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Tyrosine Structural Changes Detected during the Photoactivation of Rhodopsin*

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We present the first Fourier transform infrared (FTIR) analysis of an isotope-labeled eukaryotic membrane protein. A combination of isotope labeling and FTIR difference spectroscopy was used to investigate the possible involvement of tyrosines in the photoactivation of rhodopsin (Rho). Rho → MII difference spectra were obtained at 10 °C for unlabeled recombinant Rho and isotope-labeled L-[*ring*-$^2H_4$]Tyr-Rho expressed in *Spodoptera frugiperda* cells grown on a stringent culture medium containing enriched L-[*ring*-$^2H_4$]Tyr and isolated using a His_6 tag. A comparison of these difference spectra revealed reproducible changes in bands that correspond to tyrosine and tyrosinate vibrational modes. A similar pattern of tyrosine/tyrosinate bands has also been observed in the bR → M transition in bacteriorhodopsin, although the sign of the bands is reversed. In bacteriorhodopsin, these bands were assigned to Tyr-185, which along with Pro-186 in the F-helix, may form a hinge that facilitates α-helix movement.

Elucidation of the mechanism of photoactivation of rhodopsin (Rho),[1] the light receptor in vision, remains an important problem in biology (1). Rhodopsin is an integral membrane protein found in the disc photoreceptor membranes of rod outer segments (2, 3) with a core structure consisting of seven transmembrane α-helices (4–7). Upon light absorption, the rhodopsin chromophore, 11-*cis*-retinal, rapidly isomerizes to an all-*trans* configuration (8, 9) followed by a series of thermal transitions (Batho → Lumi → Meta I → Meta II) (10, 11). Signal transduction occurs upon formation of the Meta II intermediate, which binds and activates the G-protein transducin (12, 13). Because rhodopsin is a G-protein-coupled receptor, elucidation of its molecular mechanism is likely to be of general importance for the vast suprafamily of G-protein-coupled receptors, which include the β-adrenergic receptor (14, 15) and olfactory receptors (16).

Thus far, bR is the only IMP with a seven-helix transmembrane motif whose structure has been elucidated at atomic resolution (17–19). This structure confirmed key features of an earlier "spectroscopic" model based in part on site-directed mutagenesis and FTIR difference spectroscopy (20–23). For example, a retinal binding pocket was predicted on the basis of FTIR, UV-visible spectroscopy, and site-directed mutagenesis (20–24), which is formed in part from several residues on the F-helix (helix 6 in rhodopsin), including two tryptophans (Trp-182, Trp-189), a proline (Pro-186), and a tyrosine (Tyr-185). These residues, along with several others from the C-helix (Trp-86, Thr-89, and Asp-85) are in close proximity to the retinal chromophore and act to constrain its structure in an all-*trans* configuration. In addition, these residues are in a good position to couple retinal isomerization to protein changes involved in proton transport, including a change in the structure and orientation of the F-helix.

A comparable combination of Trp, Pro, and Tyr residues (W*X*PY) is fully conserved in helix 6 of all visual pigments except the blue pigments where it is Y*X*PY. It is possible that, although the tyrosine-proline sequence is reversed, a similar coupling between chromophore isomerization and protein exists in the two systems (22) despite their different functions and evolutionary pathways. To investigate this possibility, we have used for the first time a combination of FTIR difference spectroscopy and isotope labeling to analyze tyrosine structural changes in rhodopsin. A comparison with earlier results obtained for bacteriorhodopsin reveals that a similar, although reversed, pattern of tyrosine/tyrosinate changes does occur. This reversed pattern might be explained if the all-*trans* to *cis* transition of the chromophore in both systems is coupled to similar changes in adjacent residues in agreement with an earlier suggestion (25).

EXPERIMENTAL PROCEDURES

Preparation of Isotope-labeled Rhodopsin—Wild-type hexahistidine-tagged rhodopsin derived from bovine rhodopsin was produced and purified as described previously (26). The hexahistidine tag introduces a slight redshift in the λ_{max} of rhodopsin from 498 to 500 nm. For the purpose of isotope labeling, *Spodoptera frugiperda* (Sf9) cells (ATCC: CRL-1711) were grown and subsequently infected in a customized serum-free and protein-free medium partially depleted in L-Tyr (Biowhittaker, Walkersville, MD) to which an additional 2 mM L-[*ring*-$^2H_4$]Tyr (Cambridge Isotope Laboratories, Andover, MA) was added. To determine label incorporation levels, total proteoliposome fractions were treated with Pronase to generate free amino acids. Subsequent gas chromatography-mass spectrometry analyses of two different samples revealed that 70 ± 3% of membrane protein-incorporated tyrosines

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[1] The abbreviations used are: Rho, rhodopsin; Meta II, metarhodopsin II; bR, bacteriorhodopsin; FTIR, Fourier transform infrared; L-[*ring*-$^2H_4$]Tyr, tyrosine labeled with deuterium at all ring positions; L-[*ring*-$^2H_4$]Tyr–Rho, rhodopsin containing L-[*ring*-$^2H_4$]Tyr; L-[*ring*-$^2H_4$]Tyr–bR, bacteriorhodopsin containing L-[*ring*-$^2H_4$]Tyr; WT, wild-type.

were labeled.[2] No detectable labeling was found to occur in Phe residues as expected, because in eukaryotic cells Tyr is degraded through the Krebs cycle, and thus the labels should not be incorporated into an essential amino acid like Phe.

FTIR Spectroscopy—Rhodopsin films for transmission IR spectroscopy were prepared by isopotential spin-drying (5, 27) of a 1-ml aqueous suspension of membranes containing approximately 3 nmol of unlabeled or L-[*ring*-$^2H_4$]Tyr-labeled Rho-His_6 onto a AgCl window. The film is then rehydrated prior to insertion into a sealed transmittance cell as described previously (28). Transmission FTIR difference spectra of the hydrated rhodopsin films were recorded at 10 °C using methods similar to those reported previously (29, 30). Briefly, the H_2O content of the sample was monitored by measuring the intensity ratio of the 3400-cm^{-1} band (O–H stretch mode) to the protein Amide II band near 1545 cm^{-1}. The Rho → Meta II difference spectra were obtained as follows: the sample was photobleached for 3 min using light from a 150-watt tungsten illuminator (model 180, Dolan-Jenner Industries, Lawrence, MA) filtered by a 500-nm long-pass filter (Corion Corp., Holliston, MA) and several heat filters and transmitted to the sample with an annular optical fiber. Spectra were recorded at 8 cm^{-1} resolution and 5-min intervals for several hours before and after illumination (1350 scans for each spectrum) on a Bio-Rad FTS-60A spectrometer (Bio-Rad, Digilab Division, Cambridge, MA) equipped with a mercury-cadmium-teluride detector. Each difference spectrum shown represents a subtraction of the spectrum recorded immediately before the light is turned on from the spectrum recorded immediately after illumination. The larger set of spectra are recorded prior to and after illumination to monitor sample stability, hydration, and Meta II decay.

FTIR transmission spectra of tyrosine and L-[*ring*-$^2H_4$]Tyr-saturated solutions were measured at room temperature at pH 12. Each sample was prepared by dissolving Tyr or L-[*ring*-$^2H_4$]Tyr to saturation in a borate buffer that was titrated using KOH to pH 12. A small drop (~7 μl) of this solution was placed on a CaF_2 window, and a second CaF_2 window was pressed against it to form a uniform thin film. The windows were inserted into a liquid sample cell (Harrick Scientific Corp., Ossining, NY; TFC-M32) and mounted in a Nicolet 740 FTIR spectrometer (Nicolet Instruments, Madison, WI) equipped with a triglyceride sulfate detector. Spectra were collected consisting of 512 scans. An identical method was used to record a spectrum of the buffer, which was interactively subtracted from the absorption spectrum of the sample using the 3400-cm^{-1} water band as a reference.

RESULTS

Fig. 1 shows a comparison of the Rho → Meta II difference spectra from WT and L-[*ring*-$^2H_4$]Tyr-Rho in the region from 1800 to 1000 cm^{-1}. To assess the reliability of assignments using tyrosine isotope labeling, three samples of both WT and L-[*ring*-$^2H_4$]Tyr-Rho were measured independently, and the resulting data are shown in Fig. 1. All spectra show very similar features, reflecting changes of the retinylidene chromophore, which undergoes an 11-*cis* to all-*trans* conformational transition, as well as structural changes of the protein required to create a binding site for the G-protein transducin. For example, negative bands have been assigned to the Rho chromophore on the basis of comparison with results from resonance Raman spectroscopy at 1548 cm^{-1} (−) (C=C ethylenic stretch) and 1238 cm^{-1} (−) (C_{12}–C_{13} stretch mode)[3] (31–34). In contrast, few bands have been assigned thus far to specific amino acids except in the 1700–1800-cm^{-1} region (C=O stretch modes of Asp and Glu carboxylic acid groups (28, 35)) and near 2550 cm^{-1} (the SH stretch mode of cysteine residues (36)).

As seen in Fig. 1, the introduction of L-[*ring*-$^2H_4$]Tyr into Rho produces several highly reproducible changes in the region between 1800 and 1000 cm^{-1}. In particular, several bands are altered with a change in intensity, reflecting an isotope-induced shift in frequency, including those near 1595, 1570, 1517, 1423, 1373, 1348, 1275, 1253, 1221 (sh), and 1178 cm^{-1}. There are also bands near 1643 and 1535 cm^{-1} whose intensities are not reproducible within each set and are therefore not assigned

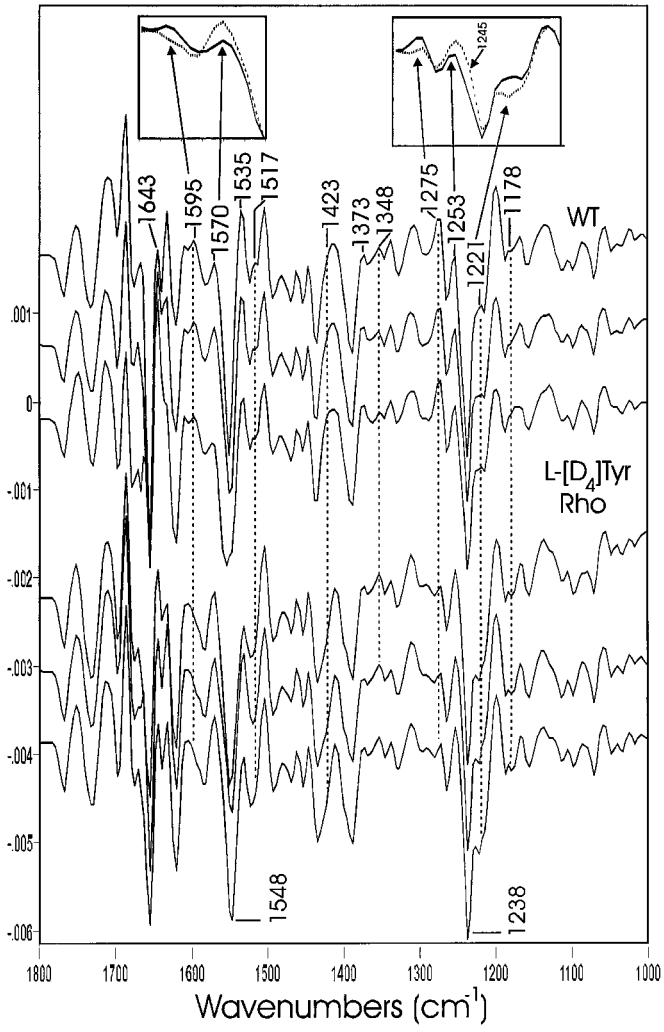

FIG. 1. **FTIR difference spectra of the Rho → Meta II transition recorded at 10 °C for three independent samples of WT and three independent samples of L-[*ring*-$^2H_4$]Tyr-Rho, hydrated with H_2O.** The *Y* scale shown is for the WT spectrum. *Insets*, expansion along the *x* axis of the lower traces in the regions 1610–1560 cm^{-1} (*left box*) and 1290–1190 cm^{-1} (*right box*). (Note the *Y* scale is compressed for the *right inset*). The L-[*ring*-$^2H_4$]Tyr-Rho difference spectrum is shown as *dashed line*. *WT*, unlabeled His-tagged rhodopsin.

to tyrosine vibrations. This variability is most likely due to sample instability observed in the highly absorbing amide I and II regions as well as small differences in sample preparation which can give rise to polarization effects.

In general, the changes we have assigned to tyrosine vibrations can be caused either by an isotope-induced frequency shift of a tyrosine/tyrosinate vibrational mode out of a particular region or by a shift of a labeled tyrosine/tyrosinate mode into that region of the spectrum. Detailed assignments can be made on the basis of comparison with Tyr and L-[*ring*-$^2H_4$]Tyr model compounds. For example, a decrease in intensity at 1595 cm^{-1} and increase at 1570 cm^{-1} due to L-[*ring*-$^2H_4$]Tyr incorporation (Fig. 1, *inset*) agrees well with the isotope induced downshift of the 1598 cm^{-1} ring stretching mode of tyrosinate to 1569 cm^{-1} in L-[*ring*-$^2H_4$]Tyr measured at pH 12 (Fig. 2). Similarly, a drop in intensity near 1275 cm^{-1} and increase near 1253 cm^{-1} (Fig. 1, *inset*) (this increase appears as a maximum at 1245 cm^{-1} in a double difference spectrum; not shown) agrees qualitatively with the isotope-induced shift of a band at 1271 cm^{-1} in L-tyrosinate to 1238 cm^{-1} in L-[*ring*-$^2H_4$]Tyr (Fig. 2). Previously this band was assigned to the C–O^- stretch mode of tyrosinate (37, 38). Overall, these data, and in particular the appearance

[2] C. H. W. Klaassen and W. J. DeGrip, unpublished results.

[3] The sign shown next to the frequency in parenthesis (*e.g.* + or −) indicates whether the band is positive or negative; sh denotes shoulder).

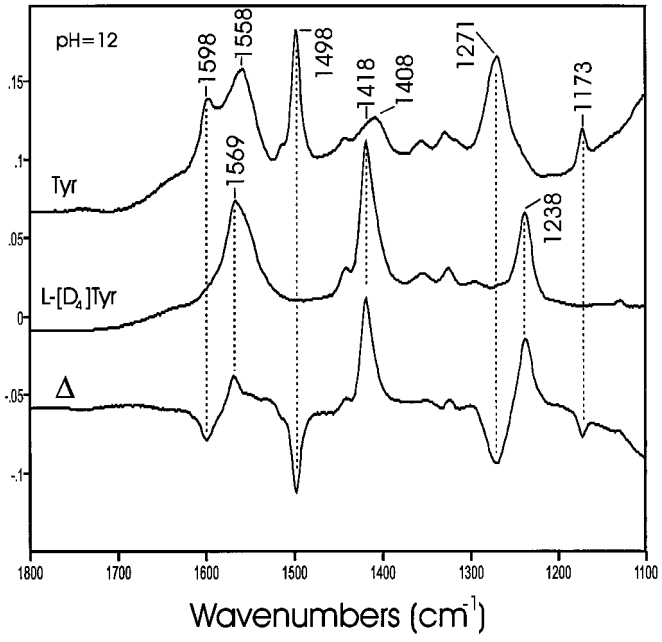

FIG. 2. **FTIR absorption of solutions of L-tyrosine and L-[*ring*-$^2H_4$]Tyr recorded at pH 12 (see "Experimental Procedures" for details).**

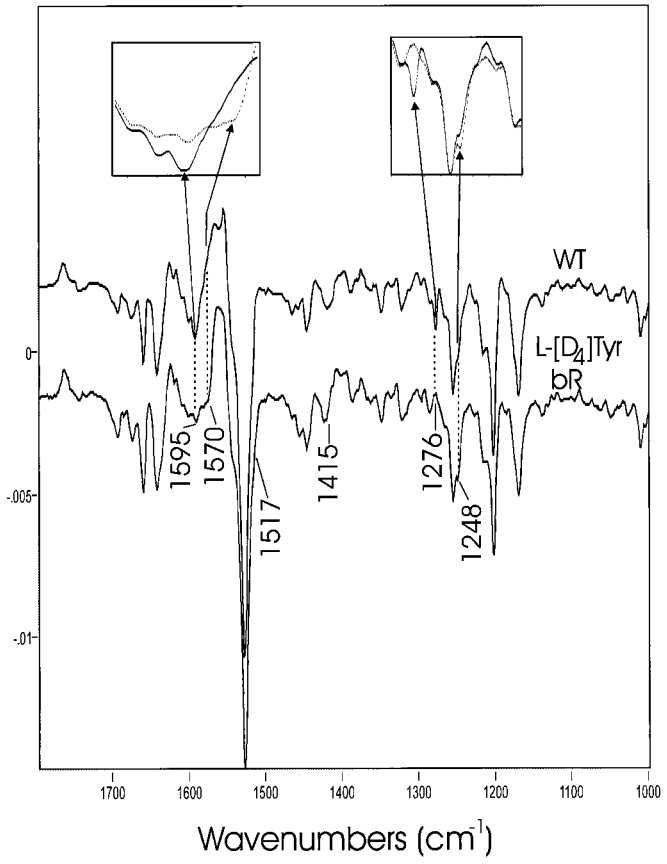

FIG. 3. **FTIR difference spectra of the bR → M difference spectrum of WT and L-[*ring*-$^2H_4$]Tyr-bR hydrated with H_2O as reported previously (41).** *Insets*, expansion of the lower traces in the regions 1615–1565 cm^{-1} (*left box*) and 1290–1190 cm^{-1} (*right box*). The L-[*ring*-$^2H_4$]Tyr-bR difference spectrum is shown as a *dashed line*.

of positive bands characteristic of (labeled) tyrosinate, indicate that a tyrosine residue is partially deprotonated in the Meta II state. This might reflect a partial deprotonation of a tyrosine residue in Rho upon formation of Meta II (see below) or, alternatively, a change in environment of an existing tyrosinate in Rho upon Meta II formation.

Additional bands that exhibit reproducible isotope-induced changes are assignable to vibrational modes found in tyrosine or L-[*ring*-$^2H_4$]Tyr. However, the pattern is complex and may reflect one or more tyrosines that undergo a change in environment or protonation state during Meta II formation. For example, the positive 1517-cm^{-1} band, which drops in intensity, is close to the frequency of an intense tyrosine ring vibration in L-Tyr (37, 39) and therefore reflects a tyrosine in the Meta II state. Other bands that match known tyrosine frequencies are at 1373 cm^{-1} (not assigned), 1250 cm^{-1} (bending mode of tyrosine hydroxyl group), and 1179 cm^{-1} (ring CH bending mode). The 1373- and 1179-cm^{-1} bands are positive and therefore most likely reflect tyrosine(s) in Meta II. The 1373-cm^{-1} band shifts to 1348 cm^{-1} in L-[*ring*-$^2H_4$]Tyr-Rho in agreement with published spectra (38). However, the increased intensity at 1250 cm^{-1} may reflect a change in a tyrosine residue in the unbleached state of Rho. Part of the intensity changes in this region were already attributed to the downshift of a positive tyrosinate band from 1277 cm^{-1} to near 1248 cm^{-1} in the Meta II state (see above). However, a prominent tyrosine mode due to the bending vibration of the tyrosine hydroxyl also occurs at this frequency and downshifts in L-[*ring*-$^2H_4$]Tyr to 1226 cm^{-1} (38) in good agreement with the change observed at 1221 cm^{-1} in L-[*ring*-$^2H_4$]Tyr-Rho. This band is not likely to be due to residual Meta I, which exhibits a band near 1215 cm^{-1} (30, 40), because no increase in the corresponding Meta I specific band at 950 cm^{-1} is observed (not shown).

Finally, a prominent drop in intensity at 1423 cm^{-1} (Fig. 1) in L-[*ring*-$^2H_4$]Tyr-Rho might be due to the isotope-induced downshift of negative tyrosine ring mode from the 1515 cm^{-1} region. A band due to such a ring mode has been previously identified in the L-[*ring*-$^2H_4$]Tyr-labeled Photosystem II in the Tyr_D to Tyr_{D^*} FTIR difference spectrum (38). Alternatively, this change might be due to a mode observed at 1418 cm^{-1} in L-[*ring*-$^2H_4$]Tyr at high pH, which downshifts from 1498 cm^{-1} in L-Tyr at high pH (Fig. 2). This would indicate that a (partially) ionized tyrosine exists in Rho. However, the loss of the corresponding unlabeled tyrosinate vibration at 1498 cm^{-1} would lead to an increase in intensity near 1498 cm^{-1} in L-[ring-$^2H_4$]Tyr-Rho, which is not clearly observed, but could be partially masked by the loss of the positive band at 1517 cm^{-1}.

Strikingly, there is a similar pattern of tyrosinate-related spectral changes observed in the bR → M difference spectrum of bR (41) and the Rho → Meta II difference spectrum of Rho when L-[*ring*-$^2H_4$]Tyr is incorporated, although the sign of the bands is reversed. Similar to the results reported above for Rho, L-[*ring*-$^2H_4$]Tyr labeling causes a downshift of a band from 1590 to 1570 cm^{-1} in the bR difference spectrum (Fig. 3, *inset*), although the downshifted bands are positive in the Rho spectrum and negative in the bR spectrum. Also, similar to rhodopsin a band at 1276 cm^{-1} appears to shift to near 1248 cm^{-1} in the bR → M difference spectrum (Fig. 3, *inset*). Additional peaks were assigned to tyrosine vibrations previously in the bR → M difference spectrum of bR (41) at 1517 cm^{-1} (−) (tyrosine) and 1419–1410 cm^{-1} (+) (L-[*ring*-$^2H_4$]tyrosine). Here again, the frequencies are similar to bands observed in the Rho → Meta II difference spectrum, although the signs of the bands are reversed.

DISCUSSION

FTIR difference spectroscopy has been used extensively to study conformational changes in membrane proteins (42–44). In the case of rhodopsin, structural changes of the retinylidene chromophore and protonation and/or hydrogen bonding changes of Asp, Glu, and Cys residues have been detected at

different stages of the photoactivation cascade (28, 30, 35, 36, 45–51). In some cases, assignment of vibrational bands to individual amino acid residues in rhodopsin was facilitated by site-directed mutagenesis (28, 35). FTIR difference spectroscopy can also be used to study changes in the environment of individual water molecules in rhodopsin during the different steps in the photoactivation cascade (52–54).

In this work, FTIR difference spectroscopy and amino acid isotope labeling were combined for the first time to analyze structural changes of tyrosines in rhodopsin. Isotope labeling was accomplished by growing Sf9 insect cells on a stringent medium containing L-[*ring*-$^2H_4$]Tyr. Tyrosine bands can be identified and assigned on the basis of the isotope induced shifts and by comparison with a series of model tyrosine compounds at high and low pH. Our results clearly demonstrate that a set of bands in the Rho → Meta II difference spectra can be assigned to vibrational modes of tyrosine and tyrosinate. On this basis, it can be concluded that one or more tyrosines participate in the rhodopsin photocascade up to formation of the Meta II intermediate and that at least a partial protonation of a tyrosinate is involved. A possible involvement of tyrosine residues was previously proposed on the basis of UV difference spectroscopy (55) and site-specific mutagenesis (56) and also suggested by a recent study using UV resonance Raman spectroscopy (57).

The similarity between the pattern of isotope-induced changes observed in bacteriorhodopsin and rhodopsin is particularly interesting. Earlier studies based on tyrosine isotope labels (41), site-directed mutagenesis (21), and site-directed isotope labeling (58) concluded that tyrosine bands identified in the FTIR difference spectra of bacteriorhodopsin arose from protonation changes in Tyr-185 located in the F-helix. Subsequent FTIR measurements on bR containing a ^{13}C isotope label in the C1 position of Tyr-185 indicated that the Tyr-185/Pro-186 peptide bond buried in the center of the F-helix undergoes some type of structural rearrangement (59). An interesting possibility is that the Tyr-185/Pro-186 bond serves as a hinge, which gives rise to the apparent tilting of the F-helix late in the bR photocycle (60, 61).

The present findings indicate that a similar, although reversed, pattern of tyrosine/tyrosinate changes occurs also upon photoactivation of rhodopsin. Although on the basis of this study we cannot assign these changes to any particular tyrosine(s) in rhodopsin, an interesting candidate is Tyr-268. This residue is also positioned adjacent to a Pro residue (Pro-267). It is well known that Pro residues break α-helical structure and can function as kinks allowing independent movement of adjacent helical segments. The existence of a Tyr/Pro pair in the center of helix 6 in bacteriorhodopsin (Tyr-185, Pro-186) and a Pro/Tyr pair in rhodopsin (Pro-267, Tyr-268) raises the possibility that such a pair acts synergistically independent of the order of these residues, thereby forming a potential hinge for movements in helix 6 in response to chromophore isomerization. Indeed, several lines of evidence have been presented that a rigid body movement involving part of helix 6 takes place during photoactivation of rhodopsin (62–64). Also, recent structural models for rhodopsin (*e.g.* Refs. 64 and 65) place Tyr-268 close to Glu-113, suggesting a possible role in the deprotonation of the Schiff base upon Meta II formation, which could be related to the (partial) protonation indicated by our data. In fact, mutagenesis studies show that replacement of Tyr-268 by Phe perturbs ligand binding and activation of rhodopsin (56) and that the adjacent Trp-265 also is involved structurally in formation of Meta II (66, 67). Actually, this region probably is one of the hot spots in rhodopsin activation, because in the vicinity of Tyr-268 are also residues Gly-121 and Phe-261, which form a synergistic functional unit with the 9-methyl group of the chromophore (68). Mutation of these residues in most cases has a profound effect on dark and/or rest activity of rhodopsin (68, 69). However, to definitely identify Tyr-268 as (one of) the residue(s) responsible for the changes we observe additional site-specific mutagenesis and/or site-directed isotope labeling will be required. Because mutagenesis tends to perturb functionally or structurally active sites, site-directed isotope labeling will be the preferred method.

Our results suggest a common element for the primary mechanism of rhodopsin and bacteriorhodopsin that goes beyond the general features of a retinylidene chromophore and a seven-helix transmembrane domain. In particular, it establishes that similar tyrosine/tyrosinate changes occur during activation of both proteins. In fact, the reversal of the signs of tyrosine/tyrosinate assigned bands may be related to the respective role of the all-*trans* chromophore in the activation of the rhodopsin and bacteriorhodopsin systems as indicated by FTIR studies on the effects of removal of the all-*trans* chromophore in rhodopsin (29) and bacteriorhodopsin (25). If these similarities arise in changes in the F-helix (helix 6), then it is possible that a common mechanism of chromophore-protein coupling exists in both proteins. This is a very important issue that will be addressed by subsequent studies using a combination of FTIR difference spectroscopy, mutagenesis, and isotope labeling.

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