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Tryptophan Perturbation in the L Intermediate of Bacteriorhodopsin: Fourier Transform Infrared Analysis with Indole-^{15}N Shift†

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ABSTRACT: In the photoreaction of bacteriorhodopsin, the L intermediate shows an intense band at 3486 cm^{-1} which is unaffected by ^2H_2O (Maeda, A., Sasaki, J., Shichida, Y., & Yoshizawa, T. (1992) *Biochemistry 31*, 462–467]. This band is shifted to 3477 cm^{-1} by [*indole*-^{15}N]tryptophan substitution and therefore is assigned to the N–H stretching vibration of the indole of tryptophan. Free indole in carbon tetrachloride shows its N–H stretching vibration at 3491 cm^{-1} [Fuson, N., Josien, M.-L., Powell, R. L., & Utterback, E. (1952) *J. Chem. Phys. 20*, 145–152]. Thus, it is suggested that at least one tryptophan residue in the L intermediate is not hydrogen bonded.

Bacteriorhodopsin in *Halobacterium halobium* is a protein which transports protons across the cell membrane in a cyclic photochemical reaction initiated by the absorption of light by its *all-trans*-retinylidene chromophore (Stoeckenius et al., 1979). The photocycle of the light-adapted form of bacteriorhodopsin (BR)[1] is composed of a series of intermediates, J, K, KL, L, M, N, and O (Mathies et al., 1991). Among them L is the first intermediate which shows significant perturbations in the carboxylic acid residues (Engelhard et al., 1985), leading to the release of a proton to the external medium.

The formation of L is essential for the transport function. The 13-cis form of bacteriorhodopsin, which is inactive for the proton pumping, does not yield an L-like intermediate (Iwasa et al., 1981). The formation of the L-like intermediate in the photocycle of halorhodopsin, a light-driven chloride pump in the same microorganism, is dependent on chloride (Ogurusu et al., 1982) and is correlated with anion-transporting activity (Lanyi et al., 1990).

Maeda et al. (1992a,b) first analyzed the O–H and N–H stretching vibrational modes for the difference FTIR spectrum of L to BR. Some of these were assigned to the O–H stretching mode of water, on the basis of the $H_2{}^{18}O$ shift, and others to the O–H stretching modes of the carboxy groups in Asp^{96} and Asp^{115}, by intensity decrease in mutant bacteriorhodopsin. A sharp band at 3486 cm^{-1}, however, could not be assigned in either fashion. It was therefore thought to be the N–H stretching vibration of the peptide backbone or a tryptophan side chain.

The indole nitrogen of tryptophan is frequently involved in H-bonding in proteins (Ippolito et al., 1990) and in some cases takes part in substrate binding, for example, in lysozyme (Phillips, 1967), and maltodextrin receptor (Spurlino et al., 1992). In bacteriorhodopsin, several tryptophan residues surround the chromophore (Henderson et al., 1990), and the mutation of some of these residues drastically affects the L-to-M conversion (Wu et al., 1992) and proton pumping (Mogi et al., 1989).

In the present paper, we examined the possibility that the 3486-cm^{-1} band is the N–H stretching mode of an indole

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[1] Abbreviation: BR, *all-trans*-bacteriorhodopsin.

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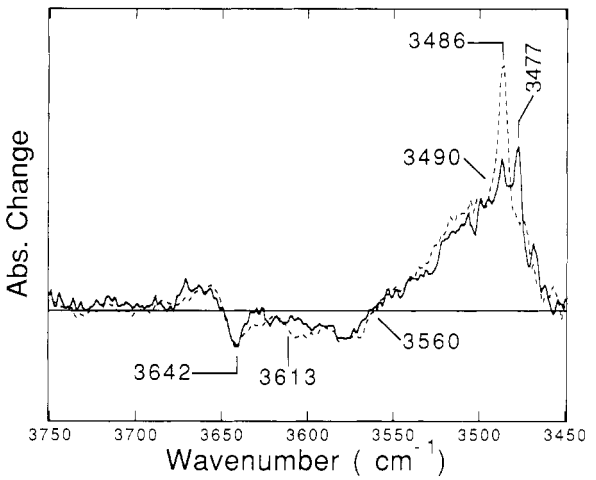

FIGURE 1: Comparison of the L/BR difference spectrum in the 3750–3450-cm^{-1} region of [indole-^{15}N]tryptophan-labeled bacteriorhodopsin (solid line) with that of unlabeled bacteriorhodopsin (dotted line). The full scale of the vertical axis is 0.0051 for the spectrum of the solid line and 0.0068 for the spectrum of the dotted line. The horizontal line is the base line.

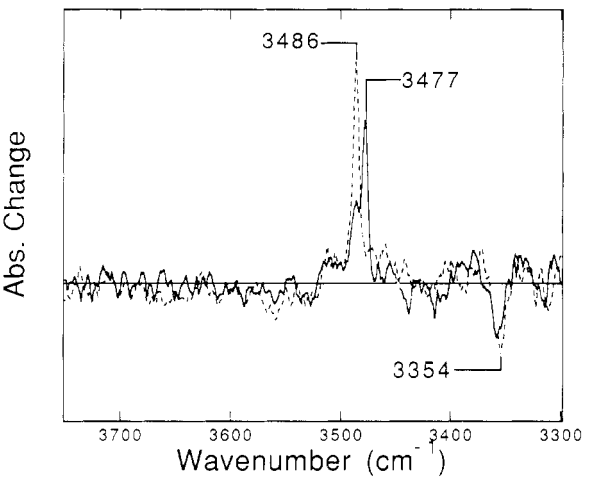

FIGURE 2: Comparison of the L/BR difference spectrum in the 3750–3300-cm^{-1} region of [indole-^{15}N]tryptophan-labeled bacteriorhodopsin ^2H_2O (solid line) with that of unlabeled bacteriorhodopsin ^2H_2O (dotted line). The full scale of the vertical axis is 0.0028 for the spectrum of the solid line and 0.0051 for the spectrum of the dotted line. The horizontal line is the base line.

group. This is demonstrated here by use of [*indole*-^{15}N]-tryptophan-labeled bacteriorhodopsin.

MATERIALS AND METHODS

[*indole*-^{15}N]Tryptophan-labeled bacteriorhodopsin was prepared by growing *H. halobium* (JW-3) in a defined medium similar to that of Gochnauer and Kushner (1969), except that the D-amino acids and NH_4Cl were omitted and 0.1 g/L [^{15}N]-anthranilic acid was added. The purple membrane was isolated by the method of Oesterhelt and Stoeckenius (1974). The distribution of [U-^{14}C]anthranilic acid similarly incorporated into BR was examined by lipid extraction and amino acid analysis. Ninety-seven percent of the radioactivity in the purple membranes was found in the protein, with negligible amounts in amino acids other than tryptophans, and the specific radioactivity indicated that tryptophan were 60% labeled.

The sample manipulation for low-temperature spectroscopy and the procedures for recording difference FTIR spectra were described previously (Maeda et al., 1991, 1992a).

RESULTS

The difference FTIR spectrum of L and BR was calculated from spectra before and after 1-min irradiation of BR at 170 K with >600-nm light. Figure 1 shows L/BR spectra in the 3750–3450-cm^{-1} region for [*indole*-^{15}N]tryptophan-labeled bacteriorhodopsin (solid line) and unlabeled bacteriorhodopsin (dotted line). Intensities were adjusted in the 1800–800-cm^{-1} region where both spectra are identical in shape. With indole-^{15}N substitution about 70% of the 3486-cm^{-1} band disappears, and a new band appears at 3477 cm^{-1}. The 3486-cm^{-1} band is thus assigned to the N–H stretching vibration of the indole moiety of the tryptophan residue. The 9-cm^{-1} shift is nearly coincident with the calculated shift on the basis of the mass effect for the N–H stretching vibration. The 70% substitution by [*indole*-^{15}N]tryptophan is also consistent with the 60% labeling estimated radiochemically (see Materials and Methods).

In contrast to L on the positive side, no correspondingly shifted band is observed on the negative side for BR in this frequency region. All other bands were assigned previously (Maeda et al., 1992a,b). A negative band at 3642 cm^{-1} is due to water. A wide negative band between 3613 and 3560 cm^{-1} and a positive band between 3560 and 3490 cm^{-1}, both of which disappear upon ^2H_2O exchange (see below), are composed of the O–H stretching vibrational bands of water, Asp^{96}, and Asp^{115} (Maeda et al., 1992a,b).

The spectral region below 3450 cm^{-1} is not presented in Figure 1 because of excessive noise due to the strong absorbance of water. Replacement of ^2H_2O removes this strong absorbance of the water and makes it possible to record the spectrum from the 3450- to 3300-cm^{-1} region, although the stretching modes of exchangeable O–H and N–H groups also disappear from these regions. Figure 2 shows the L/BR spectra in the 3750–3300-cm^{-1} region of [*indole*-^{15}N]tryptophan-labeled bacteriorhodopsin (solid line) and unlabeled bacteriorhodopsin (dotted line), both of which are humidified with ^2H_2O. Except for the sharp positive band at 3486 cm^{-1} (dotted line) or 3477 cm^{-1} (solid line), all the positive and negative bands in the 3750–3450-cm^{-1} region disappear. The fact that the 3486-cm^{-1} band is insensitive to ^2H_2O indicates that its location is inaccessible to water. The 3486-cm^{-1} band shows a shift by 9 cm^{-1} upon substitution with [*indole*-^{15}N]tryptophan as in H_2O and can be assigned to the indole N–H of tryptophan. In the region between 3450 and 3300 cm^{-1}, only a negative band at 3354 cm^{-1} can be seen. It does not shift upon substitution with [*indole*-^{15}N]tryptophan and is also insensitive to ^2H_2O. It is probably due to another stretching vibration of N–H, which is located at a site inaccessible to water. No other negative band with comparable intensity was noticed.

No band corresponding to the 3486-cm^{-1} band in the L/BR spectrum was found in the M/BR and N/BR spectra above 3450 cm^{-1} (Maeda et al., 1992a). To see whether the corresponding band is present in the 3450–3300-cm^{-1} region, the M/BR and N/BR spectra for both unlabeled and [*indole*-^{15}N]tryptophan-labeled bacteriorhodopsin were recorded for the sample in ^2H_2O (not shown). These spectra do not show any bands except for a negative band at 3354 cm^{-1}. Thus, the perturbation of the indole N–H of tryptophan was found in L, but not in M and N. Changes occur in parallel with the perturbations of water and Asp^{96} (Maeda et al., 1992a,b). In contrast, the unidentified negative band at 3354 cm^{-1} persists through L, M, and N.

No ^{15}N shifts were detected between 1800 and 800 cm^{-1} in the L/BR, M/BR, and N/BR spectra of native bacteriorhodopsin and in the M_N/BR spectrum of Asp^{96} → Asn

bacteriorhodopsin at pH 10 (Sasaki et al., 1992).

DISCUSSION

The frequency region above 3000 cm^{-1} exhibits the vibrational bands of O–H and N–H stretching modes. In the 3750–3450-cm^{-1} region of the L/BR spectrum of bacteriorhodopsin, Maeda et al. (1992a,b) showed the perturbation of vibrational bands, some of which were identified as the O–H stretching vibration of water by the replacement of H_2O with ^2H_2O or $H_2{}^{18}O$, and of carboxyl groups by substituting asparagine for Asp^{96} or Asp^{115}. The 3486-cm^{-1} band, however, was not affected at all by these changes. In the present experiments, we used [*indole*-^{15}N]tryptophan-labeled bacteriorhodopsin to show that this vibrational mode is due to the N–H stretching mode of an indole moiety of the tryptophan residue.

Fuson et al. (1952) have shown a sharp band at 3491 cm^{-1} with a molar extinction of 140 for a dilute solution of indole in carbon tetrachloride. This band was assigned to the stretching mode of the indole N–H which is free from H-bonding. With increasing concentration of indole in the same solvent, the intensity of this band decreased gradually and an additional band appeared at a frequency about 80 cm^{-1} lower, with greater bandwidth and molar extinction. These results indicate that the N–H bond is affected by the H-bonding involved in the formation of the indole dimer. The frequency value of 3486 cm^{-1} of L is very close to that of dilute indole in carbon tetrachloride, 3491 cm^{-1}. This suggests that the indole N–H of at least one tryptophan residue in L is free from H-bonding. Furthermore, the fact that the 3486-cm^{-1} band is unaffected by ^2H_2O substitution indicates that this tryptophan residue is located in a region of the protein inaccessible to water.

As stated above, such a band can be seen only for L. If the same tryptophan is H-bonding in BR, the corresponding vibrational band must be found in the frequency region above 3300 cm^{-1}. We searched for a negative BR band which is insensitive to ^2H_2O exchange in the region above 3300 cm^{-1}, where the bands with comparable intensities will be found above the noise level, if present. No additional bands are found, however.

Two possibile reasons for this can be considered. One is that increase in the molar extinction is induced upon formation of L. Increase in the infrared intensity could be due to greater polarization of the indole N–H bond of tryptophan. The molar extinction of about 400 of this band in L is three times greater than the value of a single tryptophan in carbon tetrachloride. UV spectral changes due to the perturbation of tryptophan residues in L have been reported (Kuschmitz & Hess, 1982). Contrarily, no corresponding infrared intensity changes were observed for M and N in spite of a UV change of tryptophan in these intermediates (Wu et al., 1991).

A change in the indole N–H orientation is the other preferential explanation for the absence of the corresponding mode in BR and the greater intensity of the band in L because the purple membrane may be oriented in the dried film (Clark et al., 1980). Measurement of the dichoric ratio upon photoreaction will clarify this point.

Bacteriorhodopsin has eight tryptophans. Among them Trp^{182}, which is located on the cytoplasmic side and is surrounded by the hydrophobic residues, is the most likely to have the indole N–H in a water-inaccessible site. Also, Trp^{182} is the only residue responsible for the UV change in the photocycle (Wu et al., 1991). Mutation of Trp^{182} decreases the rate of the L-to-M reaction, suggesting its importance for the instability of L (Wu et al., 1992). Mutation of Asp^{96} or Asp^{115}, which is close to Trp^{182} causes 1–2-cm^{-1} shifts of the 3486-cm^{-1} band. We suggest that the tryptophan residue which is responsible for the vibrational mode at 3486 cm^{-1} is Trp^{182}, though a systematic study of the tryptophan mutants is required.

The results presented in this paper strongly suggest that one or more indole groups are not involved in H-bonding in L. This is a characteristic feature for L but not for M or N. This is correlated with the change in either polarization or orientation of the indole N–H of the tryptophan residue. These should be determined in future experiments.

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