Mechanism of Proton Pumping in Bacteriorhodopsin by Solid-State NMR: The Protonation State of Tyrosine in the Light-Adapted and M States†

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ABSTRACT: Solid-state ^{13}C NMR spectra were employed to characterize the protonation state of tyrosine in the light-adapted (bR_{568}) and M states of bacteriorhodopsin (bR). Difference spectra (isotopically labeled bR minus natural-abundance bR) were obtained for [4′-^{13}C]Tyr-labeled bR, regenerated with [14-^{13}C]retinal as an internal marker to identify the photocycle states. The [14-^{13}C]retinal has distinct chemical shifts for bR_{555}, for bR_{568}, and for the M intermediate generated and thermally trapped at pH 10 in the presence of 0.3 M KCl or 0.5 M guanidine. Previous work has demonstrated that tyrosine and tyrosinate are easily distinguished on the basis of the chemical shift of the 4′-^{13}C label and that both NMR signals are detectable in dark-adapted bR, although the tyrosinate signal is only present at pH values greater than 12. In the present work, we show that neither the light-adapted form of bR prepared at pH 7 or 10 nor the M state thermally trapped at −80 °C in 0.3 M KCl pH 10, or in 0.5 M guanidine pH 10, shows any detectable tyrosinate. In addition, after the M samples were briefly warmed (~30 s), no tyrosinate was observed. However, small (1−2 ppm) changes in the structure or dispersion in the Tyr peak were observed in the M state phototrapped by either method. These changes were reversible when the sample was warmed, although on a time scale slower than the relaxation of the retinal back to the bR_{568} conformer. Such small changes may be interpreted as alterations in the environment or the hydrogen bonding of some of the tyrosines, but not as evidence for the formation of tyrosinate. In summary, these data indicate that no stable tyrosinate is found in the dark-adapted, light-adapted, or M states of bacteriorhodopsin.

Bacteriorhodopsin (bR),[1] the purple membrane protein from *Halobacterium halobium*, is a light-driven proton pump. Seven membrane-bound helices encapsulate the retinal chromophore, which is linked by a Schiff base to Lys-216. A recent electron diffraction study provides information on the location of aromatic residues in the protein; these data together with previous biochemical and spectroscopic characterizations provide us with a low-resolution structure for the protein (Henderson et al., 1990).

Cyclic photoisomerization of the retinal is thought to be the heart of a mechanism that couples uphill, unidirectional proton flow to the absorption of visible light. A recent paper summarizes the conformations of the retinal involved in the photocycle, as indicated by a wealth of vibrational and NMR data, and presents a model for the coupling of these isomerizations to directional proton flow (Ames et al., 1989). The ideas that are central to most models proposed for proton pumping during the photocycle involve the following: (i) transient deprotonation of the retinal Schiff base in the M intermediate based on a decrease in the affinity of the Schiff base for protons (its "effective pK_a") to allow the Schiff base (which otherwise has a pK_a greater than 12) to transfer protons to aspartate (which presumably has a pK_a between 3 and 6) or to water; (ii) changes in the orientation of the retinal Schiff base due to changes in the conformation of the Lys-216 side chain and/or conformational changes in other parts of the protein [see Fodor et al. (1988)] to enforce the directionality of proton flow. These ideas constitute a framework for understanding the unidirectional proton translocation, although the molecular details remain to be elucidated.

Many experiments have suggested that amino acid side groups mediate proton transfer between the buried Schiff base and the membrane surface. Proposed mechanisms for proton conduction in bR and other proteins have varied with respect to the degree of localization of the deprotonated or protonated sites. In one limit, the process is envisioned as involving discrete intermediates with unusual protonation states that might be trapped at low temperature, quite analogous to the situation for electron transfer in the photosynthetic reaction centers. At the other extreme, models for more delocalized

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[1] Abbreviations: bR, bacteriorhodopsin; bR_{568}, light-adapted bR, also the component of dark-adapted bacteriorhodopsin with *all-trans*-retinal; bR_{555}, component of dark-adapted bR with 13-*cis*-retinal; CHES, 2-(cyclohexylamino)ethanesulfonic acid; FTIR, Fourier transform infrared spectroscopy; HEPES, *N*-(2-hydroxyethyl)piperazine-*N*′-2-ethanesulfonic acid; MAS, magic-angle spinning; NIR, near-infrared; NMR, nuclear magnetic resonance spectroscopy; SSNMR, solid-state NMR; Tyr^-, tyrosinate; UV/VIS, ultraviolet and visible spectroscopy; UVRR, ultraviolet resonance Raman spectroscopy.

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protonation "defects" have been proposed in which an extended chain of participants may form a "proton wire" (Nagle & Tristam-Nagle, 1983). Many spectroscopic studies have been aimed at detecting changes in the protonation state or hydrogen-bonding environment; these experiments are usually conceived in terms of the former picture. Among the handful of possible amino acid participants in proton transfer in bR, only aspartate and tyrosine have been implicated by spectroscopic, chemical, or mutagenic experiments. Use of site-specific mutants has eliminated an obligatory role for many of the amino acids and has indicated a role for Asp-85, -96, and possibly -212 (Mogi et al., 1987; Braiman et al., 1988a; Stern et al., 1989). Of particular interest for the present study is the possible involvement of Tyr-185; the substitution of this residue by phenylalanine affects steady-state rates of proton pumping (Braiman et al., 1988b). Aspartates-85, -96, and -212 and Tyr-185 are well situated in the current model of bR to assist in proton transport (Henderson et al., 1990).

In the present work, we focus on the protonation state of tyrosine in the photocycle. Many early studies of the kinetics of UV/VIS fluorescence and absorption have been interpreted in terms of changes in the protonation state of tyrosine, although the specific order of events or proposed mechanisms differ from one study to another (Rosenbach et al., 1982; Kalisky et al., 1981; Hess & Kushmitz, 1979; Hanamoto et al., 1984; Fukumoto et al., 1984). More recent experiments utilizing FTIR have suggested that Tyr-185 is deprotonated in the dark- to light-adapted transition, reprotonated in K and L, and again deprotonated in M (Braiman et al., 1988b; Dollinger et al., 1986; Rothschild et al., 1986; Roepe et al., 1987).

In view of this previous work, we have been interested in studying the solid-state NMR spectra of [4′-^{13}C]Tyr-bR in various states of the photocycle. Previously, we demonstrated the ability of NMR to detect both tyrosine and tyrosinate in dark-adapted bR and discriminate unambiguously between the two, with assignments based on model compounds (Herzfeld et al., 1990). Our data indicated that in dark-adapted bR no tyrosinates are found at or below pH 12, a conclusion which has recently been confirmed by UVRR spectroscopy (Ames et al., 1990). Deprotonation of half of the tyrosines occurs at pH 13, possibly related to denaturation of the protein. The virtue of this experimental approach is that it distinguishes between tyrosine and tyrosinate, without ambiguity from environmental or hydrogen-bonding effects.

In the current work, we address the protonation state in light-adapted bR and M generated under a variety of conditions and cryogenically stabilized. Since these studies require extensive signal-averaging and very dense samples, it has been necessary to cryogenically trap the intermediates of interest. Therefore, we have focused on intermediates that are likely to be stable, relevant to proton transfer, and for which previous work has indicated a role for tyrosine.

Because this study focuses on intermediates of the photocycle which are unstable at room temperature, we have used an internal marker, the [14-^{13}C]retinal, to quantitate the states present in each sample. The chemical shift of this marker is sensitive to the state of the retinal and therefore indicates the amounts of bR_{555} (110.5 ppm) and bR_{568} (122.0 ppm) which are present. In the case of the cryogenically trapped M intermediate, two [14-^{13}C]retinal chemical shifts of 125 and 115 ppm are observed, as previously reported (Smith et al., 1989). The 125 ppm shift arises from a state, previously called $M_{Gdn\text{-}HCl}$, which probably resembles the transient M species observed at room temperature (Ames et al., 1989). We note here for the first time that this state is also trapped as the major species of the M state formed at low temperature in the presence of salt without guanidine. We therefore designate this state M. The state giving rise to the 115 ppm shift, previously called M_{NaCl} and here referred to as "X", is under further investigation.

We conclude that for the dark- and light-adapted states, at neutral to mildly basic pH values, and in a variety of salt conditions, no tyrosinate is formed. We have phototrapped the M intermediate at low temperatures (−60 °C) and in these samples, no tyrosinate was observed prior to illumination, after trapping the M state, or after a 30-s thawing period during which the phototrapped M state decayed. Since our experimental conditions are capable of stabilizing the deprotonated Schiff base in the M intermediate, it is likely that a putative tyrosinate intermediate may be stabilized as well; for this reason, we considered the cryotrapped M state to provide a relevant comparison with photocycling intermediates observed at room temperature. Our conclusion that there is no tyrosinate in the M photointermediate is consistent with recently reported UVRR results (Ames & Mathies, 1991).

Materials and Methods

[4′-^{13}C]Tyr-bR was prepared as described previously (Herzfeld et al., 1990). The labeling efficiency was such that the Tyr residues were approximately 60% ^{13}C-labeled. Purple membrane labeled with [4′-^{13}C]Tyr was bleached by incubation at 1 mg/mL bR in 0.5 M hydroxylamine hydrochloride, pH 8.1, at 35 °C overnight in darkness. The bleached membrane was subsequently washed 3 times with 50 mM HEPES buffer, pH 6.5, pelleting for 30 min at 30000g after each wash to recover the membranes. The bleached membranes were resuspended to 1 mg/mL in distilled water. Aliquots of the [14-^{13}C]retinal (Pardoen et al., 1984) at 1 mg/mL in dry ethanol were added to the bR, and the regeneration was monitored spectroscopically to ensure that the bR was completely regenerated. Excess retinal and retinal oxime were removed from this solution by resuspending the membranes in a solution containing 1 mM NaN_3 and 2% bovine serum albumin (BSA) (Sigma Chemical Co., St. Louis, MO), incubating this mixture for 1–5 h, and then collecting the membranes by centrifugation at 30000g for 30 min. This washing procedure was repeated 15 times, so that no retinal oxime was apparent in the absorption spectrum. Finally, the membranes were washed repeatedly with aqueous 1 mM NaN_3 to remove the BSA.

For light-adapted spectra, samples were prepared by first adjusting the pH in the presence of 1 mM NaN_3 with either 4 mM HEPES buffer (Sigma Chemical Co., St. Louis, MO) for pH 7.0, or 50 mM CHES buffer (Aldrich Chemical Co., Milwaukee, WI) for pH 10.0. For phototrapping the M intermediate, variations of two earlier procedures were employed (Smith et al., 1989); samples were equilibrated at 1 mg/mL protein in a solution containing either 0.5 M guanidine hydrochloride adjusted with KOH to pH 10 or 0.3 M KCl in a 50 mM CHES buffer at pH 10.0. After suspension in buffer, each sample was pelleted at 30000g for 2 h. The resulting pellet was packed into a single-crystal sapphire rotor (Doty Scientific, Columbia, SC) for illumination and NMR measurements. For light-adaptation, samples were illuminated in a water/ice bath for 20–30 min with a 500-W incandescent lamp, the light from which was focused and filtered with a water bath to remove near-IR components. For the M intermediate, previously light-adapted samples were suspended in a dry ice/ethanol bath maintained at −60 °C and illuminated with the same lamp, except that a color filter was in-

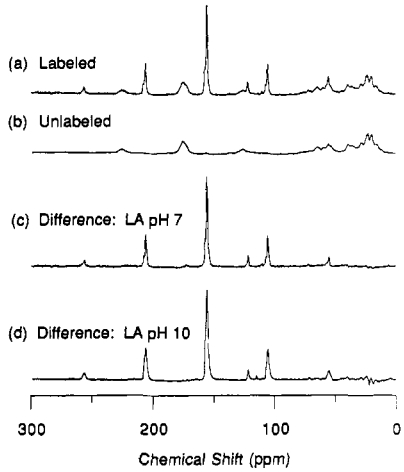

FIGURE 1: ^{13}C MAS spectra of (a) [4′-^{13}C]Tyr, [14-^{13}C]retinal-bR at pH 7 in the light-adapted state, (b) unlabeled bR (with only natural-abundance ^{13}C), (c) the pH 7 light-adapted difference spectrum (a minus b) which shows only the labeled tyrosine and retinal, and (d) the pH 10 light-adapted difference spectrum. Spectra were collected at −30 °C, with a spinning speed of 4.0 kHz. The largest line in the difference spectra, at 156 ppm, is due to the [4′-^{13}C]Tyr label which arises from all 11 tyrosines in bR. The 4-kHz spinning speed results in sidebands at 4 and 8 kHz above and below the main peak. The signal at 122 ppm in the difference spectra (c and d) is due to the [14-^{13}C]retinal and indicates that the retinal is primarily in the bR_{568} form (i.e., is effectively light-adapted). The second small peak in this region in the pH 10 spectrum (d) is at the position seen for the previously reported M_{NaCl} species, here referred to as "X", and corresponds to roughly 20% of the sample. If a tyrosinate were present in light-adapted bR, it would give rise to a signal at 164–168 ppm, and one tyrosinate per bR would contribute a signal roughly the size of the 14-^{13}C marker at 122 ppm.

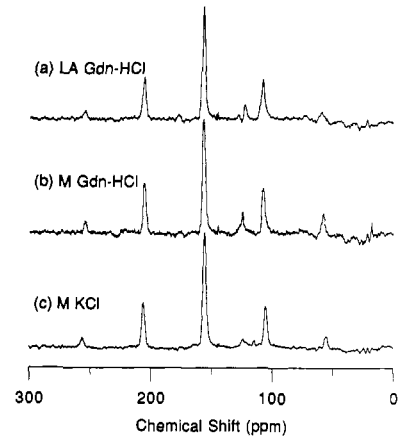

FIGURE 2: ^{13}C MAS difference spectra of [4′-^{13}C]Tyr, [14-^{13}C]-retinal-bR under two conditions which stabilize the M intermediate: at pH 10 in the presence of 0.5 M guanidine hydrochloride in (a) the light-adapted state and (b) the M intermediate, and at pH 10 in the presence of 0.3 M KCl in (c) the M intermediate. Spectra were collected at −80 °C with a spinning speed of 3.9 kHz. The signal due to the [14-^{13}C]retinal appears at 122 ppm in the top spectrum (a), indicating that the sample is in the bR_{568} form, and at 125 ppm in the middle spectrum (b), indicating that the sample is in the M intermediate. In spectrum c, the signal due to the [14-^{13}C]retinal appears at 125 and 115 ppm, indicating that the sample contains both previously reported forms of the M intermediate.

serted that absorbed light with wavelengths less than 540 nm. Upon illumination at low temperature, the M samples were tan in color, indicating little residual bR_{568}.

Solid-state NMR spectra were acquired on a home-built spectrometer operating with ^{13}C and ^{1}H frequencies of 79.8 and 317.5 MHz, respectively. The ^{13}C and ^{1}H $\pi/2$ pulse lengths were approximately 6 and 3 μs, respectively. Cross-polarization was used with a mixing time of 2 ms, an acquisition time of 20 ms, a recycle delay of 3 s, and standard phase cycling techniques. Approximately 15 000–30 000 transients were accumulated for each spectrum. Sample temperatures of approximately −30 °C (light-adapted) and −80 °C (M) were maintained during signal-averaging to avoid loss of the trapped states and to quench any proton exchange processes which might occur. Spectra of natural-abundance bR were collected under similar conditions. For subtraction, both spectra were Fourier-transformed, phased, frequency-referenced, and scaled appropriately. The resulting difference spectra contain only the signals due to the [4′-^{13}C]Tyr and [14-^{13}C]retinal labels.

RESULTS

A ^{13}C MAS spectrum of [4′-^{13}C]Tyr, [14-^{13}C]retinal doubly labeled bR that was light-adapted at pH 7 is shown in Figure 1a. Figure 1b displays a similarly collected spectrum of natural-abundance bR. Finally, Figure 1c shows the difference of the two spectra, with lines solely due to the Tyr and retinal ^{13}C labels. The Tyr centerband at 156 ppm is the most prominent line, and is flanked by sidebands spaced at the rotor frequency, 4.0 kHz (~50 ppm). These lines arise from the 11 Tyr residues in bR which exhibit relatively little chemical shift dispersion. The resolved shoulder, which we estimate to be due to 2 or 3 of the 11 tyrosines, is very similar in size and position to that observed for dark-adapted samples (Herzfeld et al., 1990). The small peak at 122 ppm arises from the [14-^{13}C]retinal label, and serves as an internal standard to confirm that the sample is greater than 85% light-adapted, as well as an approximate intensity standard. If one tyrosinate per bR were present in this sample, we would observe an additional signal at 164–168 ppm (Herzfeld et al., 1990) with an intensity similar to that of the [14-^{13}C]retinal label. Integrated intensities of this region of the spectrum are significantly less than 1% of the integrated intensity of the tyrosine main peak, corresponding to less than approximately 0.1 tyrosinate per bR molecule.

In Figure 1d, we present another difference spectrum of [4′-^{13}C]Tyr, [14-^{13}C]retinal doubly labeled bR for a sample which was light-adapted at pH 10. Again, the strongest signal in the spectrum is the centerband of the 11 tyrosines in bR. At this pH, there is poorer resolution in the tyrosine line because of an increase in the inhomogeneous line width. This effect is presumably due to the pH of the sample, and was previously observed in a study of the titration of bR (Herzfeld et al., 1990). Nevertheless, the Tyr centerband is asymmetric (see Figure 3b) due to an unresolved shoulder. The less intense lines at 122 and 115 ppm are due to the 14-^{13}C label on the retinal for bR in the light-adapted and the "X" (previously called M_{NaCl}) states, respectively. On the basis of integrated intensities, the sample contains approximately 80% bR_{568} and 20% "X". Finally, there is no detectable tyrosinate peak in the sample, and we estimate that the integrated intensity in the tyrosinate region is at most 1% of the main tyrosine peak, corresponding to an upper limit of 0.1 Tyr^- per bR. Although the [4′-^{13}C]Tyr centerband and sidebands are somewhat broader at pH 10 than at pH 7, the chemical shift value is unchanged. We conclude from these two spectra that light-adapted bR does not contain a significant amount of tyrosinate at neutral or moderately basic pH values.

Figure 2 shows difference spectra of the [4′-^{13}C]Tyr, [14-^{13}C]retinal doubly labeled bR under two different conditions which stabilize the M intermediate. A sample prepared in 0.5 M guanidine at pH 10 was first light-adapted at 0 °C (Figure 2a) and then phototrapped in the M intermediate by continuous illumination at −60 °C (Figure 2b). The position of the [14-^{13}C]retinal peak at 122 ppm in Figure 2a confirms that

the sample was greater than 80% light-adapted, and in Figure 2b the peak at 125 ppm indicates that greater than 80% of the sample was in the M intermediate previously referred to as $M_{Gdn\text{-}HCl}$ (Smith et al., 1989). In both spectra, no tyrosinate is detected, and the integrated intensity in the region expected for a tyrosinate (164–168 ppm) is less than 3% of the main tyrosine peak. Thus, we conclude that no stable tyrosinate is formed under these conditions. Upon brief thawing (ca. 30 s), the sample returned to a deep purple color, and spectra of the "warmed M" sample were recorded. These spectra (not shown) indicated that the majority of the sample returned to the bR_{568} state and there was still no indication of formation of tyrosinate.

M samples have also been trapped and characterized in this laboratory under conditions of high salt and high pH. By using difference spectroscopy, we now observe the M intermediate in such samples, along with the species that was previously reported as M_{NaCl}, and is here referred to as "X". This "X" species was thought to differ from M with respect to the conformation of the retinal about the C=N double bond (Smith et al., 1989), and its exact structure and role in the photocycle are under further investigation. The chemical shift values for the [14-^{13}C]retinal in M and "X" are 125 and 115 ppm, respectively. Figure 2c shows the spectrum of the [4'-^{13}C]Tyr, [14-^{13}C]retinal doubly labeled bR sample after continuous illumination at −60 °C in the presence of 0.3 M KCl at pH 10. Under these conditions, we phototrap the majority of the sample in the M intermediate, with a substantial amount of "X" also present. In this spectrum, we observe no tyrosinate. Upon warming, the [14-^{13}C]retinal signal returns to 122 ppm, indicating a relaxation to bR_{568}, and again no tyrosinate is observed (data not shown).

In Figure 3, we display the expanded centerband region for the [4'-^{13}C]Tyr (center column) and for the [14-^{13}C]retinal (right column) from the five difference spectra in Figures 1 and 2. The chemical shifts for the [14-^{13}C]retinal in the bR_{568}, bR_{555}, "X", and M states and the expected position of a Tyr^- signal are marked above the spectra as a guide. The expanded centerband region for Tyr clearly shows structure or dispersion in the light-adapted states, with the majority of the Tyr peak appearing at 156 ppm, and 2–3 of the 11 Tyr's appearing at 158 ppm. Although the light-adapted samples at pH 10 have broader Tyr lines than at pH 7, the dispersion in shifts is still clearly visible in all cases. Interestingly, this structure differs for both M samples. The Tyr peak in the M states is broader than that in the light-adapted state and is symmetric; this structure would be expected if 4–5 of the 11 Tyr's were at 158 ppm and the remainder at 156 ppm. In other words, the change in structure suggests that one to three of the Tyr's shift from approximately 156 to 158 ppm in the light-adapted to M transition. First-derivative spectra of the Tyr centerband region, displayed in the left-hand column of Figure 3, more explicitly reveal the shoulder at 158 ppm adjacent to a primary line at 156 ppm in the light-adapted samples; in contrast, a broad, symmetric line from 156 to 158 ppm is observed in the M samples. These changes were reversible when the sample was warmed for an extended period and light-adapted again; however, when only briefly warmed, it appeared that the changes in the Tyr structure recover on a time scale that is slower than the recovery of the retinal peak to the bR_{568} form (data not shown).

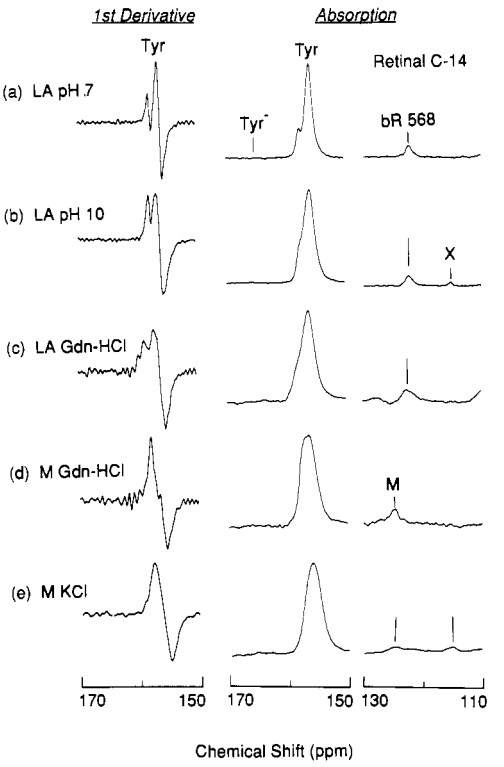

FIGURE 3: Center and right columns show expanded centerband difference spectra from Figures 1 and 2, with line intensities to scale. Note that the chemical shift axis omits the region from 130 to 150 ppm. The [14-^{13}C]retinal positions for bR_{568}, bR_{555}, M (previously $M_{Gdn\text{-}HCl}$), and "X" (previously M_{NaCl}) are indicated. If a tyrosinate ion were formed in any of these samples, it would give rise to a line at 164–168 ppm, as indicated by the position of the "Tyr^-" label. The signal should be approximately the same intensity as the corresponding retinal peak. The left column displays the first derivatives of the tyrosine centerband regions for the same spectra. A shoulder, accounting for 2 or 3 of the 11 tyrosines, is clearly visible at 158 ppm, next to the main Tyr peak at 156 ppm, in all of the light-adapted spectra. Spectra of the M samples display a broad but more symmetric peak covering the same range, from 158 to 156 ppm. Such changes in structure were reversible on warming.

Discussion

The intermediates that we studied, light-adapted and M, were chosen on the basis of experimental accessibility as well as evidence for tyrosinate from many previous studies [most recently, Braiman et al. (1989)]. In addition, the light-adapted spectrum at high pH in guanidine was intended to address the hypothesis that, prerequisite to forming M in a low-temperature illumination, the solvent conditions must generate a stable tyrosinate. Hypothetically, a tyrosinate so generated might facilitate the formation of M [see, for example, Kalisky et al. (1981)].

The solid-state NMR method is a reasonably direct approach to observing tyrosine protonation in bR. Our data show no evidence for tyrosinate in either the light-adapted state or the M intermediate prepared under two different conditions. If a tyrosinate were present in these samples, we expect that it would be readily detectable at approximately 164 ppm (Herzfeld et al., 1990). On the other hand, changes in the hydrogen-bonding environment would give rise to small changes in the chemical shift and would be rather difficult to analyze by this method. For example, the dispersion in the tyrosine centerband in the light-adapted spectra and the changes in this structure for M, as compared with light-adapted, could be due to a variety of environmental factors, and we cannot assign such variations uniquely. This degree of variation of Tyr resonances is seen in other proteins (de Groot et al., 1990).

NMR characterization of the light-adapted and M intermediates required phototrapping at low temperatures using illumination from a broad-band high-power VIS/NIR source

with a long (20–30 min) accumulation time. To quantitate our ability to phototrap the light-adapted and M intermediates, and to compare their configurations to those observed at room temperature during photocycling, the [4′-^{13}C]Tyr-bR was regenerated with a [14-^{13}C]retinal which serves as a marker of the photointermediates. The chemical shift for the [14-^{13}C]retinal is quite sensitive to the configuration and protonation state of the Schiff base, as has been discussed in detail previously (Smith et al., 1989). This retinal marker indicates essentially complete conversion to the bR_{568} form during light adaptation at pH 7 or 10. In these studies, we have trapped M under two different conditions: the M intermediate was stabilized at pH 10 with either guanidine hydrochloride or KCl. When trapped in the presence of guanidine at pH 10, we obtain exclusively the configuration of the retinal in which the Schiff base has been assigned as C=N anti (Smith et al., 1989); this form is believed to resemble that observed during room temperature photocycling at neutral pH values in the absence of guanidine (Ames et al., 1989). Illumination in the presence of KCl at pH 10 produces a mixture of M and another photointermediate, here labeled "X", which is under further investigation. These markers indicate fairly efficient conversion of the sample to the M intermediate, despite an optically dense sample.

Spectra of the light-adapted state at −30 °C show no tyrosinate. Furthermore, no changes are seen in spectra of the light-adapted state from 0 to −100 °C (data not shown). This eliminates the possibility of line broadening due to intermediate exchange. In our previous study of dark-adapted bR, we carried out experiments at −120 °C for this purpose (Herzfeld et al., 1990). Tyrosinates that have been implicated in the light-adapted state are thought to be stable at room temperature and lower temperatures, so our conclusion that there are no tyrosinates in light-adapted bR at pH 7 or 10 is rather definitive. In addition, we can conclude that the presence of a stable tyrosinate in the light- or dark-adapted samples is not a prerequisite for forming M.

NMR spectra of the M intermediate also revealed no tyrosinate, with no dramatic changes between −100 and −50 °C (spectra not shown). We suggest that conditions of low temperature capable of stabilizing the M intermediate would also be likely to stabilize a putative tyrosinate intermediate, in view of the fact that the deprotonated Schiff base, which is stable at these temperatures, is a much stronger base than a tyrosinate. Therefore, our inability to trap tyrosinate under the conditions which trap the M configuration of retinal suggests that when M is formed at low temperature no tyrosinate ion is formed with it. Furthermore, the low temperature will quench any proton exchange which might broaden a putative tyrosinate signal. Although M does not contain a tyrosinate ion under either of the conditions used to stabilize the M intermediate, there are changes in the environment of some of the tyrosines, perhaps due to changes in hydrogen bonding, that give rise to shifts of 1–2 ppm.

Recent FTIR and kinetic studies of bR mutants indicate a role for Tyr-185 in proton conduction in bR (Braiman et al., 1988b). Our data indicate that Tyr-185 should not be pictured as deprotonated in either the M, the dark-, or the light-adapted states. Participation of tyrosine in hydrogen-bonding changes, without deprotonation, would be consistent with the changes we observe in the dispersion of the Tyr peak accompanying M formation. Our data would also be consistent with the participation of tyrosine in a more delocalized deprotonation "defect", if such a situation resulted in a species more like tyrosine than like tyrosinate. Hydrogen bonds and hydrogen-bonding networks may involve dynamic exchange processes with low potential barriers. If such a process were fast on the NMR time scale and slow on the FTIR time scale, then the FTIR and NMR results could be reconciled in a hydrogen-bonding context. However, in such an interpretation, the small size of the NMR shift indicates that the equilibrium strongly favors tyrosine protonation.

Although the change in the Tyr signal is reversible following a long warming period, we observed that a brief thawing (~30 s) resulted in a sample in which the retinal had returned to the bR_{568} form but the structure in the Tyr signal had not fully recovered (data not shown). This difference in the time scale of recovery indicates that the change sensed by the Tyr is unlikely to be simply the protonation state of the retinal Schiff base but is more likely to be due to another environmental effect such as an ionizable amino acid or a larger conformational change. The recent model of bR places Tyr-185 in close proximity to Asp-212, but not in close contact with the retinal (Henderson et al., 1990). These interactions between ionizable amino acids are appealing prospects because of their possible relation to a proton conduction pathway. Unfortunately, in NMR experiments, such interactions generally cause small changes in chemical shifts, and interpretation of these shifts is difficult.

Conclusions

Using ^{13}C NMR MAS difference spectra, we do not detect tyrosinate in light-adapted bR at pH 7, at pH 10, in 0.3 M KCl at pH 10, or in 0.5 M guanidine at pH 10. Furthermore, we do not observe tyrosinate in cryogenically (−80 °C) photoaccumulated M samples prepared at pH 10 in 0.3 M KCl or 0.5 M guanidine. Small changes in the structure of the Tyr peak occur on converting light-adapted samples to M at low temperature; these shifts probably reflect changes in the environment or hydrogen bonding of the Tyr and could be due to a nearby charged amino acid but probably not to the protonation state of the retinal Schiff base. Finally, our difference spectra indicate that the C=N anti form of retinal (as observed in the M formed transiently at room temperature) is the major species present in M samples cryogenically prepared in both the presence and absence of guanidine.

Registry No. Tyr, 60-18-4; H, 12408-02-5.

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Interaction of Forskolin with the P-Glycoprotein Multidrug Transporter†

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ABSTRACT: Forskolin and 1,9-dideoxyforskolin, an analogue that does not activate adenylyl cyclase, were tested for their ability to enhance the cytotoxic effects of adriamycin in human ovarian carcinoma cells, SKOV3, which are sensitive to adriamycin and express low levels of P-glycoprotein, and a variant cell line, SKVLB, which overexpresses the P-glycoprotein and has the multidrug resistance (MDR) phenotype. Forskolin and 1,9-dideoxyforskolin both increased the cytotoxic effects of adriamycin in SKVLB cells, yet had no effect on SKOV3 cells. Two photoactive derivatives of forskolin have been synthesized, 7-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]-7-deacetylforskolin, ^{125}I-7-AIPP-Fsk, and 6-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]forskolin, ^{125}I-6-AIPP-Fsk, which exhibit specificity for labeling the glucose transporter and adenylyl cyclase, respectively (Morris et al., 1991). Both photolabels identified a 140-kDa protein in membranes from SKVLB cells whose labeling was inhibited by forskolin and 1,9-dideoxyforskolin. There was no specific labeling of proteins in membranes from the SKOV3 cells. The overexpressed 140-kDa protein in SKVLB membranes was identified as the P-glycoprotein by immunoblot analysis and immunoprecipitation using anti-P-glycoprotein antiserum. Total inhibition of photolabeling of the P-glycoprotein was observed with verapamil, nifedipine, diltiazem, and vinbalastine, and partial inhibition was observed with colchicine and cytochalasin B. Forskolin was less effective at inhibiting the photolabeling of the P-glycoprotein than 1,9-dideoxyforskolin or a lipophilic derivative of forskolin. The data are consistent with forskolin binding to the P-glycoprotein analogous to that of other chemosensitizing drugs that have been shown to partially reverse MDR. The ability of forskolin photolabels to specifically label the glucose transporter, the adenylyl cyclase, and the P-glycoprotein suggests that these proteins may share a common binding domain for forskolin analogues.

Multidrug resistance (MDR)[1] is a phenomenon whereby tumor cells acquire resistance to a variety of structurally and functionally unrelated cytotoxic drugs (Endicott & Ling, 1989). This frequently is associated with the overexpression of the P-glycoprotein, an integral membrane glycoprotein whose molecular weight has been reported to be between 130 and 180 kDa (Juliano & Ling, 1976). The protein is believed to act as an energy-dependent efflux pump capable of transporting drugs out of a cell (Willingham et al., 1986). Thus, overexpression of the P-glycoprotein would maintain a low intracellular concentration of cytotoxic drugs, which would be associated with reduced cytotoxic consequences.

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[1] Abbreviations: MDR, multidrug resistant; ^{125}I-AIPPS, *N*-[3-(4-azido-3-[^{125}I]iodophenyl)propionyl]succinimide; ^{125}I-6-AIPP-Fsk, 6-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)propionamido]ethyl]carbamyl]forskolin; ^{125}I-7-AIPP-Fsk, 7-*O*-[[2-[3-(4-azido-3-[^{125}I]iodophenyl)-propionamido]ethyl]carbamyl]-7-deacetyl forskolin; 6-HPP-Fsk, 6-*O*-[[2-[3-(4-hydroxyphenyl)propionamido]ethyl]carbamyl]forskolin.

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