Tyrosine and Carboxyl Protonation Changes in the Bacteriorhodopsin Photocycle. 2. Tyrosines-26 and -64†

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Received January 26, 1987; Revised Manuscript Received May 26, 1987

ABSTRACT: Low-temperature Fourier transform infrared (FTIR) and UV difference spectroscopies combined with selective tyrosine nitration and tyrosine isotopic labeling have been used to investigate the participation of tyrosines-26 and -64 in the bacteriorhodopsin (bR) photocycle. Nitration of Tyr-26 has no detectable effect on the FTIR or UV difference spectra of the $BR_{570} \rightarrow K_{630}$ or $BR_{570} \rightarrow M_{412}$ transitions. In contrast, nitration of Tyr-64 causes changes in both the FTIR and UV spectra of these transitions. However, this nitration does not alter tyrosine peaks in the FTIR difference spectra which have previously been associated with the protonation of a tyrosinate by K_{630} and the deprotonation of a tyrosine by M_{412} [Roepe, P., Ahl, P. L., Das Gupta, S. K., Herzfeld, J., & Rothschild, K. J. (1987) *Biochemistry* (preceding paper in this issue)]. Instead, Tyr-64 nitration appears to affect other tyrosine peaks. These results and changes in UV difference spectra upon Tyr-64 nitration are consistent with the deprotonation of Tyr-64 by M_{412} as concluded previously [Scherrer, P., & Stoeckenius, W. (1985) *Biochemistry 24*, 7733–7740]. Effects on chromophore vibrations caused by Tyr-64 nitration are unaltered upon reducing the nitrotyrosine to aminotyrosine with sodium dithionite. Finally, nitro-Tyr-64 causes a shift in the frequency of a positive peak at 1739 cm^{-1} in the $BR_{570} \rightarrow M_{412}$ FTIR difference spectrum which reflects the protonation of a carboxyl-containing residue [Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400–407; Roepe, P., Ahl, P. L., Das Gupta, S. K., Herzfeld, J., & Rothschild, K. J. (1987) *Biochemistry* (preceding paper in this issue)]. The shift does not occur for samples containing amino-Tyr-64. These data suggest that Tyr-64 may interact with this carboxyl group.

The integral membrane protein bacteriorhodopsin (bR)[1] found in the purple membrane (PM) of *Halobacteria halobium* functions as a light-driven proton pump. Actively transported protons produce an electrochemical potential that drives the synthesis of ATP (Racker & Stoeckenius, 1974; Danon & Stoeckenius, 1974). Both the primary sequence (Ovchinnikov et al., 1979; Khorana et al., 1979; Dunn et al., 1981) and the three-dimensional structure within 7-Å resolution (Henderson & Unwin, 1975) have been determined. However, the molecular mechanism of bR light-driven proton transport remains to be elucidated.

Fourier transform infrared (FTIR) difference spectroscopy has been used by several groups to study chemical changes occurring during the bR photocycle [for a review, see Rothschild (1986)]. In contrast to resonance Raman spectroscopy, which only provides detailed information about the bR chromophore [e.g., Callender et al. (1976), Stockburger et al. (1979), Braiman and Mathies (1982), Smith et al. (1985)], FTIR difference spectroscopy can detect changes in single chemical groups of the protein and chromophore components of bR (Rothschild, 1986).

Recently, L-[*ring*-$^2H_4$]tyrosine was incorporated into the bR primary sequence, and tyrosine contributions to the FTIR $BR_{570} \rightarrow K_{630}$ difference spectrum were identified (Rothschild et al., 1986). Analysis of the bR and bR-[$^2H_4$]Tyr difference spectra obtained in both H_2O and ^2H_2O as well as the study of model compounds led to the conclusion that a tyrosinate group protonates by K_{630}. Further studies indicated that a tyrosine group deprotonated by M_{412} (Roepe et al., 1986, 1987). Separate FTIR studies by Dollinger et al. (1986) led to similar conclusions. A key question raised by these results is the position in the primary sequence of these tyrosine groups.

We report here on a study that combines isotopic and specific chemical labeling of tyrosine residues in the bR primary sequence with FTIR and UV difference spectroscopy. The ability to selectively nitrate either tyrosine-26 or tyrosine-64 at near 85% efficiency with tetranitromethane (TNM) (Scherrer & Stoeckenius, 1984) and to selectively reduce the

† Supported by a grant from the NSF to K.J.R. and grants from the NIH to R.A.B. and J.H.

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[1] Abbreviations: FTIR, Fourier transform infrared; bR, bacteriorhodopsin; HOOP, hydrogen out of plane; PM, purple membrane; ATP, adenosine 5′-triphosphate; L-[*ring*-$^2H_4$]Tyr, ring-perdeuteriated L-tyrosine; bR-[$^2H_4$]Tyr, bacteriorhodopsin containing ring-perdeuteriated L-tyrosines; TNM, tetranitromethane; bR-N64, bacteriorhodopsin selectively nitrated at tyrosine-64; *o*-NO_2-Tyr, *o*-nitro-L-tyrosine; *o*-NH_2-Tyr, *o*-amino-L-tyrosine; L-Tyr, L-tyrosine; poly(L-Tyr,Glu), poly(L-tyrosine,L-glutamic acid) (random copolymer); bR-N26, bacteriorhodopsin selectively nitrated at tyrosine-26; bR-[$^2H_4$]Tyr-N26 and bR-[$^2H_4$]Tyr-N64, bR-[$^2H_4$]Tyr nitrated at designated tyrosines.

0006-2960/87/0426-6708$01.50/0

nitrotyrosines to aminotyrosines with sodium dithionite has allowed us to compare the FTIR and UV difference spectra of both the BR → K and BR → M[2] transitions for unmodified bR and bR containing nitro- or aminotyrosine-26 or -64. In order to facilitate assignment of vibrational changes due to tyrosine nitration, we have also studied bR containing deuteriated tyrosines which was subsequently nitrated at tyrosine-26 or -64.

It has previously been deduced on the basis of flash kinetic measurements that nitrotyrosine-64 deprotonates during M_{412} formation at room temperature (Scherrer & Stoeckenius, 1985). From these data, and because the modifying reagent TNM reacts with the tyrosinate ion, it was inferred that tyrosine-64 deprotonates during M_{412} formation. Earlier studies (Rosenbach et al., 1982) based on the chemical modifications of tyrosine by Lemke and Oesterhelt (1981) concluded that Tyr-26 was also directly involved in the bR photocycle. Since FTIR difference spectroscopy (Roepe et al., 1987) detects the protonation of tyrosinate during the BR_{570} → K_{630} transition and the deprotonation of tyrosine during the L_{550} → M_{412} transition, we wished to determine if either Tyr-26 or Tyr-64 might be responsible for these signals.

As discussed in this paper, our results clearly indicate that Tyr-26 is not involved in the bR photocycle, up to and including the formation of M_{412}. In contrast, our data are consistent with Tyr-64 at least partially deprotonating by M_{412}. However, this observed tyrosine change is distinct from the FTIR difference signals originating from other, as yet unidentified, tyrosine group(s) that protonate at K_{630} and deprotonate at M_{412}. Hence it appears that as many as three tyrosine residues are involved in the formation of the M_{412} intermediate(s) during the bR photocycle. We also present evidence that indicates that nitrated Tyr-64 interacts with a carboxyl group that protonates at M_{412}.

Materials and Methods

Materials. Purple membrane (PM) from *H. halobium* (strain ET1001) was isolated as described by Oesterhelt and Stoeckenius (1973). BR was selectively nitrated at tyrosines-26 and -64 by the method of Scherrer and Stoeckenius (1984). Nitrotyrosines were reduced to aminotyrosines as described. The percentage of specific Tyr-26 or Tyr-64 modification was verified by amino acid sequencing. Selective nitration and subsequent reduction of bR containing ring-perdeuteriated tyrosines (bR-[$^2H_4$]Tyr) were done according to identical procedures. The production and characterization of bR-[$^2H_4$]Tyr have been described previously (Rothschild et al., 1986; Roepe et al., 1987). Model tyrosine compounds were obtained from Sigma and used without further purification.

FTIR Spectroscopy. Partially oriented PM films were deposited on AgCl substrate by the isopotential spin dry method (Clark et al., 1980) or by slow air-drying of a concentrated PM suspension. FTIR difference spectra of the BR_{570} → K_{630} and BR_{570} → M_{412} transitions were obtained under photo-steady-state conditions as described in detail elsewhere (Rothschild et al., 1984, 1985; Roepe et al., 1987). In general, difference spectra were computed by subtracting a BR_{570} spectrum obtained at either 81 or 250 K from a spectrum of bR obtained at the same temperature while the bR film was illuminated as previously described (Rothschild et al., 1984; Roepe et al., 1987) to enhance the concentration of K_{630} or M_{412}, respectively. Up to 50 individual differences were averaged together to increase the signal-to-noise ratio of the presented spectra. For bR-N64, the wavelength of the illumination used for K photoreversal was blue shifted 30 nm (Ditric 620-nm broad-band filter) from that normally used for K_{630} photoreversal in order to compensate for the blue-shifted visible absorption maximum (Scherrer & Stoeckenius, 1984). Under these conditions, photoreversal was complete within 15 min. Temperature stability during an experiment was normally within 0.1 K. This stability combined with the averaging technique resulted in a noise level for the difference spectra near 10^{-4} OD, which was sufficient to reliably detect the single group alterations discussed herein.

UV Spectroscopy. Low-temperature UV spectroscopy was performed as described previously (Rothschild et al., 1986; Roepe et al., 1987) at several temperatures and pH. The BR → K measurements were made on humidified membrane films at 79 K. The BR → M difference spectra were obtained at 220 K on PM suspensions in 70% glycerol. When necessary, pH was adjusted with dilute HCl or NaOH.

Results

Infrared Absorption Spectra of Modified Tyrosines. In order to characterize nitration-induced changes in tyrosine regions of the FTIR difference spectra, model compounds including *o*-NO_2-L-Tyr and *o*-NH_2-L-Tyr were studied at high and low pH. Although definitive vibrational assignments of these compounds will only be available after normal-mode analysis and more extensive model compound studies, it was found that most vibrational frequencies were shifted relative to tyrosine due to the chemical modifications. For example, when model spectra of L-Tyr and *o*-NO_2-L-Tyr from pH 2 solution were compared, an apparent shift from 1516 to 1530 cm^{-1} (cf. Figure 1A) of an aromatic C=C stretch and annihilation of Fermi reasonance peaks (ring breath coupled to ring bend overtone) at 832 and 855 cm^{-1} (data not shown) were observed for the nitrotyrosine compound. The prominent band at 1245 cm^{-1} assigned to the phenoxyl C–OH stretch and OH bend (Jakobsen & Brewer, 1962) appears to shift at least 15 cm^{-1}, probably to 1260 cm^{-1} (cf. Figure 1A). In the case of ionized compounds, apparent shifts from 1500 to 1508 cm^{-1} and 1270 to 1253 (or 1281) cm^{-1} were observed due to the nitrate label (cf. Figure 1B). *o*-Amino labeling of tyrosine appears to cause a 2–4-cm^{-1} shift of the aromatic C=C stretch, at least a 20-cm^{-1} shift of the phenoxyl C–O stretch line in the protonated form, and at least an 11-cm^{-1} shift of this band in the ionized form (cf. Figure 1). These shifts are in qualitative agreement with the published spectra of benzene, toluene, phenol, and their appropriately modified derivatives (Pouchert, 1981).

UV Spectra of Modified Tyrosines. Figure 2 shows the difference spectra produced between high- and neutral-pH solutions of poly(L-Tyr,Glu) (solid line), *o*-NO_2-L-Tyr (dotted line), and *o*-NH_2-L-Tyr (dashed line). The absorbance changes for each correspond to sample concentrations of 0.13 mM. Positive tyrosinate peaks occur for the normal tyrosine compound at 244 and 294 nm, whereas corresponding peaks for the amino sample are at 245 and 306 nm. Notice that the nitro compound does not exhibit a positive peak near 300 nm upon ionization. Thus, monitoring the loss of this peak in the UV difference spectra of various transitions can be used to identify tyrosinate contributions to the difference spectra that are due to a nitrated group(s).

[2] Lack of a subscript on an intermediate symbol denotes a reference to intermediates that have shifted λ_{max} values because of Tyr-64 nitration. For example, the light-adapted form of bR is designated BR_{570} because of its 570-nm absorbance, but the "light-adapted" form of bR-N64 has a λ_{max} of 535 nm (Scherrer & Stoeckenius, 1984). Therefore, when reference to transitions of both bR and bR-N64 are made in a sentence, the symbol "BR" is used in order to avoid ambiguity.

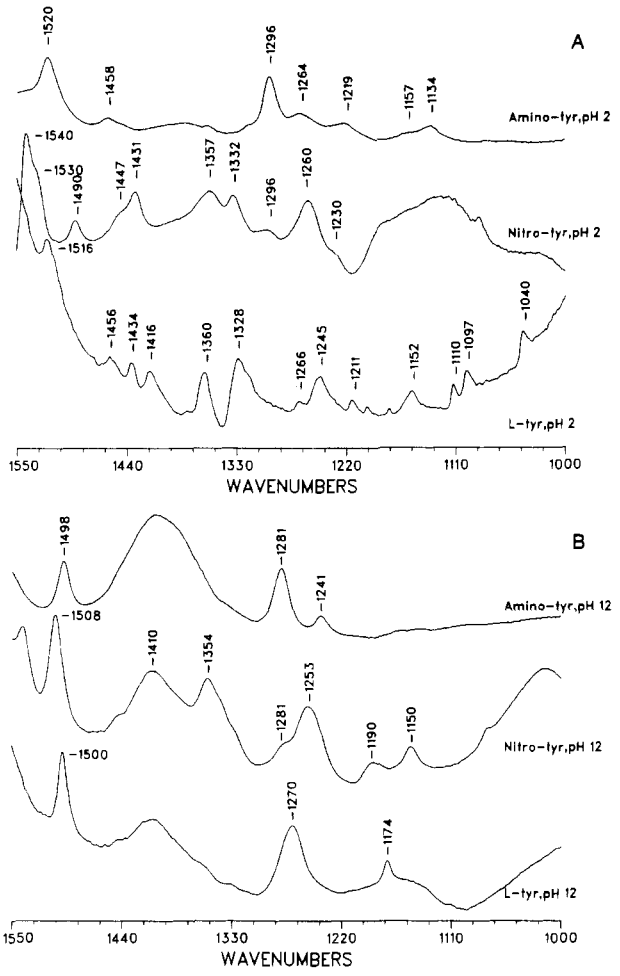

FIGURE 1: Model FTIR spectra of o-NH_2-Tyr (top), o-NO_2-Tyr, (middle), and L-Tyr (bottom) in protonated (A) and ionized (B) forms, taken in solution between two CaF_2 windows with a Nicolet 60-SX spectrometer at 2-cm^{-1} resolution. In each case 100 interferograms were coadded and multiplied by a Happ–Genzel instrument function before Fourier transformation.

Effects of Tyr-26 Nitration on BR → K and BR → M Difference Spectra. Figure 3 compares the $BR_{570} \rightarrow K_{630}$ FTIR difference spectra for bR-N26 and normal bR. Positive peaks reflect the formation of K_{630} and negative peaks the loss of BR_{570}. *It is found within the limits of our signal to noise that there are no differences between the normal and bR-N26 spectra.* In particular, positive peaks at 1514 and 1456 cm^{-1} which have been assigned in part to C=C aromatic ring stretching vibrations of tyrosine; peaks at 1276 cm^{-1} (−) and 1250–1240 cm^{-1} (+) which contain tyrosinate and tyrosine phenolic C–O stretching character, respectively; and peaks in the 800-cm^{-1} region at 853, 842, 833, and 827 cm^{-1} which are due to various components of the Fermi reasonance doublet (Siamwiza, 1974; Siamwiza et al., 1975) are unaltered in the bR-N26 sample.

Low-temperature UV–vis $BR_{570} \rightarrow K_{630}$ difference spectra of bR and bR-N26 humidified membrane films were also indistinguishable (data not shown). There were no changes in position or intensity of near-UV peaks previously assigned to tyrosine protonation and tryptophan hydrogen-bonding alteration(s) (Rothschild et al., 1986).

Figure 4 compares $BR_{570} \rightarrow M_{412}$ FTIR difference spectra for bR and bR-N26. Again, these spectra were found to be identical within our signal to noise. In particular, peaks at 1495, 1271, and 839 cm^{-1} associated with the formation of a tyrosinate (Roepe et al., 1987) and a negative peak at 1517 cm^{-1} associated with the loss of a tyrosine in BR_{570} during the formation of M_{412} do not change in intensity and/or frequency.

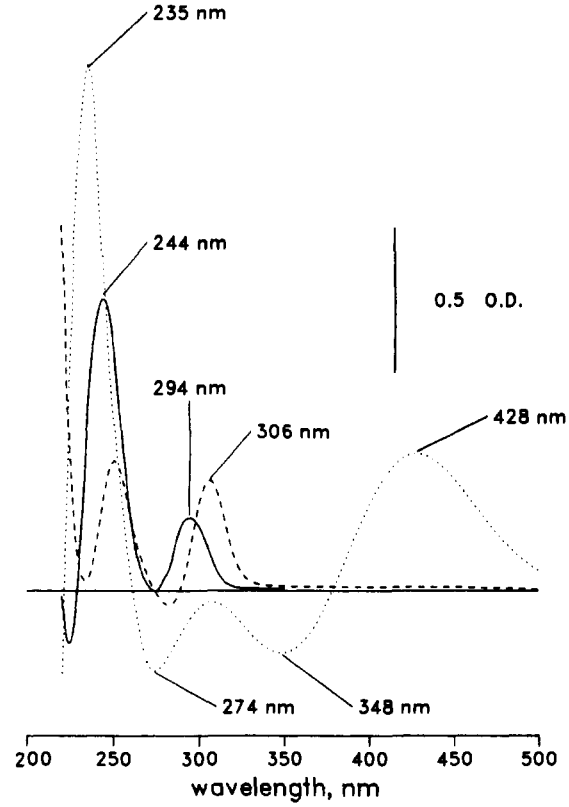

FIGURE 2: Model compound UV–vis titration spectra of poly(L-Tyr,Glu) (random copolymer; solid line), o-NO_2-Tyr (dotted line), and o-NH_2-Tyr (dashed line). Individual spectra were recorded with a Cary 219 spectrophotometer at 1-nm resolution. The presented difference spectra were computed by subtracting a spectrum of the compound in pH 11 solution from a spectrum of the same sample at pH 6. The absorbance changes correspond to concentrations of 0.13 mM at the two pH's.

UV–vis $BR_{570} \rightarrow M_{412}$ difference spectra obtained at 220 K of either glycerol suspensions or films of bR and bR-N26 were essentially identical (data not shown). Positive peaks at 240 and 300 nm assigned to the production of a tyrosinate species during M_{412} formation, as well as positive peaks at 288 and 297 nm assigned to tryptophan hydrogen-bonding changes, did not change in frequency or intensity.

Effects of Tyr-64 Nitration on BR and K Chromophore Vibrational Modes. Figure 5 compares BR → K FTIR difference spectra for bR and bR-N64. Figure 6 shows expanded regions containing tyrosine contributions. The top comparison in these expansions is between bR and bR-N64, the middle between bR-N64 and bR-[$^2H_4$]Tyr-N64, and the bottom between bR and bR-[$^2H_4$]Tyr.

The largest alterations in the difference spectrum of bR-N64 relative to the normal bR spectrum are the shifts of several prominent chromophore lines. These shifts were verified by direct comparison with the resonance Raman spectra of bR and bR-N64 (Earnest and Rothschild, unpublished results), which exhibit chromophore vibrations but not protein vibrations.

It is found that the negative peaks at 1640 and 1530 cm^{-1} as well as positive peaks at 1609 and 1515 cm^{-1} assigned to the Schiff base C=C and ethylenic C=C stretches of BR_{570} and K_{630}, respectively (Rothschild et al., 1984), all appear to upshift about 10 cm^{-1} (cf. Figure 5). These data fit the linear correlations established between the visible absorption maxima of photocycle intermediates and their respective C=N and C=C stretching frequencies (Doukas et al., 1978; Rothschild et al., 1984). Since the ground-state λ_{max} of the bR-N64

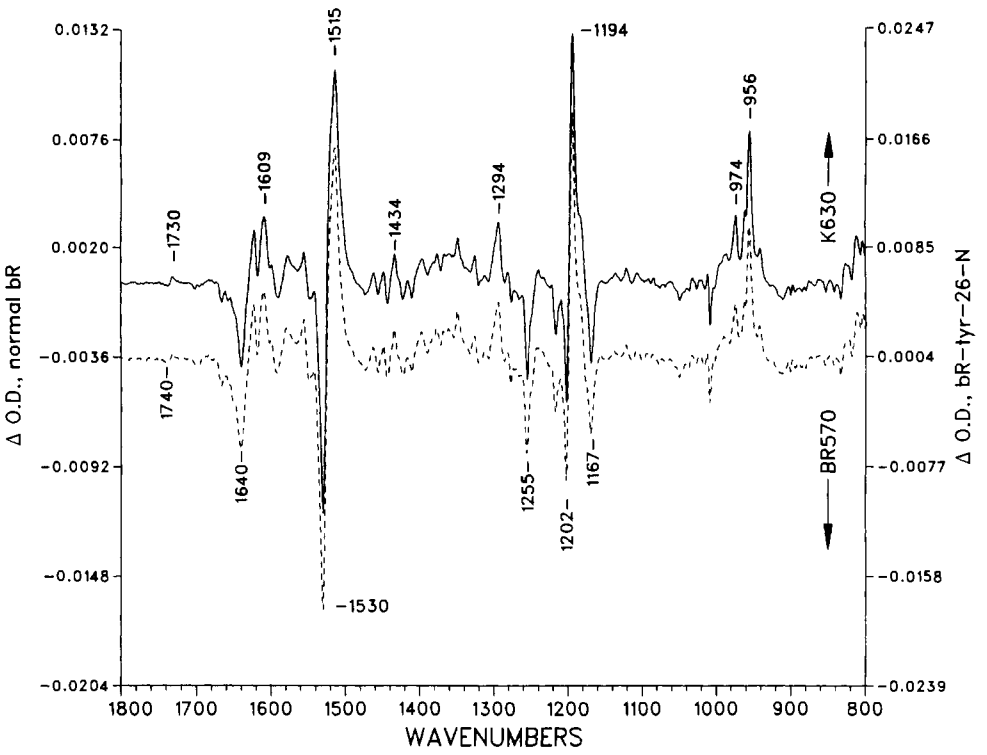

FIGURE 3: FTIR difference spectra of the $BR_{570} \rightarrow K_{630}$ transition at 81 K for normal bR (top, solid line) and bR-N26 (bottom, dashed line). A more detailed analysis of these spectra, including comparisons between expansions of these spectra in regions containing tyrosine vibrations, is given in Roepe (1987). Spectra were taken on a Nicolet MX-1 spectrometer interfaced to a Nicolet 1200S data analysis and control system at 2-cm^{-1} resolution. Each 15-min scan results in the acquisition of 480 interferograms, each of which contains 16K data points. A Happ–Genzel instrument line shape is applied to each interferogram before Fourier transformation. Experimental variables such as scan time, temperature, and illumination wavelength were controlled by using a program developed in our laboratory. (See Materials and Methods for further details.)

sample is blue shifted 30 nm, we would expect a C=C stretch in BR_{570} of $\approx$1540 cm^{-1} and a C=N stretch of $\approx$1650 cm^{-1}. Table I lists frequencies of the major chromophore lines, as well as their assignments, for bR and the modified samples. Detailed tabulation of *all* localized chromophore line shifts will only become available after study of bR-N64 samples containing isotopically labeled retinals. It is also noted that the strong positive line at 956 cm^{-1}, most likely due to the K_{630} C_7–H and C_8–H out-of-plane wags of the chromophore[3] (Braiman, 1983), is almost completely gone in the nitrated difference spectrum. This could possibly indicate a localized perturbation of the chromophore due to Tyr-64 nitration near the C_7–C_8 region of the chromophore. Interestingly, all of these effects are reproduced in the bR-NH_2-Tyr-64 spectrum (data not shown).

Effects of Tyr-64 Nitration on BR and K Tyrosine Vibrations. By carefully comparing shifts due to tyrosine ring deuteriation, Tyr-64 nitration, and tyrosine ring perdeuteriation *plus* Tyr-64 nitration (Figure 6), it is possible to identify those peaks that arise from Tyr-64 vibrations in the difference spectra. *Notably, the frequencies of all major tyrosine and tyrosinate peaks previously assigned in the* $BR_{570} \rightarrow K_{630}$ *difference spectrum are not significantly affected by Tyr-64 nitration.* This is evident by comparing the bottom two overlays in Figure 6, panels A–C, where it is found that the effects of [$^2H_4$]Tyr incorporation are very similar for bR and bR-N64. For example, the negative tyrosinate peak at 1277 cm^{-1} is upshifted less than 1 cm^{-1} in bR-N64 but is completely gone in the bR-[$^2H_4$]Tyr-N64 difference spectrum. Negative peaks associated with tyrosinate Fermi reasonances at 853, 842, and 833 cm^{-1} are still evident in bR-N64. Two peaks at 1456 and 1248 cm^{-1} assigned to masked positive tyrosine contributions are still found in bR-N64 and shift out due to isotopic labeling. In one case, a positive tyrosine peak at 1514 cm^{-1} which was hidden by the stronger ethylenic mode of the K chromophore is unmasked due to the shift of the chromophore vibration. This peak clearly shifts away due to [$^2H_4$]Tyr incorporation (cf. Figure 6A). *Since the frequencies of all these tyrosine modes are shifted considerably due to nitration in model compounds (cf. Figure 1), it can be concluded that the tyrosine group that gives rise to these signals in the* $BR_{570} \rightarrow K_{630}$ *transition is not Tyr-64.*

Nitration of Tyr-64 has a major effect on the chromophore contributions to the UV–vis BR $\rightarrow$ K difference spectrum as would be expected on the basis of the IR observations. These changes make interpretation of the UV difference spectrum difficult. However, close scrutiny of the unaltered bR and the bR-N64 BR $\rightarrow$ K UV difference spectra (cf. Figure 7) indicates there are several important similarities between the two. Both spectra show large negative peaks at 244 nm, indicating a tyrosinate protonation. Thus it appears the UV tyrosinate protonation signal we have observed in the $BR_{570} \rightarrow K_{630}$ difference spectrum is not affected by Tyr-64 nitration, in agreement with our FTIR results. Additionally, the tryptophan perturbation peaks at 289 and 294 nm are still evident in the nitrated sample and do not appear to shift in frequency. There are several new peaks in the bR-N64 spectrum including a positive peak between 272 and 275 nm, which presumably cancels out a negative peak normally seen at 272 nm, and a positive at 336 nm. These are likely to be chromophore peaks, as the model nitrotyrosine titration spectrum (cf. Figure 2) does not reveal strong new peaks at these wavelengths.

Effects of Tyr-64 Nitration on BR $\rightarrow$ M Difference Spectra.

[3] Although an unequivocal assignment of this HOOP mode is difficult on the basis of the available data, it can be concluded on the basis of published spectra of bR isotopically labeled at retinal hydrogens that the line has no C_{11}, C_{12}, or C_{14} hydrogen wag character (Braiman, 1983).

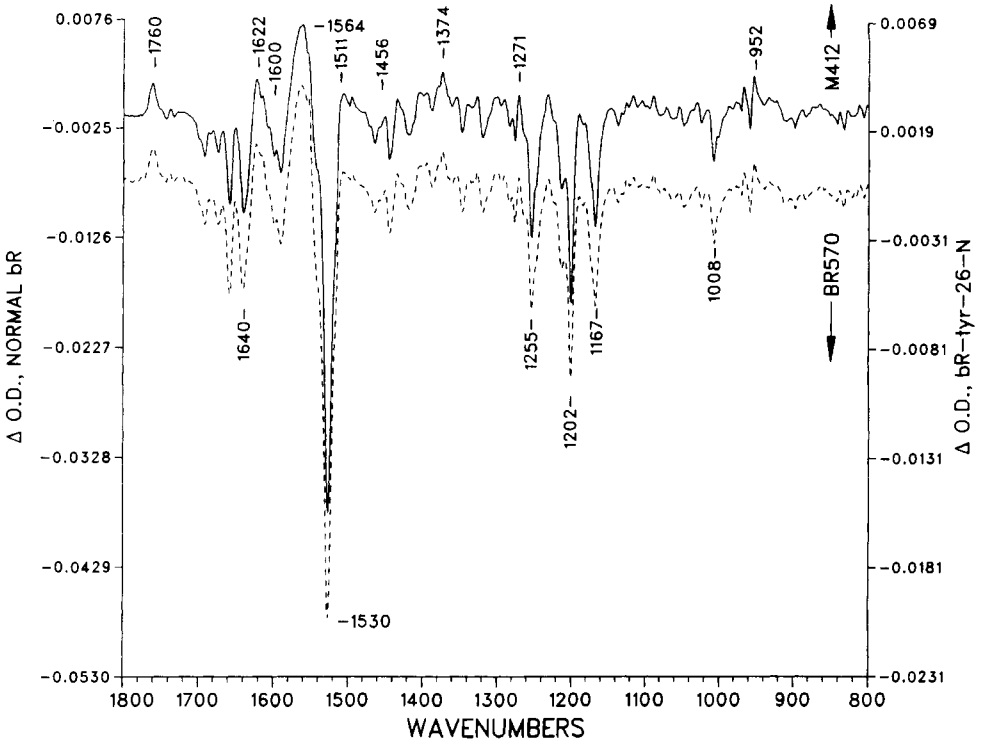

FIGURE 4: Spectra as in Figure 3, but of the $BR_{570} \rightarrow M_{412}$ transition at 250 K. Spectra were obtained under photo-steady-state conditions. (See Materials and Methods for further details.)

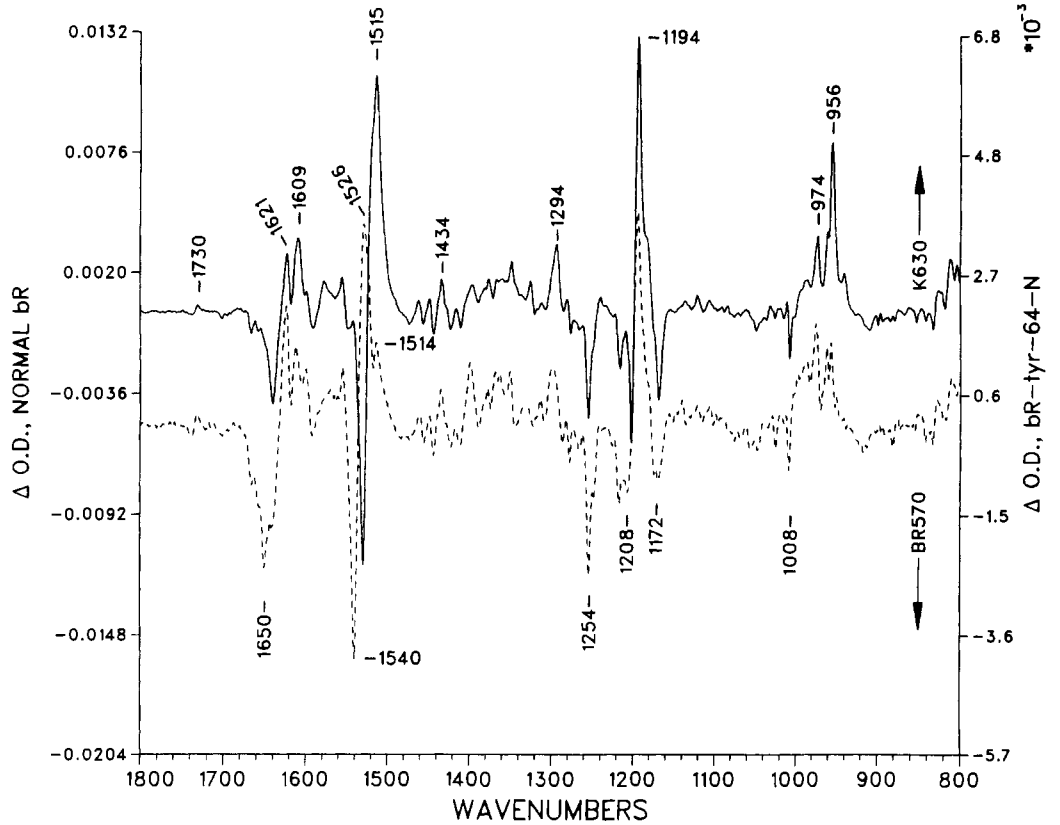

FIGURE 5: Spectra as in Figure 3, but for normal bR (top, solid line) and bR-N64 (bottom, dashed line).

Figure 8 compares BR → M FTIR difference spectra for bR and bR-N64 recorded under photo-steady-state conditions at 250 K (see preceding paper in this issue). In agreement with results from resonance Raman spectroscopy of the M intermediate (Earnest and Rothschild, unpublished results), we find only a small shift in the vibrational frequencies of the C=C and C=N stretching modes of the M chromophore, implying that the chromophore effects produced by Tyr-64 nitration are more pronounced for protonated Schiff base intermediates (cf. Table I). This agrees with previous conclusions based on the visible spectra (Scherrer & Stoeckenius, 1985).

We also find that all of the major tyrosine alterations previously identified as occurring between BR_{570} and M_{412} (Roepe et al., 1987) appear at a similar frequency in both bR and bR-N64 difference spectra. This can be seen by comparing the effects of isotope labeling for both samples (cf.

Table I: Major Chromophore Alterations[a]

	BR_{570} (cm^{-1})	K_{630} (cm^{-1})	M_{412} (cm^{-1})	assignment
normal bR	1640	1610	1627	C_{15}=N stretch
	1530	1515	1564	C=C ethylenic stretch
	1255			C_{12}–C_{13}, C_{14}–C_{15} stretches
	1216			C_8–C_9 stretch
	1202	1194		C_{14}–C_{15}, C_{12}–C_{13} stretches
	1167			C_{10}–C_{11} stretch
	1008			19-CH_3 rock
		974		C_{15}, N HOOPs
		956		C_7, C_8 HOOPs
bR-N64	1650	1620	1629	C_{15}=N stretch
	1540	1525	1565 (br)[b]	C=C ethylenic stretch
	1255			C_{12}–C_{13}, C_{14}–C_{15} stretches
	1216			C_8–C_9 stretch
	1208 (br)	1194 (red.)		C_{14}–C_{15}, C_{12}–C_{13} stretches
	1172			C_{10}–C_{11} stretch
	1010			19-CH_3 rock
		976		C_{15}, N HOOPs
		957 (80% red.)		C_7, C_8 HOOPs
bR-NH_2-Tyr-64	1650	1620	1629	C_{15}=N stretch
	1540	1525	1565 (br)	C=C ethylenic stretch
	1255			C_{12}–C_{13}, C_{14}–C_{15} stretches
	1216			C_8–C_9 stretch
	1208 (br)	1194 (red.)		C_{14}–C_{15}, C_{12}–C_{13} stretches
	1172			C_{10}–C_{11} stretch
	1010			19-CH_3 rock
		976		C_{15}, N HOOPs
		957 (80% red.)		C_7, C_8 HOOPs
bR-N26	1640	1610	1627	C_{15}=N stretch
	1530	1515	1564	C=C ethylenic stretch
	1255			C_{12}–C_{13}, C_{14}–C_{15} stretches
	1202	1194		C_{14}–C_{15}, C_{12}–C_{13} stretches
	1167			C_{10}–C_{11} stretch
	1008			19-CH_3 rock
		974		C_{15}, N HOOPs
		956		C_7, C_8 HOOPs

[a] Tabulated chromophore vibrations for bR, bR-Tyr-N64, and bR-Tyr-N26. Assignments are from Smith et al. (1985), Braiman (1983), and previous work with isotopically labeled retinals. Note: For bR-Tyr-N64 the wavelength maxima of the intermediates are shifted [see Scherrer and Stoeckenius (1985)]. [b] Abbreviations: br, broad; red., reduced.

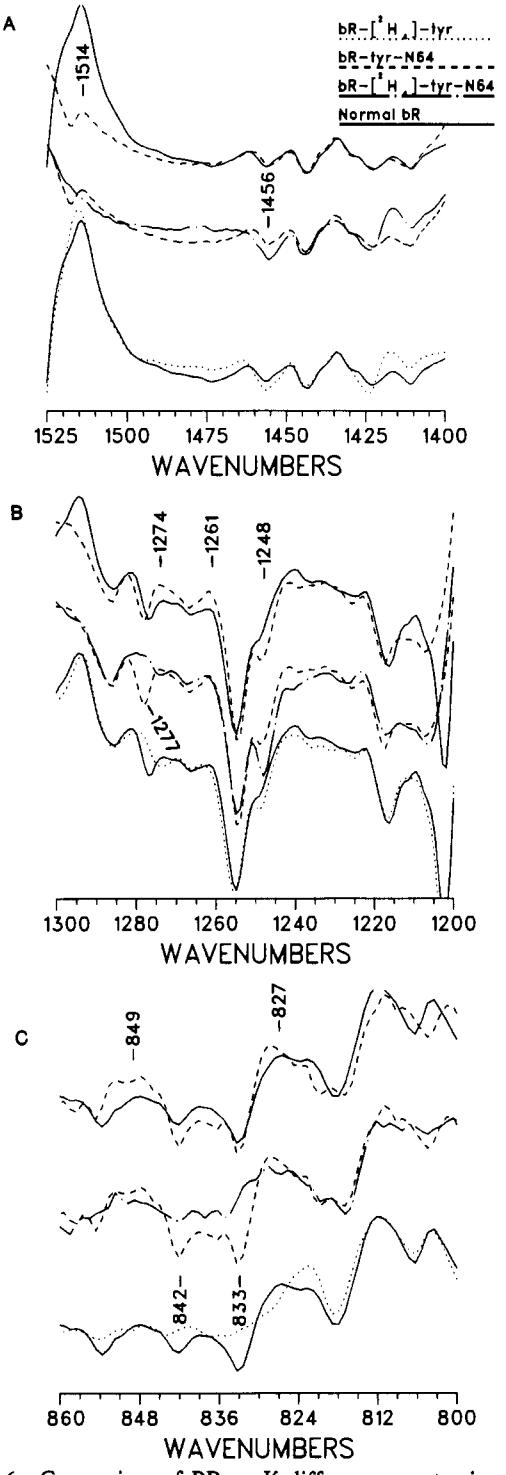

FIGURE 6: Comparison of BR → K difference spectra in regions containing tyrosine contributions for bR (top, solid line) and bR-N64 (top, dashed line); for bR-N64 (middle, dashed line) and bR-[$^2H_4$]Tyr-N64 (middle, dashed–dotted line); and for bR (bottom, solid line) and bR-[$^2H_4$]Tyr (bottom, dotted line). The spectra were obtained as in Figure 3.

Figure 9). Not only are all the tyrosine components identified in the BR_{570} to K_{630} transition still present, but new peaks associated with the deprotonation of a second tyrosine at 1271 cm^{-1} (+) and 1517 cm^{-1} (−) are evident. Again the positive peak at 1511 cm^{-1} and the negative at 1517 cm^{-1} are more easily seen in the bR-N64 sample due to the upshift in frequency of the BR_{570} ethylenic vibration (cf. Figure 9A). Hence we conclude that the tyrosine deprotonation signals previously identified in the BR_{570} → M_{412} difference spectrum (Roepe et al., 1987) are not due to Tyr-64.

Involvement of Tyr-64 in the BR_{570} → M_{412} Transition. It has previously been observed on the basis of room temperature kinetic measurements that nitro-Tyr-64 undergoes a deprotonation during the formation of M (Scherrer & Stoeckenius, 1985). On the basis of these data it was suggested that Tyr-64 similarly deprotonates. Since the pK_a of o-NO_2-Tyr is 2 units lower than that of Tyr, explicit identification of Tyr-64 peaks is needed to conclusively assign the deprotonation to the BR_{570} → M_{412} transition. While the present FTIR and UV difference measurements at low temperature do not conclusively demonstrate a Tyr-64 deprotonation, our data are consistent with such a deprotonation at the M_{412} stage of the photocycle.

For example, in the FTIR BR → M spectra the small positive peak at 1280 cm^{-1} disappears upon nitration (cf. Figure 9B). Nitration may cause it to downshift to near 1249 cm^{-1}, as a small intensity increase is seen at this frequency.

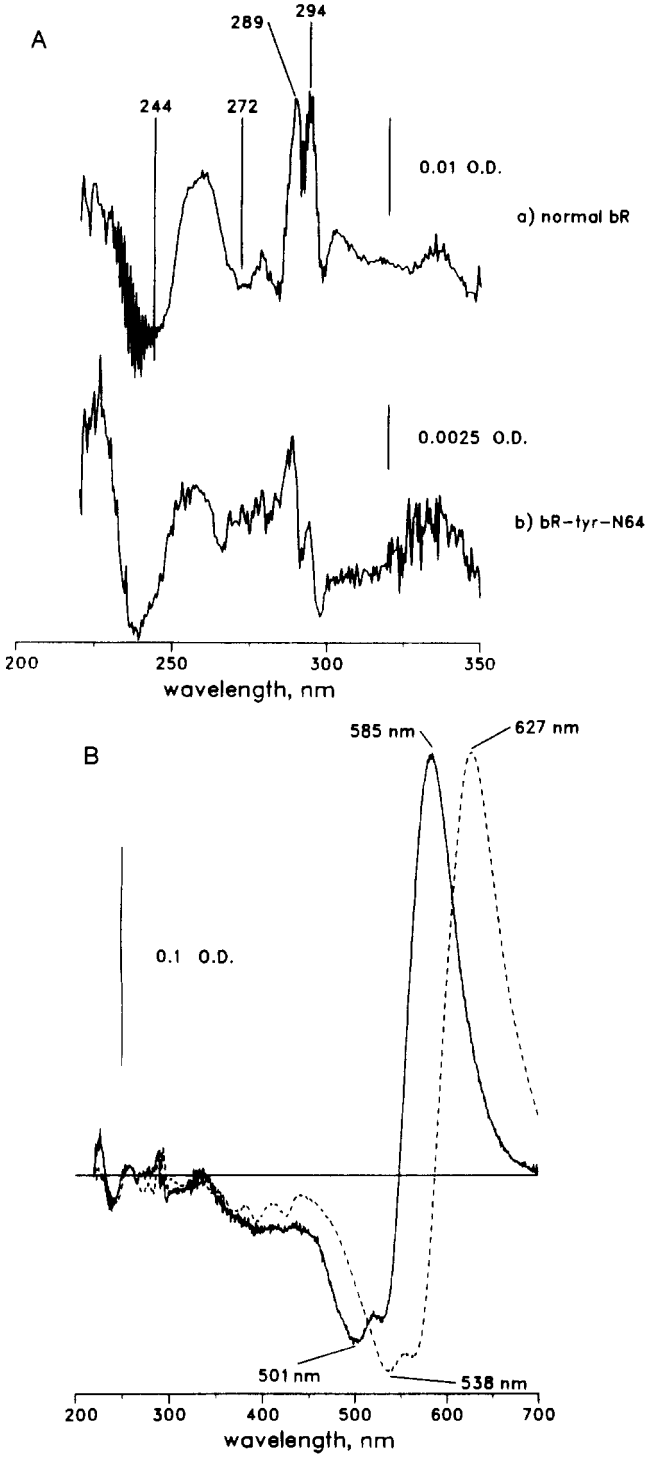

FIGURE 7: (A) BR → K UV difference spectra for bR (top) and bR-N64 (bottom). Spectra were recorded with humidified PM films at 79 K as described in Roepe et al. (1987). The spectral bandwidth was 1 nm. (B) Entire UV–vis region to 700 nm. Dashed line is normal bR, and solid line is bR-NO_2-Tyr-64.

The 1280-cm^{-1} peak is also shifted in the spectrum of the reduced sample (data not shown). It is likely that this peak is due to a tyrosinate C–O^- stretch, as it would be expected to downshift ≈25 cm^{-1} further upon $^2H_4$ substitution (Rothschild et al., 1986; Roepe et al., 1987), and a new positive peak near 1222 cm^{-1} in the bR-[$^2H_4$]Tyr-N64 spectrum is indeed seen concomitant with a decrease in positive intensity at 1249 cm^{-1} relative to the bR-N64 spectrum (cf. Figure 9B, middle).

Additionally, the 842-cm^{-1} tyrosine peak (cf. Figure 9C) previously identified by isotopic labeling (Roepe et al., 1987) is observed to decrease in intensity by approximately 80% upon Tyr-64 nitration, while the stronger 833-cm^{-1} peak is unchanged. Again, these effects are reproduced in the reduced sample (data not shown). This peak is thus attributed to a Tyr-64 vibration that appears in the difference spectrum. This would most likely indicate that the 842- and 833-cm^{-1} negative peaks in the M_{412} difference arise from separate groups. Since $^2H_4$ labeling of bR-N64 does not produce additional changes at these frequencies, we can exclude the possibility that Tyr-N64 peaks have shifted *to* 1280 and 842 cm^{-1}.

Figure 10 compares the UV–visible BR → M difference spectra obtained at 220 K from 70% glycerol suspensions of bR and bR-N64 at pH 9.5. Under these conditions, the M photoproduct produced with both samples is thermally stable during the 10-min scanning period. It has previously been shown (Roepe et al., 1987) that the BR_{570} → M_{412} FTIR difference spectrum obtained at this temperature and pH under thermal steady-state conditions exhibits tyrosinate peaks of the same intensity as those seen in a spectrum of this transition obtained at 250 K and pH 7.0 under photo-steady-state conditions. It is seen that the normal sample shows a clear absorbance increase centered at about 300 nm which is decreased in the bR-N64 spectrum. This data and the FTIR results are consistent with the elimination of a Tyr-64 deprotonation signal in the UV difference spectrum due to Tyr-64 nitration. In particular, nitration does not appear to affect the tyrosinate that gives rise to the positive 1495-, 1271-, or 839-cm^{-1} peaks in the presented FTIR difference spectrum (cf. Figures 8 and 9), since the intensities of these IR peaks are not decreased in the bR-Tyr-N64 sample.

Effects of Nitration on Carboxyl Frequencies. An unexpected effect of Tyr-64 nitration is observed on the frequency of the 1739-cm^{-1} positive peak in the BR → M difference spectrum (cf. Figure 11) which downshifts 2 cm^{-1}. Reduction of nitro-Tyr-64 to amino-Tyr-64 restores the peak to its original frequency. This peak has previously been assigned to the protonation of a carboxylate group during the formation of M_{412}. In contrast, the larger 1761-cm^{-1} peak which is also associated with the protonation of a carboxylate group is unaffected. As discussed later, these data may indicate an electrostatic interaction between nitrated Tyr-64 and a carboxyl group in the M_{412} intermediate.

Discussion

Several conclusions can be drawn from the above results:

(*i*) *Tyrosine-26 Is Not Involved Directly in the Photocycle.* A previous study (Rosenbach et al., 1982) concluded that Tyr-26 is involved in the photocycle and that both its pK_a and its protonation state influenced M_{412} formation. However, we find the FTIR difference spectra of bR and bR-N26 for both the BR_{570} → K_{630} and BR_{570} → M_{412} transitions to be identical. In addition, BR_{570} → L_{550} FTIR difference spectra (data not shown) are unaffected by Tyr-26 nitration, eliminating the possibility that a reversible change of Tyr-26 occurs between K_{630} and M_{412}. UV difference measurements of the bR-N26 sample are also identical with corresponding bR measurements. In view of the sensitivity of these measurements, it is unlikely that Tyr-26 undergoes significant changes in hydrogen bonding, orientation relative to the membrane plane, or protonation state during the formation of M_{412} at 250 K.

The discrepancy between our results and the visible absorption measurements of Rosenbach et al. may stem from the fact that their samples also contained amino-Tyr-64. The FTIR difference spectra for bR-N64 and bR-NH_2-Tyr-64 are very similar as are the visible absorption (Scherrer & Stoeckenius, 1985) and resonance Raman measurements

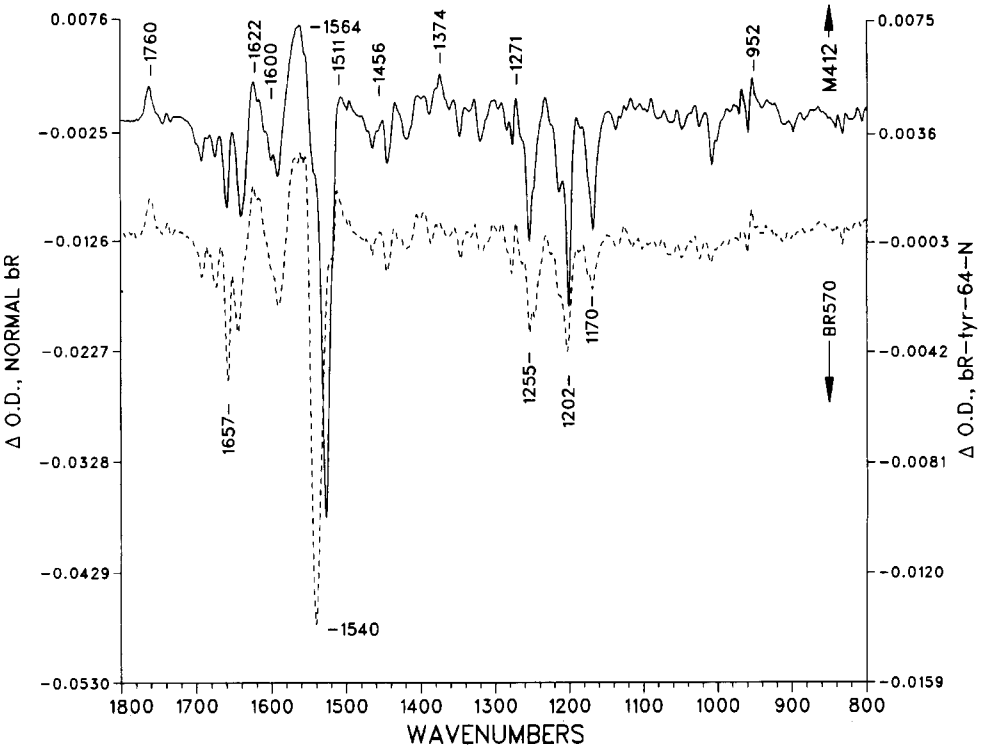

FIGURE 8: Comparison of bR and bR-N64 BR → M FTIR difference spectra obtained at 250 K as described under Materials and Methods.

(Earnest and Rothschild, unpublished results). This indicates that the effects previously observed are most likely attributable to amination of Tyr-64 and not nitration of Tyr-26.

(ii) Tyrosine-64 Participates Directly in the Photocycle. Identification of Tyr-64 contributions to the FTIR difference spectra for $BR_{570} \rightarrow K_{630}$ and $BR_{570} \rightarrow M_{412}$ transitions indicates that Tyr-64 is either directly or indirectly involved in these photocycle steps. It is difficult on the basis of the present data to unequivocally determine the nature of the Tyr-64 alterations. We do find, however, that the changes produced in the UV and FTIR difference spectra of the $BR_{570} \rightarrow M_{412}$ transition upon Tyr-64 nitration are consistent with the elimination of signals representing a Tyr-64 deprotonation. These signals are distinct from those previously identified (Roepe et al., 1987) which also indicate a tyrosine deprotonation. These results, along with those published previously (Rothschild et al., 1986), indicate that as many as three tyrosines change protonation state during the bR photocycle.

The fact that we do not identify corresponding positive nitrotyrosinate signals in the visible difference spectrum may be due to overlap of visible chromophore peaks or a blocking of nitro-Tyr-64 deprotonation at 220 K. In fact, recent flash kinetic measurements of bR-64 and bR-NH_2-Tyr-64 in the 425-nm region (Roepe, Ahl, and Rothschild, unpublished results) indicate that lowering the temperature does partially block this event.

(iii) bR-N64 Contains an All-trans Chromophore. We have found that all of the chromophoric vibrations in the conformationally sensitive fingerprint region (cf. Table I) have very similar frequencies in bR and bR-N64. If the nitration of Tyr-64 caused retinal to assume a different (e.g., 13-cis) conformation, we would expect to observe frequency shifts in many of these peaks including those at 1255, 1202, and 1167 cm^{-1}. While the intensities of these peaks change, and both the C=C and C=N stretching frequencies upshift in bR-N64, this does not necessarily require a change in the retinal isomeric structure and is likely to be due to altered electron delocalization along the polyene chain.

(iv) The bR-N64 K Intermediate Displays an Altered HOOP Region. The similarity of the fingerprint region for the K intermediate in bR and bR-N64 and in particular the invariance of the 1194-cm^{-1} peak and the shoulder near 1180 cm^{-1} indicates that Tyr-64 nitration does not alter the 13-cis conformation of the K_{630} chromophore. However, the intense peak at 956 cm^{-1}, which has been previously attributed to the HOOP mode involving the C_7–H and C_8–H wags (Braiman, 1983), is considerably reduced in intensity. Since the intense 956-cm^{-1} peak in resonance Raman spectra has been linked to partial twisting around single bonds in the K_{630} chromophore (Braiman & Mathies, 1982), its reduction may indicate the chromophore is relaxed slightly in the K state of bR-N64 relative to K_{630}. However, it is not completely clear how single bond twists should affect the IR intensity of a HOOP mode. Alternatively, this reduction may reflect a change in the chromophore electron distribution due to decreased charge delocalization (indicated by the blue shift of the absorption maximum). In any case, this is likely to be a very localized effect, as other HOOP modes in this region (i.e., at 974, 960, and 940 cm^{-1}) are unaltered in intensity.

(v) Tyr-64 Nitration Strongly Perturbs the Chromophore without Producing a Significant Change in Its Conformation. Both nitration and amination of Tyr-64 produce very similar changes in the FTIR chromophore peaks, as well as in the UV–vis absorption peaks. However, a nitro group and an amino group have different dipole moments. Hence, it is unlikely that the addition of the groups to Tyr-64 alters the chromophore absorption via direct through-space electrostatic field perturbation.

(vi) Nitrated Tyr-64 Interacts Electrostatically with a Carboxyl Group. Nitration of Tyr-64 causes a 2-cm^{-1} downshift in the carboxyl peak at 1739 cm^{-1}, which has been assigned to the protonation of a glutamate group (Engelhard et al., 1985), although recently (Eisenstein et al., 1987), this interpretation has been questioned on the basis of results from bacteriorhodopsin containing isotopically labeled carboxyl groups. Since reduction of the strong electron-withdrawing

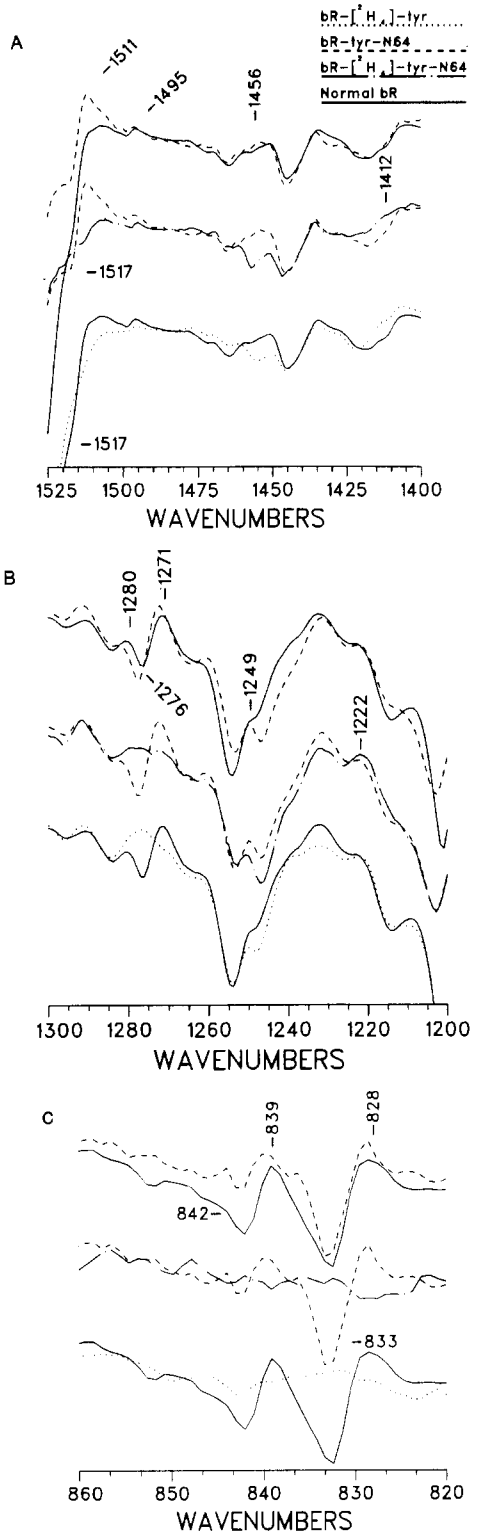

FIGURE 9: As in Figure 8 for the BR → M transition at 250 K. The top comparison is between bR (solid line) and bR-N64 (dashed line), the middle between bR-N64 (dashed line) and bR-[$^2H_4$]Tyr-N64 (dashed–dotted line), and the bottom between bR (solid line) and bR-[$^2H_4$]Tyr (dotted line).

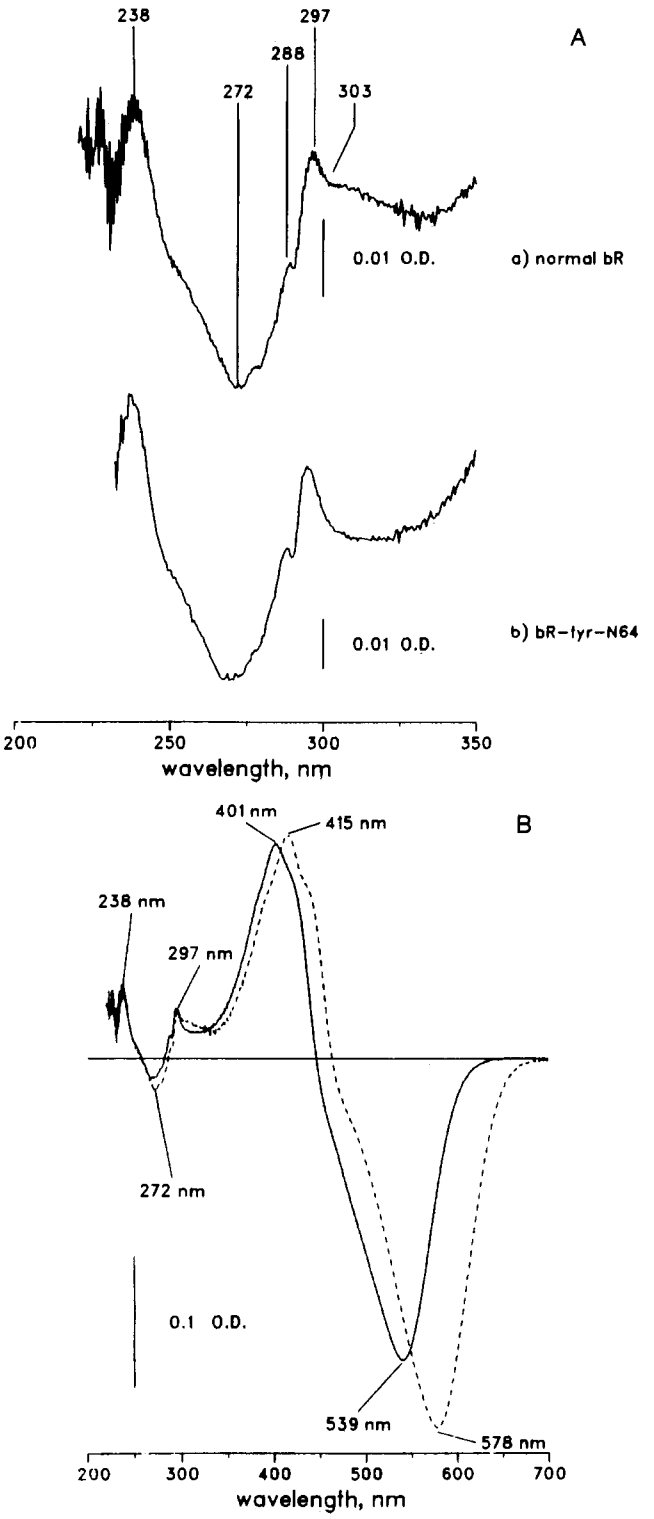

FIGURE 10: (A) BR → M UV difference spectra for bR (top) and bR-N64 (bottom). Spectra were obtained with PM suspensions as described under Materials and Methods. (B) Entire UV–vis region to 700 nm. Dashed line is normal bR, and solid line is bR-NO_2-Tyr-64.

nitro group to an amino group reverses the shift, the effect is likely to be electrostatic. However, the difference in size between the nitro group and amino group may also be involved in this effect.

SUMMARY

In conclusion, previously observed IR signals representing the protonation changes of one or two tyrosines during the bR photocycle are not significantly shifted in frequency by nitration of Tyr-26 or Tyr-64. This indicates that these events are localized to an as yet unidentified tyrosine residue(s). However, a comparison of the IR and UV differences for bR, bR-N64, and bR-[$^2H_4$]Tyr-N64 is consistent with a Tyr-64 deprotonation in agreement with a previous study at room temperature (Scherrer & Stoeckenius, 1985). Hence, we conclude that as many as three tyrosines change protonation state during M_{412} formation.

Future studies will be aimed at further identifying the location of specific tyrosine and carboxyl residues which have

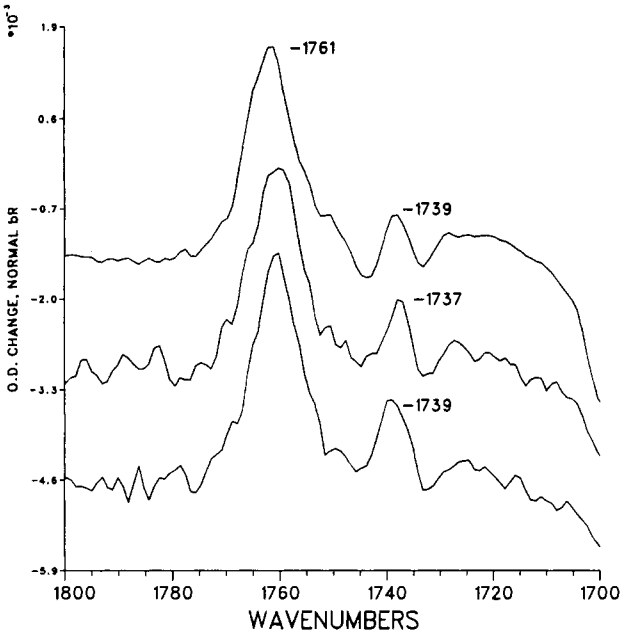

FIGURE 11: Comparison of BR → M difference spectra in the carboxyl region for bR (top), bR-N64 (middle), and bR-NH_2-Tyr-64 (bottom).

been observed to participate in the bR photocycle. In this regard, studies now under way that involve a combination of bR site-specific mutagenesis and FTIR and UV-vis difference spectroscopy will be useful.

ACKNOWLEDGMENTS

We thank T. N. Earnest for obtaining reasonance Raman spectra of bR-N64, M. Tran, J. Gillespie, and J. W. Courtemanche for invaluable technical assistance, and G. Cordrey for help in preparing the manuscript. We also thank Drs. T. Nakayama, M. S. Braiman, and S. O. Smith for helpful discussion.

Registry No. L-Tyr, 60-18-4; *o*-NH_2-L-Tyr, 19040-11-0; *o*-NO_2-L-Tyr, 110012-34-5; poly(L-Tyr,Glu), 31325-39-0.

REFERENCES

Bagley, K., Dollinger, G., Eisenstein, L., Singh, A. K., & Zimanyi, L. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 4972–4976.

Braiman, M. (1983) Ph.D. Dissertation, University of California, Berkeley, CA.

Braiman, M., & Mathies, R. (1982) *Proc. Natl. Acad. Sci. U.S.A. 79*, 403–407.

Callender, R. H., Doukas, A. G., Crouch, R., & Nakanishi, K. (1976) *Biochemistry 15*, 1621–1629.

Clark, N. A., Rothschild, K. J., Luippold, D., & Simmons, B. (1980) *Biophys. J. 31*, 65–96.

Curry, B., Broek, A., Lugtenburg, J., & Mathies, R. (1982) *J. Am. Chem. Soc. 104*, 5274–5286.

Danon, A., & Stoeckenius, W. (1974) *Proc. Natl. Acad. Sci. U.S.A. 71*, 1234–1237.

Dollinger, G., Eisenstein, L., Lin, S.-L., Nakanishi, K., & Termini, J. (1986) *Biochemistry 25*, 6524–6533.

Doukas, A. G., Alton, B., Callender, R. H., & Ebrey, T. G. (1978) *Biochemistry 17*, 2430–2435.

Dunn, R., McCoy, J., Simsek, M., Majumdar, A., Chang, S. H., Rajbhandary, U. L., & Khorana, H. G. (1981) *Proc. Natl. Acad. Sci. U.S.A. 78*, 6744–6748.

Eisenstein, L., Lin, S.-L., Nakanishi, K., Odashima, K., Konno, K., & Ding, W. (1987) *Biophys. J. 51*, 133a.

Engelhard, M., Gerwert, K., Hess, B., Kreutz, W., & Siebert, F. (1985) *Biochemistry 24*, 400–407.

Henderson, R., & Unwin, P. N. T. (1975) *Nature (London) 257*, 28–32.

Jakobsen, R. J., & Brewer, E. J. (1962) *Appl. Spectrosc. 16*, 32–35.

Khorana, H. G., Gerber, G. E., Herlihy, W. C., Gray, C. P., Anderegg, R. J., Nihei, K., & Biemann, K. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5046–5050.

Lemke, H. D., & Oesterhelt, D. (1981) *Eur. J. Biochem. 115*, 595–604.

Oesterhelt, D., & Stoeckenius, W. (1973) *Methods Enzymol. 31*, 667–678.

Ovchinnikov, Yu. A., Abdulev, N. G., Feigina, M. Yu., Kieslev, A. V., & Lobanov, N. A. (1979) *FEBS Lett. 100*, 219–224.

Pouchert, C. J. (1981) *The Aldrich Library of Infrared Spectra*, 3rd ed., Aldrich Chemical Co., Milwaukee.

Racker, E., & Stoeckenius, W. (1974) *J. Biol. Chem. 249*, 662–667.

Roepe, P. (1987) Ph.D. Dissertation, Boston University, Boston, MA.

Roepe, P., & Rothschild, K. J. (1987) *Bull. Am. Phys. Soc. 32*, 410.

Roepe, P., Ahl, P. L., Scherrer, P., Herzfeld, J. H., Bogomolni, R., & Rothschild, K. J. (1986) *Biophys. J. 49*, 479a.

Roepe, P., Ahl, P. L., Das Gupta, S. K., Herzfeld, J., & Rothschild, K. J. (1987) *Biochemistry* (preceding paper in this issue).

Rosenbach, V., Goldberg, R., Gilon, C., & Ottolenghi, M. (1982) *Photochem. Photobiol. 36*, 197–201.

Rothschild, K. J. (1986) *Methods Enzymol. 127*, 343–353.

Rothschild, K. J., Roepe, P., Lugtenburg, J., & Pardoen, J. A. (1984) *Biochemistry 23*, 6103–6109.

Rothschild, K. J., Roepe, P., & Gillespie, J. (1985) *Biochim. Biophys. Acta 808*, 140–148.

Rothschild, K. J., Roepe, P., Ahl, P. L., Earnest, T. N., Bogomolni, R. A., Das Gupta, S. K., Mulliken, C. M., & Herzfeld, J. (1986) *Proc. Natl. Acad. Sci. U.S.A. 83*, 347–351.

Scherrer, P., & Stoeckenius, W. (1984) *Biochemistry 23*, 6195–6202.

Scherrer, P., & Stoeckenius, W. (1985) *Biochemistry 24*, 7733–7740.

Siamwiza, M. N. (1974) Ph.D. Dissertation, Massachusetts Institute of Technology, Cambridge, MA.

Siamwiza, M. N., Lord, R. C., Chen, M. C., Takamatsu, T., Harada, I., Matsuura, H., & Shimanouchi, T. (1975) *Biochemistry 14*, 4870–4876.

Smith, S. O., Myers, A. B., Mathies, R., Pardoen, J. A., Winkel, C., Van Den Berg, E. M. M., & Lugtenburg, J. (1985) *Biophys. J. 47*, 653–664.

Stockburger, M., Klusmann, W., Gattermann, H., Massig, G., & Peters, R. (1979) *Biochemistry 18*, 4886–4900.

Trewhella, J., Anderson, S., Fox, R., Gogol, E., Khan, S., & Engelman, D. (1983) *Biophys. J. 42*, 233–241.