RESONANCE RAMAN SPECTRA OF THE ACIDIFIED AND DEIONIZED FORMS OF BACTERIORHODOPSIN

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ABSTRACT The 568-nm absorption band of light-adapted bacteriorhodopsin (BR) shifts to 605 nm at pH 2, forming BR^{A}_{605}, and it shifts back to 565 nm at pH 0, forming BR^{A}_{565}. We have obtained resonance Raman spectra of BR^{A}_{605} and BR^{A}_{565} using purple membrane samples that have been suspended in a rotating Raman cell with a polyacrylamide gel. Raman spectra were also obtained of purple membrane in deionized solutions (BR^{D}_{605}). The spectra of BR^{A}_{605} and BR^{D}_{605} are very similar, and they correspond closely with the Raman spectrum of dark-adapted BR, which contains an approximately equal mixture of 13-*cis* and all-*trans* retinal protonated Schiff-base chromophores. This shows that BR^{A}_{605} and BR^{D}_{605} are not homogeneous molecular species but contain a mixture of pigment molecules with both 13-*cis* and all-*trans* retinal isomers. The Raman spectrum of BR^{A}_{565} is nearly identical to that of light-adapted BR, demonstrating that BR^{A}_{565} contains an all-*trans* protonated Schiff-base chromophore. These data provide constraints on the possible structural changes that can be invoked to explain the spectral shifts induced in the acid and deionized species.

Bacteriorhodopsin (BR), a retinal-protein complex in the purple membrane of *Halobacterium halobium*, functions as a light-driven proton pump (1, 2). Light absorption by the covalently attached retinal chromophore results in a cyclic photochemical reaction that is coupled to the translocation of protons across the bacterial cell membrane (3). The chromophore in light-adapted BR_{568} is an all-*trans* protonated Schiff base, while the dark-adapted pigment (BR_{560}) contains a 60:40 mixture of the 13-*cis* and all-*trans* isomers (4–7). The photoreaction kinetics and visible absorption spectrum of purple membrane are sensitive to changes in both pH and ionic strength (8–14). At pH 2 or in deionized solutions, the absorption band shifts from 568 to 605 nm, forming pigments that we will refer to as BR^{A}_{605} (acid 605) or BR^{D}_{605} (deionized 605), respectively. Further reduction in the pH to 0 causes a blue shift of the absorption, forming a pigment denoted BR^{A}_{565} (acid 565).

A number of hypotheses have been advanced to explain these spectroscopic changes. Formation of BR^{A}_{605} may result from protonation of a negative protein counterion associated with the retinal-lysine Schiff base (10, 11), whereas proposals for the formation of BR^{A}_{565} include protonation of a negative protein group near the ionone ring of the chromophore (15), or association of a soluble anion with the positively charged Schiff-base nitrogen (11). Other studies have suggested that BR^{A}_{605} results from the accumulation of species like O_{640} (9, 11, 16) or K_{625} (17).

Resonance Raman spectroscopy is a direct method for studying retinal chromophore structure in pigments (18, 19). By selecting an exciting laser wavelength that lies within the visible absorption band, scattering from the chromophore alone can be strongly enhanced. The resulting spectra are sensitive to changes in chromophore geometry and environment. We report here resonance Raman spectra of purple membrane obtained at pH 2, pH 0, and at low ionic strength. The spectra of BR^{A}_{605} and BR^{D}_{605} are nearly identical to one another, and they are both very similar to the spectrum of BR_{560}. This indicates that mild acidification or suspension in deionized water results in "dark adaptation" of the sample. The resonance Raman spectrum of BR^{A}_{565} is remarkably similar to that of BR_{568}, demonstrating that further acidification reverses this transition or "light-adapts" the sample.

Resonance Raman spectra of BR^{A}_{605} and BR^{A}_{565} were obtained with purple membrane cast in polyacrylamide gels (7.5% acrylamide, 0.2% bisacrylamide, 0.03% tetramethylethylacrylamide, 0.04 M Tris, 0.024% ammonium persulfate, and ~3 mg/ml of BR). It was advantageous to use the acrylamide gel because it prevented the aggregation of the purple membrane that otherwise occurs at low pH. After addition of ammonium persulfate to initiate polymerization, the solution was poured into the cell used for the Raman experiments. Spinning the cell while the solution polymerized produced a thin gel on the cell's inside surface. The pH of the gel was adjusted by adding 0.1 M potassium phthalate (pH 2.0) or 1.0 M HCl (pH 0.0) buffer to the Raman cell in the dark at room temperature.

Absorption spectra taken on small sections of the gel before and after each Raman experiment confirmed that the pigment had the appropriate absorption maximum. To avoid contributions from photoproducts of BR^{A}_{605} and BR^{A}_{565}, the rotational frequency of the sample cell (ν = 40 Hz) and the laser power (P) were adjusted to minimize photolysis of the pigment. For a single pass through the laser beam, the photoalteration parameter, $F = (3.824 \times 10^{-21})\ \epsilon\phi\ P\nu^{-1}a^{-1}$, gives the fraction of molecules that photoreact (20). For BR^{A}_{605} we used 20 mW of 514.5-nm laser excitation that was cylindrically focused on the sample. Based on an extinction coefficient (ϵ) of 19,000 $M^{-1}cm^{-1}$ at 514 nm (10), a flow speed (v) given by $2\pi r\nu$ = 250 cm/s, a beam diameter (a) along the unfocused dimension of the cylindrical beam of 0.15 cm, and assuming a quantum yield (ϕ) for photoreaction of 0.3, we calculate a photoalteration parameter of 0.03. This corresponds to ~3% of the sample photoreacting as the sample passes through the laser beam. For BR^{A}_{565}, the calculated photoalteration was 0.07, using the conditions given above except with an extinction coefficient of 42,000 $M^{-1}cm^{-1}$ at 514 nm (10). The photocycle kinetics of the acid forms of purple membrane in polyacrylamide gels have been measured (10) and in each case the half-time for decay back to the original pigment after photolysis is 10–20 ms. Contributions to the Raman spectrum from photoproducts are minimized since only a small fraction of the photolyzed pigment has not returned to its parent during the 25 ms rotational period of the cell.

The deionized samples were prepared by passage of membrane suspensions through a cation exchange column as described by Kimura et al. (13). Raman spectra of deionized and native purple membrane were obtained by recirculating the sample through a capillary at 300 cm/s and exciting with a cylindrically focused 514.5 nm laser. In this case, the laser power (20 mW) and the sample flow speed were adjusted to reduce the photoalteration parameter F to ~0.03 for BR^{D}_{605} and 0.05 for BR_{568}. Spectra were also obtained of BR^{D}_{605} with and without illumination of the sample reservoir using a 100-W incandescent light. These data were very similar, showing no tendency for BR^{D}_{605} to light-adapt. Spectra of dark-adapted BR were obtained by decreasing the laser power to ~5 mW, yielding F = 0.01.

Raman spectra of BR^{A}_{605} and BR^{D}_{605} are shown in Fig. 1 *A* and *B*. The spectra are both dominated by a broad ethylenic stretching mode at 1,518 cm^{-1} and fingerprint modes at 1,171, 1,184, and 1,200 cm^{-1}. The width of the ethylenic line (~28 cm^{-1} full width at half maximum) suggests that the BR^{A}_{605} and BR^{D}_{605} pigments contain a mixture of *cis* and *trans* chromophores. For comparison, BR_{560}, which contains a mixture of 13-*cis* and all-*trans* chromophores, has an ethylenic width of 23 cm^{-1}, while BR_{568}, which contains only an all-*trans* chromophore, has a width of 14 cm^{-1} (Fig. 1 *C* and *D*). Furthermore, the close similarity of the fingerprint regions of both BR^{A}_{605} and BR^{D}_{605} with that of BR_{560} suggests that these pigments

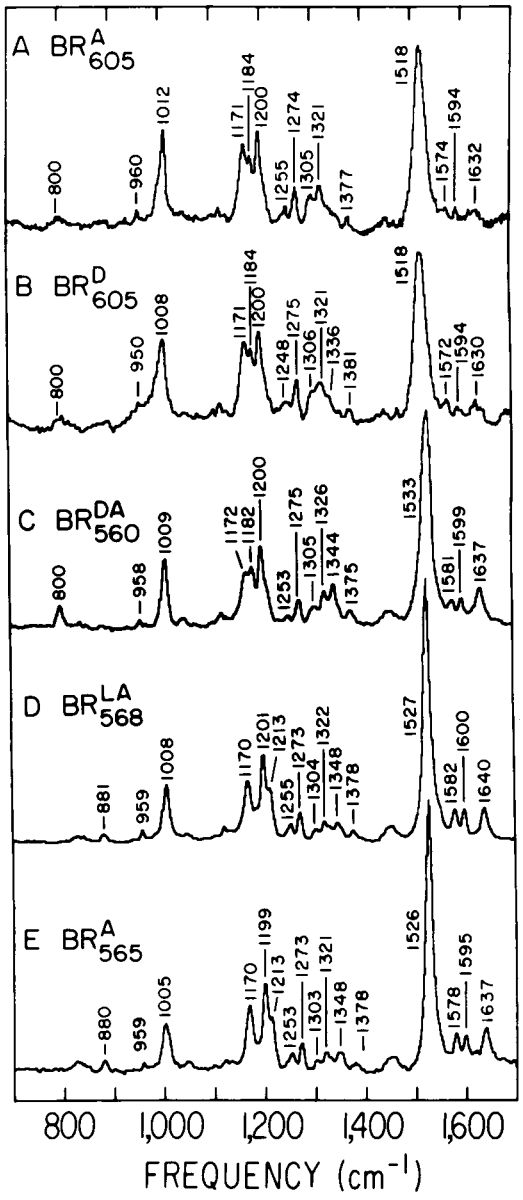

FIGURE 1 (*A*) This part shows a rotating cell resonance Raman spectrum of BR^{A}_{605} obtained using purple membrane cast in a polyacrylamide gel at pH 2. (*B*) The spectrum of BR^{D}_{605} was obtained using a flowing sample of purple membrane at pH 7 in deionized water. (*C*) A spectrum of dark-adapted BR_{560} at pH 7 (10 mM HEPES) was obtained as in *B*. (*D*) A spectrum of light-adapted BR_{568} at pH 7 (10 mM HEPES) was obtained as in *B*. (*E*) This part shows a rotating cell-resonance Raman spectrum of BR^{A}_{565} obtained using purple membrane cast in a polyacrylamide gel at pH 0. All spectra were obtained with the Raman spectrometer described in reference 24 using 514.5-nm excitation. The spectral resolution is 4 cm^{-1}.

contain a very similar mixture of all-*trans* and 13-*cis* chromophores. The relative intensity of the 1,184-cm^{-1} line to the 1,171 and 1,200-cm^{-1} lines is a sensitive measure of the 13-*cis*/all-*trans* isomer ratio. This is because BR_{548}, the 13-*cis* component of dark-adapted BR, has a strong 1,184-cm^{-1} line, while BR_{568} has intense lines at 1,170 and 1,200 cm^{-1} but no significant scattering at

~1,184 cm^{-1} (21–23). The relative intensities of the 1,171, 1,184, and 1,200 cm^{-1} lines of BR^{A}_{605} and BR^{D}_{605} indicate that these species contain approximately equal mixtures of all-*trans* and 13-*cis* chromophores. This is supported by the observation that the BR^{A}_{605} and BR^{D}_{605} spectra can be accurately described by summing spectra of BR_{548} and BR_{568} with relative percentages of 40 and 60, respectively.

The Raman spectrum of BR^{A}_{565} shown in Fig. 1 *E* is nearly identical to that of BR_{568} in Fig. 1 *D*. This demonstrates that BR^{A}_{565} contains an all-*trans* protonated Schiff-base chromophore. The width of the ethylenic stretching mode at 1,526 cm^{-1} is narrow (~15 cm^{-1}) consistent with the idea that we are observing a single molecular species. Note also that the 1,184-cm^{-1} fingerprint line diagnostic of 13-*cis* chromophores is absent.

Our results are generally consistent with previous studies, although several early speculations are shown to be incorrect. First, the chromophore composition that we estimate for BR^{A}_{605} and BR^{A}_{565} from our in situ Raman experiments is in agreement with chemical extraction results (10, 11). Mowery et al. (10) extracted ~40% 13-*cis* and 60% all-*trans* chromophores from purple membrane at pH 2.0 (BR^{A}_{605}) and ~91% all-*trans* and 9% 13-*cis* chromophores at pH −0.03 (BR^{A}_{565}). Similar extraction results were obtained by Fischer and Oesterhelt (11). There are no reports of chromophore extraction from BR^{D}_{605}. However, the observation that the BR^{A}_{605} and BR^{D}_{605} pigments have similar Raman spectra is consistent with circular dichroism studies that suggest that these two pigments exhibit similar protein conformational changes (13). Finally, it has been proposed that BR's O_{640} intermediate is identical to BR^{A}_{605} since its absorption exhibits a similar pH and temperature dependence (9, 11, 16). Our results show that there is no significant relationship between the structure of the chromophore in the BR^{A}_{605} pigment and O_{640}. O_{640} has a conformationally distorted all-*trans* protonated Schiff-base chromophore (24), while BR^{A}_{605} contains an approximately equal mixture of relaxed 13-*cis* and all-*trans* chromophores.

The vibrational spectrum of the retinal chromophore is sensitive not only to the chromophore's double bond configuration, but also to its protein environment. Therefore, the close similarity of both the frequencies and intensities of the vibrational modes in the Raman spectra of BR^{A}_{605} and BR^{D}_{605} indicates that very similar protein perturbations exist in each pigment. This suggests that the mechanism for red-shifting the absorption band in BR^{A}_{605} and BR^{D}_{605} is the same. One possibility is that both acidification to pH 2.0 and reduction of the ionic strength at pH 7 result in protein conformational changes that lead to the removal of the Schiff-base counterion. We cannot tell whether this results from neutralization of the negative charge or protein conformational changes that simply displace the charge. The lower frequencies of the C=N stretching vibration at 1,632 cm^{-1} and of the C=C stretching modes at 1,518, 1,574 and 1,594 cm^{-1} in BR^{A}_{605} and BR^{D}_{605}, relative to BR_{560}, support this proposal since either mechanism should result in increased delocalization of the π-electrons that would lower π-bond orders. An attractive hypothesis is that suspension of purple membrane in low pH or deionized solutions displaces a divalent cation from its protein binding site (13), inducing the similar protein structural changes that we have observed in BR^{A}_{605} and BR^{D}_{605}.

The alternative possibility, that twists about C—C or C=C bonds are responsible for the bathochromic shift in BR^{A}_{605} and BR^{D}_{605}, can be rejected. The Raman intensity of the vinyl hydrogen out-of-plane (HOOP) vibrations is strongly enhanced when a retinal chromophore is torsionally distorted (25). Intense HOOP modes are observed in the Raman spectra of the primary photoproducts of BR and rhodopsin, and in O_{640}. These pigments have chromophore structures that have not relaxed to a planar geometry following double bond isomerization (24–26). In BR^{A}_{605} and BR^{D}_{605}, however, only weak lines are observed at 800 and ~955 cm^{-1} in the HOOP spectral region, which are similar in frequency and intensity to those observed in BR_{560}. The absence of strong HOOP vibrations in these pigments rules out the possibility that C—C or C=C twists generate the red-shifted absorption. This suggests that the mechanism for the formation of the 13-*cis* component of the 605 nm pigments is analogous to that for the formation of the 13-*cis* component of dark-adapted BR. We have recently shown that in dark adaptation, distortion of the chromophore is minimized by performing a simultaneous "bicycle pedal" isomerization about the C_{13}=C_{14} and C=N bonds (27, 28).

We turn now to the molecular mechanism of the acid-induced spectral changes in BR^{A}_{565}. The close similarity between the vibrational spectrum of BR^{A}_{565} and BR_{568} indicates that the effects produced by lowering the pH to 2 are reversed at pH 0. This suggests that further acidification restores the negative charge (or reverses the protein counterion motion) that was discussed above. Fischer and Oesterhelt proposed that restoration of the negative charge near the Schiff base may occur by selective anion binding (11). However, it has recently been shown that the formation of BR^{A}_{565} is unaffected by suspension in deionized solutions (13). The alternative proposal in which the $BR^{A}_{605} \rightarrow BR^{A}_{565}$ transition is associated with the titration of a negative protein perturbation near the ionone ring also appears unlikely. This would require that a retinal chromophore with no Schiff-base or ionone-ring charge perturbations (BR^{A}_{565}) have Raman spectral frequencies and intensities that are nearly identical to a retinal chromophore that has both (BR_{568}).

In summary, the spectra of BR^{A}_{605} and BR^{D}_{605} provide an in situ demonstration that these pigments contain an approximately equal mixture of 13-*cis* and all-*trans* chromophores. Further lowering of the pH to 0 reverses the effects generated at pH 2, yielding a pigment containing predominantly the C_{13}=C_{14} *trans* isomer. In future work

on the acidified and deionized forms of BR, it will be necessary to take into account the mixed isomeric composition in BR^{A}_{605} and BR^{D}_{605}, plus the fact that BR^{A}_{605} and BR^{D}_{605} (as well as BR_{568} and BR^{A}_{565}) have similar chromophore environments.

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REFERENCES

1. Stoeckenius, W., and R. A. Bogomolni. 1982. Bacteriorhodopsin and related pigments of halobacteria. *Annu. Rev. Biochem.* 51:587–616.
2. Birge, R. R. 1981. Photophysics of light transduction in rhodopsin and bacteriorhodopsin. *Annu. Rev. Biophys. Bioeng.* 10:315–354.
3. Lozier, R. H., R. A. Bogomolni, and W. Stoeckenius. 1975. Bacteriorhodopsin: A light-driven proton pump in *Halobacterium halobium*. *Biophys. J.* 15:955–962.
4. Braiman, M., and R. Mathies. 1980. Resonance Raman evidence for an all-trans to 13-cis isomerization in the proton-pumping cycle of bacteriorhodopsin. *Biochemistry.* 19:5421–5428.
5. Pettei, M. J., A. P. Yudd, K. Nakanishi, R. Henselman, and W. Stoeckenius. 1977. Identification of retinal isomers isolated from bacteriorhodopsin. *Biochemistry.* 16:1955–1959.
6. Aton, B., A. G. Doukas, R. H. Callender, B. Becher, and T. G. Ebrey. 1977. Resonance Raman studies of the purple membrane. *Biochemistry.* 16:2995–2999.
7. Sperling, W., P. Carl, C. N. Rafferty, and N. A. Dencher. 1977. Photochemistry and dark equilibrium of retinal isomers and bacteriorhodopsin isomers. *Biophys. Struct. Mech.* 3:79–94.
8. Oesterhelt, D., and W. Stoeckenius. 1971. Rhodopsin-like protein from the purple membrane of *Halobacterium halobium*. *Nat. New Biol.* 233:149–152.
9. 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.
10. 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.
11. Fischer, U., and D. Oesterhelt. 1979. Chromophore equilibria in bacteriorhodopsin. *Biophys. J.* 28:211–230.
12. Kobayashi, T., H. Ohtani, J. Iwai, A. Ikegami, and H. Uchiki. 1983. Effect of pH on the photoreaction cycles of bacteriorhodopsin. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 162:197–200.
13. 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.
14. 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.
15. Nakanishi, K., V. Balogh-Nair, M. Arnaboldi, 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.
16. Lam, E., I. Fry, L. Packer, and Y. Mukohata. 1982. Comparison of the O_{640} photo-intermediate and acid-induced species in membrane patches from *Halobacterium halobium* S_9 and R_1mW strains. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 146:106–110.
17. Tsuji, K., and K. Rosenheck. 1979. The low pH species of bacteriorhodopsin: Structure and proton pump activity. *FEBS (Fed. Eur. Biochem. Soc.) Lett.* 98:368–372.
18. Mathies, R. 1979. Biological applications of resonance Raman spectroscopy in the visible and ultraviolet: Visual pigments, purple membrane and nucleic acids. *Chem. Biochem. Appl. Lasers.* 4:55–99.
19. Callender, R., and B. Honig. 1977. Resonance Raman studies of visual pigments. *Annu. Rev. Biophys. Bioeng.* 6:33–55.
20. Mathies, R., A. R. Oseroff, and L. Stryer. 1976. Rapid-flow resonance Raman spectroscopy of photolabile molecules: Rhodopsin and Isorhodopsin. *Proc. Natl. Acad. Sci. USA.* 73:1–5.
21. Terner, J., C-L. Hsieh, and M. A. El-Sayed. 1979. Time-resolved resonance Raman characterization of the bL_{550} intermediate and the two dark-adapted bR^{DA}_{560} forms of bacteriorhodopsin. *Biophys. J.* 26:527–541.
22. Aton, B., A. G. Doukas, R. H. Callender, B. Becher, and T. G. Ebrey. 1979. Resonance Raman study of the dark-adapted form of the purple membrane protein. *Biochim. Biophys. Acta.* 576:424–428.
23. Braiman, M. S. 1983. Structural changes in bacteriorhodopsin's chromophore during its proton-pumping photocycle, Ph.D. thesis. University of California, Berkeley, CA.
24. Smith, S. O., J. A. Pardoen, P. P. J. Mulder, B. Curry, J. Lugtenburg, and R. Mathies. 1983. Chromophore structure in bacteriorhodopsin's O_{640} photointermediate. *Biochemistry.* 22:6141–6148.
25. Eyring, G., B. Curry, A. Broek, J. Lugtenburg, and R. Mathies. 1982. Assignment and interpretation of hydrogen-out-of-plane vibrations in the resonance Raman spectra of rhodopsin and bathorhodopsin. *Biochemistry.* 21:384–393.
26. Braiman, M., and R. Mathies. 1982. Resonance Raman spectra of bacteriorhodopsin's primary photoproduct: Evidence for a distorted 13-*cis* retinal chromophore. *Proc. Natl. Acad. Sci. USA.* 79:403–407.
27. Smith, S. O., A. B. Myers, J. A. Pardoen, C. Winkel, P. P. J. Mulder, J. Lugtenburg, and R. Mathies. 1984. Determination of retinal Schiff base configuration in bacteriorhodopsin. *Proc. Natl. Acad. Sci. USA.* 81:2055–2059.
28. Harbison, G. S., S. O. Smith, J. A. Pardoen, C. Winkel, J. Lugtenburg, J. Herzfeld, R. Mathies, and R. G. Griffin. 1984. Dark-adapted bacteriorhodopsin contains 13-*cis*, 15-*syn* and all-*trans*, 15-*anti* retinal Schiff bases. *Proc. Natl. Acad. Sci. USA.* 81:1706–1709.