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Fourier Transform Infrared Difference Spectroscopy of Bacteriorhodopsin and Its Photoproducts Regenerated with Deuterated Tyrosine†

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ABSTRACT: Fourier transform infrared (FTIR) difference spectroscopy has been used to detect the vibrational modes due to tyrosine residues in the protein that change in position or intensity between light-adapted bacteriorhodopsin (LA) and other species, namely, the K and M intermediates and dark-adapted bacteriorhodopsin (DA). To aid in the identification of the bands that change in these various species, the FTIR spectra of the free amino acids Tyr-d_0, Tyr-d_2 (^{2}H at positions ortho to OH), and Tyr-d_4 (^{2}H at positions ortho and meta to OH) were measured in H_2O and D_2O at low and high pH. The characteristic frequencies of the Tyr species obtained in this manner were then used to identify the changes in protonation state of the tyrosine residues in the various bacteriorhodopsin species. The two diagnostically most useful bands were the ~1480-cm^{-1} band of Tyr(OH)-d_2 and the ~1277-cm^{-1} band of Tyr(O$^-$)-d_0. Mainly by observing the appearance or disappearance of these bands in the difference spectra of pigments incorporating the tyrosine isotopes, it was possible to identify the following: in LA, one tyrosine and one tyrosinate; in the K intermediate, two tyrosines; in the M intermediate, one tyrosine and one tyrosinate; and in DA, two tyrosines. Since these residues were observed in the difference spectra K/LA, M/LA, and DA/LA, they represent the tyrosine or tyrosinate groups that most likely undergo changes in protonation state due to the conversions. These changes are most likely linked to the proton translocation process of bacteriorhodopsin.

Bacteriorhodopsin (BR)[1] is the sole protein contained in the purple membrane from the bacteria *Halobacterium halobium*. It has been the focus of intense investigation primarily because it functions as a light-activated proton pump, an event that takes place upon the isomerization of retinal. The potential difference that occurs as a result of proton pumping provides the energy for the synthesis of ATP. Synthetic vesicles incorporating BR protein along with ATPase are seen to produce ATP in the presence of ADP and inorganic phosphate upon illumination, thus supporting the chemiosmotic hypothesis of Mitchell (1961). This field has been extensively reviewed (Stoeckenius & Bogomolni, 1982; Dencher, 1983). Proton translocation appears to be the penultimate event in the functioning of bioenergetic membrane proteins and is also observed to occur in the thylakoid membranes of chloroplasts and in mitochondrial subfragments (Boyer et al., 1977).

Extensive information exists on the conformation of retinal in BR as a function of its photocycle and in relation to the proton pumping, but almost nothing is known about the all important apoprotein participation in this process. In light of the wealth of structural information on BR, the relative ease

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‡ Deceased Aug 14, 1985. This paper is dedicated to the memory of Laura Eisenstein.

[1] Abbreviations: FTIR, Fourier transform infrared; BR, bacteriorhodopsin; BR^{LA} or LA, light-adapted bacteriorhodopsin; BR^{DA} or DA, dark-adapted bacteriorhodopsin; Tyr, tyrosine; Tyr(OH), tyrosine; Tyr(O$^-$), tyrosinate.

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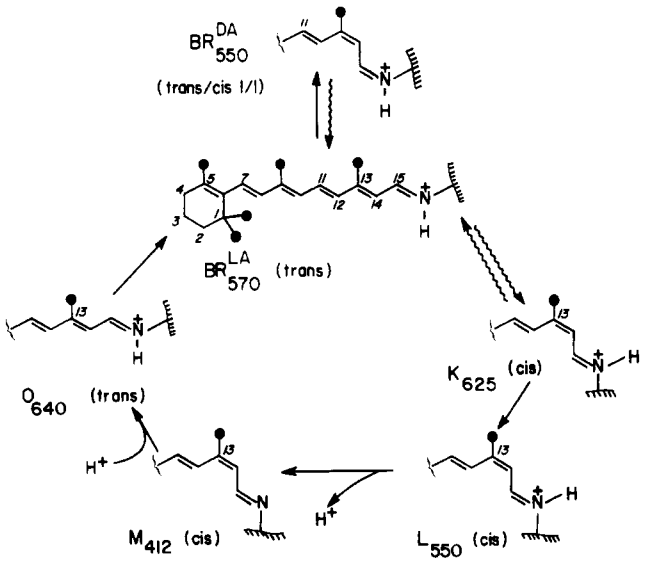


FIGURE 1: Light-adapted and dark-adapted photocycles of bacteriorhodopsin, LA, and DA. The subscript numerals denote absorption maxima, while the trans/cis notations refer to the 13-ene. Wavy and straight arrows, respectively, indicate photochemical and thermal transformations. The Schiff base linkages are depicted in the syn form for DA and anti form for LA.

of isolation, and the large yields of protein produced, it constitutes an ideal system for the study of a proton pumping mechanism. It is possible that an understanding of this process might lead to general structural requirements for proteins that translocate protons in terms of the apoprotein H^+ conduction mechanisms.

In order to discuss the proton pumping event for the specific case of BR, the nature of the attachment of the chromophore to the apoprotein as well as the photocycle needs to be considered briefly. The chromophore in BR is covalently attached via a Schiff base to Lys-216 (Bayley et al., 1981) and is found to be protonated in the LA_{570}, K_{625}, L_{550}, and O_{640} photocycle intermediates as shown by resonance Raman and FTIR studies (Lewis et al., 1974; Aton et al., 1977; Rothschild & Marrero, 1982; Bagley et al., 1982; Smith et al., 1983). The formation of the K intermediate (Figure 1, λ_{max} 625 nm) is the only step in the photocycle that requires light; the other discrete intermediates are short-lived thermal reaction products along the decay path back to light-adapted BR_{570} (LA). Figure 1 also indicates the state of protonation of the Schiff base for the various intermediates, information that has been obtained primarily by resonance Raman and FTIR studies. The conformation of the 6,7-bond and configurations of the Schiff base linkages reflect the recent findings obtained by resonance Raman (Smith et al., 1984) and solid-state ^{13}C NMR (Harbison et al., 1984, 1985). In the dark-adapted species BR_{560} half of the chromophores in the protein thermally isomerize from *all-trans*-retinal to the 13-cis isomer (Pettei et al., 1977).

After the L intermediate, the Schiff base becomes deprotonated, resulting in the formation of the blue-shifted M_{412} intermediate. The proton pumping event is observed to occur approximately concomitant with the formation of the M intermediate ($\sim$40 μs), and it is generally assumed that it is the Schiff base deprotonation which triggers the ion movement. However, this proton is not sufficient to account for the observed pumping stoichiometry, which is known to be approximately two protons per photocycle (Stoeckenius & Bogomolni, 1982), since the retinal chromophore contains only one exchangeable proton ($C{=}NH^+$). This would seem to imply that the source of the second proton must be an amino acid residue within the protein interior. A tyrosine residue situated near the ionone ring of retinal has been hypothesized in this regard (Seltzer & Ehrenson, 1984).

In principle, any amphoteric amino acid residue can participate in a proton translocation process, such as lysine, arginine, aspartic acid, glutamic acid, or tyrosine. The protonated Schiff base of retinal in BR has a counterion which must play some role in the formation of the M_{412} intermediate, and an anionic residue such as aspartate or tyrosinate may act as the primary receiver in the deprotonation event. Some evidence has been presented that correlates the Schiff base deprotonation and the formation of M_{412} with the protonation of an aspartate residue (Siebert et al., 1982), while hypothetical models based on CPK models of BR have demonstrated the feasibility of tyrosine and carboxylic amino acid participation in a proton translocating network of hydrogen bonds (Merz & Zundel, 1981). In an attempt to determine which amino acids participate in proton translocation either during the ion transduction process or as a source for the second pumped proton, we have undertaken Fourier transform infrared (FTIR) difference studies on several isotopically labeled BR proteins.

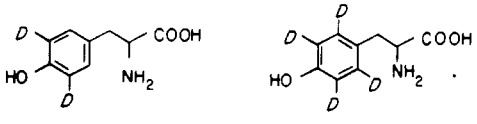
FTIR difference spectroscopy allows the study of protonation changes of amino acid residues in functional BR as it is a noninvasive absorption technique and can reliably reproduce absorbance changes due to a change in environment or protonation of a single residue ($\Delta A \geq 0.0015$). Since the technique involves subtraction between two spectra, the resulting difference spectra tend to be very complex, consisting of numerous positive and negative bands of varying intensities; careful analysis of the lines allows one to simultaneously acquire information on both retinal and the apoprotein. Where there are no vibrational changes for either the apoprotein or retinal, the generated difference spectra are a flat line with near zero absorbance throughout most of the infrared region (Chang et al., 1985). The FTIR difference technique and the implementation of procedures for trapping and generating difference spectra for the various BR photocycle intermediates have previously been described (Bagley et al., 1982; Rothschild & Marrero, 1982; Siebert & Mantele, 1983).

Studies on chemically modified tyrosines in intact BR have pointed to a pivotal role for protonation changes of this residue in the photocycle. Iodinated BR was shown to dramatically increase the yield of M_{412} within the pH range 7–9, as indicated by kinetic resonance Raman experiments; this would be anticipated for a participating catalytic group with a lowered pK, such as iodinated tyrosine (Gogel & Lewis, 1981). It had previously been shown that monoiodinated and diiodinated tyrosines stabilize the lifetime of the M_{412}, presumably because reprotonation of this residue is required for M decay (Konishi & Packer, 1978). A highly specific nitration reaction for Tyr-64 resulting in a color change (570 $\rightarrow$ 535 nm) has recently been described, but this specific reaction only occurs in the light at pH 6 (Scherrer & Stoeckenius, 1984). This seems to indicate a light-catalyzed deprotonation for Tyr-64, as tetranitromethane will not react with protonated phenols (Riordan et al., 1966; Bruice et al., 1968). Light-activated deprotonation of a tyrosine has been discussed in connection with hypothetical proton pumping mechanisms (Kalisky et al., 1981; Seltzer & Ehrenson, 1984).

Protonation changes of tyrosine residues during the photocycle of BR have been observed by several groups using kinetic UV absorbance techniques (Bogomolni et al., 1978; Hess & Kuschmitz, 1979; Rafferty, 1979; Kuschmitz & Hess, 1982; Hanamoto et al., 1984). These studies were performed by initiating the photocycle with a pulse of light and detecting

transient signals at 275 and 296 nm, respectively, where Tyr(OH) and Tyr(O^-) absorb maximally. The deprotonation of a tyrosine was shown to be correlated with the rise of M and reprotonation of a tyrosine residue with the decay of that species. However, there is some discrepancy as to whether tyrosine deprotonation is a prerequisite for the formation of M. The bulk of evidence would indicate at least that Tyr(OH)/Tyr(O^-) interactions play a pivotal role in the proton pumping process.

In an attempt to clarify the role of tyrosine in the photocycle, as either a catalytic group in M formation or a participating residue in proton translocation, or both, we have undertaken a study of isotopically labeled tyrosines in BR. Vibrational analysis of BR proteins by FTIR has proven valuable in the identification of protonation changes of tyrosine residues during the photocycle. We have synthesized $^2H_2$ and $^2H_4$ (Tyr-d_2, Tyr-d_4) labeled tyrosines I and II via exchange reactions and



I II

have studied the vibrational spectra of these model compounds in solution at high and low pH in order to assign the characteristic frequencies of these species. Both isotopically labeled tyrosines display unique vibrational spectra at high and low pH, with no observable overlap of characteristic frequencies. This is highly important if one is going to assign tyrosine/tyrosinate vibrational frequencies in the protein, and in general, we have considered assignments most reliable only if they could be ascribed to intense lines in the model compound spectra. Tyrosine-labeled BR was then prepared by rearing *Halobacterium* in a synthetic media containing the deuterium analogues of tyrosine, Tyr-d_2 (I) and Tyr-d_4 (II).

Materials and Methods

Sampling and Spectroscopic Measurements. The BR sampling and FTIR measurements of various BR species were carried out as described earlier (Bagley et al., 1982, 1985) except that the BR film was dried on a Cleartran window (ZnS, Harrick Scientific Co.) and the cell was sealed with an antireflection window coated with Ge to increase the IR transmission (Vintein Coating No. 40401, Exotic Inc.).

Preparation of L-[ϵ_1,ϵ_2-$^2H_2$]*Tyrosine* (*Tyr-d_2*) (*I*). This amino acid derivative was prepared via an acid-catalyzed (D_2SO_4, DCl) exchange reaction of commercially available L-tyrosine basically according to the published procedure of Matthews et al. (1977).

Preparation of L-[$\epsilon_1,\epsilon_2,\delta_1,\delta_2$-$^2H_4$]*Tyrosine* (*Tyr-$d_4$*) (*II*). This compound cannot be made via an acid-catalyzed exchange reaction, as the δ_1 and δ_2 protons are deactivated toward H^+ exchange; $\epsilon_1,\epsilon_2,\delta_1,\delta_2$-phenylalanine can be prepared via a heterogeneous exchange reaction involving either $^2H_2$ gas or ^2H_2O and a zerovalent metal catalyst, such as Pt^0 or Ni^0 (Calf et al., 1966), although an analogous synthesis of Tyr-d_4 has not been reported as far as we are aware. A chemical synthesis of Tyr-d_4 has been reported from Phe-d_5, although with low yield. In light of this, we explored the possibility of synthesizing this material via Pt^0-catalyzed exchange in ^2H_2O.

Finely divided Pt^0 was prepared essentially by the method of Calf and Garnett (1964) employing $NaBH_4$ reduction of PtO_2 (Adams catalyst) in degassed H_2O under argon. The resulting black precipitate was rinsed with H_2O twice, then with ^2H_2O prior to use. Pt^0 should not be vacuum dried, as it is pyrophoric when dry. Finely divided Pt^0 (200 mg) was placed in 110 mL of 99.9% ^2H_2O to which was added 450 mg of L-tyrosine. This was refluxed at 108 °C under argon for 48 h. After this time, the $\epsilon_1\epsilon_2$-protons were completely exchanged, and the $\delta_1\delta_2$-protons were exchanged to the extent of 60%. The reaction was first filtered while hot to remove the catalyst and then cooled to room temperature. After addition of decolorizing carbon (Norit), the solution was reheated to boiling, let stand until it cooled to room temperature, and then left in a cold room overnight to give crystals of Tyr-d_4 in ~80% yield.

The reproducibility of this exchange reaction exhibits slight variability in deuterium incorporation, and this probably depends on the activity of the catalyst preparation. Repetition of this procedure, by either recycling the used catalyst with fresh $NaBH_4$ or preparing a new batch for tyrosine that had undergone one exchange process, resulted in Tyr-d_4 that displayed a deuterium content of 88–90% for the $\delta_1\delta_2$-protons, with the $\epsilon_1\epsilon_2$-positions exhaustively exchanged. Material corresponding to 100% D exchange for the $\epsilon_1\epsilon_2$-protons and 65% for the $\delta_1\delta_2$-protons was employed in the model compound studies, while tyrosine that had been subjected to two exchange cycles (88% $\delta_1\delta_2$-exchange) was used in culture. The tyr-d_2 employed was of 99.5% isotopic enrichment at $\epsilon_1\epsilon_2$.

Removal of 1 mL of the heterogeneous reaction mixture followed by cooling and dissolution of the precipitated tyrosine with 100 μL of NaOD was used to monitor the exchange process by ^1H NMR. Deuterium incorporation was assessed by comparing the integral heights of the aromatic $\epsilon_1\epsilon_2$-protons (6.42 ppm, d, 2 H) and $\delta_1\delta_2$-protons (6.83 ppm, d, 2 H) with that of the aliphatic $\beta_1\beta_2$-protons, which do not exchange under the reaction condition ($\beta_1\beta_2$, 2.7 ppm, dd, and 2.5 ppm, dd, 2 H).

Model Compound Analysis. The synthetic tyrosines were measured at high (>13) and low (<0.5) pH in water, employing an attenuated total reflectance circle cell (Barnes Analytical Co.) equipped with a germanium crystal for low pH measurements and a zinc selenide crystal for high pH measurements.

Preparation of Tyr-d_2- and Tyr-d_4-BR. H. halobium (RM) cultures were obtained from agar slants containing Bacto-Peptone as an amino acid source. These were used to inoculate 1 L of a synthetic medium. A medium described by Onishi was found to be optimal for these purposes (Onishi et al., 1965). Supplementing the Onishi media requirement for tyrosine with either of the deuterated analogues is sufficient to ensure maximal incorporation. It has been previously demonstrated that radiolabeled tyrosine is incorporated intact, i.e., without scrambling into other amino acids or diluted by de novo biosynthesis via the metabolic and catabolic pathways (Kinsey et al., 1981).

Results

Model Compound Spectra (*Figure 2*). Tyr(OH) and Tyr(O^-) spectra measured at high and low pH, respectively, for Tyr-d_0, -d_2, and -d_4 are shown in Figure 2. The Tyr-d_4 sample was a mixture of 65% d_4 and 35% d_2; the d_4 spectra (Figure 2e,f) therefore show bands due to the d_2 species which are indicated by check marks in Figure 2e (at 1478 cm^{-1}) and f (at 1453 cm^{-1}). However, the isotopically labeled tyrosines employed in incorporation studies were of higher isotopic purity, >99.5% for d_2 and 85% for the d_4 species.

The purpose of measuring model spectra was to identify strong bands that would be of diagnostic value in detecting changes in tyrosine/tyrosinate residues in the BR preparations containing the various isotopic tyrosines. Therefore, although

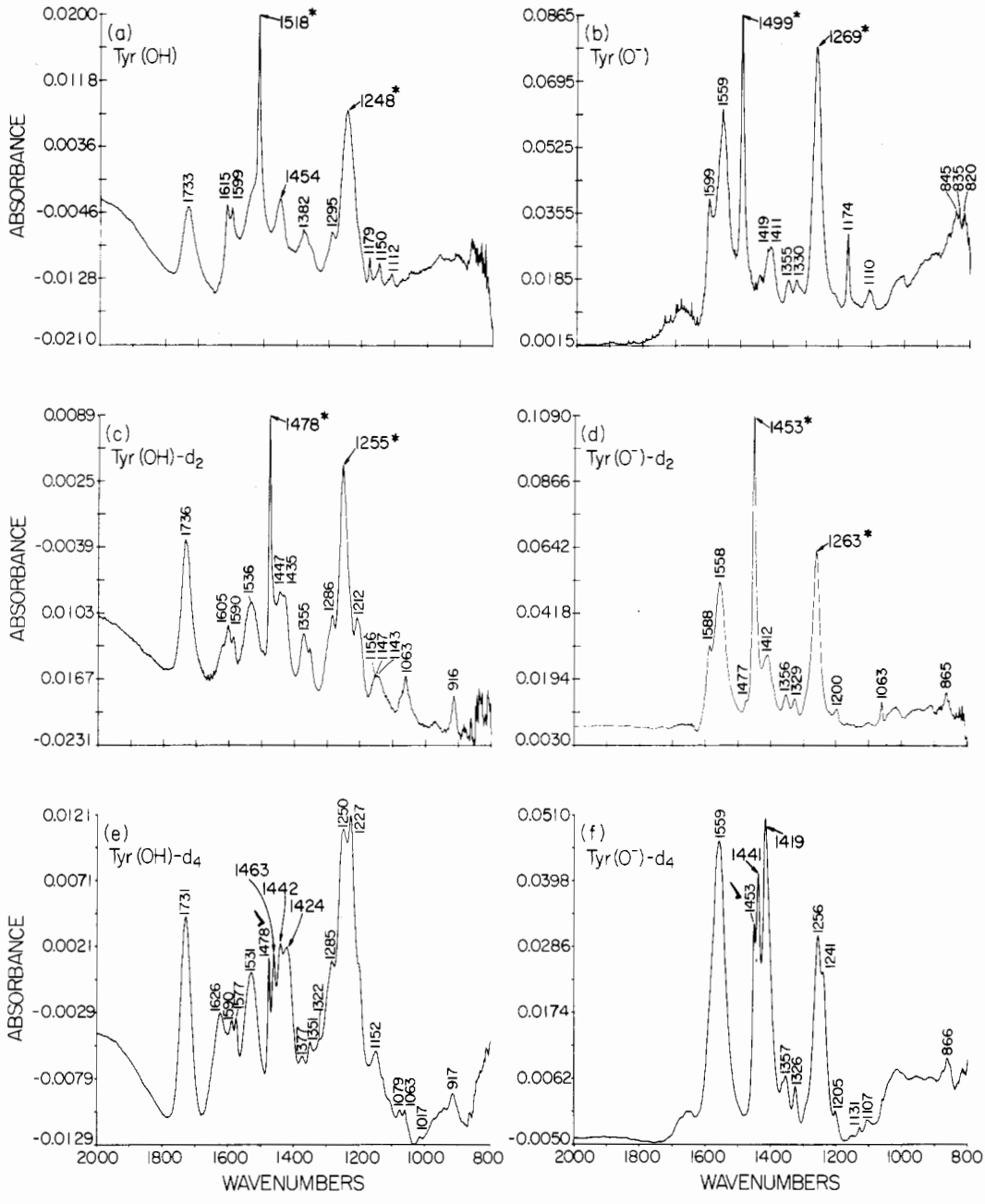


FIGURE 2: (a), (c), and (e), FTIR of tyrosine-d_0, -d_2, and -d_4 in H_2O, pH 0.5; (b), (d), and (f), FTIR of tyrosinate-d_0, -d_2, and -d_4 in H_2O, pH 13. Characteristic frequencies are asterisked, the two diagnostically most useful being the 1478-cm^{-1} in (c) and the 1269-cm^{-1} band in (b). The positions of these characteristic frequencies in D_2O were unchanged. The checked bonds at 1478 cm^{-1} in (e) and 1453 cm^{-1} in (f) are due to residual d_2 species in the sample.

some diagnostic vibrational bands in tyrosines in polypeptides have been assigned (Bendit, 1967), only the very strong tyrosine bands that would be observable in BR need to be discussed here. It is seen that two major bands (asterisked) can be identified for Tyr-d_0 and -d_2 in Figure 2a–d. The two diagnostically most important are the 1478-cm^{-1} band for Tyr(OH)-d_2 (2c) and the 1269-cm^{-1} band for Tyr(O^-) (2b). The characteristic frequencies for the d_4 species, however, are not as obvious as in the d_0 and d_2 species. The following trends were noted:

(1) The skeletal C=C stretch (aromatic), which we will denote νC=C, undergoes a ca. 45-cm^{-1} shift to low frequencies in the d_2 species, i.e., 1518 (Figure 2a) vs. 1478 cm^{-1} (2c) and 1499 (2b) vs. 1453 cm^{-1} (2d).

(2) The νC=C bands are also shifted 20 cm^{-1} to lower frequencies upon deprotonation, i.e., 1518 (2a) vs. 1499 cm^{-1} (2b) and 1478 (2c) vs. 1453 cm^{-1} (2d).

(3) The νC=C bands for Tyr(OH)-d_4 (2e), presumably the cluster of bands at 1463–1424 cm^{-1}, are not as conspicuous as in the d_0 and d_2 species.

It will be seen in the following that the two strong frequencies in Figure 2 (underscored), namely, the 1269-cm^{-1} band of Tyr(O^-) (2b), was assigned to a phenolate C–O^- (Pinchas, 1972), and the 1478-cm^{-1} band of Tyr(OH)-d_2 (2c) were of great diagnostic value. The fate of tyrosine and tyrosinate groups were mostly followed by looking for the appearance or disappearance of these two bands in the appropriate BR species.

K/LA Spectra (Figure 3, Table I).[2] The FTIR difference

[2] While this paper was in review, an FTIR study dealing with the BR → K transition appeared (Rothschild et al., 1986). The conclusion that a tyrosinate group protonates in this step is in agreement with that in this paper (Table I).

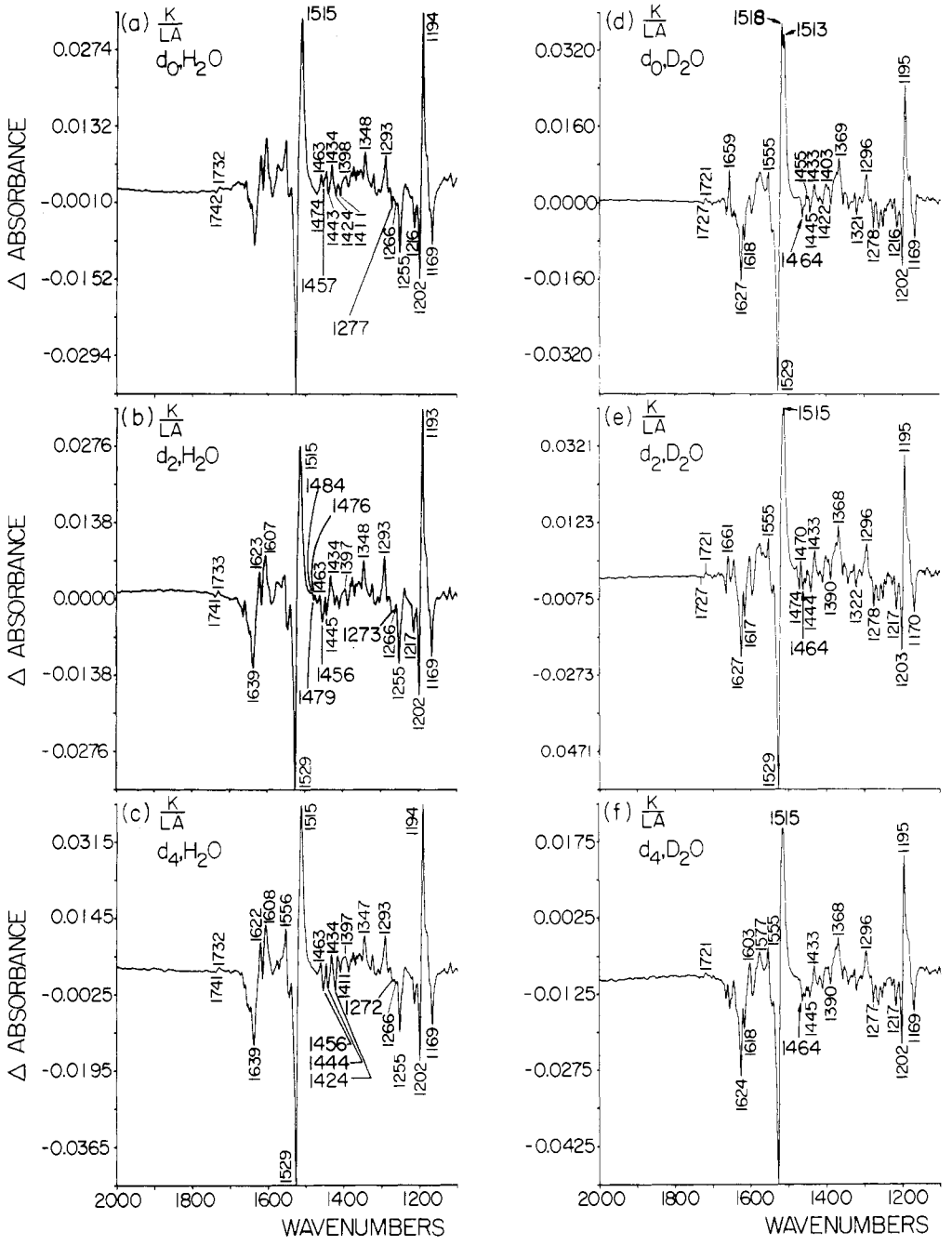


FIGURE 3: FTIR different spectra between K intermediate and light-adapted bacteriorhodopsin LA. (a) Hydrated film of native BR. (b) Hydrated film of BR regenerated with Tyr-d_2. (c) Hydrated film of BR regenerated with Tyr-d_4. (d) Deuterated film of native BR. (e) Deuterated film of BR regenerated with Tyr-d_2. (f) Deuterated film of BR regenerated with Tyr-d_4.

spectrum of K/LA for a protein sample containing Tyr-d_0 is shown in Figure 3a. The positive absorbance mode shows vibrations ascribable to K, while those in the negative absorbance mode correspond to the initial light-adapted BR_{570}. The inset includes the region where tyrosine νC=C vibrations may be expected based upon the model compound studies. Figure 3b shows the K/LA spectrum for BR isolated from *Halobacterium* reared on the Onishi medium containing Tyr-d_2. It is immediately apparent that there are dramatic changes for the region corresponding to the νC=C modes; the changes are ascribable solely to substitution with the deuterated analogues. The major conclusions obtained by comparing the difference FTIR spectra of BR containing Tyr-d_0, -d_2, and -d_4 can be summarized as follows:

(1) In the Tyr-d_2 K spectrum (Figure 3b), two new peaks are found at 1484 and 1476 cm^{-1}, while a new BR^{LA} vibration is seen at 1479 cm^{-1}. Comparison with the model compound spectra in Figure 2 shows that these vibrations arise from Tyr(OH)-d_2 which displays an intense line at 1478 cm^{-1}. In agreement with their assignments, these lines are absent from

Table I: Changes in Tyr Residues in Various Bacteriorhodopsins[a]

BR	Tyr residues		bands (cm^{-1}) obsd in isotopic species
DA	Tyr (OH)		
		Tyr (OH)	1480 (d_2, Figure 5b)
LA	Tyr (OH)		1479 (d_2, Figures 3b and 4b)
		Tyr (O^-)	1277 (d_0, Figures 3a, 4a, and 5a)
K	Tyr (OH)		1476 (d_2, Figure 3b)
		Tyr (OH)	1484 (d_2, Figure 3b)
M	Tyr (OH)		1475 (d_2, Figure 4b)
		Tyr (O^-)	

[a] Two tyrosine residues are tabulated in a staggered manner simply for the ease of following the changes; it does not necessarily mean that the Tyr residues appearing in the same column are the same.

the Tyr-d_0 (Figure 3a) and the Tyr-d_4 spectra (Figure 3c).

(2) The Tyr-d_0 LA spectrum contains a vibration at 1277 cm^{-1} (Figure 3a), which appears to shift down to ~1273 in Tyr-d_2 LA (2b) and becomes barely discernible in the Tyr-d_4 LA spectrum (2c). We assign the Tyr-d_0 vibration at 1277 cm^{-1} to the phenolate C–O^- stretch of Tyr(O^-), which occurs between 1270–1281 cm^{-1} (Pinchas, 1972). Similar changes are seen to occur for the 1277-cm^{-1} line in the LA half of the M/LA and DA/LA difference spectra (Figures 4 and 5), indicating *the presence of a tyrosinate residue in LA*. In the model compounds, the 1269-cm^{-1} peak is seen to shift to 1263 cm^{-1} upon substitution with Tyr-d_2 (Figure 2b,d); however, in Tyr-d_2 LA (Figure 3b), the band at 1273 cm^{-1} is not as clear as the -d_0 1277-cm^{-1} band, owing to other absorbances in that region. It is the disappearance of the Tyr(O^-)-d_0 1277-cm^{-1} peak upon substitution with either d_0 or d_4 tyrosine that identifies this vibration as tyrosinate in the Tyr-d_0 LA spectra.

(3) For BR substituted with Tyr-d_2 (Figure 3b), one can identify a prominent peak at 1456 cm^{-1}. It is tempting to conclude that this corresponds to the 1453-cm^{-1} tyrosinate vibration in the model compound spectrum (Figure 2d). However, this vibration is also present at 1457 cm^{-1} in Tyr-d_0 LA (Figure 2a), although with much less intensity, and is still prominent upon substitution with Tyr-d_4 at 1456 cm^{-1} (Figure 2c); this is inconsistent with its assignment to a tyrosinate, since it does not shift upon Tyr-d_2 → Tyr-d_4 substitution. Most likely, this vibration is a retinal mode, because it is present in the resonance Raman spectra of LA (Pande et al., 1981; Braiman & Mathies, 1980).

The large intensity enhancement accompanying substitution by either of the deuterated tyrosines can be accounted for by removal of a positive K band which originally was around 1456 cm^{-1}; substitution with Tyr-d_2 and Tyr-d_4 shifts this K absorbance to lower wavenumbers, thus revealing the LA (negative) vibration at the same frequency. It should be obvious that since the spectra are generated by subtraction, overlapping vibrations for the positive and negative species will partially or totally cancel each other, depending on their relative intensities. We assign this Tyr-d_0 K vibration at ~1456 cm^{-1} which overlaps with the retinal band in this region to a protonated tyrosine; it appears as a moderately intense peak at 1454 cm^{-1} in the reference spectrum (Figure 2a). This argument supports our previous discussion for the presence of protonated tyrosines in K.

(4) The aforementioned chromophore line at 1456 cm^{-1} is seen to shift to 1465 cm^{-1} in the resonance Raman spectra upon exchange in D_2O (Pande et al., 1981). This is most likely due to the retinal Schiff base H^+/D^+ exchange. The presence of a 1464-cm^{-1} line in the D_2O exchanged Tyr-d_0, -d_2, and -d_4 K/LA spectra (Figure 3d–f) is accounted for by this shifted chromophore line.

(5) An interesting observation in the Tyr-d_0 K spectrum (Figure 3a) is that the ethylenic chromophore line at 1515 cm^{-1} in H_2O splits into two at 1513 and 1518 cm^{-1} when measured in D_2O (Figure 3d). This splitting has been noticed before (Bagley et al., 1982; Siebert & Mantele, 1983), but the origin was not accounted for. The splitting is not observed in the D_2O exchanged resonance Raman spectra, which implies that it is an apoprotein rather than a chromophore effect. Neither the Tyr-d_2 nor the -d_4 K spectra (Figure 3e,f) display this ethylenic line-splitting in D_2O, thus indicating that substitution with isotopically labeled tyrosine removes the D_2O splitting in the K spectrum. It is seen that Tyr(OH)-d_0 has an intense band at 1518 cm^{-1} (Figure 2a). This demonstrates that the assigned ethylenic line in the FTIR difference spectra of K at 1515 cm^{-1} contains some absorbance due to Tyr(OH); in the Tyr(OH)-d_2 and Tyr(OH)-d_4 spectra (Figure 2c,e), this line is shifted to 1478 and 1463 cm^{-1}, respectively, and hence, splitting of the ethylenic 1515-cm^{-1} band disappears (Figure 3e,f).

M/LA Spectra (*Figure 4*). The major observations concerning the M/LA spectra are summarized below.

(1) In the Tyr-d_2 M spectrum (Figure 4b), a protonated tyrosine line is prominent at 1475 cm^{-1}. This vibration is not apparent in the Tyr-d_0 M spectrum (Figure 4e); upon substitution with Tyr-d_4 (Figure 4c), it is seen to disappear or greatly diminish in size, thus supporting its assignment as a protonated tyrosine line. It is important to note that the 1475-cm^{-1} vibration in the Tyr-d_2 M (Figure 4b) corresponds closely to the 1476-cm^{-1} line in the Tyr-d_2 K (Figure 3b). The appearance of this line in both the K and M spectra, at the same frequency, suggests that the protonated tyrosine at 1476 cm^{-1} in K undergoes neither deprotonation nor environmental changes during the transition of K → M. However, the other Tyr(OH)-d_2 that was identified in K at 1484 cm^{-1} (Figure 3b) is absent in the Tyr-d_2 M spectrum (Figure 4b).

(2) An LA tyrosine vibration at 1479 cm^{-1} is again present in the Tyr-d_2 M/LA difference spectrum (Figure 4b), as in the case of K/LA (Figure 3b). It is not seen in the Tyr-d_0 or Tyr-d_4 M/LA spectra (Figure 4a,c), therefore supporting its assignment as Tyr(OH).

(3) The trends seen in Figure 3a–c for the 1277-cm^{-1} LA line in the K/LA spectra accompanying substitution with Tyr-d_2 → Tyr-d_4 are also found in the M/LA spectra (Figure 4a–c), thus confirming assignment of the 1276-cm^{-1} line (Figure 4a) to a tyrosinate in LA.

(4) A rather prominent vibration at 1456 cm^{-1} (or 1454 cm^{-1}) for LA in the M/LA different spectra is observed for the Tyr-d_2 and Tyr-d_4 proteins (Figure 4b,c) but is absent in the Tyr-d_0 species (Figure 4a). The same argument applied above for this vibration in the K/LA spectra applies here also and supports the presence of a protonated tyrosine band in M in the M/LA difference spectrum. This vibration is also seen to shift upon D_2O exchange to 1461 cm^{-1} in both the Tyr-d_2 (spectrum not shown) and Tyr-d_4 protein samples (Figure 4d).

(5) The absence of the 1484-cm^{-1} Tyr(OH) band in Tyr-d_2 M (Figure 4b) implies that one of the two Tyr(OH)s assigned in Tyr-d_2 K (1484 and 1476 cm^{-1}) becomes deprotonated during the K → M transition.

DA/LA Spectra (*Figure 5*, *Table I*). The identification of amino acids that are associated with the thermal transition to the dark-adapted species is required in order to clarify the fact that the DA cycles, yet pumps no protons. The important observations and assignments for the DA/LA can be summarized as follows:

(1) Comparison of the expanded regions between 1511 and 1445 cm^{-1} for the Tyr-d_0 and Tyr-d_2 DA (Figure 5a,b) show changes ascribable to a protonated tyrosine $\nu C{=}C$ vibration. Figure 5b of the Tyr-d_2 species exhibits a new vibration at 1480 cm^{-1} in the positive absorbance mode (DA), this new line corresponding closely to the 1478-cm^{-1} vibration of Tyr-(OH)-d_2 (Figure 2c). It is absent in the Tyr-d_0 DA and Tyr-d_4 DA spectra (Figure 5a,c), thus demonstrating that it most likely arises from a Tyr(OH)-d_2 residue. Namely, a tyrosine residue becomes protonated in the transition of LA → DA. The appearance of a 1456-cm^{-1} retinal line upon substitution with deuterated tyrosines (Figure 5b,c) is also in agreement with the presence of a protonated tyrosine absorption in DA that overlaps the 1456-cm^{-1} retinal band.

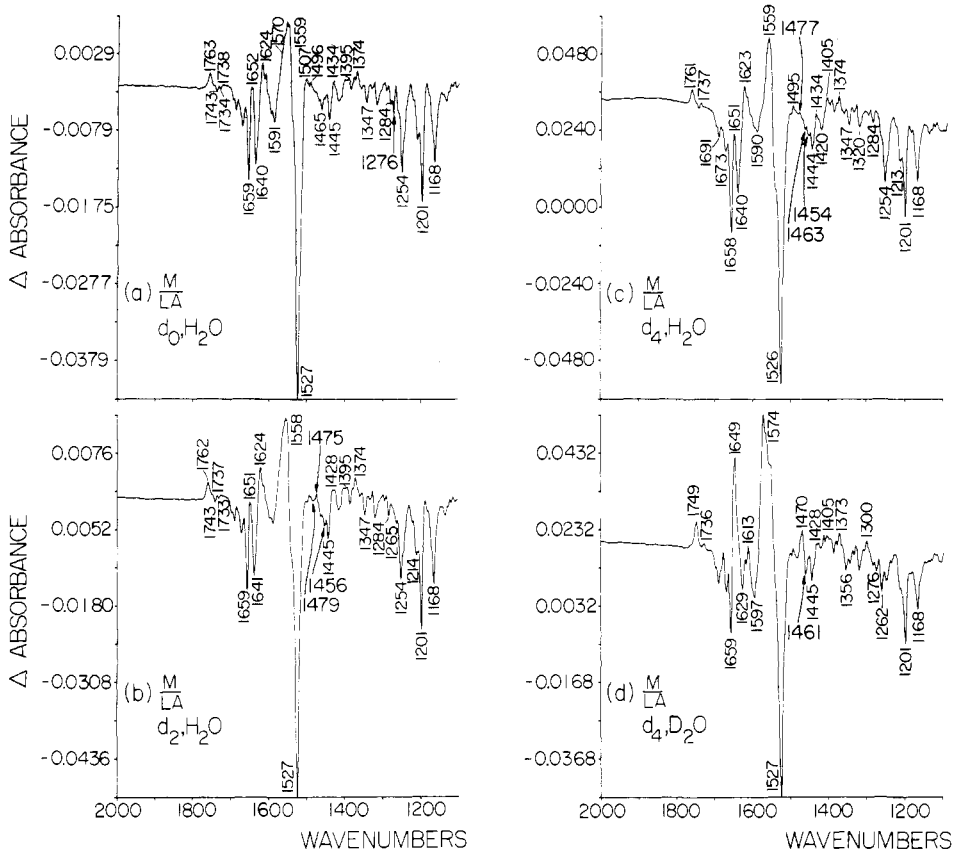


FIGURE 4: FTIR difference spectra between M intermediate and light-adapted bacteriorhodopsin LA. Insets show the expanded region between 1500–1250 cm^{-1}. The peaks discussed in the text are denoted by black dots. (a) Hydrated film of native BR. (b) Hydrated film of BR regenerated with Tyr-d_2. (c) Hydrated film of BR regenerated with Tyr-d_4. (d) Deuterated film of BR regenerated with Tyr-d_4.

(2) The 1276-cm^{-1} vibration in Tyr-d_0 LA (Figure 5a) undergoes sequential changes similar to those described previously in the series $d_0 \rightarrow d_2 \rightarrow d_4$, once again demonstrating the presence of Tyr(O^-) in the LA species (Figure 5b,c).

Discussion

The use of isotopically labeled tyrosines was indispensable for interpreting the small but reproducible absorbance changes that occur in the FTIR upon biosynthetic incorporation of these compounds into BR. The isotopic shifts caused by introduction of deuterium in the tyrosine residue is reflected in the BR spectra as appearance/disappearance of characteristic frequencies in the difference FTIR of Tyr-d_0 BR, Tyr-d_2 BR, and Tyr-d_4 BR. From these changes it has been possible to follow the conversions between tyrosine and tyrosinate residues in the various BR species. The two bands most useful were the 1270-cm^{-1} Tyr($O)^-$-d_0 (Figure 2b) and ~1478-cm^{-1} Tyr-(OH)-d_2 bands (Figure 2c) because of their intensity and appearance in a region where other strong absorptions are absent; the assignments to these species were corroborated by the absence of these bands in other isotopically labeled BR species.

FTIR difference spectroscopy therefore provides information on apoprotein or retinal vibrations that have undergone a change in environment and/or protonation state between the subtracted intermediates. For the specific case of tyrosine residues discussed here, we feel that the changes upon substitution with deuterated residues in the difference spectra indicate real protonation changes for these residues rather than vibrational shifts of residues in the same protonation state caused by environmental perturbations during a given transition. One of the more surprising findings from this work is the close correspondence observed between the characteristic model compound peaks and those identifiable as new vibrational features in the labeled protein spectra. The new vibrational features that appear in the labeled proteins coincide almost exactly with those predicted from the model tyrosines at high and low pH. This suggests that the vibrational bands employed in our study are relatively insensitive to environmental changes and indicate primarily protonation changes. In support of this, FTIR studies on the polypeptide poly(L-Tyr-L-Glu) at high and low pH demonstrate the same vibrational features for tyrosine as for the isolated compounds in solution (data not shown).

For example, the polypeptide bands at high pH (compare these with those in Figure 2b) are at 1499, 1271, and 1174 cm^{-1}. The respective vibrations in the model compound are at 1499, 1269, and 1174 cm^{-1}. An analogous situation exists for the protonated tyrosine models. Since these key vibrational features [e.g., 1270 of Tyr(O^-)] are unchanged in the model compound, polypeptide, and BR protein, we must infer that they are relatively insensitive to local environment and are highly dependent upon protonation state.

Our data are consistent with previously published experimental data that demonstrate kinetic protonation changes for tyrosine during the photocycle (see above). However, our data go beyond these results in that transitions other than the slow-forming M_{412} are amenable to FTIR studies, enabling us to probe for the first time tyrosine changes in the BR → K, BR → M, and BR → DA.

Results of the present studies are summarized in Table I. Two tyrosine residues are tabulated in a staggered manner simply for ease of discussion; as mentioned above, FTIR studies do not permit us to follow the fate of individual tyrosine residues, and hence the staggering should not be taken necessarily to represent the fate of two specific residues.

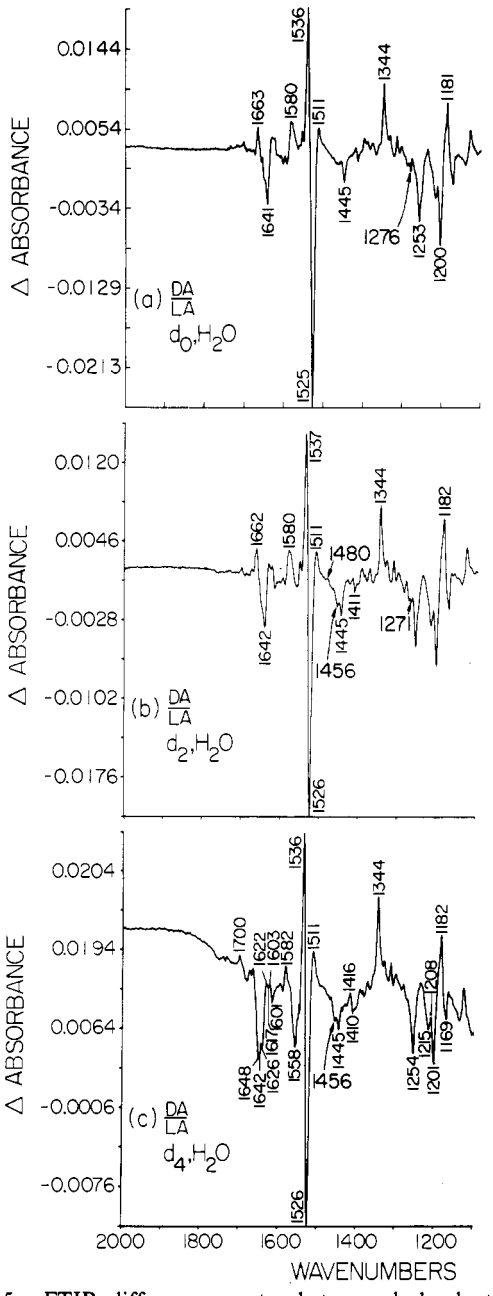


FIGURE 5: FTIR difference spectra between dark-adapted and light-adapted bacteriorhodopsin, DA, and LA. (a) Hydrated film of native BR. (b) Hydrated film of BR regenerated with Tyr-d_2. (c) Hydrated film of BR regenerated with Tyr-d_4.

The K/LA data point to the presence of a tyrosine and a tyrosinate band in LA at 1479 cm^{-1} in -d_2 LA (Figure 3b) and 1277 cm^{-1} in -d_0 LA (Figure 3a), respectively. The protonated Tyr-d_2 band at 1479 cm^{-1} for LA is also found in the difference data of d_2 M/LA (Figure 4b); however, we find no evidence for this tyrosine band in LA from the DA/LA spectra (Figure 5). This may imply that the Tyr(OH) residue which appears at 1479 cm^{-1} in -d_2 LA (Figures 3b and 4b) experiences no environmental perturbation upon thermal transition to the dark-adapted species (DA) and hence cancels out in the observed difference spectra.

The spectra of the Tyr-d_4 series in K/LA (Figure 3c,f) were used to check for the disappearance of bands assigned to Tyr-d_2 rather than to look for bands positively assignable to Tyr-d_4. This is due to complications arising from overlap of Tyr-(OH)-d_4 bands at 1463, 1442, and 1424 cm^{-1} (Figure 2e) and Tyr(O^-)-d_4 bands at 1441 and 1419 cm^{-1} (Figure 2f) with Tyr-d_0 bacteriorhodopsin bands appearing in the same region but not due to tyrosine (see Figures 3a and 4a). If the new lines arising from Tyr-d_4 substitution are of the same sign as apoprotein vibrations of Tyr-d_0 BR, intensity enhancement of these lines should be observed. This is what is found experimentally in the Tyr-d_4 K/LA spectrum (Figure 3c); the 1444- and 1424-cm^{-1} lines of LA are seen to undergo a definite increase in intensity as compared to these vibrations in the Tyr-d_0 spectrum (Figure 3b). This observation supports the assignment of a protonated tyrosine in LA. However, in general, we have avoided assignments based upon apparent changes in intensity and have relied almost exclusively on frequency shifts for isotopically labeled tyrosines in order to interpret our data. Our interpretations are therefore somewhat conservative in this regard.

The above-mentioned 1277-cm^{-1} tyrosinate band for LA in the Tyr-d_0 K/LA spectrum (Figure 3a) is also consistently observed in difference spectra Tyr-d_0 M/LA (Figure 4a) and DA/LA (Figure 5a). Thus, changes due to a tyrosine and a tyrosinate residue in LA have been observed in its transition to other species, DA, K and M. On the other hand, the Tyr-d_2 K/LA spectrum (Figure 3b) argues for the presence of at least two tyrosine residues appearing at 1476 and 1484 cm^{-1}. One possibility consistent with, but not necessarily proven by the observations of Tyr(OH)/Tyr(O^-) lines in LA and Tyr-(OH)/Tyr(OH) lines in K is that of protonation of a tyrosinate residue during the transition of LA → K. This might account for the observation of a deuterium isotope effect during the formation of the K intermediate (Applebury et al., 1978; Downer et al., 1984). This protonation event only accounts for one of the two different tyrosines in K. The other protonated tyrosine found in K might be the one revealed in LA at 1479 cm^{-1}, which upon the transition of LA → K experiences an environmental change to shift its frequency to 1476 or 1484 cm^{-1} (Figure 3b).

It is rather striking that out of three lines assigned to tyrosine in the Tyr-d_2 K/LA spectrum (Figure 3b), namely, 1484 (K), 1479 (LA), and 1476 (K), two are also seen in the Tyr-d_2 M/LA spectrum (Figure 4b) at 1479 (LA) and 1475 cm^{-1} (M). The 1479- and 1475-cm^{-1} lines may belong to the same tyrosine group in LA and M, which is in slightly different environments in the two BR species. The 1475-cm^{-1} M line is possibly the same as the 1476-cm^{-1} tyrosine in K which has undergone neither an environmental nor a protonation change during the transition to M. Absence of the 1484-cm^{-1} K vibration in the M spectrum suggests deprotonation of that residue during formation of the M intermediate. This would be consistent with the current notion of a tyrosine deprotonation upon formation of M. Since Tyr-64 reacts with tetranitromethane only in the light (Scherrer & Stoeckenius, 1984), it is tempting to speculate that it is this residue that deprotonates upon M formation. Recent near-UV results suggest that this indeed is the case (P. Scherrer, private communication). FTIR experiments on Tyr-d_2 in which Tyr-64 has been nitrated are underway to test this hypothesis by using the tyrosine/tyrosinate bands assigned in the difference FTIR of isotopically labeled BR.

The occurrence of a tyrosinate in light-adapted BR at or near neutral pH is rather remarkable. To reduce the pK of a tyrosine from its solution value of around 10 to less than 7 requires a very strong perturbation such as a positive charge within a few Å of the phenolic ring. Similarly intriguing is the protonation of this residue upon dark adaptation. It is possible that this tyrosine is a proton acceptor in the light-adapted cycle and that its protonation upon dark adaptation is what inhibits the dark-adapted cycle from pumping protons.

The exact role of tyrosines in the proton transduction mechanism and the presumed link between tyrosine/tyrosinate and chromophore motions must await further studies. We have independently verified tyrosine participation in the photocycle by vibrational analysis. We have confined our discussion in this paper to changes associated with the tyrosine in bacteriorhodopsin. However, FTIR spectroscopy has shown that other residues are also involved in the proton pumping (Rothschild et al., 1981; Bagley et al., 1982; Maentele & Kreutz 1982; Engelhard et al., 1985; Dollinger et al., submitted for publication). For example, in Figure 4a for Tyr-d_0 M/LA (in H_2O) a band at 1763 cm^{-1} in the region where monomeric carboxyl groups appear is seen to shift to 1479 cm^{-1} when measured in D_2O (Figure 3e). Therefore, this band must be due to the terminal carboxyl group in either Asp or Glu. The fact that the change is associated with Asp has been shown by shifts to 1721 (H_2O) and 1711 cm^{-1} (D_2O) in the M/LA spectra of BR incorporated with [4-^{13}C]aspartic acid (Engelhard et al., 1985; Dollinger et al., submitted for publication).

Our findings described above should be useful in defining requirements for any proton pumping scheme and perhaps shed some light on events responsible for the transition to the dark-adpated species. These studies also demonstrate the usefulness of FTIR difference spectroscopy for the detection of apoprotein changes. The small absorbance changes we have assigned on the basis of model compound studies correspond to approximately one residue for each tyrosine vibration.

Registry No. I, 30811-19-9; II, 62595-14-6; L-Tyr, 60-18-4; D_2, 7782-39-0.

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Reversible Self-Association of Bovine Growth Hormone during Equilibrium Unfolding

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ABSTRACT: Previous investigations have shown that bovine growth hormone (bGH, somatotropin) unfolds through a reversible multistate process with at least one stable equilibrium intermediate. In extending our knowledge of the folding process for bGH, we demonstrate that a self-associated form of partially denatured bGH is formed during equilibrium unfolding experiments. The self-associated species has been identified by hydrodynamic measurements (size exclusion high-performance liquid chromatography and static and dynamic light scattering) and by measurements of the bGH concentration dependence of aromatic amino acid spectral properties (fluorescence, second-derivative absorption, and circular dichroism). The apparent maximum concentration for self-association occurs when bGH is partially denatured, i.e., at 3.7 M guanidine hydrochloride or 8.5 M urea, and its formation is reversible. Some of the properties of the self-associated species include (a) quenched tryptophan fluorescence, (b) increased tryptophan circular dichroism intensity at 300 nm, (c) polar tryptophan environment, and (d) a weight-average radius of about 5 nm. The self-association of bGH is mediated by specific intermolecular interactions with little increase in molecular size occurring above the saturation level of 4 mg/mL bGH. These phenomena have important implications for the design and interpretation of folding experiments in vitro and may have physiological consequences.

One approach to studying the forces that stabilize the three-dimensional structure of native proteins is to use physicochemical methods to characterize the structural rearrangements of a protein as it folds or unfolds. We (Brems et al., 1985) and others (Holladay et al., 1974) have reported previously that bovine growth hormone (bGH, somatotropin)[1] unfolds in guanidine hydrochloride (Gdn-HCl) through a reversible multistate process with stable equilibrium intermediates. Interpretations of spectral data from acid-induced unfolding of bGH have also invoked equilibrium intermediates (Burger et al., 1966). The structural characteristics of these intermediates, however, have not been addressed and should provide insights into the relative stability of structural elements in native bGH and into the folding mechanism for bGH. In addition, it is important to establish whether the unfolding process is the same in urea (a nonionic chemical denaturant), in Gdn-HCl (an ionic chemical denaturant), and in acid.

In order to study the structural properties of intermediates which occur during bGH unfolding, we have used spectroscopic probes as indicators of the different chemical species which are present at different concentrations of denaturant. While conducting these studies, we became aware of a dependence on bGH concentration for the circular dichroism (CD) and fluorescence intensities under partially denaturing conditions. A detailed investigation of the causes of these phenomena has been undertaken by using optical spectroscopy and hydrodynamic measurements.

Applications of dynamic light scattering (photon correlation spectroscopy, PCS) to the study of protein denaturation have been reported previously (Rimai et al., 1970; Dubin et al., 1973; Nicoli & Benedek, 1976; Wang et al., 1980; Nystrom & Roots, 1982) and have provided measurements of the increase in molecular size upon denaturation. The experimental results for bGH provide these data and, in addition, indicate that the protein self-associates reversibly when partially denatured through specific protein–protein interactions. Our results emphasize the utility of monitoring molecular size in equilibrium denaturation studies and provide the impetus to determine the role played by the self-associated intermediate or intermediates in the kinetic pathway of folding. A partial characterization of the associated intermediate is discussed in the following paper in this issue (Brems et al., 1986).

Experimental Procedures

Materials

Pituitary-derived bGH was obtained from A. F. Parlow (UCLA, Los Angeles, CA); ultrapure Gdn-HCl and ultrapure urea were from Schwarz/Mann (Cleveland, OH). All other materials were analytical grade.

Methods

Buffer Conditions. All solutions were prepared in 50 mM ammonium bicarbonate buffer (pH 8.5). Denaturants were

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[1] Abbreviations: bGH, bovine growth hormone; Gdn-HCl, guanidine hydrochloride; HPLC, high-performance liquid chromatography; PCS, photon correlation spectroscopy; R_θ, Rayleigh ratio.

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