Deprotonation of Tyrosines in Bacteriorhodopsin As Studied by Fourier Transform Infrared Spectroscopy with Deuterium and Nitrate Labeling†

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ABSTRACT: Fourier transform infrared (FTIR) difference spectra are presented for bacteriorhodopsin (BR) at low temperature. Previous FTIR measurements have identified several tyrosine residues that change their absorption characteristics between light-adapted BR and dark-adapted BR, or between intermediates K and M [Dollinger, G., Eisenstein, L., Lin, S.-L., Nakanishi, K., Odashima, K., & Termini, J. (1986) *Methods Enzymol. 127*, 649–662]. These changes were explained by protonation/deprotonation of tyrosine moieties and perturbation of the protein environment surrounding tyrosines. A tyrosine deprotonation was observed to occur between intermediates K and M. The present studies confine the deprotonation to being between intermediates L and M and show that no tyrosines undergo changes between the K and the L states. Evidence is presented that none of the tyrosines undergoing changes at low temperature can be assigned to tyrosine-64. The environmental changes of these tyrosines are discussed in relation to the proton pumping mechanism. Their spatial relation to the chromophore is also discussed. At least two tyrosines are suggested to reside close to the retinal binding site. The reactive groups of the nitrated tyrosine-64 are speculated to be remote from the Schiff base and the active tyrosines but can possibly interact sterically with the ionone ring of the retinal.

Bacteriorhodopsin (BR)[1] is the sole protein in the purple membrane from *Halobacterium halobium*. It harvests light energy to pump protons from the inside to the outside of the cell, establishing a pH gradient that is utilized by the cell to drive metabolic processes such as ATP synthesis [for reviews, see Stoeckenius et al. (1979) and Stoeckenius and Bogomolni (1982)]. The photocycle of BR includes at least four intermediates (K, L, M, and O) and involves the translocation of one to two protons across the membrane (Stoeckenius & Bogomolni, 1982):

$$BR_{568} \xrightarrow{h\nu} K_{625} \longrightarrow L_{550} \xrightarrow{-H^+} M_{412} \xrightarrow{+H^+} O_{640}$$

(the subscripts refer to wavelength maxima, in nanometers). The molecular mechanism of the light-driven proton translocation is under intensive investigation. A complete understanding of the pumping function requires details of correlation between the proton motion and the conformational changes of the molecule. BR has a prosthetic retinal group as its chromophore, linked to Lys-216 of the apoprotein through a Schiff base. It is known that during the photocycle the chromophore undergoes trans–cis isomerizations as well as changes in the protonation state of the Schiff base. In the light-adapted BR the retinal is in the all-trans form and has a protonated Schiff base (PSB) linkage. In the K intermediate, retinal is isomerized to a distorted 13-cis form; this isomerization is completed in the L state. The Schiff base proton is subsequently released, leading to the M state. Before returning to BR, the retinal regains the Schiff base proton and is isomerized to the all-trans form in the red-shifted intermediate O. When BR is placed in the dark, it thermally equilibrates to the dark-adapted (DA) BR, a state in which the chromophore consists of a 6:4 (Pettei et al., 1977; Maeda et al., 1977; Harbison et al., 1984) or 2:1 (Scherrer et al., 1987) mixture of 13-cis and all-trans retinal.

While investigations of the chromophore have made considerable progress in recent years (Smith et al., 1985), the study of the much more complicated apoprotein moiety is still in an early stage. Spectroscopic studies of the apoprotein have focused on amino acids with terminal carboxylic groups or phenolic groups (Bagley et al., 1982; Rothschild & Marrero, 1982; Siebert et al., 1983; Engelhard et al., 1985; Hess & Kuschmitz, 1979; Hanamoto et al., 1984; Dollinger et al., 1986a,b; Rothschild et al., 1985) because of their proton-transfer capabilities. A tyrosine deprotonation was reported to occur in concomitance with the formation of M (Hess & Kuschmitz, 1979; Hanamoto et al., 1984). Specific chemical modifications of Tyr-64 and Tyr-26 in BR have also been exploited to clarify the role of tyrosines (Lemke & Oesterhelt, 1981; Lemke et al., 1982; Rosenbach et al., 1982; Scherrer & Stoeckenius, 1984, 1985).

Isotopic labeling has been successfully used in the FTIR studies of amino acid residues in BR (Engelhard et al., 1985; Dollinger et al., 1986a,b; Rothschild et al., 1985). Using low-temperature FTIR difference spectra of native BR and

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‖ Deceased Aug 14, 1985. This paper is dedicated to Laura Eisenstein, who initiated the project.

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[1] Abbreviations: FTIR, Fourier transform infrared; BR, bacteriorhodopsin; LA, light-adapted BR; DA, dark-adapted BR; Tyr(OH), tyrosine; Tyr(O^-), tyrosinate; BR-N64, bacteriorhodopsin labeled with 3-nitro-Tyr-64; PSB, protonated Schiff base.

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BR incorporated with deuteriated tyrosine residues, Dollinger et al. (1986b) detected two tyrosine residues that undergo changes in the protonation states and the environment during the photocycle of BR. A 1478-cm^{-1} band, attributed to one of the C=C ring-stretching vibrations in 3,5-dideuterio-L-tyrosine, and a 1269-cm^{-1} band, attributed to the C–O^- stretching in tyrosinate, were found to be of diagnostic value for identification of tyrosine and tyrosinate residues, respectively (Dollinger et al., 1986b). It was noted that a 1484-cm^{-1} line present in the K intermediate in the K/LA difference spectrum was absent in the M intermediate in the M/LA difference spectrum, and this change was attributed to a deprotonation process during the K to M transformation. In this paper we present evidence that this deprotonation occurs between the L and the M intermediates and examine whether any of the tyrosine residues exhibiting changes in the FTIR studies is Tyr-64; we also discuss the implication of our findings for the structure of BR.

Materials and Methods

Preparation of 3,5-dideuterio-L-tyrosine and BR samples with native and deuterium-labeled tyrosines was carried out as described earlier (Dollinger et al., 1986b). Specific nitration of Tyr-64 for both BR samples was carried out with tetranitromethane (TNM) at pH 6 in light according to the procedure of Scherrer and Stoeckenius (1984). The completeness of nitration was estimated by both visible and infrared spectroscopy to be >90%.

We will denote L-tyrosine as Tyr-d_0, 3,5-dideuterio-L-tyrosine as Tyr-d_2, their corresponding BR samples as Tyr-d_0-BR and Tyr-d_2-BR, respectively, and the nitrated samples as Tyr-d_0-BR-N64 and Tyr-d_2-BR-N64, respectively. The protonated state and ionic state of a tyrosine will be specified by postfixes (OH) and (O^-), respectively.

FTIR experiments were carried out on a Sirius 100 FTIR spectrometer (Mattson Instruments Inc., Madison, WI). BR sampling and generation of K/LA and M/LA difference spectra were achieved as described in Dollinger et al. (1986b), except that the M/LA spectra of nitrated samples were measured at 270 K. To obtain the L/LA difference spectrum, the sample was light-adapted at room temperature and then cooled down to 160 K. After the LA spectrum was measured, the sample was illuminated with green light (530 nm for native BR and 500 nm for nitrated BR) and then with red light (700 nm for native BR and 650 nm for nitrated BR) for 3 min each. A second spectrum was taken. The subtraction of the LA spectrum from the second spectrum produced the L/LA difference spectrum. Each single spectrum is an average of a 10-min measurement with 2-cm^{-1} resolution. Under these experimental conditions, root mean square noise level in the 2000–1900-cm^{-1} region was within a few 10^{-5} OD units. Absorption lines to be discussed were at least 1 order of magnitude higher than the noise.

In the difference spectra the negative absorbance represents LA vibration modes, while the positive absorbance represents the K, L, or M vibration modes. If any spectral changes were observed only between samples prepared with different isotopes, the changing spectral characteristics were interpreted as absorption from isotope-related vibration modes. Absorption bands in BR difference spectra that have noticeable sensitivity to tyrosine isotope substitution are therefore of diagnostic value in identifying tyrosine changes in the BR spectra. Two of such modes chosen to clarify the complexity of the difference spectra were the Tyr(OH)-d_2 C=C stretching at ca. 1480 cm^{-1} and Tyr(O^-)-d_0 C–O^- stretching at ca. 1277 cm^{-1}. They were selected also on the basis of their unambiguous reflection of

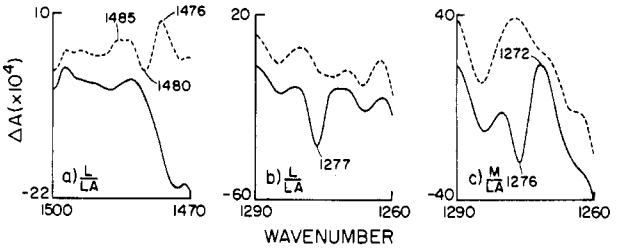

FIGURE 1: Difference spectra of Tyr-d_2-BR (broken line) and Tyr-d_0-BR (solid line) (a) between L and LA in the 1500–1470-cm^{-1} region, (b) between L and LA in the 1290–1260-cm^{-1} region, and (c) between M and LA in the 1290–1260-cm^{-1} region. The marked peaks are identified as Tyr(OH)-d_2 ring C=C stretch at 1478 cm^{-1} and Tyr(O^-)-d_0 phenolic C–O^- stretch at 1269 cm^{-1}. The data indicate a tyrosine protonation in the L → M transformation.

the tyrosine protonation states, as demonstrated before in a model compound study (Dollinger et al., 1986b). IR spectra of nitrated tyrosine were obtained at pH (pD) ~1 and ~13 to verify the influence of nitration on the corresponding bands. Tyr(OH)-d_2 and Tyr(O^-)-d_0 bands in the BR-N64 difference spectra, identified by the above-mentioned deuterium-labeling method, were compared with those identified in native BR to demonstrate the influence of Tyr-64 nitration.

Results and Discussion

Tyrosine Deprotonation during the L to M Transition. The new FTIR difference spectra between species L and LA and the lower frequency region for the difference between species M and LA are depicted in Figure 1, while the current and previous results (Dollinger et al., 1986b) are summarized in Figure 2. In all cases, the analyses are derived from the two characteristic frequencies, i.e., the 1478-cm^{-1} C=C stretch of Tyr(OH)-d_2 and the 1269-cm^{-1} C–O^- stretch of Tyr(O^-)-d_0.

(1) LA → DA: The presence of a tyrosinate peak at 1276 cm^{-1} in LA and a tyrosine peak at 1480 cm^{-1} in DA was interpreted as a *protonation* during dark adaptation.

(2) LA → K: One tyrosine 1479 cm^{-1} and one tyrosinate 1277 cm^{-1} change to two tyrosine peaks at 1484 and 1476 cm^{-1}; thus this change involves *protonation* of one tyrosine and environmental change at another tyrosine.

(3) LA → L: The result shows that a tyrosine and a tyrosinate peak at 1480 and 1277 cm^{-1} change to two tyrosine peaks at 1485 and 1476 cm^{-1}; namely, protonation states and environments of the Tyr residues in L remain practically unaltered from those in K.

(4) LA → M: A positive tyrosine band is observed at 1272 cm^{-1} in the M spectrum. This peak is not found in the L spectrum, and hence it means that *a tyrosine is formed during the L to M transformation.* This conclusion is consistent with the results of Hanamoto et al. (1984), obtained by kinetic UV spectroscopy.

An unusual incident is the presence of a tyrosinate in LA despite the fact that all titratable tyrosine residues of BR have pK_a's >11.0 in the dark (Scherrer & Stoeckenius, 1984). To remove the hydroxyl hydrogen of the tyrosine at neutral pH requires a very strong perturbation such as the movement of a remote positive charge to within a few angstroms (Dollinger et al., 1986b). This postulated positive charge can be any of the protein-bound inorganic cations or positively charged amino acid residues, e.g., arginine or lysine. It has been suggested that tyrosinate might be the counteranion of the PSB (Rothschild et al., 1985). However, this assumption is unlikely in the light of the results concerning the LA → DA transition. It is known that a 13-cis/trans isomerization of the retinal is involved between the LA and DA species, i.e., LA is all-trans

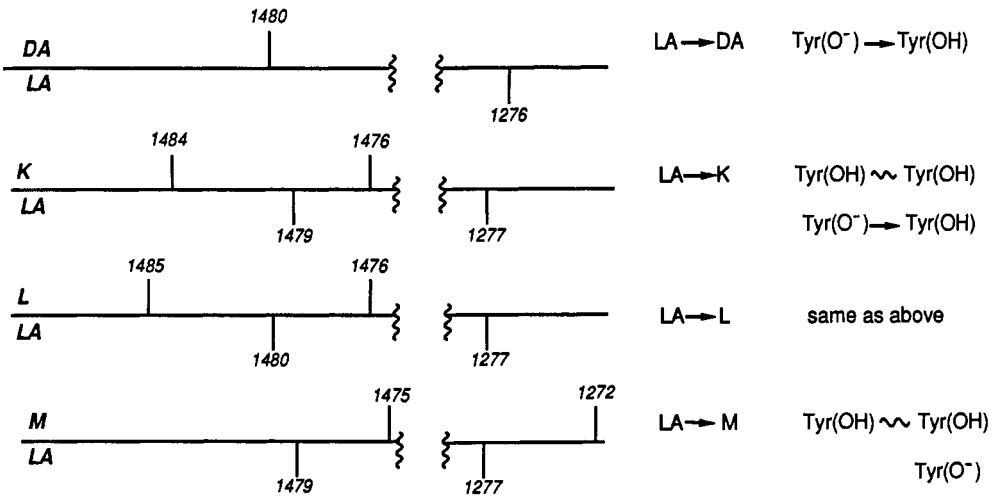

FIGURE 2: Summary of tyrosine protonation/deprotonation changes detected by FTIR difference spectroscopy between various BR species. Tyrosine species separated by a wavy line simply denote environmental changes whereas those separated by arrows denote protonation or deprotonation. The two diagnostic bands used for analyses, the 1478-cm^{-1} C=C stretch in Tyr(OH)-d_2 and the 1269-cm^{-1} C–O^- stretch in Tyr(O^-)-d_0 (Dollinger et al., 1986b), are depicted schematically.

but DA is a ca. 6:4 mixture of all-trans and 13-cis isomers (Pettei et al., 1977; Maeda et al., 1977; Harbison et al., 1984). If the tyrosinate were the counterion in both the LA and DA species, the difference spectrum should show at least an environmental difference of the Tyr(O^-) peak; in contrast, the spectrum in Figure 2 simply shows a protonation in the conversion from LA to DA. A switch of counteranions between the LA and DA species, i.e., replacement of the tyrosinate as the counteranion during dark adaptation, would be unlikely, although it would not be excluded, if one takes into account the "bicycle-pedaling" model of isomerization suggested by resonance Raman and NMR experiments (Smith et al., 1987; Harbison et al., 1984). In this model an accompanying C=N isomerization occurs that results in little displacement of the PSB and unchanged N^+–H bond orientation. The preserved PSB–counteranion relationship thus inferred has been put forward by Harbison et al. (1983, 1984) to interpret the identical ^{15}N NMR chemical shifts for both isomers in DA. Most likely, the counteranion is an aspartate residue (Dollinger et al., 1986a; Eisenstein et al., 1987).

We also propose that the tyrosinate and the tyrosine found in LA are close to the retinal, to account for their sensitivity in the LA → K transition. In this transition the protonated all-trans chromophore isomerizes to a distorted 13-cis form within the retinal binding site and cannot relax to a planar structure. The fact that the transition completes within a few picoseconds at room temperature (Downer et al., 1984) and can happen at temperatures as low as 10 K (unpublished data) supports the idea that this primary event of the photoreaction cycle involves only the protein core, consisting of the retinal and a limited volume of salt-bridged and hydrogen-bonded neighbors. The identification of tyrosine protonation and disturbance during the transition by low-temperature FTIR spectroscopy, as well as the medium deuteriation effect on picosecond kinetics (Downer et al., 1984), directly and indirectly points to the presence of tyrosines in this vicinity. The observed changes may reflect the concomitant adjustment of the chromophore binding site to the strain induced by the retinal isomerization.

The C–O^- stretch frequency of authentic tyrosinate in aqueous phase was detected at 1269 cm^{-1} (Dollinger et al., 1986b); when incorporated into a polypeptide chain, poly(LTyr-LGlu), the center of the band moved slightly higher to 1271 cm^{-1} (Dollinger et al., 1986b). The 1277-cm^{-1} location of the BR Tyr(O^-) peaks reflects shift to higher frequencies. High-frequency shifts indicate a decrease in hydrogen bonding of the phenolate ion (Pinchas, 1972). Surrounding water is thus assumed to be less accessible to the tyrosinate in LA. Indeed, a hydrophobic barrier can help prevent the tyrosinate from spontaneous reprotonation. FTIR studies of BR samples prepared in ^2H_2O showed that the ca. 1484-cm^{-1} peaks in K/L and the ca. 1476-cm^{-1} peaks in K/L/M red shifted to 1481 and 1470 cm^{-1}, respectively, while in authentic tyrosine this C=C stretching peak shifted from 1478 to 1475 cm^{-1} (Dollinger, 1986; Termini, 1986). We thus concluded that the tyrosine in question undergoes H–^2H exchange of its hydroxyl proton during the photocycle.

At least two hypothetical mechanisms can induce the H–^2H exchange. First, the environment of the tyrosine may open up to the aqueous phase as the result of protein conformational change; second, the tyrosine may be connected to a buried "proton wire" (Nagle & Tristram-Nagle, 1983). There have been several reports about titration of a tyrosine in the L → M transition (Kalisky et al., 1981; Hanamoto et al., 1984). Hanamoto et al. also proposed another titratable tyrosine as a candidate for the agent responsible for the two components of the kinetics accompanying the rise of M. If the tyrosines detected in the FTIR spectra are these two, then they actually become accessible to water no later than the L → M decay. Considering the importance of the L → M transition, opening a water channel to a tyrosine may be a significant step in the photocycle and proton pumping. Since the timing of this occurrence is not clear, the proton wiring cannot be excluded to happen at a stage before L. Functionally, an establishment of such a connection can serve as a gating system in proton translocation. There is a possibility that a premature tyrosinate protonation together with the PSB proton orientation in an unfavorable direction cuts the proton pathway in the dark photocycle which lacks proton-pumping capability (Smith et al., 1985; Dollinger et al., 1986b).

Spectra of Nitrated Bacteriorhodopsin. The nitration of Tyr-64 blue shifts the absorption maximum from 568 to ca. 535 nm (Scherrer & Stoeckenius, 1984). As expected from the empirical linear relation of the absorption maxima and the ethylenic stretch frequencies of retinal pigments (Aton et al., 1977), the chromophore C=C stretch mode of nitrated BR shifts to higher frequencies by ca. 10 cm^{-1}. Figure 3 shows the K/LA difference spectra of the native series and of the BR-N64 series. The frequencies of retinal ethylenic stretch in LA-N64 and K-N64 are at 1540 and 1528 cm^{-1}, blue

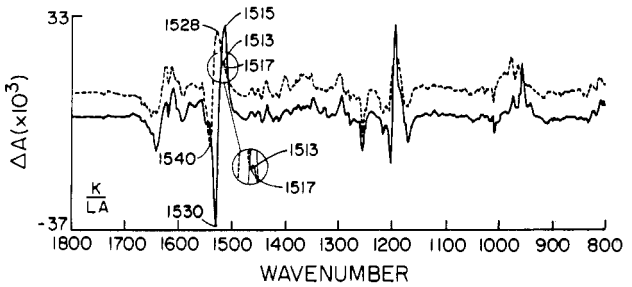

FIGURE 3: Difference spectra of Tyr-d_0-BR-N64 (broken line) and Tyr-d_0-BR (solid line) between K and LA in the 1800–800-cm^{-1} region.

shifted from 1530 and 1515 cm^{-1}, respectively. There is no perceivable residual absorption of unmodified species, thus indicating that the nitration is nearly complete. The upshift seen in the K-N64 ethylenic peak exposes the 1513–1517-cm^{-1} shoulders (Figure 3, inset) on the low-frequency side which are absent in the corresponding Tyr-d_2-BR-N64 spectrum (data not shown), an observation akin to what Dollinger et al. (1986b) found in a BR sample suspended in ^2H_2O as indication of the existence of Tyr-d_0 peaks in this region.

All four above-mentioned photointermediates were reported to exist in nitrated BR (Scherrer & Stoeckenius, 1985). Under slightly modified experimental conditions, we were able to obtain comparable K/LA and M/LA difference spectra and an L/LA difference spectrum with a substantial component of M which we will denote as (L + M)/LA. The differences between the spectra of nitrated and native samples will be discussed separately. We will base the following discussion on the comparison of spectra gathered from various sample preparations. As before, comparison of spectra of Tyr-d_0-BR-N64 and Tyr-d_2-BR-N64 reveals characteristic frequencies associated with Tyr(OH)-d_2 at 1478 cm^{-1} and Tyr(O$^-$)-d_0 at 1269 cm^{-1}. Model compound studies show that nitration causes the strongest C=C stretch mode of Tyr(OH) to blue shift by ca. 10 cm^{-1} and the C–O$^-$ stretch mode of Tyr(O$^-$) to red shift by ca. 17 cm^{-1} (unpublished data).

As shown in Figure 4, the reappearance of all of the detected Tyr-d_2 and Tyr-d_0 lines, seen in unnitrated samples (compare with Figures 1 and 2), indicates that none of the changes originates from Tyr-64. In the 1480-cm^{-1} region of the K/LA difference spectrum (Figure 4a), three peaks are prominent only in the Tyr-d_2-BR-N64 sample (broken line); two of them are K peaks at 1485 and 1475 cm^{-1}, and the third is an LA peak at 1479 cm^{-1}. The 1475- and 1479-cm^{-1} peaks still appear in the M/LA difference spectrum (Figure 4b, broken line), but the 1485-cm^{-1} peak disappears. In the lower frequency region, a 1278-cm^{-1} peak is distinctive on the LA side of both the native K/LA and M/LA difference spectra, while a positive 1272-cm^{-1} peak is present in native M (Figure 4d,e, solid lines). The L intermediate of nitrated samples was always in equilibrium with M under our experimental conditions. The positive side of Figure 4c,f is a mixture of L and M absorbance. The observation that the 1485-cm^{-1} peak is present in L + M (Figure 4c, broken line) but absent in M (Figure 4b, broken line) suggests that it comes from the L component (as in the L/LA spectrum, Figure 2). In summary, the changes observed with the nitrated samples are closely analogous to changes seen in native BR (Figure 2). Namely, for LA we detect one Tyr-d_2 peak at 1479 cm^{-1} and one Tyr-d_0 peak at 1278 cm^{-1}, for K and L two Tyr-d_2 peaks at 1485 and 1475 cm^{-1}, and for M one Tyr-d_2 peak at 1474 cm^{-1} and one Tyr-d_0 peak at 1272 cm^{-1}. In addition, all these peaks show frequency shift and amplitude change in ^2H_2O (unpublished data) similar to those observed in unnitrated samples (Dollinger et al., 1986b).

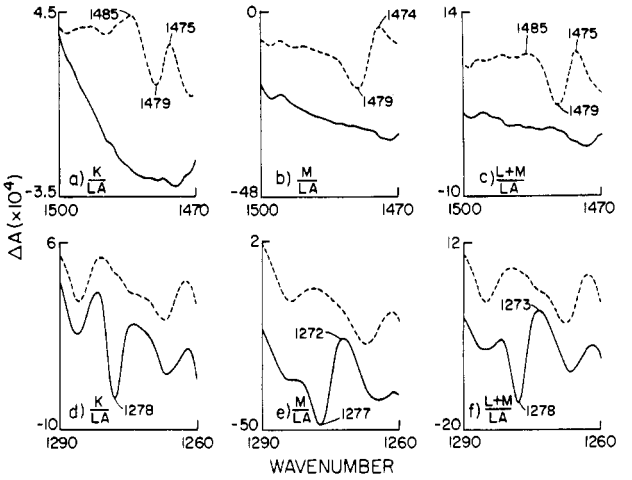

FIGURE 4: Difference spectra of Tyr-d_2-BR-N64 (broken line) and Tyr-d_0-BR-N64 (solid line), in the 1500–1470-cm^{-1} (a–c) and 1290–1260-cm^{-1} (d–f) regions, between K and LA (a, d), between M and LA (b, e), and between a mixture L + M and LA (c, f).

Comparison of the shapes of panels a, b, d, and e of Figure 4 with those of corresponding native bR spectra (Dollinger et al., 1986b) shows that these regions characteristic of Tyr residues remain unaltered whether Tyr-64 is nitrated or not. The lack of nitration effect suggests the following: (1) They are not associated with Tyr-64 modes. (2) The disturbance and protonation state changes of tyrosine residues appearing in the difference spectra are not influenced by nitration of Tyr-64; the apparent lack of interaction between Tyr-64 and the observed tyrosines suggests that they are not close to each other. As discussed before, those tyrosines are located in the vicinity of the chromophore binding site. Tyr-64 can be remote from the chromophore so that, as suggested by Scherrer and Stoeckenius (1985), its nitration affects the chromophore indirectly; e.g., the effect is mediated by apoprotein conformational change. Another possibility is that the phenolic ring of Tyr-64 is located in a protein pocket near the ionyl ring; the additional bulk of the nitro group sterically distorts the planar configuration of the chromophore, which would account for the effects reported by Scherrer and Stoeckenius (1984, 1985). A nonplanar ring–chain configuration could account for the ca. 30-nm absorption maximum blue shift (Honig et al., 1976). The observation that both nitro-Tyr-64 and amino-Tyr-64 cause strong but similar spectroscopic and kinetic effects on BR despite their difference in 3 pK_a units can also be explained by assuming that the active site of Tyr-64 is remote from the PSB. The configuration change induced by nitration in situ probably locks the retinal inside the protein pocket by the steric effect and/or protein conformational change, thus leading to the extremely low retinal extraction ratio [5% in the case of BR-N64 vs 60–80% in the case of BR (Scherrer & Stoeckenius, 1985)].

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