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Time-Resolved Resonance Raman Characterization of the bO_{640} Intermediate of Bacteriorhodopsin. Reprotonation of the Schiff Base†

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ABSTRACT: The resonance Raman spectrum of photolyzed bacteriorhodopsin under conditions known to increase the concentration of the bO_{640} intermediate in both H_2O and D_2O is presented. By use of computer subtraction techniques and a knowledge of the Raman spectra of the unphotolyzed bacteriorhodopsin as well as the other intermediates in the cycle, a qualitative spectrum of bO_{640} is determined. The shift of a band at 1630 cm^{-1} in H_2O to 1616 cm^{-1} in D_2O suggests that the Schiff base of bO_{640} is protonated. Additional bands at 947, 965, and 992 cm^{-1} that appear only in D_2O suspensions confirm that a proton is coupled to the retinal chromophore of bO_{640}. The reprotonation of the Schiff base thus occurs during the bM_{412} to bO_{640} step. The fingerprint region, sensitive to the isomeric configuration of the retinal chromophore of bO_{640}, is dissimilar to the fingerprint regions of published model compounds and other forms of bacteriorhodopsin.

Upon illumination, bacteriorhodopsin, a retinal protein complex similar in structure to the visual pigments (Oesterhelt & Stoeckenius, 1971), has been shown to undergo a cyclic transformation through several intermediates (Lozier et al., 1975). The net effect of the cycle is the transport of protons from the inside of the bacterial cell membrane to the outside, which sets up an electrochemical gradient that is thought to drive the synthesis of ATP (Oesterhelt, 1976). The intermediates of bacteriorhodopsin (Figure 1) were first characterized by time-resolved optical absorptions [for a recent review see Stoeckenius et al. (1979)] and are now being characterized by resonance Raman spectroscopy. The vibrational spectra are capable of providing more detailed structural information than are the broad absorption bands. In particular, the protonation state of the Schiff base linkage between the retinal and the lysine residue and the isomeric configuration of the retinal have received the most attention. At the present time, complete resonance Raman spectra have been obtained of the bR_{560}^{DA} (Terner & El-Sayed, 1978; Marcus & Lewis, 1978; Terner et al., 1979a), bR_{570} (Terner et al., 1977; Aton et al., 1977; Marcus & Lewis, 1978), bL_{550} (Terner et al., 1979a), bM_{412} (Terner et al., 1977; Aton et al., 1977; Marcus & Lewis, 1978), and bK_{590} (Terner et al., 1979b) components of the cycle. In this paper we present the complete resonance Raman spectrum of the retinal chromophore of the bO_{640} intermediate and discuss the evidence for its protonation state as well as conformation changes during the cycle.

Experimental Procedures

Materials

Samples. Purple membrane isolated from *Halobacterium halobium* R_1 was the gift of Dr. R. A. Bogomolni and Professor W. Stoeckenius of the University of California, San Francisco, San Francisco, CA. Refrigerated samples were illuminated by a tungsten lamp for several weeks until sufficient carotenoid was photooxidized so that the carotenoid Raman bands at 1255 and 1515 cm^{-1} were no longer detectable (Terner et al., 1979a). Samples were placed in short lengths of melting point capillary tubes at approximately 50 μM in distilled water or D_2O (Bio-Rad). The capillaries were mounted inside a flowing-water jacket and maintained at 40 °C. Considerable enhancement of some of the Raman bands was observed at this temperature, relative to room temperature. Since the optical absorption of the bO_{640} intermediate was similarly enhanced (Lozier et al., 1975), we have assigned the corresponding Raman bands to the bO_{640} intermediate.

Apparatus. The excitation system used for this experiment consisted of a Spectra-Physics Model 375 dye laser pumped

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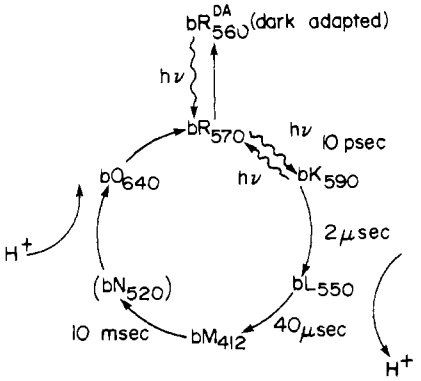

FIGURE 1: The photoinduced cycle of bacteriorhodopsin according to Lozier et al. (1975). The subscripts are the absorption maxima in nanometers. The cycle is believed to be more complex than this simple scheme.

by a Spectra-Physics Model 171 argon ion laser and two mechanical choppers driven by line-synchronized 30-Hz motors. An etalon was used to narrow the frequency of the dye laser emission to 0.5 Å. A five-sided dispersing prism and a diaphragm were used to eliminate dye fluorescence and interference fringes.

The detection system consisted of a Spex 0.5-m spectrograph fitted with a 64 × 64 mm^2 1200 groove/mm ruled grating and a Princeton Applied Research Corp. (PARC) optical multichannel analyzer (OMA).[1] The resolution was 5 cm^{-1}. The OMA consisted of a Model 1205 A console, a Model 1205 D silicon intensified target (SIT) vidicon in a Model 1212 cooled housing, and a Model 1207 extended delay accessory. The operation of the OMA with a cooled SIT and extended delay accessory has been described elsewhere (Terner et al., 1979a). A glass cutoff filter was used to eliminate scattered Rayleigh radiation.

Methods

Excitation Frequency. The bO_{640} intermediate may be expected to appear as prominently in CW (continuous wave) resonance Raman spectra as the other long-lived intermediate, bM_{412} (Terner et al., 1977). However, when one tunes the laser into the bO_{640} absorption band, the Raman signal is overcome by the intrinsic red fluorescence (Lewis et al., 1976) of bacteriorhodopsin. The onset of the fluorescence is at about 6000 Å, increasing in intensity toward longer wavelength, with the result that excitation at wavelengths longer than 5550 Å produces a serious degradation in the signal to noise ratio, especially for peaks in the 1600–1650-cm^{-1} range. One is thus limited to the excitation range 5500–5550 Å in order to get as close as possible to the absorption of bO_{640}, while being able to obtain an adequate signal to noise ratio. In this range, the lasing dye was Rhodamine 560 (Exciton). It should be noted that we also have been able to observe bO_{640} bands quite clearly with the 5145-Å argon ion laser line.

Behavior of Fluorescence. We have made some observations that should be of considerable interest to resonance Raman spectroscopists studying purple membrane. It is possible to reduce the fluorescence considerably by rapidly flowing the sample through a syringe needle (Terner, 1979), with the result that very good spectra have been obtained with 5900-Å excitation. We also note that the ratio of the fluorescence to the Raman scattering intensity *increases* as incident laser power is increased in both stationary and flow experiments. The intrinsic fluorescence has been previously reported by

[1] Abbreviations used: OMA, optical multichannel analyzer; CW, continuous wave.

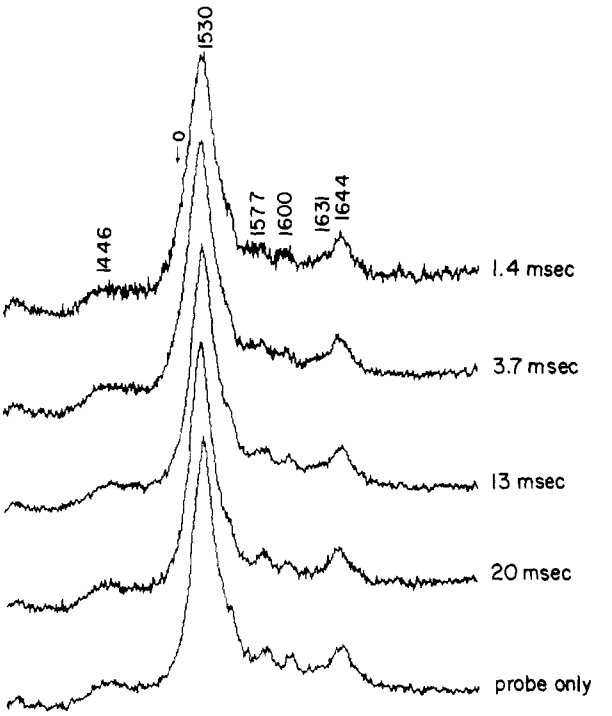

FIGURE 2: Time-resolved Raman spectrum in the region of 1400–1800 cm^{-1}. The purple membrane was maintained at 40 °C. The peak laser power was 200 mW at 5507 Å. The photolytic flash was 0.28 mJ and was followed by a 0.034-mJ probe pulse after a variable delay. The decay of the C=C stretch of bO_{640} can be seen on the low-energy side of the 1530-cm^{-1} band. The 1631-cm^{-1} band also decays on the time scale of this experiment.

Mendelsohn et al. (1974) and Lewis et al. (1976). Our observations might imply that the fluorescence is produced from an excited state of a photoproduct rather than from the excited state of bR_{570}. Gillbro and co-workers (Gillbro et al., 1977; Gillbro & Kriebel, 1977) have suggested that the fluorescence emanates from bM_{412} and bO_{640}. The red-emission lifetime has, however, been measured by Alfano et al. (1976), Hirsch et al. (1976), and Shapiro et al. (1978) to be in the picosecond range, from which they concluded that the emission is from an excited state of bR_{570}. Govindjee et al. (1978) have reached the same conclusion. Rotation of the electric vector of the polarized incident laser irradiation results in no significant increase or decrease in the fluorescence relative to the Raman band intensities, which suggests that these effects are not caused by differences in orientation of the purple membrane fragments between flowing and stationary samples.

Kinetic Measurements. The time-resolved resonance Raman spectra shown in Figures 2 and 3 were obtained by using laser pulses produced by a chopper (30 Hz) as diagramed and described by Terner et al. (1977). The chopping wheel possessed two slits which produced a 0.28-mJ photolytic flash followed at a variable delay by a 0.034-mJ proble pulse from an incident laser power of 200 mW. A second chopper placed in front of the spectrograph was adjusted in phase so that only scattered light from the probe pulse would be allowed to enter the spectrograph. It should be noted that it was not feasible to reduce the width of the probe pulse to the extent required to entirely eliminate photolysis of the sample and simultaneously obtain a good signal to noise ratio. Thus, bL_{550} bands appear in the probe pulse spectrum.

Obtaining the Resonance Raman Spectrum of bO_{640}. Although time-resolved resonance Raman spectra establishing the identity of bO_{640} were obtained by the method described in the previous paragraph, the method we have found to

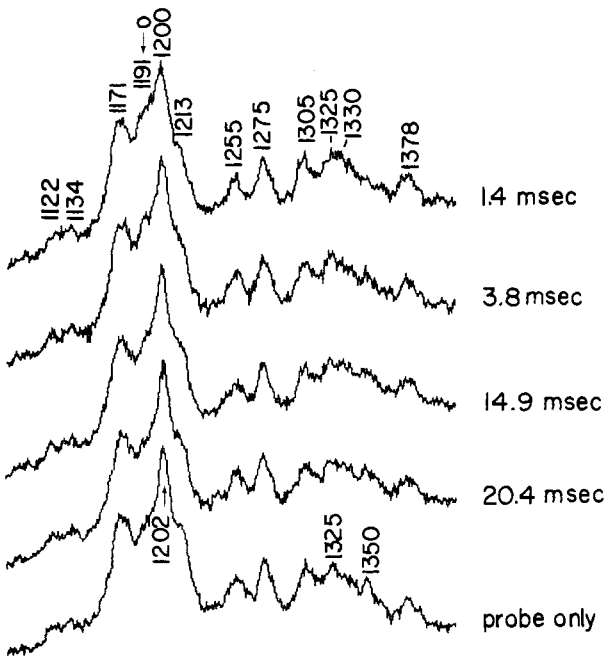

FIGURE 3: Time-resolved resonance Raman spectrum in the region 1050–1400 cm^{-1} using 200 mW at 5507 Å (40 °C). The photolytic flash was 0.28 mJ and was followed by a 0.034-mJ probe pulse after a variable delay. The decay of a bO_{640} band can be seen at 1191 cm^{-1}.

produce the clearest resonance Raman spectra of bO_{640} was as follows. We first obtained a continuous (CW) resonance Raman spectrum containing large steady-state concentrations of bO_{640} and bL_{550} superimposed upon bR_{570}. A pulsed-laser resonance Raman spectrum containing only bR_{570} and bL_{550} was then subtracted from the CW spectrum to reveal the resonance Raman spectrum of bO_{640}.

More specifically, a stationary sample at 40 °C irradiated with 80–200-mW CW laser excitation and focused to a 100-μm spot across the diameter of a melting point capillary will produce a large resonance Raman signal from bO_{640}. Superimposed on the spectrum are the spectra of bL_{550}, due to the large resonance enhancement, and that of unphotolyzed bR_{570}. The 40 °C temperature enhances the production of bO_{640} and selects against the production of bN_{520} which is prevalent at 0 °C [Lozier et al. (1975) and our observations]. To obtain a spectrum containing only bL_{550} and bR_{570} (a little bO_{640} would also usually be present), we irradiated the same sample (at 40 °C) with 0.2-ms, 0.04-mJ pulses at 30 Hz. Contributions from the resonance Raman spectrum of bM_{412} were very weak (though observable), since the excitation was 1400 Å away from the absorption maximum. Because of its short half-life relative to those of bO_{640} and bL_{550}, contributions to the spectrum from the bK_{590} intermediate (Terner et al., 1979b) were very weak and were not present in any significant amount in our spectra.

It should be noted that our method of producing large steady-state concentrations of bO_{640} by illumination with a strong CW beam could produce photoproducts other than bO_{640} and bL_{550}. It has been suggested (Hurley et al., 1978) that bM_{412} and bL_{550} form photoproducts upon illumination. From this laboratory (Terner et al., 1979a,b) we were able to minimize photoproducts by quickly flowing the irradiated sample away from the detection area. In the present experiment, this procedure was not possible, since the sample had to be kept stationary. To test for the possibility of photoproducts in the spectra, we varied the incident laser power to see if any were variable in relation to the bO_{640} or bL_{550} peaks. We were not able to detect any such behavior.

Table I[a]

bR_{570}	bO_{640}	bL_{550}	bM_{412}
			791 (m)
			819 (w)
			839 (w)
843 (w)			
	[947 (w)]		
961 (w)			
	[965 (m)]		
[979 (m)]			979 (m)
		[988 (m)]	
	[992 (s)]		
1012 (s)	1012 (s)	1012 (s)	1012 (s)
1117 (w)	1117 (w)	1122 (w)	
			1126 (w)
1132 (w)	1132 (w)	1135 (w)	
1169 (s)			
	1172 (s)		
			1180 (vs)
			1188 (s)
	1187 (vs)		
	1194 (w) L	1197 (vs)	1197 (m) L
1201 (vs)			
1211 (s)			
			1227 (m)
1254 (m)	1252 (m)	1255 (m)	
1277 (m)	1273 (m)	1275 (m)	1278 (m)
1303 (m)	1303 (m)	1309 (m)	1309 (m)
1323 (m)			
	1333 (m)	1331 (m)	1334 (m)
1380 (m)	1380 (m)	1380 (m)	1382 (m)
1452 (m)	1452 (m)	1442 (m)	1450 (m)
1520 (vs)	1530 (vs)	1547 (vs)	1540 (m) L
	1550 (m) L		1570 (m)
	1574 (w) M		
1584 (m)			
1600 (m)	1598 (m)	1597 (m)	
			1623 (m)
1645 (m)	1630 (m)	1647 (m)	
[1625 (m)]	[1616 (m)]	[1619 (m)]	

[a] A comparison of the resonance Raman frequencies of bR_{570}, bO_{640}, bL_{550}, and bM_{412} is given. The bM_{412} frequencies are from Terner et al. (1977) and Terner (1979). The bL_{550} frequencies are from Terner et al. (1979a). Absolute energies are accurate to ±5 cm^{-1}. Brackets indicate bands that have shifted or appear upon deuteration. When bands are labeled L (for bL_{550}) or M (for bM_{412}), these are foreign bands that we have not been able to completely remove from the spectra of bO_{640} or bM_{412}.

Computer Subtraction. The problems inherent in computer subtraction of Raman spectra from an OMA have been discussed previously (Terner et al., 1979a). The computer subtractions shown here are a series of arbitrarily unnormalized weighting factors to avoid inaccurate normalization. Plots where peaks have gone negative due to over subtraction have been eliminated. Though we have subtracted the bL_{550} bands, the subtraction is not complete, and residual bL_{550} contributions appear in all subtracted spectra. The known bL_{550} bands are (see Table I) discussed under Results and labeled in the figures.

Results

Kinetics. Time-resolved spectra obtained by the two-slit chopper method (Terner et al., 1977) with 5530-Å excitation are shown in Figures 2 and 3. Several features, though weakly seen, are identified as the bands of the bO_{640} intermediate by their millisecond decay times. In Figure 2 the half-width of the 1530-cm^{-1} band decreases from 28 to 22 cm^{-1}, and a band at 1520 cm^{-1} can be clearly identified upon computer subtraction of the data (Figure 4). Also, the decay of a feature at 1631 cm^{-1} can be resolved. In Figure 3 a band at 1191 cm^{-1} is clearly shown to decay. Additionally, it can be seen that the 1171-, 1255-, 1275-, 1305-, 1330-, and 1378-cm^{-1} bands

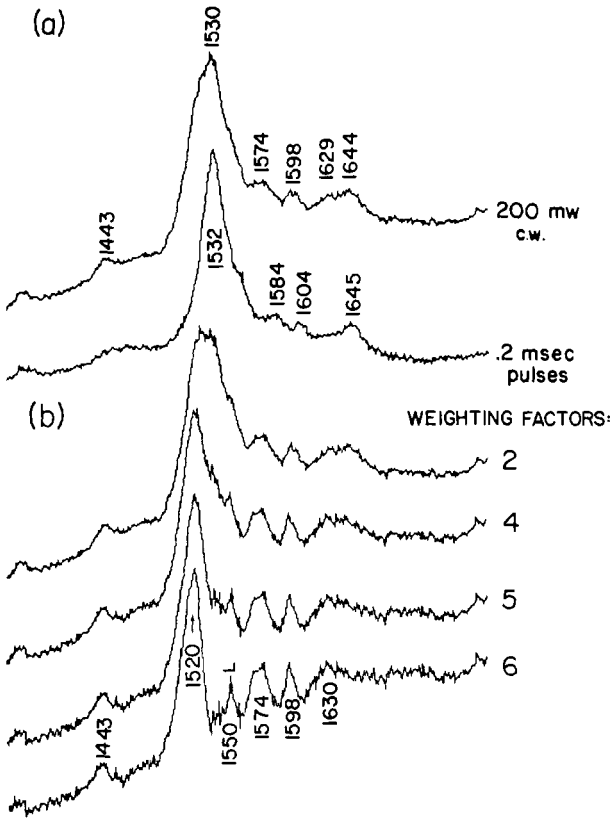

FIGURE 4: (a) A 200-mW continuous wave (CW) resonance Raman spectrum (1400–1800 cm^{-1}) of purple membrane in H_2O and of bO_{640} and bL_{550} superimposed upon bR_{570}, and a resonance Raman spectrum of bL_{550} superimposed on bR_{570} obtained with 0.2-ms, 0.04-mJ pulses (30 Hz). The temperature was 40 °C. The excitation wavelength was 5503 Å. (b) Computer subtraction of the pulsed spectrum from the CW spectrum in (a) by arbitrary weighting factors in Figure 7a to obtain the resonance Raman spectrum of bO_{640} for the region 1400–1800 cm^{-1}. A band at 1550 cm^{-1} is probably a residual of the resonance Raman spectrum of bL_{550}. The broadening of the 1630-cm^{-1} band is due to the water Raman band centered at 1640 cm^{-1}.

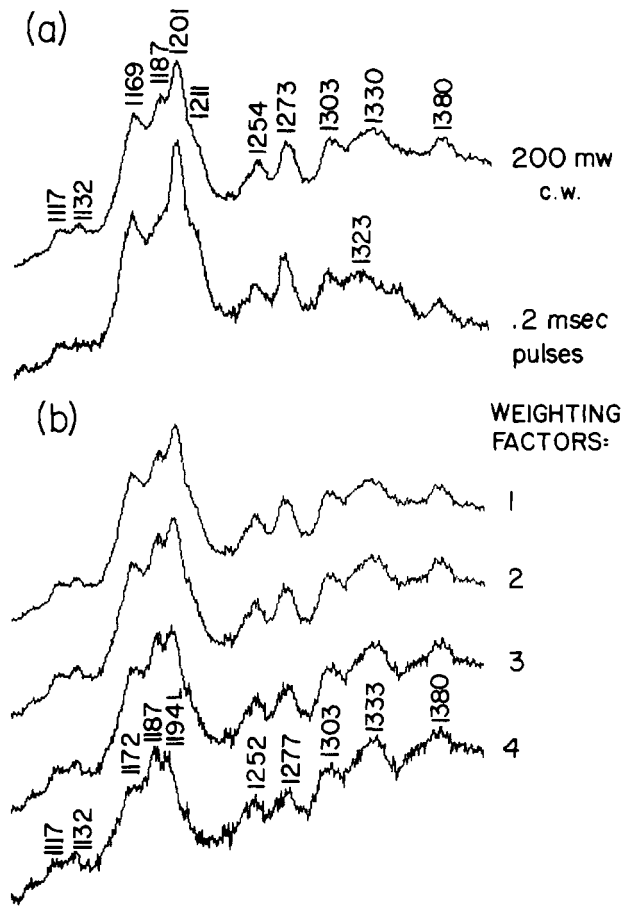

FIGURE 5: (a) A 200-mW continuous wave (CW) resonance Raman spectrum (1100–1400 cm^{-1}) of bacteriorhodopsin in H_2O containing a steady-state superposition of the resonance Raman spectra of bO_{640} and bL_{550} on that of bR_{570}, and a resonance Raman spectrum of bL_{550} superimposed upon bR_{570} obtained with 0.2-mJ pulses (30 Hz). The temperature was 40 °C. The excitation wavelength was 5503 Å. (b) Computer subtraction of the pulsed spectrum from the CW spectrum of (a) by a series of unnormalized weighting factors revealing the fingerprint region of the resonance Raman spectrum of bO_{640}. A band at 1194 cm^{-1} is a residual of the resonance Raman spectrum of bL_{550}. No new features were revealed in a D_2O suspension.

decrease in intensity, allowing the 1213-, 1325-, and 1350-cm^{-1} bands of bR_{570} to become more prominent.

The bM_{412} intermediate, which is also known to have a millisecond decay time, has been well characterized (Terner et al., 1977; Aton et al., 1977) and has Raman peaks at different positions than those for bO_{640} identified in this paper (see Table I). The bN_{520} intermediate, which may also have a decay time in the millisecond range, appears at a lower temperature (Lozier et al., 1975) than that used for the experiments described in this paper.

Resonance Raman Spectrum of bO_{640}. Spectra of bO_{640}, with bL_{550}, derived by computer subtraction are shown for purple membrane suspended in H_2O (Figures 4, 5, and 6) and D_2O (Figures 7 and 8).

(a) The 1400–1800-cm^{-1} Region. This region contains the large ethylenic stretching vibrations which range from about 1515 to 1570 cm^{-1} for the bacteriorhodopsin intermediates and can be used as monitors for detecting the presence of particular intermediates. This region also contains protonated and unprotonated C=N stretching vibrations (Heyde et al., 1971), the protonated vibrations being very sensitive to deuteration (Oseroff & Callender, 1974). As can be seen in Figure 4a, bO_{640} is most strongly evidenced by a large amount of broadening at 1520 cm^{-1} plus a band at 1629 cm^{-1}. The computer subtraction of Figure 4b shows bands of 1520, 1574, 1598, and 1630 cm^{-1}. Some or all of the intensity of the 1574-cm^{-1} band could be the C=C stretch of bM_{412}. The 1550-cm^{-1} feature is a remnant of the bL_{550} intermediate.

Lewis et al. (1974) have shown that the 1645-cm^{-1} protonated C=N stretch of bR_{570} shifts to 1625 cm^{-1} upon deuteration (as shown in the pulsed spectra of Figures 4a and 7a). The 1630-cm^{-1} band of bO_{640} also shifts to 1616 cm^{-1} (compare Figures 4b and 7b), indicating that bO_{640} also possesses a protonated Schiff base.

(b) The 1100–1400-cm^{-1} Region. This is the "fingerprint region" which has been shown to be sensitive to the isomeric configuration of the retinals (Rimai et al., 1971). This region (Figure 5) shows essentially no changes upon deuteration (Terner, 1979). The fingerprint region of the bO_{640} (Figure 5b) appears to consist of a large band at 1187 cm^{-1} with a shoulder at 1172 cm^{-1} plus bands at 1252, 1277, 1303, 1333, and 1380 cm^{-1}. The shoulder at 1194 cm^{-1} is from bL_{550} that has not been subtracted away completely.

(c) The 800–1100-cm^{-1} Region. This region is of interest because it is sensitive to deuteration (Terner et al., 1977, 1979a,b). The H_2O spectra in Figure 6 show no interesting features other than a small band at 843 cm^{-1} and the presence of the 1012-cm^{-1} band in common with all other intermediates. A number of small bands may be present between 961 and 1012 cm^{-1} but cannot be resolved. The D_2O spectra in Figure 8 on the other hand show several interesting features. In

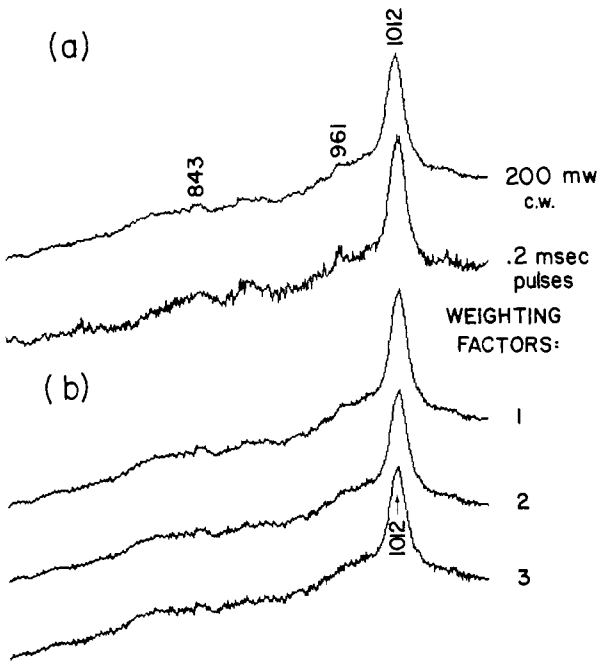

FIGURE 6: (a) A 200-mW continuous wave (CW) resonance Raman spectrum of bacteriorhodopsin in H_2O for the region 750–1100 cm^{-1} containing a superposition of the resonance Raman spectrum of bO_{640} and bL_{550} on the resonance Raman spectrum of bR_{570}, and a resonance Raman spectrum of bL_{550} superimposed upon the resonance Raman spectrum of bR_{570} obtained with 0.2-ms, 0.04-mJ pulses. The temperature was 40 °C. The excitation wavelength was 5520 A. (b) Computer subtraction of the pulsed spectrum from the CW spectrum of (a) by a series of unnormalized weighting factors revealing the resonance Raman spectrum of bO_{640} in the region 750–1100 cm^{-1} in an H_2O suspension. Compare this with the D_2O suspension of Figure 8b.

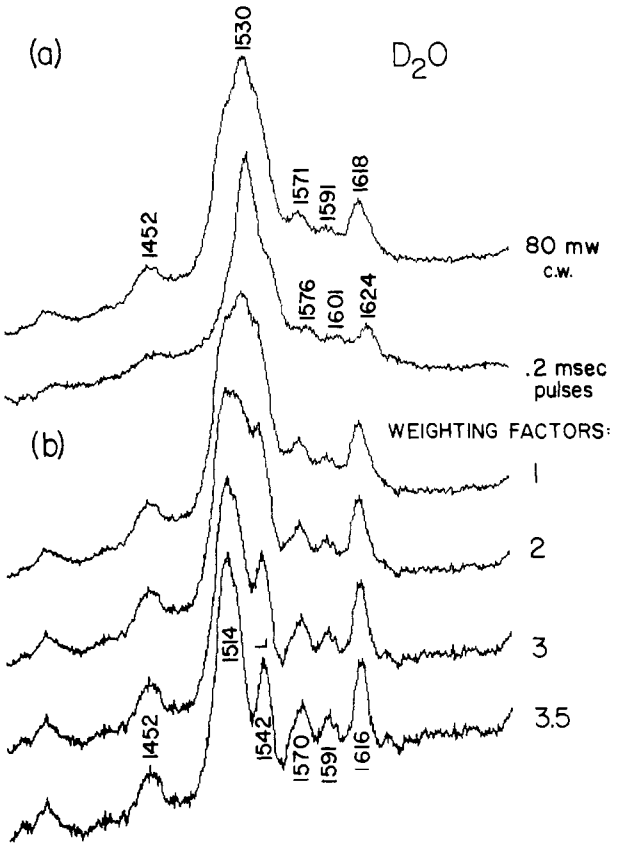

FIGURE 7: A repetition of the results shown in Figure 4 for a D_2O suspension. (a) An 80-mW continuous wave (CW) spectrum (1400–1800 cm^{-1}) of purple membrane containing the resonance Raman spectra of bO_{640} and bL_{550} superimposed upon the spectrum of bR_{570}, and a resonance Raman spectrum of bL_{550} superimposed on bR_{570} obtained with 2-ms, 0.04-mJ pulses (30 Hz). The temperature was 40 °C. The excitation frequency was 5503 A. (b) Computer subtraction of the pulsed spectrum from the CW spectrum shown in (a) by an arbitrary unnormalized weighting factor to reveal the resonance Raman spectrum of bO_{640} in D_2O for the 1400–1800-cm^{-1} region. Note that the C=N stretch has shifted to 1616 from 1630 cm^{-1} (compare this with Figure 4b).

Figure 8a, the 0.2-ms pulse spectrum shows the 979-cm^{-1} band of bR_{570} that has been found to appear upon deuteration (Terner et al., 1977, 1979a) as well as some contribution from bL_{550} at 991 cm^{-1}. In the 80-mW CW spectrum of Figure 8a new features can be clearly seen upon a steady-state buildup of bO_{640}. The computer-subtracted resonance Raman spectrum of bO_{640} (Figure 8b) shows several strong features at 947, 965, and 992 cm^{-1}. The 1012-cm^{-1} methyl stretching frequency is also present.

The appearance of three deuteration bands in this region is unusual in that bR_{570}, bL_{550}, and bR_{550}^{DA} exhibit only one band in this region (Terner et al., 1979a). We have varied laser power and temperature in order to observe relative intensity changes that might indicate the presence of photoproducts. We have not been able to observe such effects, but, because of the difficulties involved in normalization of the computer subtractions and the large number of bands in this region, the possibility of photoproducts still cannot be ruled out.

Discussion

Protonation of Schiff Base. Several deuteration effects, the shift of the band at 1630 to 1616 cm^{-1} upon suspension of the purple membrane in D_2O, as well as the appearance of several new bands at 947, 965, and 992 cm^{-1}, strongly suggest that bO_{640} is protonated, probably at the Schiff base linkage between the retinal and the lysine residue of bacterioopsin.

Deuteration studies for assignment of the C=N stretches in bacteriorhodopsin and rhodopsin have been documented (Lewis et al., 1974; Oseroff & Callender, 1974). The 14-cm^{-1} shift we have observed is smaller than previously reported shifts. It also should be pointed out that protonated Schiff bases usually have bands that are higher than 1630 cm^{-1}.

It is interesting to note that bM_{412} does not show any effect upon deuteration in either the 1600- or the 1000-cm^{-1} region, which is consistent with the assignment that bM_{412} possesses an unprotonated Schiff base (Lewis et al., 1974). The reprotonation step is thus $bM_{412} \rightarrow bO_{640}$. We have determined that deprotonation occurs between bL_{550} and bM_{412} (Terner et al., 1979a).

Isomeric Configuration. Some workers have suggested that the retinal chromophore might stay in the all-trans configuration throughout the light-adapted cycle, unlike the 11-cis to all-trans isomerization occurring in the energy transduction process of the visual pigments; however, it has been recently suggested by Pettei et al. (1977) and Aton et al. (1977) that bM_{412} might exist in the 13-cis conformation.

The fingerprint region of the retinals has been shown to be sensitive to isomeric configuration (Rimai et al., 1971). The fingerprint region of bO_{640} consists of a large peak at 1187 cm^{-1} and a shoulder at 1172 cm^{-1}. Additonally, there are bands at 1252, 1277, 1303, 1333, 1380, and 1443 cm^{-1} which are common with other forms of bacteriorhodopsin (Terner et al., 1979b). Though there are no close matches, there might be an overall general resemblance of the large band at 1187 cm^{-1} (with a 1172-cm^{-1} shoulder) of bO_{640} to the 1180-cm^{-1} band (with a 1188-cm^{-1} shoulder) of bM_{412} (Terner et al., 1977, 1979a; Terner, 1979). It must be realized, though, that

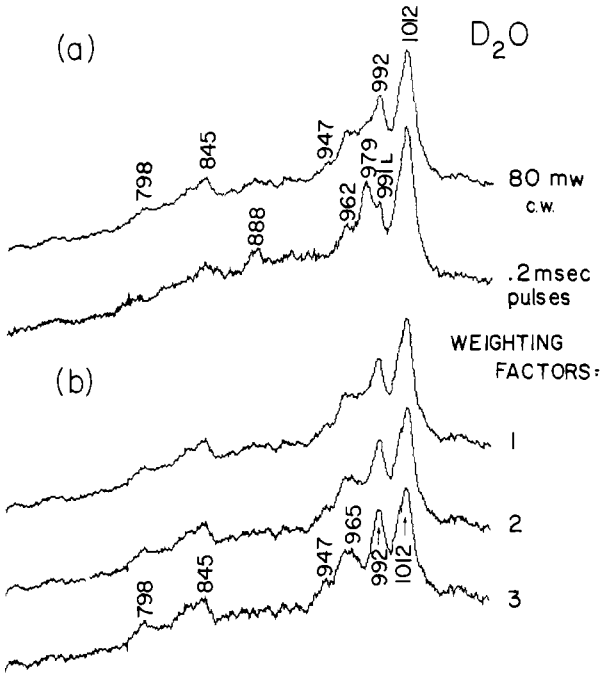

FIGURE 8: A repetition of the vibrational region shown in Figure 6 (750–1100 cm^{-1}), with a D_2O suspension. (a) An 80-mW continuous wave (CW) spectrum containing the resonance Raman spectra of bO_{640} and bL_{550} superimposed upon the resonance Raman spectrum of bR_{570}, and a resonance Raman spectrum of bL_{550} superimposed upon the resonance Raman spectrum of bR_{570} obtained with 0.2-ms, 0.04-mJ pulses. The excitation frequency was 5520 Å. (b) Computer subtraction of the pulsed spectrum from the CW spectrum of (a) by arbitrary weighting factors revealing the resonance Raman spectrum of bO_{640} for the 750–1100-cm^{-1} vibrational region in a D_2O suspension. Compare this with the results for an H_2O suspension shown in Figure 6b.

these comparisons are in the "eye of the beholder". Comparison of the spectra of the bL_{550}, bM_{412}, bR_{570}, and bR_{560}^{DA} forms with those of model compounds has been made in the listed references. Comparison of the spectrum of bO_{640} with those of published model protonated Schiff bases (Mathies et al., 1977) does not provide an unambiguous match. The correspondence with all-trans or 13-cis models might be closer than that with the 11-cis or 9-cis models, but a close match is not in evidence. The bO_{640} intermediate has one of the largest optical red shifts of all the retinal-based pigments, which might explain the difficulty encountered when trying to match the spectrum with those of model compounds. More systematic analysis is necessary, in the areas of both modeling and theory (Warshel & Karplus, 1974), before conclusions can be made about the conformation of the chromophore of the bO_{640} intermediate.

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