Circular dichroism and photocycle kinetics of partially detergent solubilized and partially retinal regenerated bacteriorhodopsin

Shuguang Wu, Elias S. Awad, and M. A. El-Sayed
Department of Chemistry and Biochemistry, University of California, Los Angeles, California 90024 USA

ABSTRACT The circular dichroism (CD) spectra and the kinetics of the M_{412} formation have been determined and compared for bacteriorhodopsin (bR) partially delipidated by the addition of Triton X-100 and partially reconstituted by the addition of retinal to apoprotein at pH 6.8 and 22°C. As the degree of delipidation increases or the fraction of reconstitution decreases, the following observations are made: (*a*) the shape of the visible CD band changes from biphasic, as found in bR, to a single monomer type band; (*b*) the CD spectra and the deprotonation rate constants change in a similar way; (*c*) the relative amplitudes of the components of the deprotonation kinetics do not change upon reconstitution, but change greatly upon delipidation. These results lead to the conclusion that the CD band shape as well as the deprotonation rate constants are sensitive to one type of perturbation, which is linked to the retinal structure within the protein environment, whereas the relative amplitudes of the components of the deprotonation kinetics are sensitive to another type, which may be linked to acid-base equilibria of the amino acid side groups within the active site.

INTRODUCTION

Bacteriorhodopsin (bR) acts as a light-driven proton pump and converts light into chemical energy by pumping protons across the bacterial membrane (1). Its biochemistry, biophysics, and photochemistry have been investigated widely (2). The chromophore, all-*trans* retinal, is bound to the protein moiety through a protonated Schiff base (PSB) linkage at the ϵ-amino group of Lys_{216} (3–6). Upon absorption of light a photocycle is initiated consisting of a number of steps over a wide range of timescales during which the PSB undergoes deprotonation followed by reprotonation back to native bR ready to start another cycle:

$$bR_{568} \xrightarrow[400\ fs]{h\nu} J_{625} \xrightarrow[5\ ps]{} K_{610} \xrightarrow[2\ \mu s]{} L_{550}$$

$$\xrightarrow[60\ \mu s]{H^+} M_{412} \xrightarrow[H^+]{} N_{520} \longrightarrow O_{640} \longrightarrow bR_{568}.$$

The removal and replacement of a proton at the active site results in the translocation of hydrogen ions across the purple membrane.

Circular dichroism (CD) spectroscopy has been applied to the field of biophysics for several decades because of its sensitivity to the conformation of biomacromolecules. It has proven to be a very useful tool in understanding the structural properties of bacteriorhodopsin (7–16), which are of importance in establishing a suitable model for the proton pumping mechanism of bR.

The bleaching of retinal in the presence of hydroxylamine and subsequent regeneration of the purple membrane have been studied by absorption and CD spectroscopies (7, 17). The results were compared with those of solubilized native bR in Triton X-100. Both preparations are found to give samples having single positive CD bands instead of the biphasic CD band of native bR. These results led to the conclusion (7, 17, 18) that the biphasic CD in bR results from exciton coupling between the retinal electronic systems in the trimer structure that was proposed by Henderson from x-ray diffraction (19) and by Unwin and Henderson from electron diffraction (20) of the solid sample. Recently a detailed discussion of the structure of bR has been given by Henderson and co-workers (21) based on high-resolution electron cryo-microscopy.

The trimer structure is destroyed either by treatment with hydroxylamine or with Triton X-100, leading in both cases to positive CD bands. The monomer is formed when the regenerated bR is at low percentage of reconstitution. According to Dencher and Heyn (22–24, 26–28), the solubilization of native bR in detergents such as Triton X-100 and β-octyl glucoside gives CD spectra similar to partially regenerated bR and it was thus assumed that the trimer structure of bR is indeed destroyed by the detergent to form the so-called monomer.

The exciton model was questioned recently (14) and a proposal was made that the biphasic feature might actually result from heterogeneity within the active site

0006-3495/91/01/70/06 $2.00

of the trimer structure (13, 14). In any case, the above two preparations seem to destroy the trimer structure of the retinal within the active site that leads to the biphasic CD. The question immediately arises as to whether or not the initial structure of the active site determines the reaction path of the bR photocycle. If it does, then the kinetics of the photocycle and the biphasic nature of the CD spectrum should change concomitantly in the above two preparations.

In this work we used transient absorption spectroscopy to monitor the formation kinetics of the M_{412} intermediate during the photocycle of bR samples prepared in two different ways. In one preparation, the bR samples are prepared by the addition of retinal to apoprotein with different ratios of retinal to bacterioopsin (bO). In the other preparation partial delipidation of bR is carried out by detergent dialysis in Triton X-100 for different lengths of time. Our results show that while the CD spectra and the deprotonation rate constants change in a similar fashion, significant differences are observed in the amplitudes of the components of the deprotonation kinetics. This suggests that different types of structural heterogeneity are responsible for the changes in the measured properties resulting from these perturbations.

MATERIALS AND METHODS

Bleaching of bR followed the method described by Oesterhelt and co-workers (24, 25) with some modifications. The purple bR was suspended in 1.0 M hydroxylamine (NH_2OH) and pH was adjusted to 8.0 with 1.0 N NaOH. The sample was then irradiated by light from a 500-W projector lamp filtered through a Corning glass filter type 3-66 and 3-67. A constant temperature of 40°C was maintained during the bleaching. The complete removal of the retinal chromophore to form the retinaloxime and bacterioopsin took ~4–5 h. The bleached samples were washed three times with deionized water to remove excess hydroxylamine by centrifuging the sample at 19,000 *g*. Finally the samples were resuspended in 0.02 M phosphate buffers at pH 6.8.

Bleached samples were regenerated by addition of different amount of all-*trans* retinal (0.6 mM in ethanol) at pH 6.8. After addition of retinal, the samples were incubated overnight in the dark at room temperature (22°C). The samples were then light-adapted with the same filter combination described above for the bleaching for 20 min. The concentration of bleached bR was calculated from the absorbance of the protein band at 280 nm to prepare the samples with different molar ratios of retinal to bacterioopsin (bO).

Solubilization of bR was carried out by mixing 0.4% (vol/vol) Triton X-100 with bR suspension in 0.02 M phosphate buffer at pH 6.8.

The steady-state absorption spectra were taken with a Hewlett-Packard 8451 diode-array spectrometer and the CD spectra of regenerated and solubilized bR at a concentration 30–60 μM were measured using a JASCO J-600 spectropolarimeter.

The experimental setup for the transient absorption as described previously (22) was used with some modification. A focused 0.5 ns 560 nm pulse generated from a N_2-pumped dye laser (LN 1000 and LN 102; Photochemical Research Associates Inc., London, Ontario, Canada) was used as a photolysis beam. The laser energy was ~50 μJ with a spot of 2 mm. The 405 nm probe beam was the output of a 100-W Hg arc lamp (Pek Labs 401, Sunnyvale, CA) passed through filters and focused into a 0.25 m monochromator (Jarrel-Ash 82-410, Waltham, MA). A photomultiplier tube (1P28A; RCA, RCA New Products Div., Lancaster, PA) was used to detect the probe light. The PMT signal was recorded by a fast response detector (Biomation 8100; Princeton Applied Research Corporation, Princeton, NJ) which was interfaced to the IBM/PC computer.

RESULTS

Fig. 1 and Fig. 2 show the absorption and CD spectra, respectively, of bR regenerated at different stages of reconstitution by addition of all-*trans* retinal to bO at pH 6.8 and 22°C. Gradual regeneration of the purple membrane with all-*trans* retinal is reflected by an increase in the 568 nm absorption band. The CD peak at ~360 nm is due to the retinaloxime which is formed in the bleaching process and is probably trapped within the protein moiety. Although a better isosbestic point is observed at 568 nm, our CD results generally agree with published CD spectra (17). It has been believed that the single positive CD band in the retinal absorption region at low percentage of regeneration is due to monomeric bR (17). As the percentage of retinal added increases, trimers are gradually expected to form. The observed unsymmetric biphasic CD band is believed to result either from the retinal-retinal (exciton) interaction between the chromophores (3) or from the heterogeneity in the bR sample (13, 14) which is sensitive to the extent of reconstitution. The differences in CD spectra for regenerated bR at different percentages are very obvious (see Fig. 2), suggesting that structural differences must exist in the samples with different percentage of regeneration.

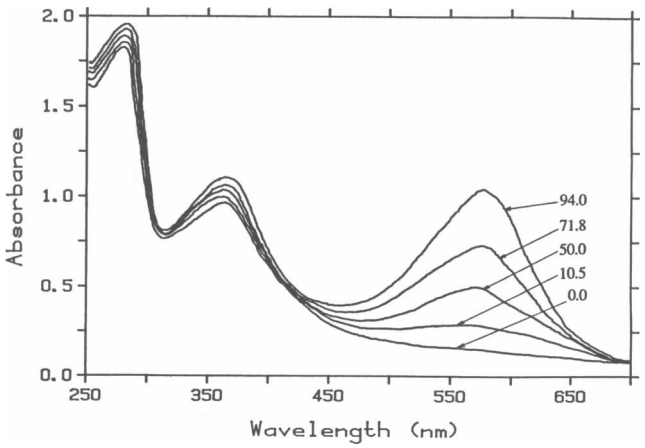

FIGURE 1 Absorption spectra of bleached bR at different stages of regeneration with all-*trans* retinal in pH 6.8 phosphate buffer solution. Percent regeneration yield is indicated in the figure.

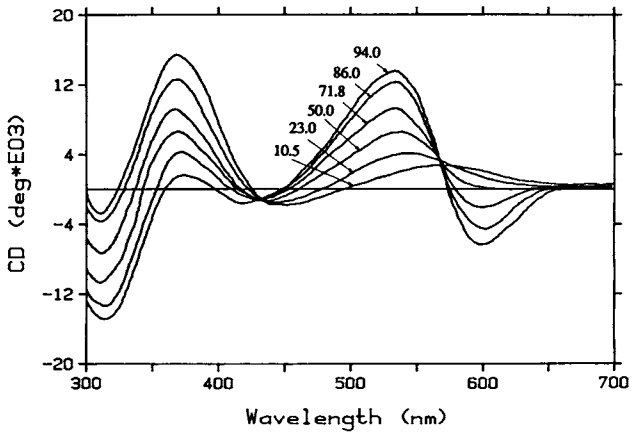

FIGURE 2 Near-UV and visible CD spectra of bleached bR at different stages of regeneration with all-*trans* retinal in pH 6.8 phosphate buffer solution. Percent generation yield is indicated in the figure.

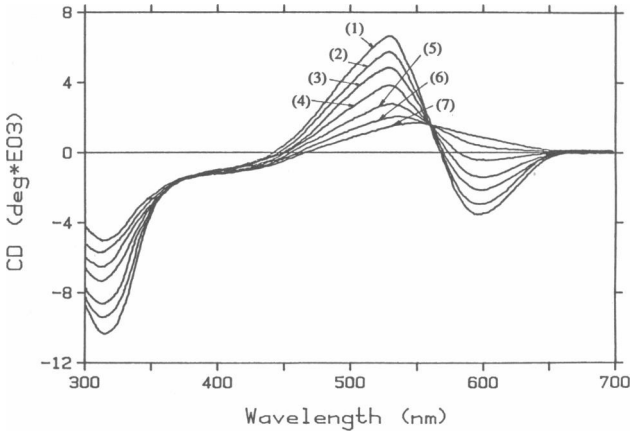

FIGURE 3 Near-UV and visible CD spectra of native bR solubilized in a 0.4% (vol/vol) Triton X-100 and 0.02 M phosphate buffer at pH 6.8 at different times after addition of Triton X-100. The spectra were recorded at the following times after mixing of bR with Triton X-100: (1) 0 min (native bR); (2) 30 min; (3) 110 min; (4) 180 min; (5) 290 min; (6) 360 min; (7) 540 min.

The CD spectra of solubilized bR in 0.4% (vol/vol) mixture of Triton X-100 and 0.02 M phosphate buffer at pH 6.8 are recorded at different times after the addition of detergent as shown in Fig. 3 and agree with previous results (18). Only a small decrease in extinction and a shift of the absorption maximum from 568 to 556 nm were observed for the solubilized samples. With increasing time after the mixing of bR and Triton X-100, the CD amplitudes decrease as does the biphasic feature of these bands and finally only a single positive CD band is observed. It should be noted that Fig. 2 and Fig. 3 show a similar trend for the change in the CD spectra upon different perturbations in the visible region. Both sample preparations reveal a successive transition from a biphasic band to a single positive band and a marked isosbestic point at ~568 nm.

However, the M_{412} formation kinetics of solubilized bR are found to be very different from those of native bR and partially regenerated bR. The two formation rate constants and pre-exponential amplitudes are summarized in Table 1. The relative amplitudes (uncertainty within 5%) and the rate constants (uncertainty within 15%) were obtained by the computer program directly from the output of the transient recorder. As the mole fraction of retinal increases, the rate constants of both the fast and the slow components decrease. No effect is observed in the relative amplitudes of these two components in these samples. This is quite different from the behavior observed for Triton treated bR. The transient rise kinetics of M_{412} formation observed at 405 nm of bR solubilized in 0.4% Triton are presented in Fig. 4. From Table 2 it can be seen that both the relative amplitudes and the rate constants of the fast and the slow components of M_{412} formation change as the time of the solubilization increases, i.e. as the trimer structure is destroyed. More noticeable is the large increase in the relative amplitude of the fast component while both rate constants only increase slightly as the extent of solubilization increases. The constancy of the relative amplitudes found in the reconstitution experiments (~75% A_{slow}) is strikingly in contrast to the large effect seen when bR is exposed to Triton X-100 (80% A_{slow} in native bR, 27% after exposure for 440 min, 0% in the limit after 10 h). In addition both rate constants k_{fast} and k_{slow} increase in value by a factor of ~2.

TABLE 1 Bi-exponential formation rate constants and pre-exponential amplitudes of M intermediate for regenerated bR at different percent reconstitution in 0.02 M pH 6.8 phosphate buffer (22 °C)

Regeneration yield (%)*	Rate constants ($10^4 s^{-1}$)		Amplitudes (%)	
	k_{fast}	k_{slow}	A_{fast}	A_{slow}
10.5	14.4	2.0	26	74
23.0	13.0	2.0	25	75
50.0	11.3	1.7	27	73
71.8	10.4	1.9	20	80
94.0	9.9	1.8	26	74
native bR	6.9	1.3	20	80

*Calculated from retinal absorbance at 568 nm with baseline correction.

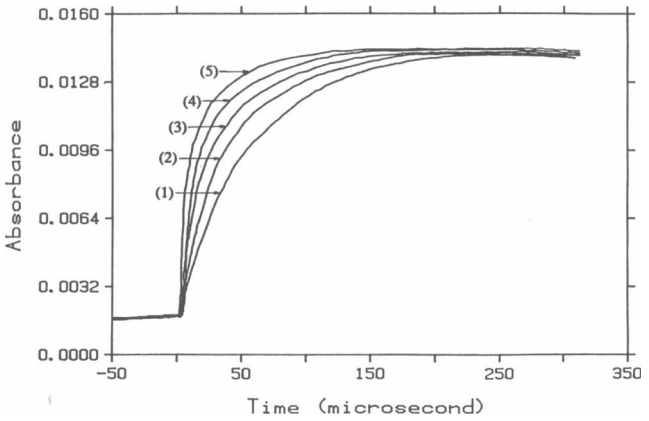

FIGURE 4 Formation kinetics of M_{412} intermediate formation of native bR solubilized in 0.4% (vol/vol) Triton X-100 and 0.02 M phosphate buffer at pH 6.8. The measurements were taken at the following times after mixing of bR with Triton X-100: (1) 0 min (native bR); (2) 20 min; (3) 100 min; (4) 210 min; (5) 375 min.

DISCUSSION

The above results can be rationalized as follows: (*a*) The fact that the two preparations affect the CD similarly could be explained by similar changes in the local retinal structure. It is possible that the trimer structure has a more compact protein configuration around the retinal that gives rise to a biphasic CD, either by exciton coupling (7, 17, 18) or by the presence of more than one retinal-protein relative conformation (13, 14). Once this structure is destroyed by removing the retinal or upon solubilization of the lipids by Triton treatment, a monomer type retinal configuration arises, leading to similar changes in the CD spectrum for the two preparations. (*b*) The kinetics, on the other hand, are controlled by the dynamics of protein conformational changes (26) during the photocycle. It is now believed that the different forms of bR present at different pH (29, 30) or temperature (18, 31, 32) result from different protein conformations. Some of these, for example, acid bR (30, 32, 33) and acid purple bR (30, 33) do not deprotonate the PSB. It can thus be concluded that the rate of the protein conformational change during the photocycle is sensitive to the conformation of the protein at the start of the photocycle. This in turn is found to be sensitive to pH, to concentration of metal cations (29, 34–36), or to types and fractions of lipids in the purple membrane (22, 23). Recently, in an attempt to explain these observations, surface pH was proposed to control the conformation of the different forms of bR (37). More recently, it was found that the pH at which the blue to purple transition occurs is sensitive to the protein structure within the active site near the retinal (27). All these observations lead to the conclusion that the protein conformation and its rate of change in bR are controlled by the coupling between the surface potential and the amino acid side groups of the protein moiety within and around the active site (27). These interactions could in principle be modulated by the different acid-base equilibria of the different amino acid side groups within the protein at different pH. At any particular pH, there could be more than one minimum for the free energy of the retinal-protein environment, i.e., different types of bR, when viewed through the narrow window of the measured retinal absorption kinetics. These different bR states would be expected to have different reaction potential surfaces and thus different rates for their deprotonation (26). As was proposed previously (26) the relative amplitudes of the slow and fast components thus give the relative amounts of the two types of bR present before the initiation of the photocycle.

When the two perturbations are applied to bR, both the CD spectrum and the deprotonation rates change similarly, but the relative amplitudes change very differently. This might be explained as follows. In both perturbations, the trimers convert into monomers. If so, then the rate of deprotonation is faster in the monomer than in the trimer. A similar observation was made in the light-to-dark adaptation of bR (38), where the reversion rate for the monomer was considerably faster than for the trimer, suggesting that the trimer structure imposes greater rigidity on the retinal environment. The fact that the two perturbations change the CD spectrum and the rate of deprotonation in a similar way, but have quite different effects on the relative amplitudes of the two

TABLE 2 Bi-exponential formation rate constants and pre-exponential amplitudes of M intermediate for native bR solubilized in Triton X-100 at pH 6.8 (22 °C)

	Rate constants ($10^4 s^{-1}$)		Amplitudes (%)	
Time*	k_{fast}	k_{slow}	A_{fast}	A_{slow}
min				
native bR	6.8	1.3	20	80
7	8.2	1.6	31	69
30	9.2	1.5	43	57
110	10.4	1.7	48	52
180	12.7	1.9	63	37
260	13.3	2.3	72	28
375	13.1	2.2	71	29
440	13.4	2.3	73	27
10 h in 5% Triton	13.8	—	100	—

*Time was recorded when the measurement was taken after addition of 0.4% Triton X-100.

components of the deprotonation process, strongly suggests that the CD band shape and the rate constants are sensitive to one type of structural perturbation whereas the relative amplitudes of the components of the deprotonation kinetics are sensitive to another type. The former is probably determined by the structure of the retinal in its protein environment, which changes when trimer is converted into monomer, whereas the latter could be determined by the acid-base equilibria of the amino acid side groups within the active site.

This work was supported by the Department of Energy (Office of Basic Energy Sciences) under grant DE-FG03-88ER13828.

Received for publication 6 June 1990 and in final form 10 September 1990.

REFERENCES

1. Stoeckenius, W., and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* 51:587–616.
2. Lanyi, J. K. 1984. Bacteriorhodopsin and related light-energy converters. *In* Bioenergetics (New Comprehensive Biochemistry). L. Ernster, editor. Elsevier/North Holland, Amsterdam. 9:315–350.
3. Oesterhelt, D., and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium. Nature (Lond.). New Biol.* 233:149–152.
4. Khorana, H. G. 1988. Bacteriorhodopsin, a membrane protein that uses light to translocate protons. *J. Biol. Chem.* 263:7439–7442.
5. Bridgen, J., and I. Walker. 1976. Photoreceptor protein from the purple membrane of *Halobacterium halobium.* Molecular weight and retinal binding site. *Biochemistry.* 15:792–798.
6. M. P. Heyn, D. Braun, N. A. Dencher, A. Fahr, M. Holz, M. Lindau, F. Seiff, I. Wallat, and J. Westerhausen. 1988. Chromophore location and charge displacement in bacteriorhodopsin. *Ber. Bunsen-ges. Phys. Chem.* 92:1045–1050.
7. Becher, B., and J. Y. Cassim. 1977. Effects of bleaching and regeneration on the purple membrane structure of *Halobacterium halobium. Biophys. J.* 19:285–297.
8. Khorana, H. G., G. E. Gerber, W. C. Herlihy, C. P. Gray, R. J. Anderegg, K. Nihei, and K. Biemann. 1979. Amino acid sequence of bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 76: 5046–5050.
9. Becher, B., and J. Y. Cassim. 1976. Effects of light adaptation on the purple membrane structure of *Halobacterium halobium. Biophys. J.* 16:1183–1200.
10. Ovchinnikov, Y. A., N. G. Abdulaev, M. Yu. Feignia, A. V. Kiselev, and N. A. Lobanov. 1979. The structural basis of the functioning of bacteriorhodopsin: an overview. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 100:219–224.
11. Huang, K.-S., H. Batley, M-J. Liao, E. London, and H. G. Khorana. 1981. Refolding of an integral membrane protein. *J. Biol. Chem.* 256:3802–3809.
12. London, E., and H. G. Khorana. 1982. Denaturation and renaturation of bacteriorhodopsin in detergents and lipid-detergent mixtures. *J. Biol. Chem.* 257:7003–7011.
13. Jang D.-J., T. Mogi, L. J. Stern, H. G. Khorana, and M. A. El-Sayed. 1990. Sensitivity of the retinal circular dichroism of bacteriorhodopsin to the mutagenetic single substitution of amino acid. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 262:155–158.
14. El-Sayed, M. A., C. T. Lin, and W. R. Mason. 1989. Is there an excitonic interaction or antenna system in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 86:5376–5379.
15. Gibson, N. J., and J. Y. Cassim. 1989. Nature of forces stabilizing the transmembrane protein bacteriorhodopsin in purple membrane. *Biophys. J.* 56:769–780.
16. Draheim, J. E., and J. Y. Cassim. 1985. Large scale global structural changes of the purple membrane during the photocycle. *Biophys. J.* 47:497–507.
17. Becher, B., and T. G. Ebrey. 1976. Evidence for chromophore-chromophore (exciton) interaction in the purple membrane of *Halobacterium halobium. Biochem. Biophys. Res. Commun.* 69: 1–6.
18. Heyn, M. P., P.-J. Bauer, and N. A. Dencher. 1975. A natural CD label to probe the structure of the purple membrane from *Halobacterium halobium* by means of exciton coupling effects. *Biochem. Biophys. Res. Commun.* 67:897–903.
19. Henderson, R. 1975. The structure of the purple membrane from *Halobacterium halobium:* analysis of the x-ray diffraction pattern. *J. Mol. Biol.* 93:123–138.
20. Unwin, P. N. T., and R. Henderson. 1975. Molecular structure determination by electron microscopy of unstained crystalline specimens. *J. Mol. Biol.* 94:425–440.
21. Henderson, R., J. M. Baldwin, T. A. Ceska, F. Zemlin, E. Beckmann, and K. H. Downing. 1990. Model for the structure of bacteriorhodopsin based on high-resolution electron cryomicroscopy. *J. Mol. Biol.* 213:899–929.
22. Dencher, N. A., and M. P. Heyn. 1978. Formation and properties of bacteriorhodopsin monomers in the non-ionic detergents octyl-β-D-glucoside and Triton X-100. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 96:322–326.
23. Heyn, M. P., and N. A. Dencher. 1982. Reconstitution of monomeric bacteriorhodopsin into phospholipid vesicles. *Methods Enzymol.* 88:31–35.
24. Dencher, N. A., and M. P. Heyn. 1982. Preparation and properties of monomeric bacteriorhodopsin. *Methods Enzymol.* 88:5–10.
25. Oesterhelt, D., and L. Schuhmann. 1974. Reconstitution of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 44:262–265.
26. Hanamoto, J., P. Dupuis, and M. A. El-Sayed. 1984. On the protein (tyrosine)-chromophore (protonated Schiff base) coupling in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 81:7083–7087.
27. Jang, D.-J., M. A. El-Sayed, L. J. Stern, T. Mogi, and H. G. Khorana. 1990. Effect of genetic modification of tyrosine-185 on the proton pump and the blue-to-purple transition in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 87:4103–4107.
28. Oesterhelt, D., L. Schuhmann, and H. Gruber. 1974. Light-dependent reaction of bacteriorhodopsin with hydroxylamine in cell suspensions of *Halobacterium halobium:* demonstration of an apo-membrane. *FEBS (Fed. Eur. Biochem. Soc.)* Lett. 44:257–261.

29. Chang, C.-H., J.-G. Chen, R. Govindjee, and T. G. Ebrey. 1985. Cation binding by bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 82:396–400.

30. Chronister, E. L., T. C. Corcoran, L. Song, and M. A. El-Sayed. 1986. On the molecular mechanisms of the Schiff base deprotonation during the bacteriorhodopsin photocycle. *Proc. Natl. Acad. Sci. USA.* 83:8580–8584.

31. Nishiya, T., I. Tabushi, and A. Maeda. 1987. Circular dichroism study of bacteriorhodopsin-lipid interaction. *Biochem. Biophys. Res. Commun.* 144:836–840.

32. Mowery, P. C., R. H. Lozier, Q. Chae, Y.-W. Tseng, and M. Taylor. 1979. Effect of acid pH on the absorption spectra and photoreactions of bacteriorhodopsin. *Biochemistry.* 18:4100–4107.

33. Chronister, E. L., and M. A. El-Sayed. 1987. Time-resolved resonance Raman spectra of the photocycle intermediates of acid and deionized bacteriorhodopsin. *Photochem. Photobiol.* 45:507–513.

34. Roux, M., M. Seigneuret, and J.-L. Rigaud. 1988. ^{31}P NMR study of the interaction of cations with purple membrane and of the purple-blue transition. *Biochemistry.* 27:7009–7015.

35. 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.

36. Szundi, I., and W. Stoeckenius. 1988. Purple-to-blue transition of bacteriorhodopsin in a neutral lipid environment. *Biophys. J.* 54:227–232.

37. Szundi, I., and W. Stoeckenius. 1989. Surface pH controls purple-to-blue transition of bacteriorhodopsin. A theoretical model of purple membrane surface. *Biophys. J.* 56:369–383.

38. Ebrey, T. G., B. Belcher, B. Mao, P. Kilbride, and B. Honig. 1977. Exciton interactions and chromophore orientation in the purple membrane. *J. Mol. Biol.* 112:377–397.