the molybdate method after ashing (25) and sugar content was determined according to Dubois et al. (26). Absorption spectra were recorded using a Perkin-Elmer Lambda 4A spectrophotometer. Formation and decay of the M intermediate was measured as described by Lozier (27). Absorption changes at 410 nm after excitation by a laser flash at 579 nm (Molectron Dye Laser) were monitored under right angle with a nonpolarized beam. To measure proton transport, bR-liposomes were dialyzed against 10 mM KCl or K_2SO_4 solutions overnight. The pH change upon illumination was recorded with a combination glass electrode (Leeds & Northrup Instruments, North Wales, PA); a Xenon lamp equipped with heat filters and a long-pass yellow filter ($\lambda > 520$ nm) served as the light source. The light response of the pH-electrode was insignificantly small. The vesicle suspension was flushed with nitrogen gas to remove CO_2 before illumination.

Egg phosphatidylcholine (EPC) was obtained from Avanti Polar Lipids (Birmingham, AL), digalactosyldiglyceride (DGDG) from Serdary Research Laboratories (Port Huron, MI), CHAPS from Sigma Chemical Co. (St. Louis, MO); they were used without further purification. All other chemicals were reagent grade commercial preparations.

RESULTS

Composition of the Substituted Preparations

After extraction of native lipids the phosphorus content of the pm had dropped from 9–10 to 3.3–3.4 P/bR, which corresponds to 1.8–2.0 phospholipid molecule per bR, estimated on the basis of the known lipid composition of pm (11). Similarly, the glycolipid content was reduced from 3 to 1–2 molecules per molecule of bR. The amount of native lipids was further lowered during the lipid exchange process to about one phospholipid per bR in DGDG-pm and about one glycolipid per bR in EPC-pm. Assuming that the native phospholipids and glycolipids were replaced to the same extent in DGDG- and EPC-pm, we calculate, based on phosphorus and sugar contents, a composition of 5 EPC, 1 native P-lipid, and 1 native glycolipid per bR in EPC-pm and 4 DGDG-, 1 native glycolipid, and 1 native P-lipid per bR in DGDG-pm. The molar protein to lipid ratio in the vesicles varied from 1:500 to 1:2,000.

Characterization of Spectral and Functional Properties of bR in the Modified Membranes

Compared with pm, the lipid-substituted membranes had a small blue shift similar to that of LD-pm with absorption maxima at 560 ± 1 nm in the light-adapted state. Whereas the LD-pm showed hardly any spectral shift during dark adaptation, the blue shift was 6–9 nm with 60 min half-time for dark adaptation in DGDG-pm, and 8–9 nm with 15–30 min half-time for EPC-pm at room temperature and pH ~ 5.8. The decrease in absorbance during dark adaptation was 5–6%, approximately half of the change observed in native membrane.

The photoreactions of bR in lipid-exchanged membranes were checked by measuring the rise and decay times of the M-intermediate. The modified membranes showed the same extent of M formation as the native pm. Half-times of the M-rise for all the modified membranes were similar or only slightly longer than those of native membranes, 30–40 μs for EPC-pm and 70 μs for DGDG-pm and the M-decay kinetics were much slower. A typical trace as monitored at 410 nm and the difference absorption spectrum are shown in Fig. 1 for DGDG-pm. The M-decay kinetics of all the modified membranes require at least three exponentials for a satisfactory fit to the data. The halftimes and amplitudes for EPC-pm were ~10 ms (40–50%), 100–150 ms (20–30%), and 400–500 ms (30–40%), whereas for DGDG-pm, they were 10–20 ms (10–20%), 150–200 ms (30–40%), and 700–800 ms (50–60%).

The absorption maximum of vesicles was between 554 and 559 nm in the light-adapted stated. It shifted towards the blue by 6–7 nm and its amplitude decreased by 2–3% during dark adaptation. Due to the enhanced light scattering and low bR concentration, the spectroscopic data for DGDG-vesicles did not allow a reliable kinetic analysis. For EPC-vesicles, the halftime of dark adaptation was around 40 min. Their M-decay kinetics were similar to EPC-pm, and the half-times and amplitudes of the components were 10 ms (20%), 80 ms (36%), and 450 ms (43%).

Bacteriorhodopsin can pump protons in the altered lipid environment. Fig. 2 shows the pH increase of DGDG-vesicle suspensions under illumination. Similar results were obtained with EPC-reconstituted vesicles. The extent of the observed pump activity is comparable with that of a similar preparation obtained from native membrane and asolectin.

Purple-to-Blue Transition of bR in Lipid-exchanged Membranes

As already observed with LD-pm (23), deionization with cation-exchange resins did not change the color of EPC-

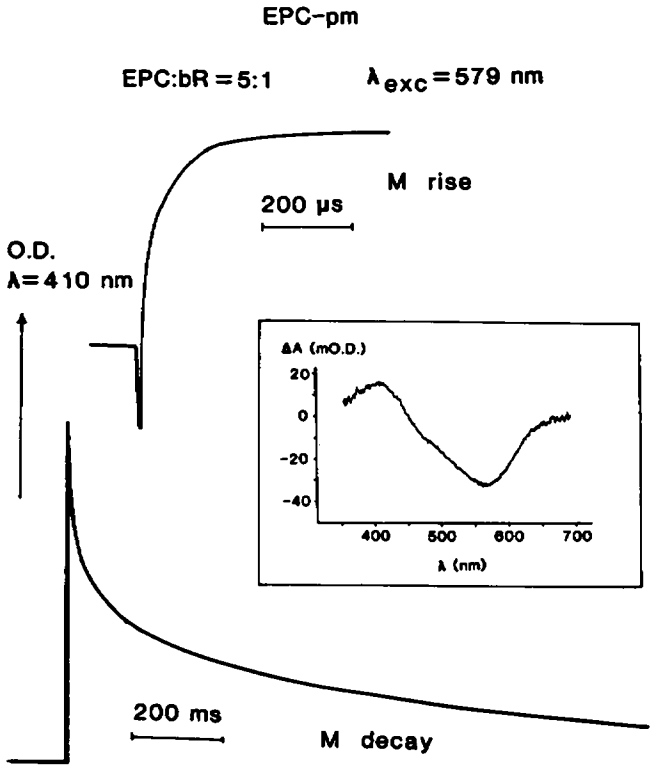

FIGURE 1 Kinetics of the M intermediate of EPC-pm suspension in distilled water at room temperature (22°C). Absorbance changes were recorded at 410 nm under right angle after excitation with dye laser at 579 nm. Insert shows the difference absorption spectra recorded with an optical multichannel analyzer 1 ms after excitation. All traces are averages of 100 flashes at 0.2 Hz repetition rate.

and DGDG-pm but a salt-independent red shift occurred upon titration with acid. Fig. 3 shows the titration with HCl at different $CaCl_2$ concentrations for DGDG-pm. The transition begins near pH 2 and there is hardly any effect of salt. The maximal color change is less than in the case of native membrane (λ_{max} = 605 nm) because the acid purple form of bR begins to dominate near pH 1.0 and is not affected by the surface charge density (15); the absorption shifts back to shorter wavelength, as already reported for

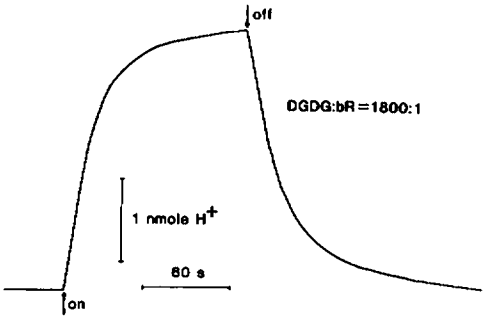

FIGURE 2 pH response of bR in DGDG vesicles at room temperature. Increase in pH was measured with a glass electrode in the presence of 10 mM KCl under N_2 atmosphere; bR concentration ~0.5 µM. Illumination with heat-filtered yellow light ($\lambda > 520$ nm) of 60 mW/cm² intensity; calibration with HCl and NaOH.

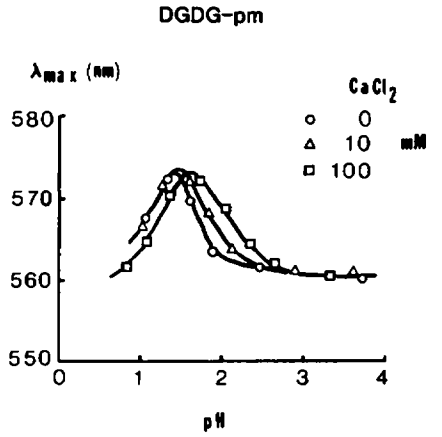

FIGURE 3 Titration of DGDG-pm with HCl in the presence of $CaCl_2$ at room temperature. Polyacrylamide gel slices (3 × 3 × 20 mm) containing the membrane fragments were incubated in 10 ml solution. Absorption spectra were recorded and the wavelength of absorption maximum (λ_{max}) plotted against bulk pH.

LD-pm (23). This is shown in Fig. 4 for the native, deionized membrane and compared with the titrations for LD-, EPC-, and DGDG-pm. The transition to the acid purple form is strongly dependent on the anion species and concentration, and chloride is most effective (13). However, the transition is apparently also affected by the lipid environment.

Taking advantage of the similar chromophore environments but different lipid surroundings of bR in the modified membranes, we carried out titrations with different acids to get more information on the action of anions and possibly on the mechanism of color change. When HNO_3 was used for titration (Fig. 5), the acid purple form began to appear at ~0.3 U lower pH than with HCl or at higher anion concentration, which allowed the LD-pm to com-

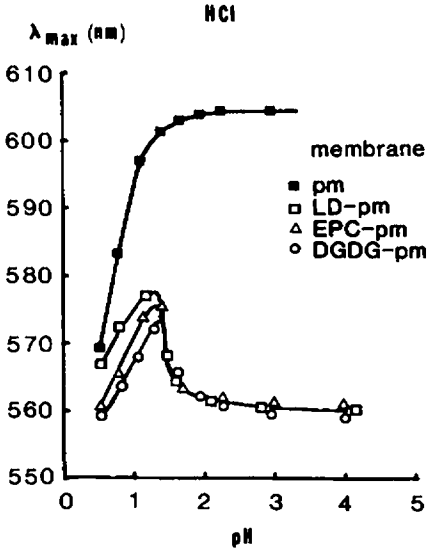

FIGURE 4 Titration of deionized membranes with HCl. Conditions were as in Fig. 3. Note that none of the modified membranes turned completely blue.

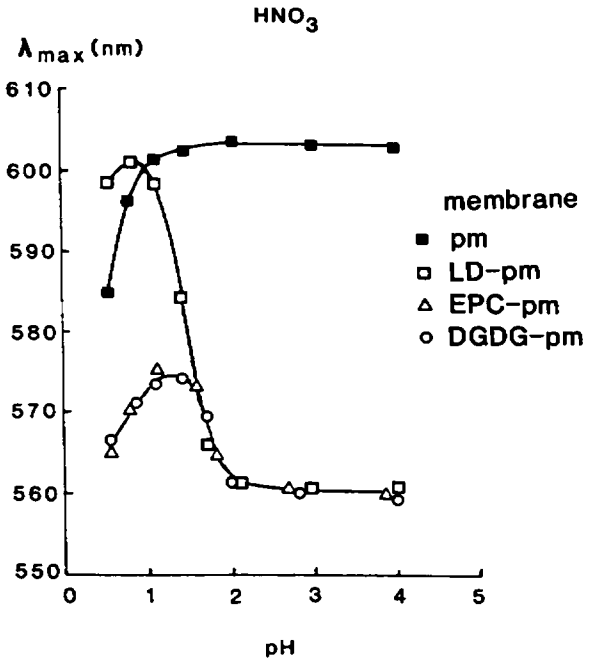

FIGURE 5 Titration of deionized membranes with HNO_3. Conditions as in Fig. 3. Note that LD-pm turned completely blue.

plete the purple-to-blue transition. However, the DGDG- and EPC-membranes remained only partially converted, which we take as an indication for a contribution of the lipid environment in the transition. This conclusion is further supported by the observation that in LD-pm the blue to acid purple transition occurs at a slightly lower pH than in deionized native pm. H_2SO_4 titration (Fig. 6) is even less effective in promoting the acid purple transition,

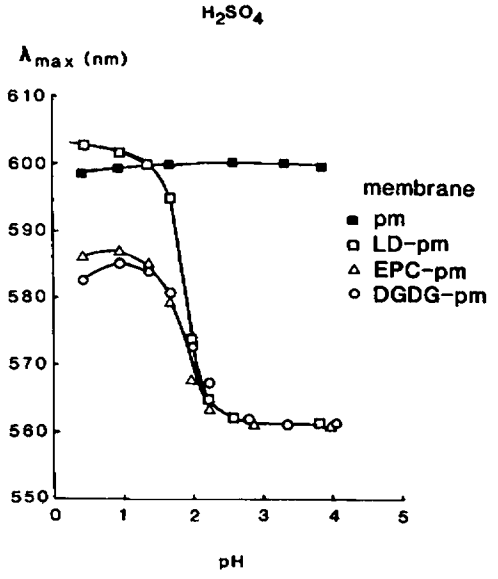

FIGURE 6 Titration of deionized membranes with H_2SO_4. Conditions as in Fig. 3. Note that practically no acid purple was formed and that LD-pm turned completely blue.

because the blue color of both the deionized native membrane and LD-pm remained practically unchanged down to pH 0.3. DGDG- and EPC-pm also showed little change in λ_{max} below pH 1.5; however, their λ_{max} only shifts to ~585 nm. Unfortunately, the titrations cannot be carried much further, because irreversible absorbance changes begin to appear.

DISCUSSION

We have shown here that incorporating neutral lipids into LD-pm, which restores its function, does not change the pK of the purple-to-blue transition and does not restore the cation effect observed in native membranes. That reconstitution of delipidated bR with an excess of the acidic native lipids shifts the pK to 4.5, even in the presence of 150 mM KCl, had already been shown by Khorana's group (28). We have confirmed that membranes reconstituted with halolipids under our experimental conditions undergo the purple-to-blue transition upon deionization. These results support our earlier conclusions that the purple-to-blue transition is controlled only by the pH at the membrane surface and that the effect of cations is due only to their shielding of negative surface charges and the resulting increase in surface pH. We note that this interpretation holds only for bR in the form of a membrane; completely delipidated and solubilized bR in lauryl sucrose has a 480 nm absorption maximum at neutral and alkaline pH, which shifts to 540 nm below pH 6.0, indicating that more extensive structural changes have occurred in this preparation. Whether or not the further red shift of its absorbance maximum with a pK of 4.0 (29) or 3.1 (30) or the 480 nm to 540 nm shift is the equivalent of the purple-to-blue transition in pm cannot be decided. Resonance Raman spectra might allow us to determine which, if any, of these chromophores corresponds to the purple or blue chromophores of native, LD-pm and neutral lipid-substituted pm.

Structural studies indicate that the protein lattice of the partially delipidated purple membranes is well-ordered but has shrunk due to the removal of lipids mainly from the spaces between the bR trimers. The structure of the protein is unchanged at the 5–6 Å level (9, 13) and, we may assume, well below this level in the vicinity of the chromophore, which should be highly sensitive to much smaller changes (32, 33). A structural change must occur, however, at least for the purple-to-blue transition in native membrane, because the lattice structure is lost (14). We have argued before that in the blue conformation, the Schiff base counterion distance has changed and is responsible for the spectral shift. Our present results further support this conclusion.

If the blue form of bR were induced by protonation of a negative counterion to the protonated Schiff base, as is often suggested, e.g. (13), it should not stop at an intermediate state in the case of DGDG- and EPC-membranes, when the proton concentration in the medium is still

increasing. The different chromophore environment of bR in these membranes cannot explain the phenomena because the LD-pm, with an apparently very similar if not identical absorption spectrum, completes the transition.

We arrive at the following explanation for the color changes of bR. At high proton concentrations on or near the membrane surface, one or more groups which stabilize its native conformation are protonated; they may be buried in the protein, but probably not very far from the surface. (Note that FTIR spectra showed only protonation changes of water-exposed carboxyl groups in the blue-to-purple transition [34]). This protonation causes an imbalance in charge–charge, dipole, or other interactions inside the protein and leads to conformational changes resulting (probably) in a small change in the Schiff base counterion distances and a red shift in the chromophore absorption. The charge imbalance caused by protonation can be reduced or completely eliminated by relatively high concentrations of some anion(s). The anion-specific effects on the conformation of polymeres seen at high anion concentrations may also be involved. They have been discussed with respect to halophile proteins by Lanyi (35). Although the chromophore and protein structure seem to be restored by anions in the acid purple form, a new set of interactions is introduced and the functional properties of bR may be altered significantly.

As we have pointed out before (23), our results do not allow the conclusion that cation-binding sites do not exist on the purple membrane or that metal cations in such sites are not required for proton translocation. The results, therefore, do not necessarily contradict a number of recent publications describing evidence for such sites (22, 36–38); they do, however, suggest some restraint in the interpretation of these data and they clearly contradict the conclusion that metal cations are required to maintain the purple state of the chromophore.

Another problem which further complicates interpretation of most data has not been addressed so far. The transition from purple to blue during acid titration, as well as the back reaction near neutral pH during cation titration, occurs via at least one intermediate state, which is difficult to distinguish from the native purple state by absorption spectroscopy (14, 39). Preliminary work in our laboratory has shown that the intermediate state(s) may be long-lived. Whereas the chromophore is apparently very close to the native conformation, this is not necessarily true for the protein; nothing is known about the functional properties of the intermediate(s). This problem should be explored further, before firm conclusions on the role of metal cations in the function of bR are drawn.

This work was supported by National Institutes of Health grant GM-27057.

Received for publication 23 December 1987 and in final form 11 April 1988.

REFERENCES

1. Oesterhelt, D., and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nat. New Biol.* 233:149–152.
2. Oesterhelt, D., and W. Stoeckenius. 1973. Functions of a new photoreceptor membrane. *Proc. Natl. Acad. Sci. USA.* 70:2853–2857.
3. Ottolenghi, M. 1980. The photochemistry of rhodopsins. *Adv. Photochem.* 12:97–200.
4. Ovchinnikov, Y. U. A., N. G. Abdulaev, and N. N. Modyanov. 1982. Structural basis of proton-translocating protein function. *Annu. Rev. Biophys. Bioeng.* 11:445–463.
5. Stoeckenius, W., and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* 52:587–615.
6. Blaurock, A. E., and W. Stoeckenius. 1971. Structure of the purple membrane. *Nat. New Biol.* 233:152–155.
7. Henderson, R., and P. N. T. Unwin. 1975. Three-dimensional model of purple membrane obtained by electron microscopy. *Nature (Lond.).* 257:28–32.
8. Jost, P. C., D. A. McMillen, W. D. Morgan, and W. Stoeckenius. 1978. Lipid-protein interactions in the purple membrane. *In* Light Transducing Membranes: Structure, Function, and Evolution. Academic Press, New York. 141–155.
9. Glaeser, R. M., J. S. Jubb, and R. Henderson. 1985. Structural comparison of native and deoxycholate-treated purple membrane. *Biophys. J.* 48:775–780.
10. Kushwaha, S. C., M. Kates, and W. G. Martin. 1975. Characterization and composition of the purple and red membrane from *Halobacterium cutirubrum*. *Can. J. Biochem.* 53:284–292.
11. Kates, M., S. C. Kushwaha, and G. D. Sprott. 1982. Lipids of purple membrane from extreme halophiles and of methanogenic bacteria. *Methods Enzymol.* 88:98–105.
12. Moore, T. A., M. E. Edgerton, G. Parr, C. Greenwood, and R. N. Perham. 1978. Studies of an acid-induced species of purple membrane from *Halobacterium halobium*. *Biochem. J.* 171:469–476.
13. Fischer, U., and D. Oesterhelt. 1979. Chromophore equilibria in bacteriorhodopsin. *Biophys. J.* 28:211–230.
14. Mowery, P. C., R. H. Lozier, Q. Chae, Y.-W. Tseng, M. Taylor, and W. Stoeckenius. 1979. Effect of acid pH on the absorption spectra and photoreactions of bacteriorhodopsin. *Biochemistry.* 18:4100–4107.
15. Kimura, Y., A. Ikegami, and W. Stoeckenius. 1984. Salt and pH-dependent changes of the purple membrane absorption spectrum. Evidence for changes in conformation of the protein. *Photochem. Photobiol.* 40:641–646.
16. Chang, C.-H., J. G. Chen, R. Govindjee, and T. Ebrey. 1985. Cation binding by bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 82:396–400.
17. Massig, G., M. Stockburger, and T. Alshuth. 1985. Structure of bacteriorhodopsin in the acidified membrane and at high ionic strength: resonance Raman study. *Can. J. Chem.* 63:2012–2017.
18. Muccio, D. D., and J. Y. Cassim. 1979. Interpretations of the effects of pH on the spectra of purple membrane. *J. Mol. Biol.* 135:595–609.
19. Smith, S. O., and R. A. Mathies. 1985. Resonance Raman spectra of the acidified and deionized forms of bacteriorhodopsin. *Biophys. J.* 47:251–254.
20. Chang, C.-H., R. Jonas, S. Melchiore, R. Govindjee, and T. G. Ebrey. 1986. Mechanism and role of divalent cation binding of bacteriorhodopsin. *Biophys. J.* 49:731–739.
21. Ariki, M., and J. K. Lanyi. 1986. Characterization of metal ion binding sites in bacteriorhodopsin. *J. Biol. Chem.* 261:8167–8174.
22. Dunach, M., M. Seigneuret, J.-L. Rigaud, and E. Padros. 1987. Characterization of the cation binding sites of the purple mem-

brane. Electron spin resonance and flash photolysis studies. *Biochemistry.* 26:1179–1186.
23. Szundi, I., and W. Stoeckenius. 1987. Effect of lipid surface charges on the purple-to-blue transition of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 84:3681–3684.
24. Oesterhelt, D., and W. Stoeckenius. 1974. Isolation of the cell membrane of *Halobacterium halobium* and its fractionation into red and purple membrane. *Methods Enzymol.* 31:667–678.
25. Ames, B. N. 1966. Assay of inorganic phosphate, total phosphate and phosphatases. *Methods Enzymol.* 8:115–118.
26. Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith. 1956. Colorimetric method for determination of sugars and related substances. *Anal. Chem.* 28:350–356.
27. Lozier, R. H. 1982. Rapid kinetic optical absorption spectroscopy of bacteriorhodopsin photocycles. *Methods Enzymol.* 88:133–162.
28. Lind, C., B. Hojeberg, and H. G. Khorana. 1981. Reconstitution of delipidated bacteriorhodopsin with endogenous polar lipids. *J. Biol. Chem.* 256:8298–8305.
29. Baribeau, J., and F. Boucher. 1985. Isolation, purification and partial characterization of stable forms of monomeric bacteriorhodopsin in lauryl sucrose. *Can. J. Biochem. Cell Biol.* 63:305–312.
30. Hiraki, K., T. Hamanaka, T. Mitsui, and Y. Kito. 1984. The absorbance spectrum of the brown holo-membrane and the comparison of pI values of bacteriorhodopsin solubilized from purple membrane and from brown holomembrane. *Biochim. Biophys. Acta.* 777:232–240.
31. Tsygannik, I. N., and J. M. Baldwin. 1987. Three-dimensional structure of deoxycholate-treated purple membrane at 6 Å resolution and molecular averaging of three crystal forms of bacteriorhodopsin. *Eur. Biophys. J.* 14:263–272.
32. Nakanishi, K., V. Balogh-Nair, M. Arnoldi, K. Tsujimoto, and B. Honig. 1980. An external point-charge model for bacteriorhodopsin to account for its purple color. *J. Am. Chem. Soc.* 102:7945–7947.
33. Blatz, P. E., J. H. Mohler, and H. V. Navangul. 1972. Anion-induced wavelength regulation of absorption maxima of Schiff bases of retinal. *Biochemistry.* 11:848–855.
34. Gerwert, K., U. M. Ganter, F. Siebert, and B. Hess. 1987. Only water-exposed carboxyl groups are protonated during the transition to the cation-free blue bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 213:39–44.
35. Lanyi, J. K. 1974. Salt-dependent properties of proteins from extremely halophilic bacteria. *Bacteriol. Rev.* 38:272–290.
36. Ariki, M., D. Magde, and J. K. Lanyi. 1987. Metal ion binding sites of bacteriorhodopsin: laser-induced lanthanide luminescence study. *J. Biol. Chem.* 262:4947–4951.
37. Corcoran, T. C., K. Z. Ismail, and M. A. El-Sayed. 1987. Evidence for the involvement of more than one metal cation in the Schiff base deprotonation process during the photocycle of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 84:4094–4098.
38. Dunach, M., M. Seigneuret, J.-L. Rigaud, and E. Padros. 1986. The relationship between the chromophore moiety and the cation binding sites in bacteriorhodopsin. *Biosci. Rep.* 6:961–966.
39. Zubov, B., K. Tsuji, and B. Hess. 1986. Transition kinetics of the conversion of blue to purple bacteriorhodopsin upon magnesium binding. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 200:226–230.