Tyrosine Protonation Changes in Bacteriorhodopsin
A FOURIER TRANSFORM INFRARED STUDY OF BR648 AND ITS PRIMARY PHOTOPRODUCT

(Received for publication, July 13, 1987)

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The structural alterations which occur in bacteriorhodopsin (BR) during dark adaptation (BR570→BR648) and the primary phototransition of the dark photocycle (BR648→K630) have been investigated by Fourier transform infrared and UV difference spectroscopy. Possible contributions of tyrosine to the Fourier transform infrared difference spectra of these transitions were assigned by incorporating ring per-deuterated tyrosine into BR. Based on these data and UV difference measurements, we conclude that a stable tyrosinate exists in BR570 at physiological temperature and that it protonates during formation of BR648. A tyrosinate protonation has also been observed at low temperature during the primary phototransition of BR570 to the red-shifted photoprodut K630. However, we now find that no tyrosine protonation change occurs during the primary phototransition of BR648 to the red-shifted intermediate K630. Through analysis of BR containing isotopically labeled retinals, it was also determined that the chromophore of K630 exits in a 13-trans,15-cis configuration. On the basis of this evidence and previous studies on the structure of the chromophore in BR570, BR648, and K630, it appears that only the 13-trans,15-trans configuration of the protonated chromophore leads to a stable tyrosinate group. It is proposed that a tyrosinate residue is stabilized due to its interaction with the Schiff base positive charge in the BR570 chromophore. Isomerization of the chromophore about either the C13=C14 or C=N bond disrupts this interaction causing a protonation of the tyrosinate.

Bacteriorhodopsin (BR), the 26-kDa retinal-containing protein of the purple membrane of Halobacteria halobium,

*This work was supported by Grant DMB-8509857 from the National Science Foundation (to K. J. R.), Grant GM 23516 from the National Institutes of Health (to J. H.), and by a grant from Zuiver Wetenschappelijk Onderzoek (Netherlands) (to J. L.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "advertisement" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

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1 The abbreviations used are: BR, bacteriorhodopsin; BR-[4H]Tyr, BR containing ring per-deuterated tyrosine; FTIR, Fourier transform infrared; HOOP, hydrogen-out-of-plane; RPSB, retinal-protonated Schiff base.

functions as a light-driven transmembrane proton pump (2). Photon absorption by the light-adapted state of purple membrane (BR570) results in the initiation of a photochemical cycle which is characterized by several distinct intermediates that have different visible absorption maxima. The formation of the early photointermediate K630 involves C13=C14 trans to cis isomerization of the retinylidene chromophore (3). Protein changes during K630 formation have been detected by FTIR and UV spectroscopy. These include a tyrosinate protonation (1, 4, 5), tryptophan perturbation (1), and the perturbation of at least one carboxyl group (6, 7).

In the dark, at least 50% of the light-adapted BR570 species converts to a blue-shifted form designated BR465. Recent resonance Raman and NMR studies indicate that in contrast to the 13-trans,15-trans configuration of the retinylidene chromophore of BR570, the BR648 chromophore exists in a 13-cis,15-cis configuration (8, 9). The photocycle of BR648, as shown in Fig. 1 (10-12), also exhibits an early red-shifted intermediate, K630. However, the "dark photocycle" which occurs upon light absorption by BR648 does not result in the formation of a blue-shifted "M"-like intermediate or result in proton pumping as found for the photocycle of the light-adapted BR570 species. In general, a comparison of the molecular changes that accompany the BR648→K630 and BR570→K630 transitions may help explain why both transitions produce a red-shifted intermediate yet only one leads to proton pumping.

In this work, we have used FTIR spectroscopy in combination with isotopic labeling of both the retinal chromophore and BR tyrosine residues in order to study the molecular changes occurring in these moieties during both dark adaptation (the BR570→BR648 transition) and during the primary phototransition of the dark photocycle (the BR648→K630 transition). In agreement with earlier reports (4, 5), it is found that dark adaptation involves the protonation of a tyrosinate group. In contrast, a tyrosine protonation is not observed during the BR570→K630 transition. We have also determined that the retinylidene chromophore in K630 exists in a 13-trans,15-cis configuration. On the basis of this evidence it appears

2 The primary photoprodut of BR465 formed at 77 K is referred to by Iwasa et al. (12) as batho-13-cis-bR in analogy to the K630 photoprodut of BR570, batho-trans-bR. The first photoproduct detected by the flash kinetic absorption studies of Dencher et al. (10) at -90 °C has been designated C and the decay product at this temperature, C, refers to the "cis" photocycle of BR648 in contrast to the "trans" photocycle of BR570. In this paper, we use the designation K630 (i.e. the K photoprodut of the dark photocycle) in order to stress the analogy to the K photoprodut of BR570. This name also avoids potential confusion which might arise from designations which imply a cis or trans structure of the intermediate, which is under investigation in these studies.
that the existence of a tyrosinate in BR depends on the existence of a 13-trans, 15-trans retinal configuration and that it is stabilized by interaction with the positive charge on the Schiff base.

**EXPERIMENTAL PROCEDURES**

**Materials**—The production of BR containing L ring per-deuterated tyrosines (BR-[\(^{13}\)C,\(^{15}\)C]Tyr) has been described previously (1, 13, 14). Purple membrane sheets containing either normal BR or BR-[\(^{13}\)C,\(^{15}\)C]Tyr were isolated by the method of Oesterhelt and Stoeckenius (15). White membrane containing bacterio-opsin was isolated from a retinal-deficient strain of *H. halobium* (JW5) as reported previously (6).

Regeneration of bacterio-opsin with \(^{13}\)C\(^{15}\)C- or \(^{13}\)C\(^{16}\)-labeled retinals (16) was done in dim red light at room temperature and was monitored with a Cary 219 UV-visible spectrophotometer. Membrane fragments were washed in distilled water and deposited on AgCl windows by the isopotential spin-dry method (17) or by slow air drying at 4 °C. Individual differences revealed that all the reactions observed were completely reversible under the illuminations used and that the existence of a tyrosinate in BR depends on the photointermediates, this illumination was shut off at 273 ps.

**FTIR Difference Spectroscopy**—BR\(_{570}\)→BR\(_{548}\) difference spectra were computed by subtracting an FTIR spectrum of a hydrated purple membrane film recorded at 310 K while the film was being cooled rapidly to 81 K/s to 81 cm\(^{-1}\) from that for a completely dark-adapted film. The procedure used to compute the pure BR\(_{570}\) difference spectrum is described under "Results." Comparison of individual differences revealed that all the reactions observed were completely reversible under the illuminations used and that the intermediates observed were thermally stable at 81 K. Light adaptation of films was accomplished by illuminating the sample with 550-nm light for 15 min from a spectrum taken at the same temperature while the same film was in the dark for 15 min. As many as 25 of these differences were averaged together. By comparing the intensities of peaks in successive individual differences, it was found that dark adaptation of the film was complete before the end of a 15-min dark scan at 310 K.

BR\(_{507}\)→BR\(_{548}\) FTIR difference spectra were obtained as reported previously (1, 6, 14) from completely light-adapted films at 81 K, and an analogous procedure was used to obtain spectra at 81 K from a dark-adapted film. The procedure used to compute the pure BR\(_{507}\)→K\(_{560}\) difference spectrum is described under "Results." Comparison of individual differences revealed that all the reactions observed were completely reversible under the illuminations used and that the intermediates observed were thermally stable at 81 K. Light adaptation of films was accomplished by illuminating the sample with 550-nm light for 15 min at 310 K. In order to avoid trapping undesired photointermediates, this illumination was shut off at 273 K while the film was being cooled rapidly (\(\approx 2\) K/s) to 81 K. All measurements were made on a Nicolet MX-1 spectrometer interfaced to a Nicolet 1200DS data analysis terminal, and experimental variables were controlled using a Forth program developed in our laboratory. Each FTIR scan was 15 min long and resulted in the acquisition of 480 interferograms which were multiplied by a Happ-Genzel instrument function before Fourier transformation. Interferometer mirror velocity was 0.85 s/cm, and final effective spectral resolution was 2 cm\(^{-1}\).

**FTIR-UV-visible Difference Spectroscopy**—The UV-visible measurements were made as previously described (1, 13, 14) on fully hydrated purple membrane films. These films were formed by slow air drying of a concentrated purple membrane suspension on a quartz substrate which was then hydrated and sealed in a specially constructed cell.

The spectra were recorded at 1-nm resolution with a Cary 219 spectrophotometer equipped with a Janis (Walther, MA) liquid nitrogen cryostat and interfaced to an Apple Ile computer. Room temperature light adaptation was done with a 600-watt tungsten lamp in combination with a Schott OG475 glass filter (Duryea, PA) and two heat filters (Edmund Scientific, Camden, NJ). Difference spectra were recorded at 80 K from either light-adapted or dark-adapted fully humidified films. Samples were illuminated using a 1-kilowatt Hg-Xe lamp in combination with a monochromator and either a 540- or 640-nm narrow band interference filter (Ditric Optics, Hudson, MA).

**RESULTS**

**Dark Adaptation**

Figs. 2 and 3 compare FTIR difference spectra obtained for dark adaptation of normal BR (solid) and BR-[\(^{13}\)C,\(^{15}\)C]Tyr (dashed). These spectra represent the BR\(_{570}\)→BR\(_{548}\) reaction. Several small but reproducible changes are caused by the isotopic label. In the 1450 to 1550 cm\(^{-1}\) region (Fig. 3A), two small positive peaks, one centered at about 1510 cm\(^{-1}\) and the other at 1453 cm\(^{-1}\) (cf. Fig. 3A), are reduced in the BR-[\(^{13}\)C,\(^{15}\)C]Tyr difference spectrum, possibly shifting to near 1470 and 1415 cm\(^{-1}\) due to isotope labeling. Consistent with this interpretation, a comparison of the IR spectra of unlabeled tyrosine and [\(^{13}\)C,\(^{15}\)C]Tyr reveals that tyrosine modes at 1514 cm\(^{-1}\) (aromatic ring C=C stretching vibration(s)) and 1456 cm\(^{-1}\) shift due to ring per-deuteration (1, 13, 18) in a very similar manner to that seen in the difference spectra of BR and BR-[\(^{13}\)C,\(^{15}\)C]Tyr.

In the 1290–1210 cm\(^{-1}\) region (Fig. 3B) tyrosine labeling causes a disappearance of the negative peak at 1276 cm\(^{-1}\) concomitant with an increase in negative intensity between 1249 and 1230 cm\(^{-1}\). This effect is consistent with the \(\sim 25\) cm\(^{-1}\) downshift of the phenolate C=O stretching mode near 1270 cm\(^{-1}\) in tyrosinate due to ring per-deuteration (1, 18). An increase in positive intensity in the BR-[\(^{13}\)C,\(^{15}\)C]Tyr difference spectrum seen near 1212 cm\(^{-1}\) may also be due to the downshift of a tyrosine C=OH stretch band centered at 1234 cm\(^{-1}\) in unlabeled BR.

The **Primary Photoproduct of BR\(_{548}\)**

The Configuration of the K\(_{560}\) Chromophore—Fully dark-adapted films contain as much as 67% BR\(_{548}\) (19). In order to obtain an FTIR difference spectrum of the primary photo-reaction of BR\(_{548}\), we subtracted a difference spectrum for the primary photoreaction of a fully light-adapted film (BR\(_{507}\)→K\(_{560}\)) (Fig. 4, solid) from that for a completely dark-adapted film obtained under identical conditions. Scaling during the subtraction was done interactively, so that the ethylenic stretching modes of the BR\(_{507}\) and K\(_{560}\) chromophores at 1528 and 1515 cm\(^{-1}\), respectively, vanished. This procedure led to the disappearance of most other BR\(_{507}\) and K\(_{560}\) chromophore peaks, and it enhanced negative peaks associated with BR\(_{548}\) chromophore vibrations (20, 21) including the Schiff base C=N stretch at 1636 cm\(^{-1}\), the ethylenic C=C stretch at 1536 cm\(^{-1}\), the NH rock at 1346, and C–C stretches at 1234 and 1185 cm\(^{-1}\). Peaks in the resonance Raman spectrum of BR\(_{548}\) at 1292 and 1167 cm\(^{-1}\) assigned to C–C stretches (21) are not observed, most likely because they are cancelled by positive peaks due to K\(_{560}\) and 1205 and 1177 cm\(^{-1}\), respectively.

The positive ethylenic line at 1521 cm\(^{-1}\) falls close to the frequencies expected for a species (K\(_{560}\)) with an absorption maximum near 610 nm, based on the linear correlation between the \(\lambda_{\text{max}}\) and the C=C stretching frequencies of BR photocycle intermediates (22). Other chromophore lines at 1315, 1205, 1177, and 1004 cm\(^{-1}\) can tentatively be assigned to the 12C–H in-plane rock, the 14–15 and 10–11 C–C stretches, and the 19CH\(_3\) rock, respectively, based on previous
work with all-trans and 13-cis isotopically labeled retinals (20, 21, 23) and the data presented below. The protonated Schiff base C=N stretch of K630 is surmised to fall near 1622 cm\(^{-1}\) because of the effects in the 1660–1550 cm\(^{-1}\) region produced by \(^2\)H–C15 and \(^2\)H–N labeling (data not shown). This frequency is very close to that expected for a 610-nm absorbing species from a linear plot of the C13=N stretch versus the visible \(\lambda_{\text{max}}\) of photocycle intermediates (6).

In contrast to the intense positive hydrogen-out-of-plane (HOOP) modes observed in the 1000–800 cm\(^{-1}\) region for the primary photointermediates of rhodopsin (24) and light-adapted bacteriorhodopsin (3) (cf. Fig. 4, top) by both resonance Raman and FTIR spectroscopy (25–27) weak HOOPS are observed for K630 (cf. Fig. 4, bottom). The intensification of HOOP modes has been attributed to significant single bond twisting of the chromophore. In view of the similarity between the resonance Raman and FTIR difference spectra for the BR548+K610 transition (27), the absence of intense HOOP modes in the BR548→K630 difference spectrum may indicate a more relaxed chromophore for K630. However, other possible mechanisms for a decrease in the IR activity of these modes cannot be completely excluded at this time.

In order to deduce the configuration of the K630 chromophore, spectra of this transition were obtained for br containing isotopically labeled retinals. Fig. 5 presents expansions of the BR548→K630 spectrum in the fingerprint region for normal br in \(\text{H}_2\text{O}\) and \(\text{D}_2\text{O}\) (A and B), br regenerated with \(^1\)C14–\(^1\)C15 retinal in \(\text{H}_2\text{O}\) and \(\text{D}_2\text{O}\) (C and D), and br regenerated with \(^2\)H–C15 retinal in \(\text{H}_2\text{O}\) (E). It is known, through comparison with model retinal-protonated Schiff base (RPSB) compounds, that certain \(^2\)H–C15 effects on the fingerprint region of br spectra are highly dependent on the configuration of the chromophore C13=C15 bond (3). Also, the magnitude of the upshift of the C14–C15 single bond stretch upon \(^2\)H/H exchange at the Schiff base nitrogen is known to be highly dependent on the C13=N bond conformation (8). Thus, analysis of the spectra from the above samples can be used to determine the retinal configuration for K630.

We first note that the prominent positive peak at 1205 cm\(^{-1}\) can be assigned to the C14–C15 stretch of K630 on the basis of its shift upon \(^1\)C14→\(^1\)C15 labeling (cf. Fig. 5C). As seen in Fig. 6, this peak for a 610-nm absorbing species falls on an empirical straight line correlation between the C14–C15 stretching frequency and the \(\lambda_{\text{max}}\) of 13-trans intermediates and the 13-trans RPSB. In contrast, all of the points which correspond to intermediates known to be 13-cis (as well as the point corresponding to the 13-cis RPSB) do not fall near this line or exhibit a straight line correlation. The latter may be due to uncertainty in assignment of the C14–C15 stretch. For example, the shoulder near 1185 cm\(^{-1}\) in K630 also contains significant C14=C15 character (28); thus this point may actually fall further from the all-trans line. Uncertainty also exists about the assignment of the position of the L630 C14=C15 stretch, which has also been assigned near 1155 cm\(^{-1}\) (28) and attributed to a cis conformation about the C13=C15 bond. Since the 13-trans straight line correlation is based on only a few data points, the conclusion that the K630 species contains a 13-trans chromophore is not compelling. However, additional considerations discussed below also support this conclusion.

Table I summarizes the effects \(^2\)H–C15 substitution of the chromophore has on the K630 spectrum and compares them to the effects previously seen in spectra of the 13-cis and 13-trans RPSB model compounds (3). It is clear from examination of this table that the \(^2\)H–C15 substitution effects, which are diagnostic for the C13=C14 configuration, are more 13-trans-like than 13-cis-like. In particular, the K630 HOOP mode alterations upon \(^2\)H–C15 substitution include a decrease in intensity of the weak line at 877 cm\(^{-1}\). This is similar to the disappearance of the 870 cm\(^{-1}\) line in the trans model compound. The major HOOP effects seen in the cis model compound include the apparent shift of a line at 972 cm\(^{-1}\) to 975 cm\(^{-1}\), as well as the dramatic appearance of an intense new line at 988 cm\(^{-1}\). None of these effects are seen in the K630 spectrum.

In the cis model compound spectrum, a line at 1174 cm\(^{-1}\) shifts to 1168 cm\(^{-1}\); however, in the K630 spectrum the line at 1177 cm\(^{-1}\) does not shift upon \(^2\)H–C15 substitution (cf. Fig. 5, A and E). Furthermore, in the cis model compound, a line at 1206 cm\(^{-1}\) does not shift upon \(^2\)H–C15 substitution (3), but the 1205 cm\(^{-1}\) lines does shift in the K630 spectrum (cf. Fig. 5E). Also, the decrease at 1277 cm\(^{-1}\) concomitant with the appearance of a small peak at 1262 cm\(^{-1}\) in the \(^2\)H–C15 K630 spectrum (cf. Fig. 5E) is quite similar to effects at 1279 and 1271 cm\(^{-1}\) in the trans model compound. Finally, other effects such as the decrease at 1412 cm\(^{-1}\) and the increase at 1600 cm\(^{-1}\) are much more similar to the effects seen in the 13-trans RPSB than the 13-cis RPSB.

While complete agreement between the K630 spectrum and
the 13-trans model compound is not observed, the environment surrounding the chromophore in bR as well as the configuration of the retinal C=N bond (see below) most likely influence the electronic character of many bonds, thus affecting the frequencies and coupling of many modes. However, the generally good agreement between the \( K_{\text{lo}} \) and 13-trans RPSB model compound \(^2\)H-C\(^1\)S effects (as well as the disagreement between the \( K_{\text{lo}} \) and 13-cis model compound \(^2\)H-C\(^1\)S spectra) and the empirical correlation seen in Fig. 6 support but do not unequivocally establish a 13-trans assignment for \( K_{\text{lo}} \).

Assignment of the C\(^1\)S-C\(^1\)S stretch and observation of its behavior upon N\(^-\)H substitution can also be used to determine the C=N bond configuration (8). When the C\(^1\)S-N bond is cis, a significant upshift in the C\(^1\)S-C\(^1\)S single bond stretch of the chromophore is observed upon deuto substitution at the Schiff base nitrogen. Conversely, when the C\(^1\)S-N bond is trans, shifts of less than 5 cm\(^{-1}\) are seen because of decreased coupling between the individual modes. As noted, comparison of spectra in Fig. 5, A and C, indicates the \( K_{\text{lo}} \) C\(^1\)S-C\(^1\)S stretch is localized to a large degree in a band at 1205 cm\(^{-1}\), as this line is seen to shift down due to di-\(^13\)C labeling, very likely to near 1195 cm\(^{-1}\). (A similar 10 cm\(^{-1}\) shift has previously been observed in model compounds, cf. Ref. 21.) Comparison of spectra in Fig. 5, A and B, indicates the 1205 line has upshifted to 1205 cm\(^{-1}\) upon deuto substitution at the Schiff base nitrogen, this line (1234 cm\(^{-1}\)) and then appears to downshift to 1224 cm\(^{-1}\) for the di-\(^13\)C sample in \(^2\)H\(_2\)O (spectrum D). The magnitude of this downshift is exactly that of the 1205 cm\(^{-1}\) line upon di-\(^13\)C labeling. Since the C\(^1\)S-C\(^1\)S stretch upshifts 30 cm\(^{-1}\) upon \(^2\)H-N substitution, we conclude the C\(^1\)S-N bond is cis in \( K_{\text{lo}} \).

**Protein Groups Involved in the Formation of \( K_{\text{lo}} \)---** A notable feature of the BR\(^{\text{res}}\)---\( K_{\text{lo}} \) difference spectrum is the absence of peaks near 853, 842, and 833 cm\(^{-1}\) due to the Fermi resonance doublet of tyrosine (1) (cf. Fig. 7A, top dashed line) which have previously been associated with the protonation of a tyrosinate group in the BR\(^{\text{res}}\)---\( K_{\text{lo}} \) transition (cf. Fig. 7, solid lines). These peaks disappear upon introduction of perdeuterated tyrosine into bR, as seen in Fig. 7B (bottom dashed line). The Fermi doublet peaks are those tyrosine vibrations most sensitive to protonation changes. Their absence in the BR\(^{\text{res}}\)---\( K_{\text{lo}} \) difference spectrum indicates no tyrosine group undergoes a protonation change during this reaction.

Additional protein change(s) involving one or more peptide amide groups is indicated by the prominent negative peak at 1660 cm\(^{-1}\) and a smaller 1668 cm\(^{-1}\) band (cf. Fig. 4). The major amide I band of bR is observed at 1660 cm\(^{-1}\), and thus the peak in the difference spectrum may indicate a perturbation in amide structure during this transition. A peak at 1662 cm\(^{-1}\) is observed upon formation of BR\(^{\text{res}}\) from BR\(^{\text{lo}}\) (cf. Fig. 2) and may reflect a change in the same peptide groups. Since the C=N stretch of the BR\(^{\text{res}}\) retinal Schiff base has previously been assigned at 1636 cm\(^{-1}\) (20, 21), we can exclude the possibility that the negative 1660 cm\(^{-1}\) peak is due to this mode.

We also note that small negative and positive peaks at 1740 and 1730 cm\(^{-1}\) due to the carboxyl COOH stretches of aspartic acid or glutamic acid, seen in BR\(^{\text{lo}}\)---\( K_{\text{lo}} \) difference spectra in BR\(^{\text{lo}}\)---\( K_{\text{lo}} \) (6, 7), are also seen in the BR\(^{\text{res}}\)---\( K_{\text{lo}} \) difference spectrum (cf. Fig. 4). These peaks are due either to a hydrogen bond perturbation of a carboxyl group or the simultaneous protonation/deprotonation of two carboxyls.

**UV-visible Difference Spectra**

Fig. 8A and the expansion in Fig. 8B show the UV-visible difference spectrum for light adaptation (the BR\(^{\text{res}}\)---BR\(^{\text{lo}}\) transition). The film was kept in total darkness for over 2 h before being illuminated with yellow light (cf. "Experimental Procedures") to produce the light-adapted BR\(^{\text{lo}}\) state. Unlike the UV differences previously measured (1, 13) for the BR\(^{\text{res}}\)---\( K_{\text{lo}} \) and M\(_{412}\) transitions, the dominant feature in the UV region for light adaptation is a positive peak at 275 nm. Retinal model compound studies indicate that this peak is not due to the chromophore (30) but is consistent with a tryptophan absorption.\(^3\) Two positive peaks are also observed at 244 and 304 nm which are consistent with the deprotonation of a tyrosine during the BR\(^{\text{res}}\)---BR\(^{\text{lo}}\) transition. However, the intensity of the 243-nm peak relative to the 304-nm peak is smaller than expected on the basis of model compound tyrosine deprotonation difference spectra (1). This may be due to cancellation near 243 nm of retinal contributions at

\(^3\) P. L. Ahl and K. J. Rothschild, unpublished data.
FIG. 4. Comparison of the FTIR difference spectra of the BR6,0→K630 (solid) and BRsox→K570 (dashed) transitions in the 1800–800 cm⁻¹ region. The BR6,0→K630 spectrum is computed by subtracting the BRsox→K630 spectrum from a BR→K spectrum obtained with a completely dark-adapted film (see “Experimental Procedures”).

FIG. 5. FTIR difference spectra of the BR6,0→K630 transition in the fingerprint region for BR in H₂O (A, solid line), and in D₂O (B, dashed line), BR regenerated with tC₁₄-tC₁₅ retinal in H₂O (C, solid line), and in D₂O (D, dashed line), and BR regenerated with ²H-⁻C₁₅ retinal in H₂O (E). All spectra were obtained as described under “Experimental Procedures.”

the same wavelength which were found in difference spectra for conversion of 13-cis to all-trans protonated Schiff base retinals (30).

In Fig. 8, C and D, we compare the UV-visible difference spectra obtained under identical conditions for the primary phototransition of light-adapted (top) and dark-adapted (bottom) humidified films. In the visible region, we find a blue shift of the absorption maxima for the negative and positive peaks of dark-adapted relative to light-adapted films due to the occurrence in the dark-adapted films of the BRM⁺elO transition. In the case of the UV region (Fig. 8D), except for the “filling in” of the negative K₆₃₀ difference peak at 270 nm observed in the light-adapted difference spectra, no new major UV difference peaks are observed in the dark-adapted case. We do find, however, an apparent reduction in the doublet at 290 and 294 nm. Furthermore, interactive subtraction (not shown) of the light-adapted and dark-adapted difference spectra in Fig. 8C so that the visible region resembled previously reported BRsox→K₆₃₀ visible difference spectra (12) also revealed that the peaks near 300 and 243 nm are reduced or absent. In a previous study (1) of the BRsox→K₆₃₀ transition, the negative peak (cf. expansion in Fig. 8D, top) at 244 nm was assigned to a tyrosine protonation and the positive doublet at 290 and 294 nm to a tryptophan hydrogen bonding change on the basis of UV absorption measurements of model compounds and their perturbation difference spectra (29–31). Thus, the present results support our conclusion based on FTIR spectroscopy that a tyrosinate protonation does not occur during the primary phototransition in the dark-adapted
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DISCUSSION

The present results on dark adaptation and the primary photoreaction of BR₇₀ add to the conclusions already reached on the basis of FTIR and UV difference spectroscopy pertaining to tyrosine protonation changes occurring during the BR photocycle (1, 4, 5, 13, 14). The major conclusions of this study are summarized below.

FTIR of Bacteriorhodopsin

A comparison of a BR₇₀-K₆₃₀ difference spectrum (solid line) and a BR₄₄₆-K₆₃₀ spectrum (dashed line) in the 860-820 cm⁻¹ region. B, comparison of BR₄₄₆ (solid line) and BR-[²H₄]Tyr (dashed line) BR₄₄₆-K₆₃₀ difference spectra in the Fermi resonance region showing the disappearance in the labeled spectrum of tyrosine and tyrosinate peaks which indicate a tyrosinate protonation during the BR photocycle.
FTIR of Bacteriorhodopsin

Fig. 8. The UV-visible difference spectrum for light adaptation at room temperature in the 700–200-nm region (A) and in an expanded view of the 400–200-nm UV region (B). The spectrum shown is the average of two differences recorded from a humidified purple membrane film. Absorbance scale markers are 0.002 (A) and 0.01 absorbance unit (A). C, comparison of the UV-visible difference spectra in the 700–200-nm region; D, expanded view of the 400–200-nm UV region for the primary photoproduct of light-adapted (top) and dark-adapted (bottom) bR films at 80 K. Scale markers are 0.01 (D) and 0.05 absorbance unit (C) for both top and bottom spectra (cf. "Experimental Procedures" for further details).

Fig. 9. A summary of observed chromophore isomerizations and protein changes during the early photochemistry of BR670 and BR648. The positioning of the tyrosine group is not meant to imply it is the sole counterion to the Schiff base. Note that 15-anti and 15-syn are equivalent to 15-trans and 15-cis, respectively.

which possesses a tyrosinate ion. Since the existence of a tyrosinate would require stabilization by one or more positive groups, direct interaction of the tyrosine with the Schiff base proton as previously proposed (1) is quite plausible. If this is the case, the tyrosinate must be positioned such that the 13-trans,15-trans configuration confers a more energetically favorable interaction than any of the other three configurations. However, it is possible that additional groups in bR interact electrostatically with the Schiff base.

Examination of proposed two-dimensional folding patterns for bR (38, 39) reveals that at least one tyrosine (Tyr-185) is in a position to interact with the C=N group of the chromophore. Indeed, recent FTIR studies on a bR mutant that has Tyr-185 replaced by a phenylalanine indicate that vibrations in the BR670→K630 difference spectrum attributable to the BR570 tyrosinate are absent in the site-specific mutant (40). Thus, it is likely that it is this residue which gives rise to the tyrosinate to tyrosine signals in both the primary phototransition of light-adapted bR as well as during dark adaptation.

Finally, determination of the chromophore configuration for K630 reveals a consistent pattern for wavelength regulation in BR570, BR548, K300, and K630 whose chromophores have all four possible combinations of isomerization about the C13=C14 and C=N bonds. As seen in Fig. 9, the 13-trans,15-trans (BR570) and 13-cis,15-cis (BR548) states result in a similar orientation of the Schiff base proton, relative to the long axis of the retinal chromophore (the axis drawn along the polyene chain). The absorption maxima of these two intermediates are relatively close, being separated by only 20 nm. Similarly, the 13-cis,15-trans (K300) and 13-trans,15-cis (K630) forms, which have significantly red-shifted λmax, also have a similar N–H bond dipole orientation (relative to the long axis). A recent polarized FTIR study has determined that the average angle of the long axis of the chromophore remains fixed during the primary photochemistry of bR (37); thus, the major factor which appears to be associated with the λmax of these intermediates is the orientation of the N–H bond dipole and not the configuration about either the C13=C14 or C=N bonds.

In conclusion, these FTIR and UV difference measurements of the BR548 and K630 intermediates of bacteriorhodopsin provide new information about structural changes occurring during the photochemistry of bR. A correlation appears to exist between the configuration of the retinylidene chromophore, particularly near the 13=14 and C=N double bonds, and the state of protonation of a tyrosine group. The most consistent picture is that the positive charge of the Schiff base stabilizes a tyrosinate group, most likely Tyr-185. The movement of this charge during dark adaptation or during the primary phototransition of the light-adapted photocycle destabilizes this tyrosinate causing its protonation. In the case of the primary phototransition for the dark-adapted photocycle, a protonation signal is not observed since the tyrosinate group has already become protonated during the formation of BR548.

Additional progress will be possible by studying the effects of site-specific mutagenesis on the UV-visible (41) and FTIR difference spectra of the various bR transitions. Such studies are presently underway to determine the origin of the tyrosine signals detected during the FTIR difference spectra of the light to dark adaptation reaction and the carboxyl signals.
detected during primary phototransition of both the light and dark photocycles.

Acknowledgments—We wish to thank Drs. H. Jurgen Weber for providing the JW5 bacterial strain, J. A. Pardoen and C. Winkel for synthesizing the labeled retinals, and S. K. Das Gupta for synthesizing the labeled tyrosine. We also wish to thank M. D. Tran, J. Gillespie, and T. Earnest for technical assistance, and M. S. Brainan, H. G. Khorana, L. Stern, and S. O. Smith for helpful discussions.

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