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Protein–chromophore interactions in bacteriorhodopsin: the effects of a change in surface potential

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The chromophore retinal is bound to bacteriorhodopsin via a protonated Schiff base linkage. The retinal binding site is reported to be buried in the transmembrane portion of the protein, distant from the membrane surfaces. When bound to bacteriorhodopsin, the absorption maximum of retinal is red-shifted from 366 nm to 568 nm producing a purple color. This color persists across a wide pH range. However, when the pH is raised above 12.0, the membranes become pink in color, while at pH values of 3.0 or below, a blue color is produced. The blue color can also be obtained by removing the divalent cations bound to the surface of the protein. In this study, bacteriorhodopsin was examined by circular dichroism and absorption spectroscopy to determine if protein conformational changes were associated with the color shifts. It was found that although the retinal chromophore can be completely removed by bleaching with hydroxylamine with no significant influence on the secondary structure of the protein, a change in the surface charge of bacteriorhodopsin results in measurable conformational change in the protein, which apparently affects the nature of the retinal binding site.

Introduction

Bacteriorhodopsin is the only protein found in the purple membrane of *Halobacterium halobium*. Bacteriorhodopsin functions as a proton pump to provide for ATP synthesis under anaerobic conditions [1]. The protein consists of 248 amino acids and a chromophore, all-*trans* retinal, attached to lysine 216 via a protonated Schiff base linkage [2–6]. The secondary structure of the protein, as determined by electron microscopy and X-ray diffraction, is mainly α-helical, with seven transmembrane segments passing through the lipid bilayer of the membrane [7–10]. Fluorescence energy transfer and neutron diffraction experiments had indicated that the retinal is buried in the membrane-spanning portion of bacteriorhodopsin isolated from both the cytoplasmic and extracellular surfaces [11–17], and this has been confirmed recently when the three-dimensional structure of the molecule was determined by electron microscopy image reconstruction [10].

Although bacteriorhodopsin is functional as a monomer, in the purple membrane the protein molecules are packed as trimers on a two-dimensional hexagonal lattice [8,18,41]. The close packing and restricted mobility of the protein molecules within the membrane provide the necessary conditions for exciton coupling effects between the retinal chromophores of neighboring bacteriorhodopsin molecules [19,20,42].

The retinal chromophore is central to the proton pumping activity of the protein. Light-induced isomerization of the retinal from an all-*trans* to a 13-*cis* configuration drives the translocation of protons across the bacterial cell membrane. The photocycle events occur without cleavage of the Schiff base bond between retinal and the apoprotein. Several photocycle intermediates have been identified, each having a characteristic absorption maximum [21,22]. Protein structural changes may also occur during the photocycle [23]. The potential of the area of the protein surrounding the chromophore is also altered during the photocycle as evidenced by the protonation state changes of nearby tyrosine and aspartic acid residues [24–31].

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Shifts in the absorption maximum of bacteriorhodopsin can likewise be seen when the pH of the external medium is altered. In alkaline pH, the membrane becomes pink in color and absorbs light at 563 nm [32]. In an acidic medium, the membrane becomes blue in color and absorbs light at 605 nm [56]. The same blue color change can be produced by removing divalent cations which are bound to the surface of bacteriorhodopsin [33]. Bacteriorhodopsin has a large negative surface potential and binds up to ten divalent cations per protein molecule [34,37]. The two blue forms, acid blue and deionized blue, are not fully functional. They are capable of forming the first two photocycle intermediates, but the 13-*cis* transition state is not seen [38]. The pink form of the membrane also does not undergo the complete photocycle. The 13-*cis* transition state is formed, but reprotonation and proton pumping do not occur [40]. Although bacteriorhodopsin is nonfunctional in the blue and pink forms, it is not irreversibly denatured. By neutralizing the pH of the external medium or by the re-addition of divalent cations in the case of the deionized blue protein, the purple color of the protein can be fully restored [34–40].

The purple-to-blue transition is due to protonation of exposed side chains in an acidic medium [35–40]. The presence of cations modulates the pK_a of the transition. Deionized membrane becomes blue at pH 5.4, while native membranes become blue at pH 3.0 [35–40,43]. In alkaline media, the protonation states of surface residues would also be altered, leading to the purple-to-pink transition. These results indicate that the retinal, which is buried deep in the membrane and does not come into contact with either surface, is affected by changes in the external medium. One possible mechanism for such an effect, is a pH-induced conformational change in the protein which produces an alteration in the retinal binding pocket. In this study, circular dichroism was used to determine if such a change was occurring, and if the conformational change involved the secondary structure of the protein.

The retinal chromophore can be removed from the protein without altering the secondary structure of the protein. The tertiary and quaternary structures, however, may be affected [44]. By comparing these conformational changes to those that may accompany the purple-to-blue and purple-to-pink color changes, insight can be gained into the nature of the retinal binding site and how the conformation of the protein can govern chromophore spectral characteristics and regulate the functioning of the photocycle.

Material and Methods

Materials

Halobacterium halobium was grown and purple membranes were isolated according to the procedure of Oesterhelt and Stoeckenius [45].

The relative protein concentrations of all samples were determined by Lowry assay in the presence of 0.1% SDS [46]. The Lowry assay was calibrated on an absolute scale by comparison with the molar absorbance at 568 nm of native purple membrane.

pH effects

A series of 0.02 M sodium phosphate buffers were made to span the pH range between 2.0 and 12.5. Light-adapted purple membranes were pelleted at $70000 \times g$ for 20 min and resuspended in the buffers and incubated for at least 30 min before spectral analysis. Reversibility of the pH-induced changes was examined by regenerating purple membranes from blue and pink membranes. 1 mg of protein was suspended in 1 ml of pH 2.4 medium and incubated for 30 min. The sample was washed at $70000 \times g$ for 20 min and resuspended in 1 ml of pH 7.0 buffer. pH 12.1 pink membranes (1 mg/ml) were also prepared and incubated for 30 min. After centrifuging, the membranes were resuspended in 1 ml of pH 7.0 buffer. The samples were incubated in the pH 7.0 buffer overnight before spectral data were collected.

Deionized samples

Light-adapted purple membranes were chromatographed on a Dowex-50 cation exchange column (100–200 mesh). They were then pelleted and resuspended in double-distilled water. Reversibility of the deionization procedure was examined by titrating 1 mg of deionized blue protein with a saturated solution of calcium chloride. After an incubation of 20 minutes, the membranes were washed at $70000 \times g$ and resuspended in pH 7.0 buffer at a concentration of 1 mg/ml. The sample was incubated overnight before spectral analysis.

Bleached membranes

Isolated purple membranes were suspended at a concentration of 0.1 mg/ml in a 4.0 M solution of hydroxylamine (pH 7.0). The suspension was maintained at 10 °C and bleached with yellow light (> 500 nm). When the 568 nm absorption band of the purple complex had been reduced to 0.1% of the original value, bleaching was considered to be complete. Hydroxylamine was removed from the sample by centrifugation at $70000 \times g$ for 20 min and resuspension in doubly distilled water. Washing was repeated three times. The protein was then resuspended in doubly distilled water at a concentration of 1 mg/ml. Five independent samples of apomembrane were prepared from native membrane. Five separate controls were also run in which purple membranes were exposed to hydroxylamine but not to light, to examine any effects

of hydroxylamine on the protein in the absence of bleaching.

Half of the apomembrane sample was removed and regenerated by titration with a 1.0 mM solution of all-*trans* retinal in ethanol. Full regeneration was evidenced by the reappearance of at least 98% of the original 568 nm absorption band after an incubation period of 1 h. The retinal was in molar excess by a factor of ten. Excess retinal was removed by repeatedly washing the regenerated sample with doubly distilled water, centrifuging at $70000 \times g$ for 20 min.

Spectroscopy

Absorption spectra were recorded on a Cary 2200 spectrophotometer over the wavelength range from 700 to 190 nm with a scan rate of 10 nm/s. The spectra were stored digitally for difference calculations. The second derivatives of the spectra were overlaid on the original spectra for peak maxima identification. For absorption spectroscopy, a protein concentration of 1.0 mg/ml was used for all samples.

Circular dichroism spectra were recorded on an Aviv 60DS spectrophotometer in the range from 300 to 190 nm at 0.2 nm intervals. The spectra were obtained with the variable position photomultiplier tube placed directly adjacent to the sample cell resulting in an average acceptance angle of 90°. The instrument was calibrated with d-10-camphorsulfonic acid. The calibration was checked for optical rotation using sperm whale myoglobin and for wavelength using benzene vapor. A protein concentration of 1 mg/ml and a 0.002 cm pathlength quartz cell were used for native, apo, regenerated and control membrane samples. These parameters were also used for the alkaline pink membrane samples. The low pH blue membrane samples were suspended at 0.5 mg/ml and spectra were recorded with a 0.01 cm pathlength cell. The acidic blue membranes had a strong tendency to aggregate and the protein concentration was kept low in order to prevent the protein from precipitating. However, the low and high pH buffers absorb strongly and the protein concentrations in these buffers need to be fairly high to obtain a spectrum in the shorter pathlength cells. Several different protein concentrations, from 0.25 to 1 mg/ml, were analyzed at low pH to allow maximization of the protein signal while minimizing and establishing the extent of aggregation. The alkaline pink membranes could be prepared at a higher protein concentration since aggregation does not occur at high pH. The pH 7.0 membrane and the deionized blue membrane samples contained 0.25 mg protein/ml in 0.02 cm pathlength cells.

At least twenty-five circular dichroism scans were collected for each sample. The data points were analyzed at 0.2 nm intervals between 190 and 240 nm using a normalized, unconstrained fitting procedure [47]. The reference data set of Chang et al. [48] was used for the analysis. The average helix length used in the analyses was 26 residues. Statistical analyses were also performed on the collected data. The deviations between averaged data sets from a minimum of five different preparations were calculated to estimate the variation between samples. For each sample type, the standard deviation was computed at all measured wavelengths to determine whether significant differences existed between the various spectra.

In analyses of the secondary structure contents of membrane proteins, two optical artifacts must be considered: differential scattering and differential absorption flattening [60]. The apparent differential light scattering depends on the size of the particles and can be decreased by increasing the acceptance angle of the photodetector to include the scattered light. The effects of absorption flattening, however, cannot be eliminated mechanically. These are a consequence of the non-random distribution of chromophores in the membrane sample and are a function of the relative protein concentration within each membrane patch and the size of the patches. As these parameters are constant in all of the purple membrane samples used in this study, differential scattering and absorption flattening will not vary between samples and thus will not be the source of any differences seen between the spectra at the different pH values. However, it will mean that the secondary structures calculated for all of the membrane samples will be distorted from their correct values. If absorption flattening is present, the magnitude of the low wavelength peaks will be depressed relative to the higher wavelength peaks and consequently tend to produce a calculated structure with an increased beta and random coil content at the expense of α-helix. Therefore, the secondary structures reported in Table I are meant to be illustrative of the trends in secondary structural changes, rather than as absolute measures of the secondary structures present.

Visible region (700 to 400 nm) circular dichroism spectra were obtained at 1 nm intervals using a 1 cm pathlength quartz cell and a protein concentration of 1 mg/ml. The scans were smoothed and averaged and the standard deviations for individual samples were calculated.

Results

pH-dependent changes

Absorption spectra (Fig. 1) of native purple membranes at pH 7.0, and of blue and pink membranes, show the shifts in the absorption maximum corresponding to the color changes. The acidic and deionized blue membrane samples have an absorption maximum at 605 nm while the alkaline pink membrane absorbs light maximally at 563 nm. Native purple membranes have

TABLE I

Secondary structure calculations for purple membrane samples at various pH values

Note that the absolute values of each secondary structural type may not be accurate because of the optical effects discussed in the text, but the trends of the changes are illustrative.

Sample	λ_{max} (nm)	Percent secondary structure			
		α-helix	β-sheet	β-turn	coil
pH 2.4	604	37	0	34	29
pH 2.6	597	39	0	33	28
pH 3.0	592	45	0	30	25
pH 7.0	568	72	0	29	0
pH 11.8	563	74	0	26	0
pH 12.0	563	75	0	24	1
pH 12.1	563	77	0	21	2
Deionized	605	71	0	29	0
12.1 → 7.0	568	69	0	31	0
2.4 → 7.0	568	67	0	33	0
Deionized $+Ca^{2+}$	568	70	0	30	0

an absorption maximum centered at 568 nm. The absorption maximum of the retinal-protein complex is dependent on the pH of the external medium and varies throughout the pH range examined (Table I).

Changes associated with the protein color were examined in the far-ultraviolet circular dichroism spectra. This region (250–190 nm) of the spectra provides a view of the n to π^* and π to π^* transitions of the amide bonds of the protein. Because the arrangement of the peptide bonds' transition dipole moments with respect to each other can lead to coupled transitions if the dipoles are close enough to each other and in an ordered array, the magnitude of the interactions will differ depending on the secondary structural conformation of the protein [49]. Thus, the secondary structure of the protein can be monitored in this region.

Above pH 11.0, the peaks near 195 nm and 210 nm are decreased in magnitude and shifted to lower wavelengths relative to those at neutral pH (Fig. 2). Three different pink membrane samples were prepared at high pH: pH 11.8, 12.0 and 12.1. The spectra of these samples were nearly identical, with any variation being within the error limits of approx. 2%. However, the differences between the high pH pink samples and the pH 7.0 purple sample are significantly greater than the error levels of the measurements, and correspond to a small increase in helical content (Table I).

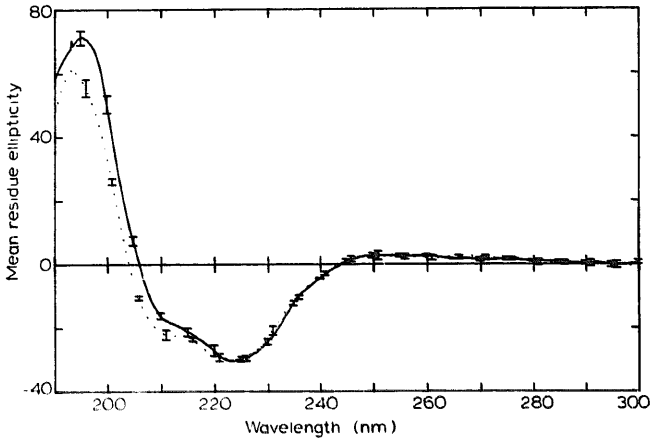

Fig. 2. Circular dichroism spectra of purple membrane samples at pH 7.0 (———) and pH 12.1 (····). The error bars indicate the reproducibility of the data.

At pH 3.0 where blue membranes are produced, the spectrum of bacteriorhodopsin also undergoes significant change relative to purple membrane at pH 7.0 (Fig. 3). There is a large decrease in ellipticity at 195 nm and a smaller decrease at 215 nm relative to the pH 7.0 sample which corresponds to a change in secondary structure. Two other low pH values were also examined: pH 2.4 and pH 2.6. The pH 2.4 and pH 2.6 spectra are nearly identical to each other, with any variations being within the error levels, but considerably different than the pH 3.0 spectra (Fig. 3). The

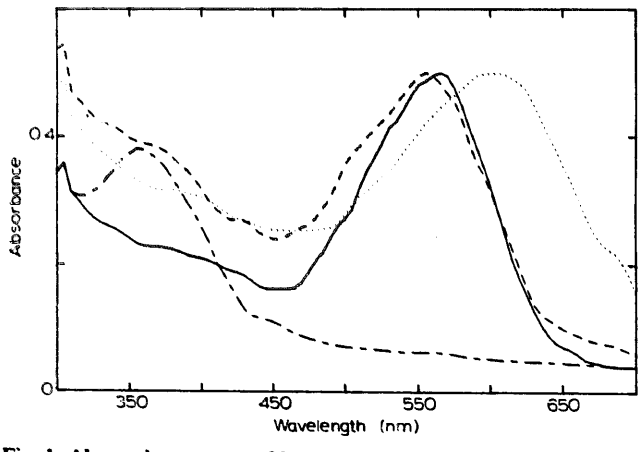

Fig. 1. Absorption spectra of bacteriorhodopsin at pH 7.0 (———), pH 2.4 (····), pH 12.0 (– –) and apo (– —) membrane samples.

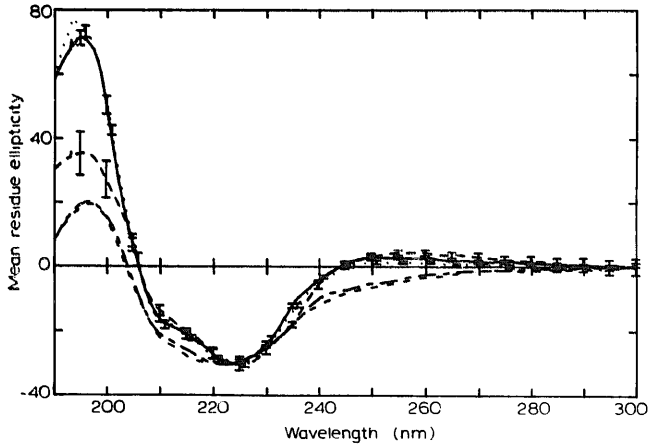

Fig. 3. Circular dichroism spectra of pH 7.0 (———) and deionized membranes (····), pH 3.0 (– – –), pH 2.6 (– —) and pH 2.4 (----) membranes. The error bars indicate the reproducibility of the data.

lowest pH samples exhibit a larger decrease in ellipticity at 195 nm and at 215 nm relative to the pH 7.0 sample. Some of the spectral differences at these pH values are undoubtedly due to the substantial aggregation of the membrane sheets, which occurs below pH 3.0 because of the loss of the negative surface potential which normally causes the sheets to repel each other. Such aggregation would lead to absorption flattening effects [60], which would tend to suppress the magnitude of the peaks and could account for the differences between the pH 3.0 and 2.4 spectra. These in turn, could produce lower apparent calculated helix contents with concomitantly increased random coil contents. However, the very low pH spectra should not be seen as reliable indicators of either secondary structure or conformational change (Table I).

In contrast to the changes seen at pH 3.0, the changes seen in the deionized blue membrane relative to purple membrane at pH 7.0 are small. There is a slight increase in ellipticity at 195 nm which leads to a small difference in calculated secondary structure (Fig. 3, Table I). Hence, deionized blue membranes are more similar to purple membranes than they are to low pH blue membranes.

The reversibility of the structural changes seen at low and high pH and due to cation removal was also examined. A blue membrane sample prepared at pH 2.4 and pink membranes at pH 12.1 were titrated to pH 7.0 and cations were restored to a sample of deionized blue membranes. All three samples regained the characteristic purple color of the native purple membranes at pH 7.0. The circular dichroism spectra of the restored samples were compared to that of a pH 7.0 purple membrane sample which had been prepared and maintained at pH 7.0. Although the spectra of the restored deionized and the restored alkaline membranes were nearly identical to that of the native pH 7.0 spectrum, the spectrum of the restored acidic membranes still retained some of the differences previously noted (Fig. 4). The peak maximum of the restored acidic membranes is shifted to 197 nm relative to the maximum centered at 195 nm seen for native purple membranes and the other restored samples. Also, there is still a marked decrease in the ellipticity of the restored acidic spectrum at 197 nm relative to the native spectrum although it is not as large as that seen in the spectrum of the low pH samples, again suggesting some of the changes at the very low pH values may be the result of irreversible aggregation.

The visible region circular dichroism spectra of the native purple, deionized blue and alkaline pink membranes were compared (Fig. 5). The spectrum of the low pH blue membranes could not be examined because of rapid aggregation of the membranes at the high protein concentrations necessary for these measurements. There are significant shifts in both the

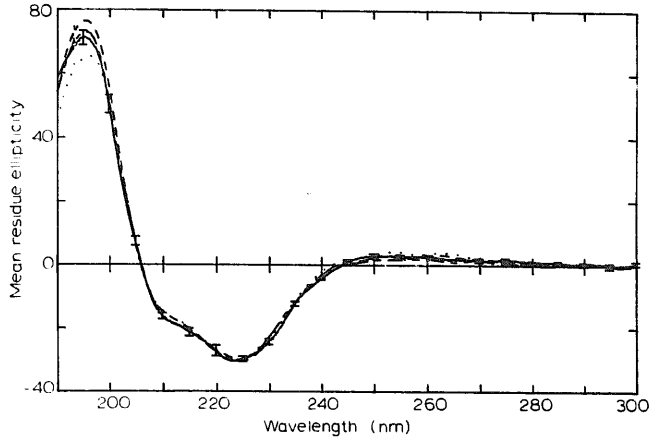

Fig. 4. Circular dichroism spectra of pH 7.0 purple membranes (———), pH 2.4 (····) and pH 12.1 (– – –) membranes shifted to neutral pH and deionized blue membranes (– —) with Ca^{2+} cations. The error bars indicate the reproducibility of the data.

negative and positive peak maxima in the blue and pink membranes relative to the native purple membranes. In the pink membranes, the positive peak was centered at 550 nm and the negative peak was centered at 620 nm. In the blue membrane sample, the positive peak was located at 570 nm and the negative peak at 660 nm. The positive peak in the native purple membrane was centered at 540 nm and the negative peak at 610 nm. The intensities of the negative peaks in the blue and pink membranes were also significantly reduced relative to the intensity of the negative peak in the purple membrane spectrum.

Chromophore-dependent changes

The results obtained when the surface potential of the purple membrane was varied can be compared to those seen when the chromophore is entirely removed. To examine the effects of chromophore removal, several membrane samples were bleached. The apomem-

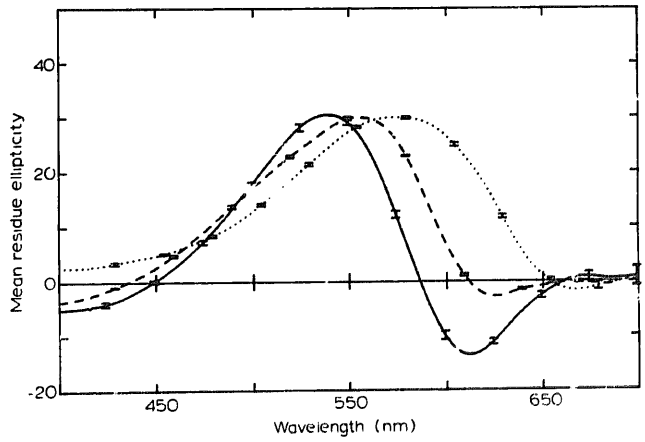

Fig. 5. Visible region of the circular dichroism spectra of native purple membranes (———), deionized blue membranes (····) and pH 12.1 pink membranes (– – –). The error bars indicate the reproducibility of the data.

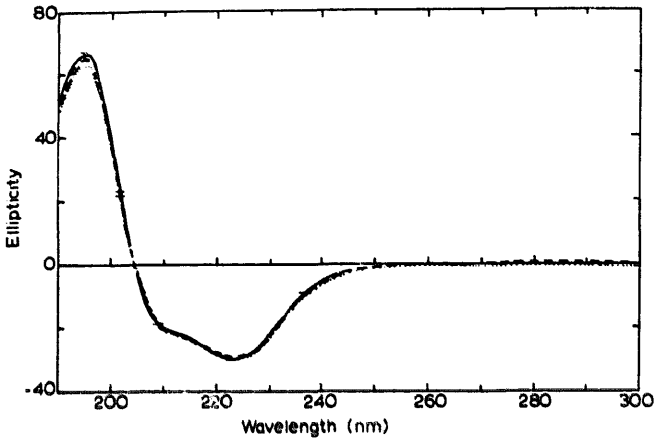

Fig. 6. Circular dichroism spectra of native (———), apo (· · · ·), control (- - -) and regenerated (— —) membrane samples. The error bars indicate the reproducibility of the data.

branes were determined to be > 99% bleached by measuring the decrease in the 568 nm absorption band (Fig. 1). The apomembranes could be regenerated to at least 98% of their original state by the addition of all-*trans* retinal. The control sample, which was exposed to hydroxylamine but not to light, showed no bleaching: no decrease was seen in the 568 nm absorption band. The circular dichroism spectrum (Fig. 6) of bacteriorhodopsin is unchanged, within the error limits (approx. 1%) of the measurements, upon bleaching. Unless there are secondary structural changes which are exactly compensated for by other changes leading to no observable net change in secondary structure, it is likely that the secondary structure is not involved in the changes seen during the bleaching phenomenon. The circular dichroism spectra of the control and regenerated samples (Fig. 6) are also unchanged from the spectrum of native bacteriorhodopsin. These experiments then establish the level of reproducibility of the measurements and set a standard for interpretation of the magnitude of pH-induced changes.

Discussion

Circular dichroism spectroscopy provides a sensitive indication of changes in secondary structures of protein molecules, particularly in largely α-helical proteins such as bacteriorhodopsin.

In this study, we have shown that although the retinal chromophore can be removed without changing the secondary structure of bacteriorhodopsin, upon varying the external pH, the conformation of the protein, as measured by circular dichroism spectroscopy, is significantly altered.

Although no changes in secondary structure are seen during the bleaching process, quaternary structural changes may occur as evidenced by the loss of crystallinity seen in the diffraction patterns [14,51]. Furthermore, previous absorption and circular dichroism studies [44] suggested such changes might also involve the tertiary structure, and studies on oriented purple membrane films [52] have indicated that the differences between native and apomembranes might be due to the tilting of helix axes.

The bleaching experiments not only serve as a control for the absence of change in the spectra, but also demonstrate the error levels for detection of the measurements. They suggest that any changes involving more than 19 amino acids could be detected by this method. In the pH experiments, secondary structural differences are detected which are considerably larger than the error levels. Because of the associated change in the retinal absorption spectra, this implies that the secondary structural changes produce some alteration in the retinal binding site, located in the transmembrane portion of the protein [10–17].

The conformational effects observed in this study using both a wide range of pH values and deionization can be compared with other observations on color changes in bacteriorhodopsin. The X-ray diffraction pattern of deionized blue membrane shows a different lattice than the native purple membrane hexagonal lattice [53], indicating quaternary and possibly tertiary structural changes may occur when the divalent cations are removed from native bacteriorhodopsin. Raman spectroscopic studies [54] also indicate that secondary structural changes occur at pH 3.0 and in deionized blue samples, although those studies suggest that the conformational changes which take place at low pH are smaller than those seen in the present circular dichroism studies of pH 3.0 membranes. Muccio and Cassim [59], using absorption, and linear and circular dichroism state that they detected tertiary structural changes at low pH values (down to pH 2.4), but no secondary structural changes. As no far UV CD spectra were shown in that work, it may be that the difference between that and this study was the sensitivity of the measurements. Those authors did, however, detect both secondary and tertiary structural changes at high pH values (> 11.8), similar to the results seen here. Kimura et al. [33] reported detecting similar changes in the near UV spectra of low pH and deionized blue membranes relative to those at neutral pH, which they attribute to changes in the interactions between the retinal and tryptophan residues. At the level of sensitivity and resolution of their spectra, however, they could detect little difference in the far UV region, in contrast to this study, and conclude that any conformational differences between native and either of the types of blue membranes is small. Studies of low pH blue membranes incorporated into 7.5% polyacrylamide gels to prevent aggregation, have shown that the visible circular dichroism spectrum is significantly different than that of the native purple membrane [55],

and far ultra-violet circular dichroism studies [56] have suggested that small local rearrangements in protein structure may occur, which may result in reduced stability of the protein. Finally, flash photolysis experiments [57] have also indicated that conformational changes may take place at low pH.

The effects produced by the alteration of the surface charge, either by suspending the protein in alkaline medium or removing the divalent cations, are entirely reversible as evidenced by the regeneration of a native type circular dichroism spectrum from the restored samples. Edgerton et al. [57] have shown that when exposed only to pH values as low as 3.2, the native spectrum is fully recoverable. In this study, we found there is a residual difference in the spectrum of the sample that was exposed to very low pH (2.4) after returning it to pH 7.0; this is attributable to some irreversible aggregation at pH 2.4.

Bacteriorhodopsin exists in two forms, light- and dark-adapted. In the absence of light, the retinal chromophores are found in both the 13-*cis* and all-*trans* configurations. The dark-adapted form has an absorption maximum at 560 nm [58]. When the protein is exposed to light, or light-adapted, all of the retinals are present as the all-*trans* isomer and proton pumping occurs. Although the absorption maxima of the alkaline pink form and the dark-adapted protein are similar, dark-adapted membranes exhibit significant exciton splitting in the visible circular dichroism spectrum [58], while the alkaline pink membrane does not as evidenced by the reduction in the intensity of the negative peak. The changes seen in alkaline media are therefore not due to dark-adaptation and represent another phenomenon.

The conformational changes in the buried section of the protein around the retinal must be the consequence of changes which take place far away, at the surface of the membrane and at the periphery of the protein molecule where protein–protein interactions occur. Changes in the surface potential due to high or low pH, or removal of the bound divalent cations result in changes in the helical portion of the protein, which might result in changes in the retinal binding site. Thus, changes in the surface potential result in long range effects and alter protein–chromophore interactions.

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References

1 Oesterhelt, D. and Stoeckenius, W. (1971) Nature New Biol. 233, 149–152.
2 Khorana, H.G., Gerber, G.E., Herlihy, W.C., Gray, C.P., Anderegg, R.J., Nihei, K. and Biemann, K. (1979) Proc. Natl. Acad. Sci. USA 76, 5046–5050.
3 Lemke, H.D. and Oesterhelt, D. (1981) FEBS Lett. 128, 255–260.
4 Mullen, E., Johnson, A.H. and Akhtar, M. (1981) FEBS Lett. 130, 187–193.
5 Bayley, H., Huang, K.-S., Radhakrishnan, R., Ross, A.H., Takagaki, Y. and Khorana, H.G. (1981) Proc. Natl. Acad. Sci. USA 78, 2225–2229.
6 Katre, N.V., Wolber, P.K., Stoeckenius, W. and Stroud, R.M. (1981) Proc. Natl. Acad. Sci. USA 78, 4068–4072.
7 Henderson, R. (1975) J. Mol. Biol. 93, 123–138.
8 Unwin, P.N.T. and Henderson, R. (1975) J. Mol. Biol. 94, 425–440.
9 Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28–31.
10 Henderson, R., Baldwin, J.M., Ceska, T.A., Zemlin, F., Beckmann, E. and Downing, K.H. (1990) J. Mol. Biol. 213, 899–929.
11 Otomo, J., Tomoika, A., Kinosita, K. Jr., Miyata, H., Takenaka, Y., Kouyama, T. and Ikegami, A. (1988) Biophys. J. 54, 57–64.
12 Kometani, T., Kinosita, K. Jr., Furono, T., Kouyama, T. and Ikegami, A. (1987) Biophys. J. 52, 509–517.
13 Kouyama, T., Kimura, Y., Kinosita, K. Jr. and Ikegami, A. (1981) J. Mol. Biol. 153, 337–359.
14 Jubb, J.S., Worcester, D.L., Crespi, H.L. and Zaccai, G. (1984) EMBO J. 3, 1455–1461.
15 Seiff, F., Wallat, I., Ermann, P. and Heyn, M.P. (1985) Proc. Natl. Acad. Sci. USA 82, 3227–3231.
16 Heyn, M.P., Westerhausen, J., Wallat, I. and Seiff, F. (1988) Proc. Natl. Acad. Sci. USA 85, 2146–2150.
17 Seiff, F., Westerhausen, J., Wallat, I. and Heyn, M.P. (1986) Proc. Natl. Acad. Sci. USA 83, 7746–7750.
18 Blaurock, A.E. and Stoeckenius, W. (1971) Nature New Biol. 233, 152–155.
19 Heyn, M.P., Bauer, P.J. and Dencher, N.A. (1975) Biochem. Biophys. Res. Commun. 67, 897–903.
20 Becher, B. and Ebrey, T.G. (1976) Biochem. Biophys. Res. Commun. 69, 1–6.
21 Lozier, R.H., Bogomolni, R.A. and Stoeckenius, W. (1975) Biophys. J. 15, 955–962.
22 Lozier, R.H. and Niederberger, W. (1977) Fed. Proc. Fed. Am. Soc. Exp. Biol. 36, 1805–1809.
23 Draheim, J.E. and Cassim, J.Y. (1985) Biophys. J. 47, 497–507.
24 Scherrer, P. and Stoeckenius, W. (1985) Biochemistry 24, 7733–7740.
25 Braiman, M.S., Mogi, T., Marti, T., Stern, L.J., Khorana, H.G. and Rothschild, K.J. (1988) Biochemistry 27, 8516–8520.
26 Marinetti, T., Subramaniam, S., Mogi, T., Marti, T. and Khorana, H.G. (1989) Proc. Natl. Acad. Sci. USA 86, 529–533.
27 Braiman, M.S., Mogi, T., Stern, L.J., Hackett, N.R., Chao, B.H., Khorana, H.G. and Rothschild, K.J. (1988) Proteins 3, 219–229.
28 Holz, M., Drachev, L.A., Mogi, T., Otto, H., Kaulen, A.D., Heyn, M.P., Skulachev, V.P. and Khorana, H.G. (1989) Proc. Natl. Acad. Sci. USA 86, 2167–2171.
29 Butt, H.J., Fendler, K., Bamberg, E., Tittor, J. and Oesterhelt, D. (1989) EMBO Journal 6, 1657–1663.
30 Renthal, R., Cothran, M., Espinoza, B., Wall, K.A. and Bernard, M. (1985) Biochemistry 24, 4275–4279.
31 Engelhard, M., Gerwert, K., Hess, B., Kreutz and W., Siebert, F. (1985) Biochemistry 24, 400–407.
32 Fischer, U. and Oesterhelt, D. (1979) Biophys. J. 28, 211–230.
33 Kimura, Y., Ikegami, A. and Stoeckenius, W. (1984) Photochem. Photobiol. 40, 641–646.

34 Chang, C.-H., Chen, J.-G., Govindjee, R. and Ebrey, T. (1985) Proc. Natl. Acad. Sci. USA 82, 396–400.
35 Dunach, M., Seigneuret, M., Rigaud, J.-L. and Padros, E. (1988) J. Biol. Chem. 263, 7555–7559.
36 Dunach, M., Seigneuret, M., Rigaud, J.-L. and Padros, E. (1987) Biochemistry 26, 1179–1186.
37 Chang, C.-H., Jonas, R., Melchiore, S., Govindjee, R. and Ebrey, T. (1986) Biophys. J. 49, 731–739.
38 Akiri, M. and Lanyi, J.K. (1986) J. Biol. Chem. 261, 8167–8174.
39 Chronister, E.L., Corcoran, T.C., Song, L. and El-Sayed, M.A. (1986) Proc. Natl. Acad. Sci. USA 83, 8580–8584.
40 Schreckenbach, T., Walckhoff, B. and Oesterhelt, D. (1977) Eur. J. Biochem. 76, 499–511.
41 Dencher, N.A. and Heyn, M.P. (1979) FEBS Lett. 108, 307–310.
42 Ebrey, T.G., Becher, B., Mao, B., Kilbride, P. and Honig, B. (1977) J. Mol. Biol. 112, 377–397.
43 Szundi, I. and Stoeckenius, W. (1989) Biophys. J. 56, 369–383.
44 Becher, B. and Cassim, J.Y. (1977) Biophys. J. 19, 285–297.
45 Oesterhelt, D. and Stoeckenius, W. (1974) Methods Enzymol. 31, 667–678.
46 Lowry, O.H., Rosebrough, N.J., Farr, L.A. and Randall, R.J. (1951) J. Biol. Chem. 193, 265–275.
47 Mao, D., Wachter, E. and Wallace, B.A. (1982) Biochemistry 21, 4960–4968.
48 Chang, C.T., Wu, C.-S.C. and Yang, J.T. (1978) Anal. Biochem. 91, 13–31.
49 Bell, J.E. (1981) in Spectroscopy in Biochemistry, Vol. 1, pp. 69–70, CRC Press, Boca Raton.
50 Swords, N.A. and Wallace, B.A. (1988) American Chemical Society, Northeast Regional Meeting, Abstracts (18), 48.
51 Swords, N.A. and Wallace, B.A. (1989) Biophys. J. 55, 386a.
52 Muccio, D.D. and Cassim, J.Y. (1979) Biophys. J. 26, 427–440.
53 Heyn, M.P., Dudda, C., Otto, H., Seiff, F. and Wallat, I. (1989) Biochemistry 28, 9166–9172.
54 Smith, S.O. and Mathies, R.A. (1985) Biophys. J. 47, 251–254.
55 Mowery, P.C., Lozier, R.H., Chae, Q., Tseng, Y.-W., Taylor, M. and Stoeckenius, W. (1979) Biochemistry 18, 4100–4107.
56 Tsuji, K. and Rosenheck, K. (1979) Febs Lett. 98, 368–372.
57 Edgerton, M.E., Moore, T.A. and Greenwood, C. (1980) Biochem. J. 189, 413–420.
58 Becher, B. and Cassim, J.Y. (1976) Biophys. J. 16, 1183–1199.
59 Muccio, D.D. and Cassim, J.Y. (1979) J. Mol. Biol. 135, 595–609.
60 Wallace, B.A. and Teeters, C.L. (1987) Biochemistry 241, 663–669.