6-1-1996

ANION INDUCED BLUE TO PURPLE TRANSITION IN BACTERIORHODOPSIN

Mrunalini M. Kapil
Harrisburg University of Science and Technology, lpattarkine@harrisburgu.edu

Anil K. Singh

Journal of Photoscience, Vol. 3, No. 2, pp. 71-76.

ANION INDUCED BLUE TO PURPLE TRANSITION IN BACTERIORHODOPSIN

ANIL K. SINGH* and MRUNALINI M. KAPIL
Department of Chemistry, Indian Institute of Technology
Bombay - 400076, INDIA

(Received 21 *June* 1996; *accepted* 14 *August*1996)

Abstract — Purple membrane (PM, λ_{max} 570 nm) of *H. halobium* on treatment with sulphuric acid changes its colour to blue (λ_{max} 608 nm). The purple chromophore can be regenerated from the blue chromophore by exogeneous addition of anions such as Cl^- and HPO_4^{2-}. Chloride ion is found to be more effective than the dibasic phosphate ion in regenerating the purple chromophore. Nevertheless, one thing common to the anion regeneration is that both Cl^- and HPO_4^{2-} show marked pH effect. At pH 1.0 the efficiency of regeneration of the purple chromophore is greater than at pH 2.0, for the same anion concentration. Fluorescence and circular dichroic studies indicate that the proteins do not undergo drastic changes at the secondary or tertiary structure level and the native structure is preserved during this transition. However, chromophoric-site interactions between retinal and the apoprotein are affected during this colour transition. A molecular mechanism is advanced for this transition.

INTRODUCTION

Bacteriorhodopsin (bR) is a photoreceptor protein in the purple membrane of *Halobacterium halobium*[1]. The chromophore of bR is a retinylidene unit covalently bound to a ε-amino group of a lysine residue through a protonated Schiff-base linkage. Recently electron cryomicroscopic studies performed at a resolution of 3.7 Å in the direction parallel to the membrane plane have greatly clarified the the tertiary structure of bR[2]. The absorption spectral properties of bR are dependent on the chemical nature of the Schiff base chromophore and its interactions with the apo-protein side chains[3]. Upon absorption of light, the light-adapted form of bacteriorhodopsin (bR^{LA}) undergoes a reaction cycle during which protons are pumped from the inside of the cell to the extracellular medium. The photocycle consists of several thermal reactions that follow the initial photon absorption. The different steps in the photocycle lead to intermediates K.L.M, and O, each with a characteristic absorption spectrum.

In acidic environment bR changes its purple colour to blue colour. The blue form of bR (B-bR) is considered to be one of the protonated intermediates appearing in the normal photocycle of bR[4]. The blue membrane lacks 'M' intermediate and it does not pump protons[5,6]. The purple colour can be restored from the blue colour by exogeneous addition of cations or anions or by raising the pH of the medium[7,8]. This transition has been studied by a variety of spectroscopic and biochemical techniques and various suggestions have been advanced to rationalize this transition in terms of conformational changes in the protein and the membrane potential[9-21].

The bR protein has received wide interest since it has some similarities to the visual pigment rhodopsin, and because of its intrinsic properties as a very simple light energy transducer. Photochemistry of *in vitro* bR has recently stimulated further extensive study of its potential application to the design of electro-optical devices[22,23]. Colour sensitivity of bR to its environment is an important property for use in the development of molecular electronic devices.

In the present work anion-induced regeneration of the purple chromophore has been studied using uv-vis absorption, fluorescent emission and circular dichroic techniques. A molecular level mechanism has been presented to explain the colour changes.

EXPERIMENTAL

General Procedure. All experiments were carried out at ambient temperature (22°-25°C). The salt (AR grade,

* To whom correspondence may be addressed.

Ranbaxy, India) solutions were prepared in triple distilled water. *Halobacterium halobium* R.M was a gift from Dr. W. Stoeckenius, California, U.S.A. The Oxoid bacteriological peptone L-37 was used in the growth medium. The organic solvents were from Spectrochem, Bombay, India. All other chemicals were from Sigma, Fluka or Aldrich. The growth of the halobacterial cells was achieved by shaking the cultures broths on 505-105 Sarit orbit environ incubator shaker (Sarit Equipment, Bombay). Wet cellulose dialysis tubes (Spectrum, U.S.A.) were used for dialyses. All the pH measurements were made on Radiometer PHM-84 pH meter equipped with GK 2410 C microelectrode. Ultracentrifugation was carried out on Beckman L8-55 M ultracentrigfuge using Type-15 Ti or SW-27 rotor. UV-vis absorption measurements were made on Shimadzu-UV-260 spectrophotometer. Lyophilization was done on a Lyophilizer Pvt. Ltd. (Bangalore) instrument. Sonication was performed on Branson B-12 sonifier. The steady state fluorescence measurements were carried out on Spex spectrofluorolog with 16810.22 mm spectrometer as single grating emission monochromator and 16800.22 m double spectrometer as double grating emission monochromator with 450 W Xe lamp as the source of radiation. Data analysis were done on DM-1B datamat interfaced with the spectrofluorimeter. Relative fluorescence quantum yields (φ_f) of the purple and blue chromophores were determined using tryptophan (φ_f = 0.135) in water as standard. The circular dichroism studies were carried out on a JASCO-J-600 spectropolarimeter. The bR used has molar absorptivity of 63000 L. M^{-1} cm^{-1} at 570 nm.

Preparation of Blue Membrane. Purple membrane (570 nm) was isolated from the cells of *H. halobium* as described earlier[24]. The aqueous suspension of purple membrane (4.7 x 10^{-6} M) was treated with appropriate amounts of concentrated sulphuric acid (0.1 M/0.01 M) depending upon the desired pH (1.0/2.0). The acid treatment gave the blue membrane with absorption band at 608 nm.

Regeneration of Purple Chromophore and Fluorescence Measurements. The blue to purple transition was effected by adding sodium salts of chloride and dibasic phosphate. These were added from stock solutions (2.5 M) of the salts prepared in triple distilled water. The additions were made using Gilson-P-20 micropippette and care was taken so that the change in total volume was kept as small as possible. The regeneration of the purple chromophore (570 nm) was monitored by uv-vis absorption and fluorescent emission measurements, (λ_{max} of excitation 280 nm).

The difference absorption spectrum of purple to blue chromophore has a maximum at 630 nm. Using the decrease in 630 nm absorbance to quantitatively reflect the blue to purple transition, the ratio of absorbance at 550 nm and 630 nm was plotted against the ratio of concentration of anions added to that of the blue membrane, in order to get the titration curves for the absorption changes. The regeneration of the fluorescence emission during the transition was monitored by plotting the ratio of the emission intensity at 330 nm and 345 nm versus the ratio of concentration of added anions to that of the concentration of blue membrane, to get the fluorescence titration cuves.

In order to study the effect of pH and anion concentration upon regeneration of the purple chromophore, the purple membrane suspensions were treated with two different concentrations of sulphuric acid viz. 0.1 M for pH 1.0 and 0.01 M for pH 2.0. The addition of anions and regeneration of purple chromophore was monitored as described above.

Circular Dichroism Measurements. In order to estimate if any significant change in the conformation was occurring during the blue to purple transition, circular dichroism measurements were undertaken at pH 1.0 using 7.12 x 10^{-6} M bacteriorhodopsin (ε-63000 L. M^{-1} cm^{-1}). Chloride (Cl^-) was used to effect the blue → purple transition. The uv-vis CD spectra were recorded in 200 to 700 nm range in order to study changes in the helical content of the protein as well as changes in the chromophoric interactions. The baseline corrections and data analysis were carried out with the help of the computer interfaced with the spectropolarimeter. The distance between the sample holder and PMT detector was kept minimum, to reduce the scattering effect.

RESULTS AND DISCUSSION

The effect of acid on purple membrane and the effect of anions on the blue membrane is summarized in the following equation :

$$\text{Native protein chromophore} \xrightarrow{H^+} \text{Acidified protein chromophore}$$
$$(\text{purple } \lambda_{max}\ 570, 280\ \text{nm}) \xleftarrow[Cl^- \text{ or } HPO_4^{2-}]{} (\text{Blue } \lambda_{max}\ 608, 280\ \text{nm})$$

The absorption and fluorescence characteristics of purple membrane, blue membrane and anion-regenerated purple membrane are given in the Table. The absorption maximum showed a red-shift from 570 nm to 608 nm upon acidification but the 280 nm band, corresponding to the absorption due to aromatic amino acid residues, remained unchanged.Upon addition of anions (Cl^- and HPO_4^{2-}), the absorption spectrum obtained for the purple chromophore is similar to that of the native one, except for a slight broadening of the 570 nm band. This is due to incomplete regeneration of the native chromophore from the acidified form.

The native bR has fluorescent emission band at 323 nm whereas the acidified form has a maximum at 326 nm due to aromatic amino acid residues of the protein. The fluorescence intensity of the blue membrane is approximately 28 time higher than that of the purple membrane. The quantum yields of fluorescence (φ_f) were found to be 0.046 and 0.051 for the purple membrane and the blue membrane

Table1. Uv-vis and fluorescence spectral data for purple (PM), blue (BM) and anion-regenerated purple membrane (APM).

Sample	Absorption λ_{max} nm, (ε, LM^{-1} CM^{-1})	Fluorescence λ_{max}, nm	φ_f
PM	280, 570 (63.000)	323	0.046
BM	280, 608 (61.500)	326	0.051
APM	280, 568-570 (63.000)	323	-

respectively. This small change in φ_f between the two proteins indicates towards insignificant change in the overall protein conformation during blue purple transition, whereby some of the fluorescent protein residues, which may be fully quenched in the native bacteriorhodopsin are either not quenched or only partially quenched after acidification. The absorption maximum of acidified protein (608 nm) is red shifted by 38 nm as compared to the native purple membrane. This red-shift can arise due to altered electro-static interactions between retinal Schiff base moiety and the surrounding apo-protein including some changes in the distance between the retinal Schiff base moiety and its counterion.

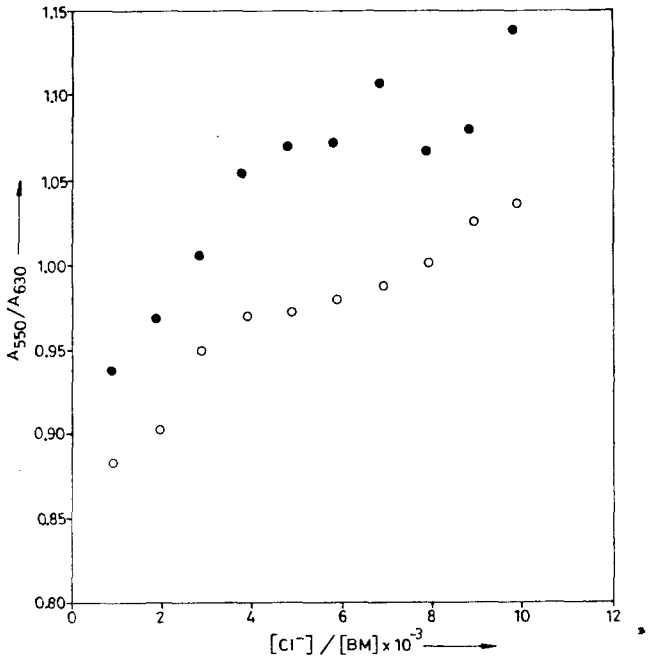

Figure 1. Effect of pH and chloride ion concentration on the regeneration of purple chromophore from blue membrane : Titration curves of absorption changes of blue membrane on addition of chloride ion (●, at pH 1.0, 0.1 M H_2SO_4 ;and, ○, at pH 2.0, 0.01 M H_2SO_4).

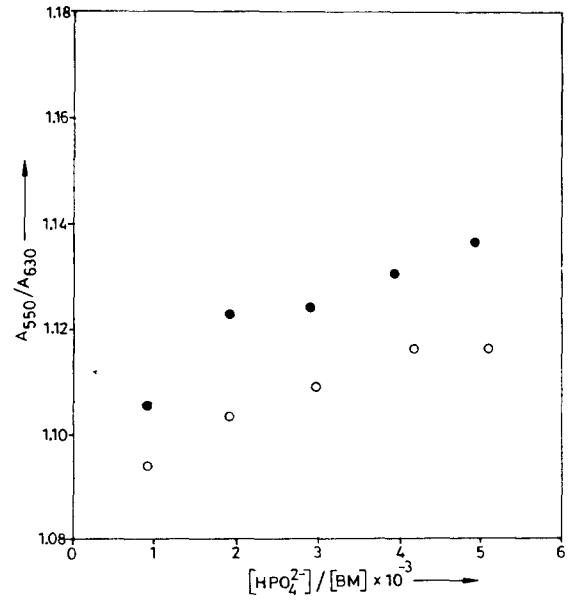

Figure 2. Effect of pH and dibasic phosphae ion concentration on the regeneration of purple chromophore from blue membrane : Titration curves of absorption changes of blue membrane on addition of phosphae ion (●, at pH 1.0, 0.1 M H_2SO_4 ; and, ○ at pH 2.0, 0.01 M H_2SO_4).

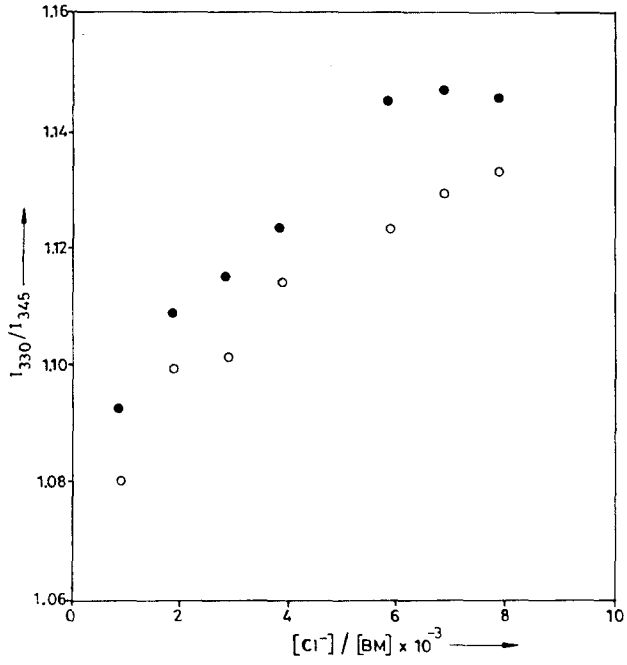

Figure 3. Effect of pH and chloride ion concentration on the regeneration of purple chromophore from blue membrane as monitored by fluorescence ratio changes at 330 and 345 nm in the protein : Titration curves of fluorescence changes of blue membrane on addition of chloride (●, at pH 1.0, 0.1 M H_2SO_4 ; and, ○ at pH 2.0, 0.01 M H_2SO_4).

The addition of anions to the blue membrane restores the absorption band at 570 nm. The titration curves for the absorption changes upon addition of chloride and dibasic phosphate are shown in Fig. 1 and Fig. 2 respectively. It was observed that at the same chloride concentration, the chloride regenerated the purple membrane more efficiently at pH 1.0 than at pH 2.0. Similar results were obtained for dibasic phosphate anions also.

Fig. 3 and 4 show the titration curves for the fluorescence changes upon addition of chloride and phosphate ions to the blue membrane respectively. Upon addition of these anions, the λ_{max} of emission slowly shifts back from 326 nm to 323 nm. There is a decrease in the emission intensity upon anion addition, and the intensity slowly approaches the original values, corresponding to the native protein.

The chloride was found to be more efficient in regenerating the purple chromophore than the dibasic phosphate. Nevertheless, one thing common to the anion regeneration is that both Cl^- and HPO_4^{2-} show a marked pH effect, i.e., at pH 1.0, the efficiency of regeneration of the purple chromophore is more as compared to that at pH 2.0, using the same anion concentration. This may be explained in terms of protonation of the purple membrane at highly acidic conditions. This pH influence on regeneration may be

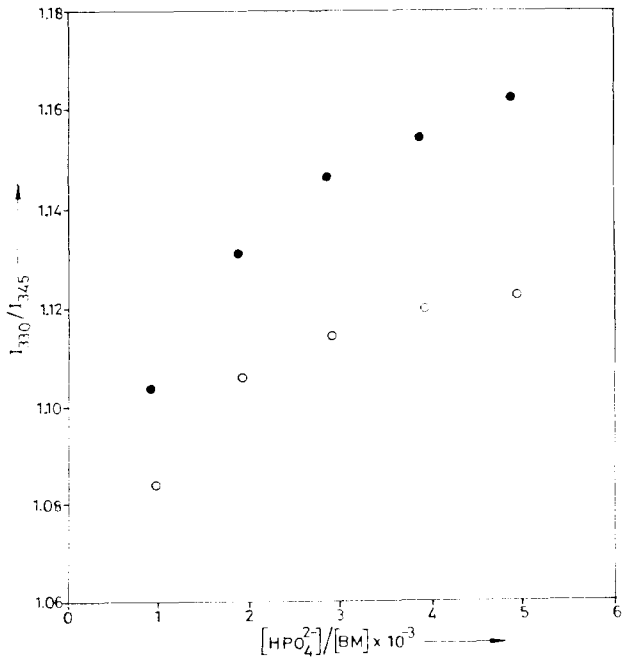

Figure 4. Effect of pH and dibasic phosphate ion concentration on the regeneration of purple chromophore from blue membrane as monitored by fluorescence intensity ratio changes at 330 nm and 345 nm (I_{330}/I_{345}) in the protein(●, at pH 1.0, 0.1 M H_2SO_4 ; and, ○ at pH 2.0, 0.01 M H_2SO_4).

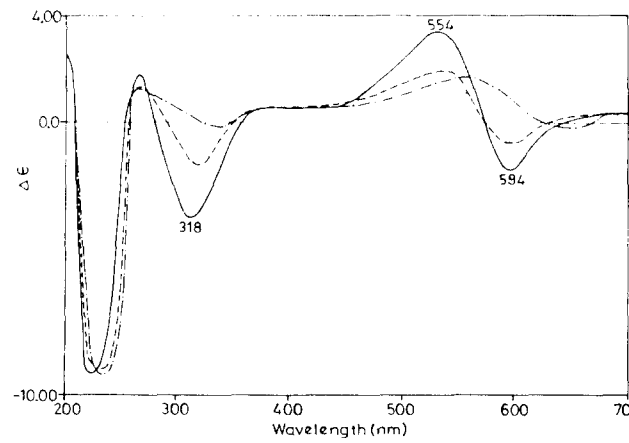

Figure 5. CD spectra of [7.12 x 10^{-6} M] purple membrane (——), blue membrane(– –), and blue membrane plus anion (----).

due to an indirect anion binding by positive charges of the membrane upon progressive protonation, rather than due to a direct acid-induced formation of 608 nm chromophore. The formation of the 608 chromophore appears to be a function of protonation of an intrinsic anion, which may be negatively charged amino acid residue in the vicinity of the retinal Schiff base moiety. This is in conformity with the studies on artificial pigments suggesting the titration of a negative or dipolar group in the vicinity of the retinal Schiff base moiety[16].

The circular dichroism spectrum of the native bacteriorhodopsin (Fig. 5) shows a positive (λ_{max} = 554 nm) and a negative (λ_{max} = 594 nm) peak of unequal amplitude in the visible region. This visible circular dichroism is believed to be resulting from the exciton interactions between the chromophores in the hexagonal lattice of bacteriorhodopsin[9]. The same characteristic bands were seen shifted to longer wavelengths upon acidification, indicating that the interactions still exist at thse pH values. The support for this comes from the fact that the cross-over point from positive to negative band in a visible-CD spectrum is near the wavelength of absorption maximum of the chromophore.

The circular dichroism spectrum in the near-UV region for both bacteriorhodopsin and its acidified form does not change significantly at lower pH values, the change being only about 10% decrease in the α-helical content. This indicates that the structure of the acidified bacteriorhodopsin molecule and its arrangement in the membrane seems to be essentially similar to that of bacteriorhodopsin at neutral pH. The spectral shift is most likely due to slight local rearrangements and changes in the protonation states of some amino acid residues in the vicinity of the

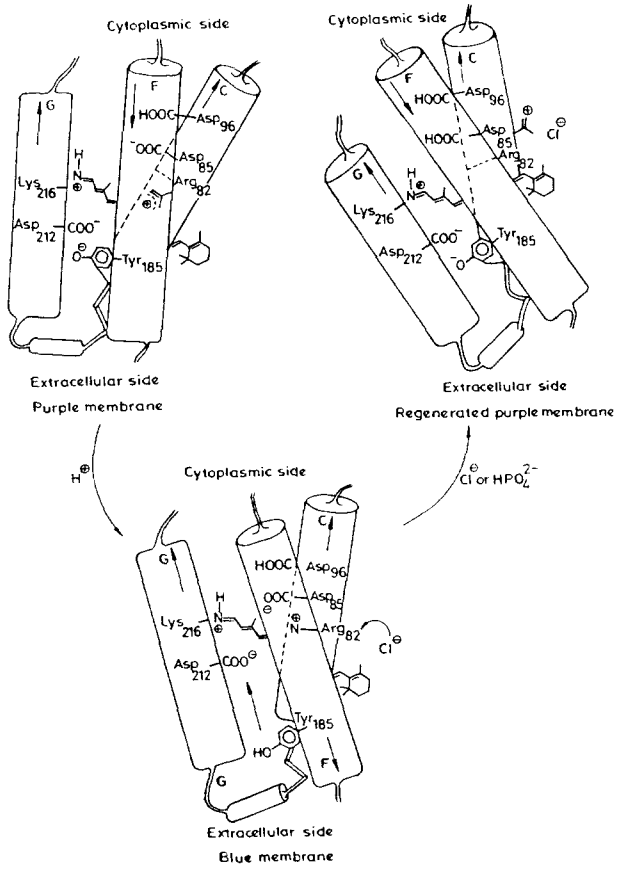

Figure 6. Schematics of the proposed molecular mechanism for blue ⇄ purple blue transition in bacteriorhodopsin. Only a few amino acid residues relevant to the present discussion are shown. C,G and F are the proteins α-helices out a total of seven (A,B,C,D,E,F and G) α-helices in bacteriorhodopsin. Retinal is attached to the ε-amino group of Lys $_{216}$, *via* a protonated Schiff base linkage in the G-helix. Asp_{212} acts as counter anion to the Schiff base at Lys $_{216}$. Tyr_{185} (F-helix) and Arg_{82} (C-helix) are some of the residue which line the retinal pocket. At lower pH (presence of $H^{\oplus}$) Tyr_{185} can be protonated disturbing its electrostatic interaction with Arg_{82}. This local charge destabilises the active site forces, but brings the Arg positive charge closer to the retinylidene stabilizing the polyene Schiff base chromophore. Anions can bind to Arg_{82} inducing conformational changes leading to ionization of nearby phenolic residue and hence regeneration of purple colour (see text for further details).

retinal Schiff base. The 318 nm CD band, which is believed to be due to the retinal-protein interactions changes at lower pH values, indicating a change in the interactions at the binding site. This suggests that the binding site molecular architecture of the acidified bacteriorhodopsin is different from that of the native bacteriorhodopsin. Thus, from the circular dichroism experiments it appears that even though 90% of the native structure is preserved during this transition, there is a definite change in the interactions between the retinal and the apo-protein most probably at the chromophoric site. Upon addition of chloride, all the characteristic bands are shifted back with peaks similar to the native bacteriorhodopsin.

The molecular mechanism of this transition can be discussed in terms of the three dimensional structure of bacteriorhodopsin[2]. A schematic diagram showing molecular phenomenon responsible for this transition is depicted in Fig. 6. It is believed that C, F and G helices are largely responsible for this transition as shown in the figure. At lower pH, Tyr_{185} can undergo protonation disturbing its electrostatic interaction with Arg_{82}. This local change destabilizes the active site forces, but brings the Arg positive charge much closer to the polyene. A greater stabilisation of the Schiff base chromophore (stabilisation of imine N as well as electrostatic stabilisation of the protein near β-ionyl ring) causes the generation of the blue species. Added of anion is most likely to bind with the positive Arg_{82} residue thereby inducing slight conformational change leading to the ionisation of phenolic residue and the regeneration of the purple colour. Thus, the anion binding brings out a change in the local electrostatic environment such that Try_{185} gets deprotonated and in turn protonated Asp_{85}. Studies on site specific mutants have convincingly shown earlier that in the acidified state Asp_{85} is protonated[18]. This leads to yet another conformational change that restores the F helix to the normal position and hence the distance between the Schiff base and its counterion is also restored. This results in the regeneration of the purple complex.

Thus, the colour of the chromophore seems to be a function of protonation states of the amino acid residues at the active site of bacteriorhodopsin and is controlled by the distance between the Schiff base and its counterion.

Acknowledgement — The authors thank DST, New Delhi for supporting research on retinal-proteins and Professor S. Mitra (TIFR, Bombay) for help in the CD measurements.

REFERENCES

1. Stoeckenius, W. and R. A. Bogomolni (1982) Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* **52**, 587-615.
2. Henderson, R., J. M. Baldwin, T. A. Ceska, H. Zemlin, E. Beckman, and K. H. Downing (1990) Model for the structure of bacteriorhodopsin based on high resolution electron cryo-microscopy. *J. Mol. Biol.* **213**, 899-929.
3. Bolag-Nair, V. B., J. D. Carriker, B. Honig, V. Kamat, M. G. Motto, K. Nakanishi, R. Sen, M. Sheeves, M. Arnaboldi and V. Tsujimoto (1981), The 'Opsin shift' in bacteriorhodopsin studies with artificial bacteriorhodopsin. *Photochem. Photobiol.* **33**, 483-488.

4. Moore, T. A., M. E. Edgerton, G. Parr, Z. Greenwood, and R. N. Perham (1978) Studies of an acid induced species of purple membrane from *Halobacterium halobium*. *Biochem. J.* **171**, 469 - 476.
5. Oesterhelt, D. and W. Stoeckenius (1973) Functions of a new photoreceptor membrane. *Proc. Natl Acad. Sci. USA*, **70**, 2853-2857.
6. Dracher, L. A., A. D. Kaulen, V. Shulachev, (1978) Time resolution of the intermediate steps in the bacteriorhodopsin linked electrogenesis. *FEBS lett*, **87**, 161-167.
7. Kimura, Y., A. Ikegami and W. Stoeckenius, (1984) Salt and pH dependent changes of the purple membrane absorption spectrum. *Photochem. Photobiol.*, **40**, 641-646.
8. Fischer, U. and D. Oesterhelt (1979) Chromophore equilibriums in bacteriorhodopsin. *Biophys. J.* **28**, 211-230.
9. Braumer, P. J., M. P. Heyn, N. A. Dencher (1973) Evidence for chromophore intenactions in the purple membrane from reconstitution experimens of the chromophore free membrane. *Biochem. Biophys. Commun.* **67**, 897-903.
10. Becher, B. and J. Y. Cassim (1976) Effects of light adaptation on the purple membrane structure of *Halobacterium halobium*. *Biophys. J.* **16**, 1183-1200.
11. Fischer, O. and D. Oesterhelt (1980) Changes the Protonasin state of bacteriorhodoption during reconstitution of bacteriorhodopsin. *Biophys. J.* **31**, 139 45.
12. Kalisky, O., J. Feitelson and M. Ottolenghi (1981) Photochemistry and fluorescence of bacteriorhodopsin excited in its 280-nm absorption band. *Biochemistry*, **20**, 205-209.
13. Smith, S. O. and R. A. Mathies (1988) Resonance Raman spectra of the acidified and deionised form of bacteriorhodopsin. *Biophys. J.* **47**, 251-254.
14. Massig, G., M. Stockburger and T. Alsherth (1985) Structure of bacteriorhodopsin in the acidified membrane and at high ionic strengh: resonance Raman study. *Can. J. Chem.* **63**, 2012-2017.
15. Mercier, G. and P. Dupius (1988) Effect of deionization on the protein fluorescence of bacteriorhodopsin. *Photochem. Photobiol.*, **47**, 433-438.
16. Albeck, A., N. Friedmann, M. Sheves, and M. Ottolengh (1989) Factors affecting the absorption maxima of acidic form of bacterirhodopsin. *Biophys. J.*, **56**, 1259-1265.
17. 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.
18. Subramaniam, S., Thomas, M. and H. G. Kharona (1990) Protonation state of Asp (Glu)-85 regulates the purple to blue transition in bacteriorhodopsin mutants Arg $_{82}$ → Ala and Asp 85 Glu. The blue form is inactive in translocation. *Proc. Natl. Acad. Sci. U.S.A.*, **87**, 1013-1017
19. Renthal, R. K., R. Shuler and R. Regalado (1990) Control of bacteriorhodopsin cold by chloride at low pH. Significance for he proton pump mechanism. *Biochem. Biophys. Acta.* **1016**, 375 -384.
20. DeGroot, H. J. M., S. O. Smith, J. Courtin, E. V. D. Berg, C. Winkel, J. Luglenburg, P. G. Griffin and J. Herzflex (1990) Solid state ^{13}C and ^{15}N NMR study of the low pH forms of bacteriorhodopsin. *Biochemistry*, **29**, 6873-6883.
21. Der, A., S. Szaraz, R. Toth-Boconadi, Z. Totaji, L. Kesthelyi and W. Stoeckenius (1991) Alternative translocation of protons and halide ions by bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* **88**, 4751-4755.
22. Birge, R. R. (1990) Photophysics and molecular electronic application of the rhodopsins. *Annu. Rev. Phys. Chem.* **41**, 683-733.
23. Brauchle, C., N. Hampp and D. Oesterhelt (1991) Optical applications of bacteriorhodopsin and its mutated variants. *Adv. Mater.* **3**, 420-428.
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.