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# Tyrosine Protonation Changes in Bacteriorhodopsin

A FOURIER TRANSFORM INFRARED STUDY OF BR<sub>548</sub> AND ITS PRIMARY PHOTOPRODUCT\*

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The structural alterations which occur in bacteriorhodopsin (bR) during dark adaptation (BR<sub>570</sub>→BR<sub>548</sub>) and the primary phototransition of the dark photocycle (BR<sub>548</sub>→K<sub>610</sub><sup>D</sup>) 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 BR<sub>570</sub> at physiological temperature and that it protonates during formation of BR<sub>548</sub>. A tyrosinate protonation has also been observed at low temperature during the primary phototransition of BR<sub>570</sub> to the red-shifted photoproduct K<sub>630</sub> (1). However, we now find that no tyrosine protonation change occurs during the primary phototransition of BR<sub>548</sub> to the red-shifted intermediate K<sub>610</sub><sup>D</sup>. Through analysis of bR containing isotopically labeled retinals, it was also determined that the chromophore of K<sub>610</sub><sup>D</sup> exists in a 13-*trans*,15-*cis* configuration. On the basis of this evidence and previous studies on the structure of the chromophore in BR<sub>570</sub>, BR<sub>548</sub>, and K<sub>630</sub>, 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 BR<sub>570</sub> chromophore. Isomerization of the chromophore about either the C<sub>13</sub>=C<sub>14</sub> or C=N bond disrupts this interaction causing a protonation of the tyrosinate.

Bacteriorhodopsin (bR),<sup>1</sup> the 26-kDa retinal-containing protein of the purple membrane of *Halobacteria halobium*,

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<sup>1</sup> The abbreviations used are: bR, bacteriorhodopsin; bR-[<sup>2</sup>H<sub>4</sub>]Tyr, bR containing ring per-deuterated tyrosines; 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 (BR<sub>570</sub>) 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 K<sub>630</sub> involves a C<sub>13</sub>=C<sub>14</sub> *trans* to *cis* isomerization of the retinylidene chromophore (3). Protein changes during K<sub>630</sub> 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 BR<sub>570</sub> species converts to a blue-shifted form designated BR<sub>548</sub>. Recent resonance Raman and NMR studies indicate that in contrast to the 13-*trans*,15-*trans* configuration of the retinylidene chromophore of BR<sub>570</sub>, the BR<sub>548</sub> chromophore exists in a 13-*cis*,15-*cis* configuration (8, 9). The photocycle of BR<sub>548</sub>, as shown in Fig. 1 (10-12), also exhibits an early red-shifted intermediate, K<sub>610</sub><sup>D</sup>.<sup>2</sup> However, the "dark photocycle" which occurs upon light absorption by BR<sub>548</sub> 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 BR<sub>570</sub> species. In general, a comparison of the molecular changes that accompany the BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> and BR<sub>570</sub>→K<sub>630</sub> 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 BR<sub>570</sub>→BR<sub>548</sub> transition) and during the primary phototransition of the dark photocycle (the BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> 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 BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> transition. We have also determined that the retinylidene chromophore in K<sub>610</sub><sup>D</sup> exists in a 13-*trans*,15-*cis* configuration. On the basis of this evidence it appears

<sup>2</sup> The primary photoproduct of BR<sub>548</sub> formed at 77 K is referred to by Iwasa *et al.* (12) as batho-13-*cis*-bR in analogy to the K<sub>630</sub> photoproduct of BR<sub>570</sub>, 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<sup>2</sup> and the decay product at this temperature, C<sub>610</sub>. C refers to the "cis" photocycle of BR<sub>548</sub> in contrast to the "trans" photocycle of BR<sub>570</sub>. In this paper, we use the designation K<sub>610</sub><sup>D</sup> (i.e. the K photoproduct of the dark photocycle) in order to stress the analogy to the K photoproduct of BR<sub>570</sub>. 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.

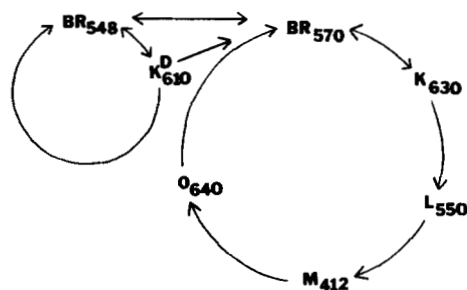


FIG. 1. A simplified representation of cyclic BR<sub>548</sub> and BR<sub>570</sub> photochemistry. The subscripts denote the visible absorption maxima of the intermediates. At room temperatures, K forms in less than 6 ps, L in 2  $\mu$ s, M in 40  $\mu$ s, and BR<sub>570</sub> reforms in  $\sim$ 10 ms. Precise rate constants for the dark cycle have not been determined.

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- $^{2}\text{H}_4$ Tyr) has been described previously (1, 13, 14). Purple membrane sheets containing either normal bR or bR- $^{2}\text{H}_4$ Tyr were isolated by the method of Oesterhelt and Stoekenius (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  $^{13}\text{C}_{14}$ ,  $^{13}\text{C}_{15}$ - or  $^2\text{H}$ -C<sub>15</sub>-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 of a concentrated suspension. Prior to loading the sample in a sealed cell, purple membrane films were rehydrated by direct exposure to liquid H<sub>2</sub>O or  $^2\text{H}_2\text{O}$  for 2–5 min, and the excess liquid was shaken off. The amount of H<sub>2</sub>O or  $^2\text{H}_2\text{O}$  in the film was monitored by comparing the integrated intensities of the bands at 3300 or 2500  $\text{cm}^{-1}$  to that of the amide I at 1660  $\text{cm}^{-1}$ . Ratios of 6:1 or higher were taken as indicative of adequate hydration.

**FTIR Difference Spectroscopy**—BR<sub>570</sub>→BR<sub>548</sub> difference spectra were computed by subtracting an FTIR spectrum of a hydrated purple membrane film recorded at 310 K while the film was illuminated by 550-nm light (550-nm broad band filter, Ditrac Optics, Hudson, MA) 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<sub>570</sub>→K<sub>630</sub> 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<sub>548</sub>→K<sub>630</sub> 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 ( $\sim$ 2 K/s) to 81 K. All measurements were made on a Nicolet MX-1 spectrometer interfaced to a Nicolet 1200S 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  $\text{cm}^{-1}$ .

**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 (Waltham, MA) liquid nitrogen cryostat and interfaced to an Apple IIe 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- $^{2}\text{H}_4$ Tyr (*dashed*). These spectra represent the BR<sub>570</sub>→BR<sub>548</sub> reaction. Several small but reproducible changes are caused by the isotopic label. In the 1450 to 1550  $\text{cm}^{-1}$  region (Fig. 3A), two small positive peaks, one centered at about 1510  $\text{cm}^{-1}$  and the other at 1453  $\text{cm}^{-1}$  (*cf.* Fig. 3A), are reduced in the bR- $^{2}\text{H}_4$ Tyr difference spectrum, possibly shifting to near 1470 and 1415  $\text{cm}^{-1}$  due to isotope labeling. Consistent with this interpretation, a comparison of the IR spectra of unlabeled tyrosine and  $^{2}\text{H}_4$ Tyr reveals that tyrosine modes at 1514  $\text{cm}^{-1}$  (aromatic ring C=C stretching vibration(s)) and 1456  $\text{cm}^{-1}$  shift due to ring per-deutero labeling (1, 13, 18) in a very similar manner to that seen in the difference spectra of bR and bR- $^{2}\text{H}_4$ Tyr.

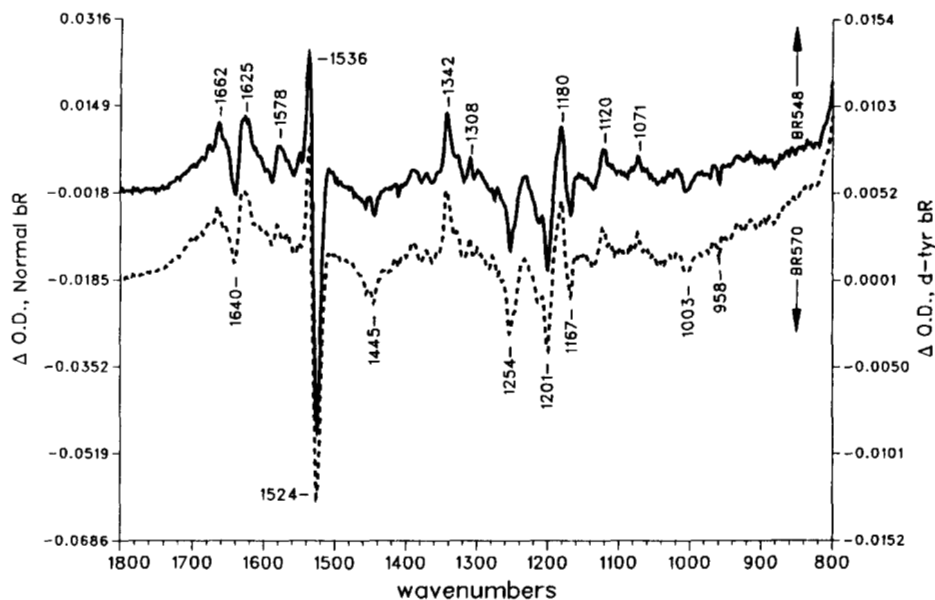
In the 1290–1210  $\text{cm}^{-1}$  region (Fig. 3B) tyrosine labeling causes a disappearance of the negative peak at 1276  $\text{cm}^{-1}$  concomitant with an increase in negative intensity between 1249 and 1230  $\text{cm}^{-1}$ . This effect is consistent with the  $\sim$ 25  $\text{cm}^{-1}$  downshift of the phenolate C–O<sup>−</sup> stretching mode near 1270  $\text{cm}^{-1}$  in tyrosinate due to ring per-deuteration (1, 18). An increase in positive intensity in the bR- $^{2}\text{H}_4$ Tyr difference spectrum seen near 1212  $\text{cm}^{-1}$  may also be due to the downshift of a tyrosine C–OH stretch band centered at 1234  $\text{cm}^{-1}$  in unlabeled bR.

##### The Primary Photoproduct of BR<sub>548</sub>

**The Configuration of the K<sub>630</sub><sup>D</sup> Chromophore**—Fully dark-adapted films contain as much as 67% BR<sub>548</sub> (19). In order to obtain an FTIR difference spectrum of the primary photo-reaction of BR<sub>548</sub>, we subtracted a difference spectrum for the primary photoreaction of a fully light-adapted film (BR<sub>570</sub>→K<sub>630</sub>) (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<sub>570</sub> and K<sub>630</sub> chromophores at 1528 and 1515  $\text{cm}^{-1}$ , respectively, vanished. This procedure led to the disappearance of most other BR<sub>570</sub> and K<sub>630</sub> chromophore peaks, and it enhanced negative peaks associated with BR<sub>548</sub> chromophore vibrations (20, 21) including the Schiff base C=N stretch at 1636  $\text{cm}^{-1}$ , the ethylenic C=C stretch at 1536  $\text{cm}^{-1}$ , the NH rock at 1346, and C–C stretches at 1234 and 1185  $\text{cm}^{-1}$ . Peaks in the resonance Raman spectrum of BR<sub>548</sub> at 1202 and 1167  $\text{cm}^{-1}$  assigned to C–C stretches (21) are not observed, most likely because they are cancelled by positive peaks due to K<sub>630</sub><sup>D</sup> and 1205 and 1177  $\text{cm}^{-1}$ , respectively.

The positive ethylenic line at 1521  $\text{cm}^{-1}$  falls close to the frequencies expected for a species (K<sub>630</sub><sup>D</sup>) 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  $\text{cm}^{-1}$  can tentatively be assigned to the 12C–H in-plane rock, the 14–15 and 10–11 C–C stretches, and the 19CH<sub>3</sub> rock, respectively, based on previous

FIG. 2. Comparison of FTIR difference spectra of the light (BR<sub>570</sub>, negative peaks) to dark (BR<sub>548</sub>, positive peaks) transition at 310 K for normal bR (solid line) and bR containing ring per-deuterated tyrosines (dashed line) in the 1800–800 cm<sup>-1</sup> region. Spectra are at 2 cm<sup>-1</sup> resolution and are unsmoothed. Scaling of the spectra is done by matching the intensities of the chromophore peaks.



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 K<sub>610</sub><sup>D</sup> is surmised to fall near 1622 cm<sup>-1</sup> because of the effects in the 1660–1550 cm<sup>-1</sup> region produced by <sup>2</sup>H-C<sub>15</sub> and <sup>2</sup>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 C<sub>15</sub>=N stretch versus the visible λ<sub>max</sub> of photocycle intermediates (6).

In contrast to the intense positive hydrogen-out-of-plane (HOOP) modes observed in the 1000–800 cm<sup>-1</sup> 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 K<sub>610</sub><sup>D</sup> (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 BR<sub>570</sub>→K<sub>630</sub> transition (27), the absence of intense HOOP modes in the BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> difference spectrum may indicate a more relaxed chromophore for K<sub>610</sub><sup>D</sup>. 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 K<sub>610</sub><sup>D</sup> chromophore, spectra of this transition were obtained for bR containing isotopically labeled retinals. Fig. 5 presents expansions of the BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> spectrum in the fingerprint region for normal bR in H<sub>2</sub>O and <sup>2</sup>H<sub>2</sub>O (A and B), bR regenerated with <sup>13</sup>C<sub>14</sub>-<sup>13</sup>C<sub>15</sub> retinal in H<sub>2</sub>O and <sup>2</sup>H<sub>2</sub>O (C and D), and bR regenerated with <sup>2</sup>H-C<sub>15</sub> retinal in H<sub>2</sub>O (E). It is known, through comparison with model retinal-protonated Schiff base (RPSB) compounds, that certain <sup>2</sup>H-C<sub>15</sub> effects on the fingerprint region of bR spectra are highly dependent on the configuration of the chromophore C<sub>13</sub>=C<sub>14</sub> bond (3). Also, the magnitude of the upshift of the C<sub>14</sub>-C<sub>15</sub> single bond stretch upon <sup>2</sup>H/H exchange at the Schiff base nitrogen is known to be highly dependent on the C<sub>15</sub>=N bond conformation (8). Thus, analysis of the spectra from the above samples can be used to determine the retinal configuration for K<sub>610</sub><sup>D</sup>.

We first note that the prominent positive peak at 1205 cm<sup>-1</sup> can be assigned to the C<sub>14</sub>-C<sub>15</sub> stretch of K<sub>610</sub><sup>D</sup> on the basis of its shift upon <sup>13</sup>C<sub>14</sub>-<sup>13</sup>C<sub>15</sub> 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 C<sub>14</sub>-C<sub>15</sub> stretching

frequency and the λ<sub>max</sub> 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 C<sub>14</sub>-C<sub>15</sub> stretch. For example, the shoulder near 1185 cm<sup>-1</sup> in K<sub>630</sub> also contains significant C<sub>14</sub>-C<sub>15</sub> 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 L<sub>550</sub> C<sub>14</sub>-C<sub>15</sub> stretch mode, which has also been assigned near 1155 cm<sup>-1</sup> (28) and attributed to a *cis* conformation about the C<sub>14</sub>-C<sub>15</sub> bond. Since the 13-*trans* straight line correlation is based on only a few data points, the conclusion that the K<sub>610</sub><sup>D</sup> species contains a 13-*trans* chromophore is not compelling. However, additional considerations discussed below also support this conclusion.

Table I summarizes the effects <sup>2</sup>H-C<sub>15</sub> substitution of the chromophore has on the K<sub>610</sub><sup>D</sup> 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 <sup>2</sup>H-C<sub>15</sub> substitution effects, which are diagnostic for the C<sub>13</sub>=C<sub>14</sub> configuration, are more 13-*trans*-like than 13-*cis*-like. In particular, the K<sub>610</sub><sup>D</sup> HOOP mode alterations upon <sup>2</sup>H-C<sub>15</sub> substitution include a decrease in intensity of the weak line at 877 cm<sup>-1</sup>. This is similar to the disappearance of the 870 cm<sup>-1</sup> 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<sup>-1</sup> to 975 cm<sup>-1</sup>, as well as the dramatic appearance of an intense new line at 988 cm<sup>-1</sup>. None of these effects are seen in the K<sub>610</sub><sup>D</sup> spectrum.

In the *cis* model compound spectrum, a line at 1174 cm<sup>-1</sup> shifts to 1168 cm<sup>-1</sup>; however, in the K<sub>610</sub><sup>D</sup> spectrum the line at 1177 cm<sup>-1</sup> does not shift upon <sup>2</sup>H-C<sub>15</sub> substitution (cf. Fig. 5, A and E). Furthermore, in the *cis* model compound, a line at 1206 cm<sup>-1</sup> does not shift upon <sup>2</sup>H-C<sub>15</sub> substitution (3), but the 1205 cm<sup>-1</sup> lines does shift in the K<sub>610</sub><sup>D</sup> spectrum (cf. Fig. 5E). Also, the decrease at 1277 cm<sup>-1</sup> concomitant with the appearance of a small peak at 1262 cm<sup>-1</sup> in the <sup>2</sup>H-C<sub>15</sub> K<sub>610</sub><sup>D</sup> spectrum (cf. Fig. 5E) is quite similar to effects at 1279 and 1271 cm<sup>-1</sup> in the *trans* model compound. Finally, other effects such as the decrease at 1412 cm<sup>-1</sup> and the increase at 1600 cm<sup>-1</sup> are much more similar to the effects seen in the 13-*trans* RPSB than the 13-*cis* RPSB.

While complete agreement between the K<sub>610</sub><sup>D</sup> spectrum and

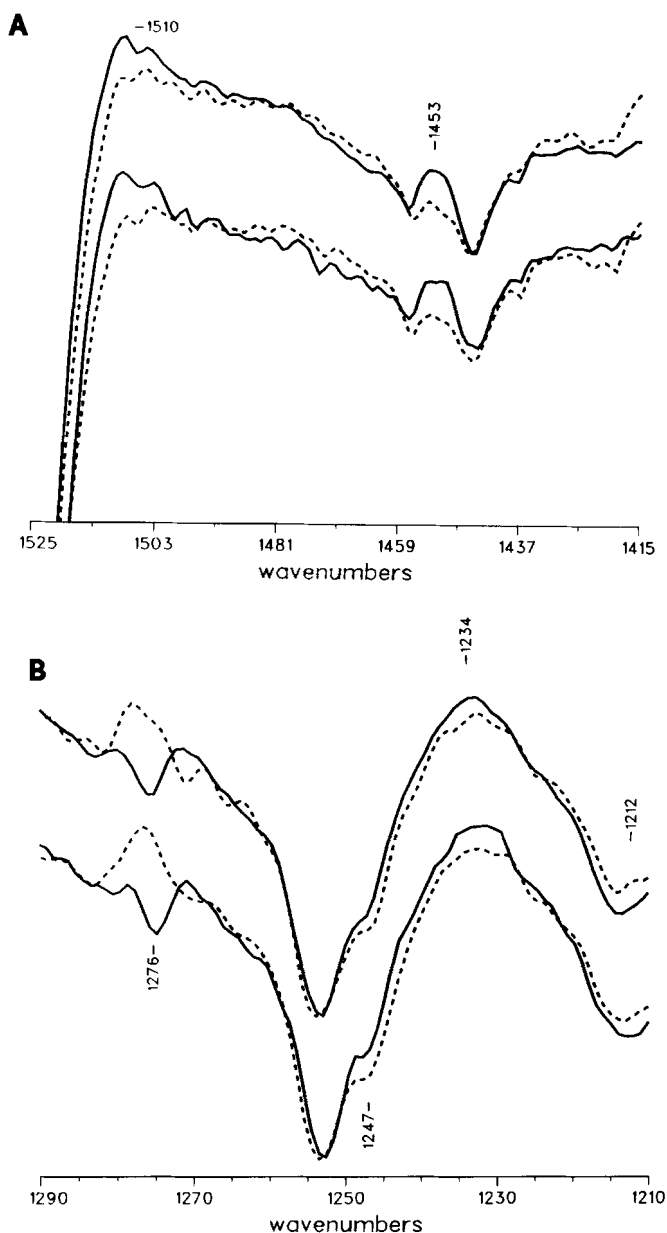


FIG. 3. A, expanded overlay of the spectra in Fig. 1 in the 1525–1415  $\text{cm}^{-1}$  region. Two additional spectra of two other samples are also shown to demonstrate reproducibility. B, same as in A but in the 1290–1210  $\text{cm}^{-1}$  region.

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  $K_{610}^D$  and 13-*trans* RPSB model compound  $^2\text{H-C}_{15}$  effects (as well as the disagreement between the  $K_{610}^D$  and 13-*cis* model compound  $^2\text{H-C}_{15}$  spectra) and the empirical correlation seen in Fig. 6 support but do not unequivocally establish a 13-*trans* assignment for  $K_{610}^D$ .

Assignment of the  $\text{C}_{14}\text{-C}_{15}$  stretch and observation of its behavior upon  $\text{N-}^2\text{H}$  substitution can also be used to determine the C=N bond configuration (8). When the  $\text{C}_{15}=\text{N}$  bond is *cis*, a significant upshift in the  $\text{C}_{14}\text{-C}_{15}$  single bond stretch of the chromophore is observed upon deuterio substitution at the Schiff base nitrogen. Conversely, when the  $\text{C}_{15}=\text{N}$  bond is *trans*, shifts of less than 5  $\text{cm}^{-1}$  are seen because of de-

creased coupling between the individual modes. As noted, comparison of spectra in Fig. 5, A and C, indicates the  $K_{610}^D$   $\text{C}_{14}\text{-C}_{15}$  stretch is localized to a large degree in a band at 1205  $\text{cm}^{-1}$ , as this line is seen to shift down due to  $\text{di-}^{13}\text{C}$  labeling, very likely to near 1195  $\text{cm}^{-1}$ . (A similar 10  $\text{cm}^{-1}$  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  $\text{cm}^{-1}$  upon deuterio substitution at the Schiff base nitrogen, this line (1234  $\text{cm}^{-1}$ ) and then appears to downshift to 1224  $\text{cm}^{-1}$  for the  $\text{di-}^{13}\text{C}$  sample in  $^2\text{H}_2\text{O}$  (spectrum D). The magnitude of this downshift is exactly that of the 1205  $\text{cm}^{-1}$  line upon  $\text{di-}^{13}\text{C}$  labeling. Since the  $\text{C}_{14}\text{-C}_{15}$  stretch upshifts 30  $\text{cm}^{-1}$  upon  $^2\text{H-N}$  substitution, we conclude the  $\text{C}_{15}=\text{N}$  bond is *cis* in  $K_{610}^D$ .

**Protein Groups Involved in the Formation of  $K_{610}^D$** —A notable feature of the  $\text{BR}_{548}\rightarrow\text{K}_{610}^D$  difference spectrum is the absence of peaks near 853, 842, and 833  $\text{cm}^{-1}$  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  $\text{BR}_{570}\rightarrow\text{K}_{630}$  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  $\text{BR}_{548}\rightarrow\text{K}_{610}^D$  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  $\text{cm}^{-1}$  and a smaller 1668  $\text{cm}^{-1}$  band (cf. Fig. 4). The major amide I band of bR is observed at 1660  $\text{cm}^{-1}$ , and thus the peak in the difference spectrum may indicate a perturbation in amide structure during this transition. A peak at 1662  $\text{cm}^{-1}$  is observed upon formation of  $\text{BR}_{548}$  from  $\text{BR}_{570}$  (cf. Fig. 2) and may reflect a change in the same peptide groups. Since the C=N stretch of the  $\text{BR}_{548}$  retinal Schiff base has previously been assigned at 1636  $\text{cm}^{-1}$  (20, 21), we can exclude the possibility that the negative 1660  $\text{cm}^{-1}$  peak is due to this mode.

We also note that small negative and positive peaks at 1740 and 1730  $\text{cm}^{-1}$  due to the carboxyl COOH stretches of aspartic acid or glutamic acid, seen in  $\text{BR}_{570}\rightarrow\text{K}_{630}$  difference spectra (6, 7), are also seen in the  $\text{BR}_{548}\rightarrow\text{K}_{610}^D$  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  $\text{BR}_{548}\rightarrow\text{BR}_{570}$  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  $\text{BR}_{570}$  state. Unlike the UV differences previously measured (1, 13) for the  $\text{BR}_{570}\rightarrow\text{K}_{630}$  and  $\text{M}_{412}$  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.<sup>3</sup> Two positive peaks are also observed at 244 and 304 nm which are consistent with the deprotonation of a tyrosine during the  $\text{BR}_{548}\rightarrow\text{BR}_{570}$  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

<sup>3</sup> P. L. Ahl and K. J. Rothschild, unpublished data.

FIG. 4. Comparison of the FTIR difference spectra of the BR<sub>570</sub>→K<sub>630</sub> (solid) and BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> (dashed) transitions in the 1800–800 cm<sup>-1</sup> region. The BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> spectrum is computed by subtracting the BR<sub>570</sub>→K<sub>630</sub> spectrum from a BR→K spectrum obtained with a completely dark-adapted film (see "Experimental Procedures").

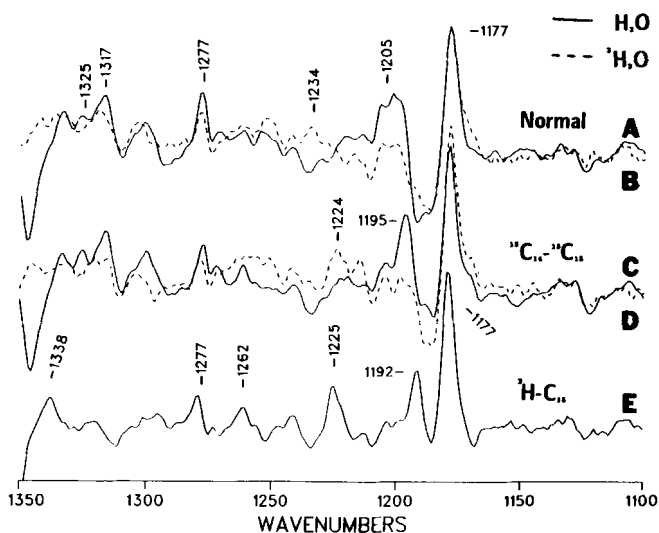
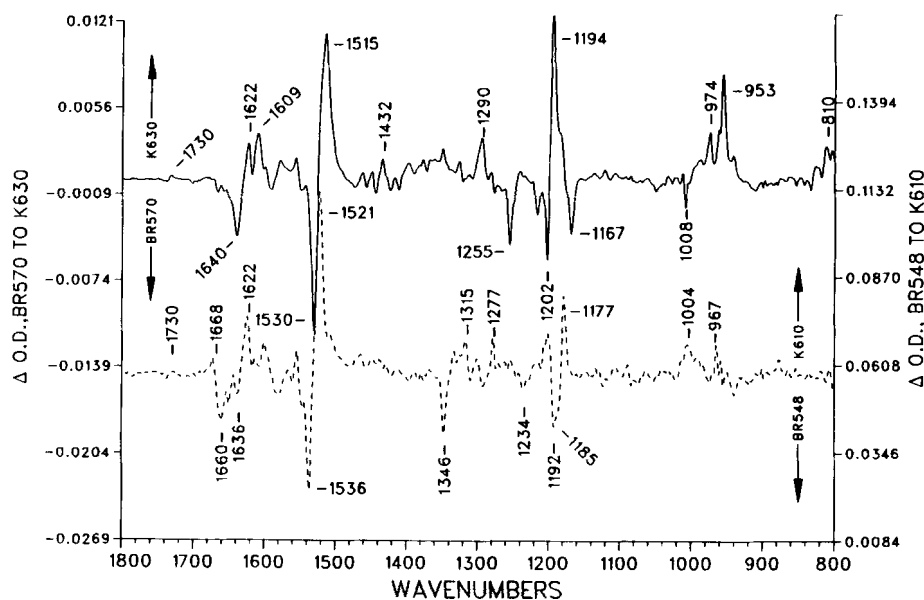


FIG. 5. FTIR difference spectra of the BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> transition in the fingerprint region for bR in H<sub>2</sub>O (A, solid line), and in <sup>2</sup>H<sub>2</sub>O (B, dashed line), bR regenerated with <sup>13</sup>C<sub>14</sub>-<sup>13</sup>C<sub>15</sub> retinal in H<sub>2</sub>O (C, solid line), and in <sup>2</sup>H<sub>2</sub>O (D, dashed line), and bR regenerated with <sup>2</sup>H-C<sub>15</sub> retinal in H<sub>2</sub>O (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 BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> transition. In the case of the UV region (Fig. 8D), except for the "filling in" of the negative K<sub>630</sub> 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 spec-

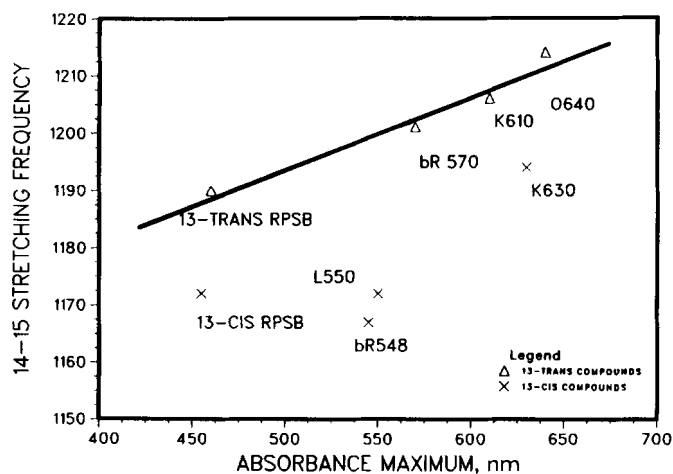


FIG. 6. Plot of the C<sub>14</sub>-C<sub>15</sub> stretching frequency versus  $\lambda_{\max}$  of 13-*trans* retinal compounds. Also shown are points which correspond to known 13-*cis* compounds. Plotted is a C<sub>14</sub>-C<sub>15</sub> assignment for the 13-*trans* intermediate O<sub>640</sub> at 1214 cm<sup>-1</sup>. Although originally C<sub>14</sub>-C<sub>15</sub> stretch character was assigned at 1198 for O<sub>640</sub> (8), data in Ref. 34 are consistent both with C<sub>14</sub>-C<sub>15</sub> character in a band at 1214 cm<sup>-1</sup> and a 13-*trans*,15-*trans* configuration for O<sub>640</sub> as originally proposed (34). C<sub>14</sub>-C<sub>15</sub> stretching character in more than one peak can occur due to mixing between individual C-C stretches (8, 20, 21, 23, 34). Data is from Refs. 20, 21, and 34 and the present work. It is seen that C<sub>14</sub>-C<sub>15</sub> stretches of 13-*cis* compounds (*i.e.* K<sub>630</sub>, C<sub>14</sub>-C<sub>15</sub> stretch at 1193 cm<sup>-1</sup>) do not fall close to this straight line correlation.

tra in Fig. 8C so that the visible region resembled previously reported BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> 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 BR<sub>570</sub>→K<sub>630</sub> 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

TABLE I  
C<sub>15</sub>-<sup>2</sup>H effects on chromophore vibrations

Tabulated are the observed shifts and intensity changes of chromophore vibrations for K<sub>610</sub><sup>D</sup>, the 13-*trans* RPSB, and the 13-*cis* RPSB upon deuterio substitution at C<sub>15</sub>. All entries are in cm<sup>-1</sup>. ↓ or ↑ indicates the peak decreases or increases significantly in intensity, respectively, upon the isotopic substitution. Model compound data is from Ref. 3.

13- <i>cis</i> RPSB		K <sub>610</sub> <sup>D</sup>		13- <i>trans</i> RPSB	
15H	15 <sup>2</sup> H	15H	15 <sup>2</sup> H	15H	15 <sup>2</sup> H
		877	875↓	870	
		957	957		
972	975 988	964	964	972	972
				1161	1164
1174	1168	1177	1177		
		1197	1192	1197	1202
1206	1206 1232	1205		1239	
		1277	1277↓ 1262	1279	1279↓ 1271 (sh)
1332		1323			
			1338		
1400		1412	1412↓↓	1410	
		1463	1463↓		1440
		1452			
				1525	
1572	1572	1522	1508	1565	1561
		1600 (sh)	1600↑		1605

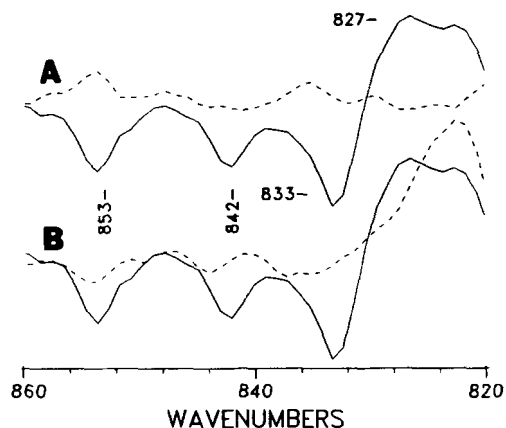


FIG. 7. A, comparison of a BR<sub>570</sub>→K<sub>630</sub> difference spectrum (solid line) and a BR<sub>548</sub>→K<sub>610</sub> spectrum (dashed line) in the 860–820 cm<sup>-1</sup> region. B, comparison of bR (solid line) and bR-[<sup>2</sup>H<sub>4</sub>]Tyr (dashed line) BR<sub>570</sub>→K<sub>630</sub> 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 K<sub>630</sub> formation (1). The same peaks eliminated by [<sup>2</sup>H<sub>4</sub>]Tyr labeling are also seen to be absent in the BR<sub>548</sub>→K<sub>610</sub> spectrum.

photocycle. Furthermore, if a tryptophan perturbation occurs for BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> it is relatively smaller than in the BR<sub>570</sub>→K<sub>630</sub> case.

#### DISCUSSION

The present results on dark adaptation and the primary photoreaction of BR<sub>548</sub> 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.

(i) *At Physiological Temperature (310 K) a Tyrosinate Group in BR<sub>570</sub> Protonates during the BR<sub>570</sub>→BR<sub>548</sub> Transition*—Features in both the FTIR and UV difference spectrum of dark adaptation support this conclusion. Furthermore, the similarity between the frequencies and intensities of tyrosine and tyrosinate peaks seen in the BR<sub>570</sub>→K<sub>630</sub> FTIR difference spectrum at 81 K and the BR<sub>570</sub>→BR<sub>548</sub> FTIR difference spectrum at 310 K indicates that one specific tyrosinate group in BR<sub>570</sub> at physiological temperature has also been deduced recently from both time-resolved FTIR (32) and FTIR-attenuated total reflection difference spectroscopy (33) of the BR<sub>570</sub>→M<sub>412</sub> transition.

(ii) *The BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> Transition Does Not Involve a Protonation Change of Tyrosine*—This conclusion is based primarily on the absence of peaks, particularly in the 900–800 cm<sup>-1</sup> region of the FTIR difference spectrum, associated with tyrosine protonation changes at various stages of the photocycle (1, 13, 14, 18). In addition, tyrosinate protonation peaks near 240 and 300 nm are not apparent in the UV difference spectrum for this transition.

(iii) *Carboxyl Group(s) of Glutamic Acid and/or Aspartic Acid Are Perturbed in the BR<sub>570</sub>→K<sub>630</sub> and BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> Transitions but Not in the BR<sub>570</sub>→BR<sub>548</sub> Transition*—This conclusion is based on the appearance of almost identical peaks in the COOH stretching region for the primary phototransition of BR<sub>548</sub> and BR<sub>570</sub>. Thus, it is likely that one or more aspartic acid and/or glutamic acid groups are perturbed in the same way, despite the fact that the chromophore configurational changes during the two transitions are quite different.

(iv) *The Conformation of K<sub>610</sub><sup>D</sup> Is 13-*trans*, 15-*cis**—This conclusion is based on the isotopic induced shifts for peaks assigned to the K<sub>610</sub><sup>D</sup> retinylidene chromophore. In particular, <sup>2</sup>H-C<sub>15</sub> retinal substitution causes shifts similar to those seen for C<sub>13</sub>=C<sub>14</sub> *trans* model compounds. Furthermore, the frequency of the C<sub>14</sub>-C<sub>15</sub> single bond stretch supports a 13-*trans* assignment. In the case of the C<sub>15</sub>=N bond, the large upshift of the C<sub>14</sub>-C<sub>15</sub> stretch upon N deuterio substitution indicates this bond is *cis*, as no large upshift of the C<sub>14</sub>-C<sub>15</sub> line is expected when the C<sub>15</sub>=N bond is *trans* (8).

(v) *Intense HOOP Lines are Absent in Spectra of the K<sub>610</sub><sup>D</sup> Chromophore*—In contrast to the BR<sub>570</sub>→K<sub>630</sub> transition and other primary retinal pigment phototransitions studied by FTIR difference spectroscopy (including the rhodopsin to bathorhodopsin transition (25)), very weak peaks in the HOOP mode region are found in the BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> difference spectrum. In resonance Raman studies, intensified HOOP modes have been associated with conformationally distorted forms of the chromophore which contain significant single bond twists (3, 24, 34). Such peaks have been observed in the primary photoproducts of BR<sub>570</sub> (3) and dry BR<sub>530</sub> (35) as well as in the O<sub>640</sub> intermediate of the bR photocycle (34). Thus, the K<sub>610</sub><sup>D</sup> chromophore may be in a more relaxed conformation, relative to K<sub>630</sub>. However, since HOOP modes are expected to be infrared active and normally produce intense absorption lines in spectra of retinal model compounds (36), it is also possible that the absence of intense peaks in the 1000–800 cm<sup>-1</sup> region of the BR<sub>548</sub>→K<sub>610</sub><sup>D</sup> spectrum indicates a cancellation of HOOP intensity, which would occur if the HOOP modes for BR<sub>548</sub> and K<sub>610</sub><sup>D</sup> were very similar in frequency and intensity.

The above conclusions are represented diagrammatically in Fig. 9. Several features are worth noting with regard to possible mechanisms for proton pumping and wavelength control of chromophore absorbance.

First, it is found that BR<sub>570</sub> is the only one of the four states

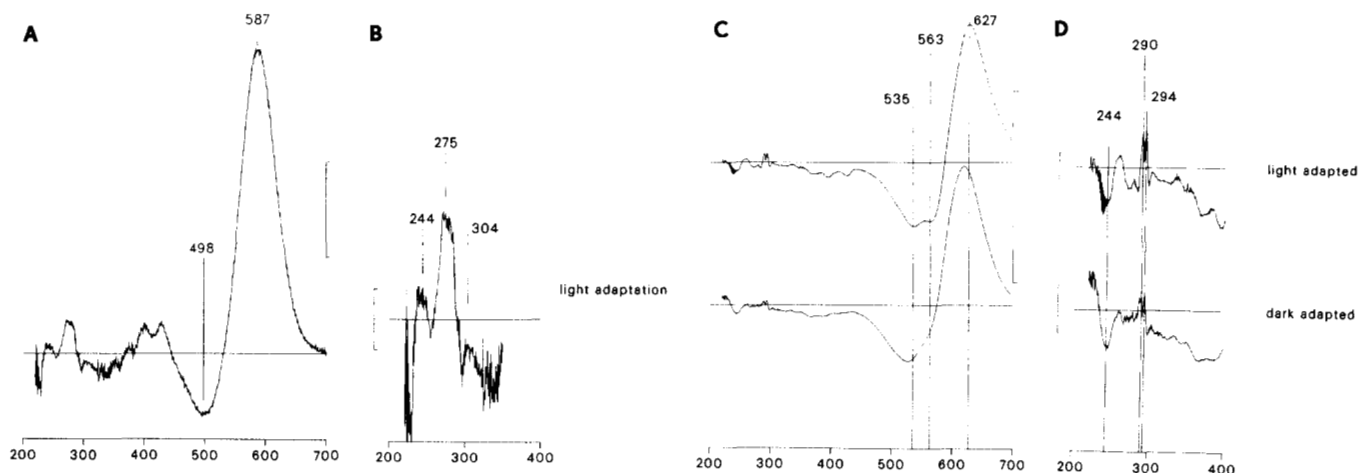


FIG. 8. The UV-visible difference spectrum for light adaptation at room temperature in the 700–200-nm region (A) and in an expanded view of the 400–200-nm UV region (B). The spectrum shown is the average of two differences recorded from a humidified purple membrane film. Absorbance scale markers are 0.002 (B) 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).

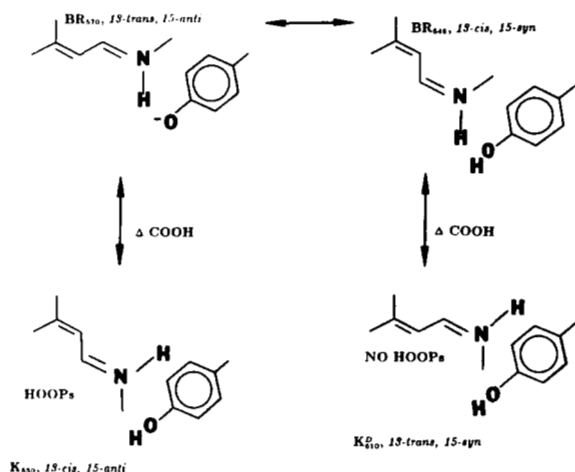


FIG. 9. A summary of observed chromophore isomerizations and protein changes during the early photochemistry of BR<sub>570</sub> and BR<sub>548</sub>. The positioning of the tyrosine group is *not* meant to imply it is the sole counterion to the Schiff base. Note that 15-*anti* and 15-*syn* are equivalent 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 tyrosinate 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 BR<sub>570</sub>→K<sub>630</sub> difference spectrum attributable to the BR<sub>570</sub> 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 K<sub>610</sub><sup>D</sup> reveals a consistent pattern for wavelength regulation in BR<sub>570</sub>, BR<sub>548</sub>, K<sub>630</sub> and K<sub>610</sub><sup>D</sup>, whose chromophores have all four possible combinations of isomerization about the C<sub>13</sub>=C<sub>14</sub> and C=N bonds. As seen in Fig. 9, the 13-*trans*,15-*trans* (BR<sub>570</sub>) and 13-*cis*,15-*cis* (BR<sub>548</sub>) 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* (K<sub>630</sub>) and 13-*trans*,15-*cis* (K<sub>610</sub><sup>D</sup>) forms, which have significantly red-shifted λ<sub>max</sub>, 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 λ<sub>max</sub> of these intermediates is the orientation of the N-H bond dipole and not the configuration about either the C<sub>13</sub>=C<sub>14</sub> or C=N bonds.

In conclusion, these FTIR and UV difference measurements of the BR<sub>548</sub> and K<sub>610</sub><sup>D</sup> 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 BR<sub>548</sub>.

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.

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