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O-H stretching vibration in Fourier transform difference infrared spectra of bacteriorhodopsin

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FTIR difference spectroscopic studies of M intermediate and LA bacteriorhodopsin in the O-H stretching region show bands at 3671 and 3641 cm^{-1}, respectively. The O-H stretching bands in this region may reflect protonation–deprotonation changes or environmental change in the tyrosine residues in bR.

Bacteriorhodopsin; M intermediate; Fourier transform infrared spectroscopy; O-H stretching vibration

1. INTRODUCTION

Bacteriorhodopsin (bR), a retinal protein in the purple membrane of *Halobacterium halobium*, is known to function as a light-driven proton pump across the plasma membrane [1]. In the photocycle, a series of intermediates (Fig. 1) has been detected and characterized by spectroscopic studies [2].

Infrared studies of the mechanism of proton pumping have revealed a great wealth of information about the changes in the amino acid residues that occur in the photocycle of bR [3–10], but so far no changes in the O-H stretching region have been associated with the transfer of proton. We report here changes in the infrared bands observed in the difference spectra between the M and LA intermediates, which are assignable to O-H stretching vibration of amino acid residues in the protein.

2. MATERIALS AND METHODS

Purple membranes were isolated from *H. halobium*, strain S9, following the procedure of Oesterhelt and Stoeckenius [11]. The apomembrane obtained by a previously published method [12] was suspended in 100 mM sodium phosphate buffer, pH 6.0. The concentration of apobR in suspension was determined by titration with all-*trans*-retinal to be 290 μM. The all-*trans* isomers of 10-, 12-, and 14-fluororetinals were generous gifts of professor Liu, University of Hawaii [13]. The analog bRs (10-fluoro-, 12-fluoro-, and 14-fluoro-bR) were regenerated by the addition of each fluororetinal (1.1 equivalent) in ethanol solution to 500 μl of apomembrane suspension. The sample pellet was made by depositing 15 μl of bR ($O.D._{560\ nm}$ = 1) on a ZnS window of a gas-tight sample cell [14]. Allowing the film to dry slowly for 8 h in a film canister provided the most transparent pellet for FTIR studies. The sample was then hydrated by equilibrating for 1 h with a drop of water at 37°C in the canister. The gas-tight sample cell was placed inside a cryostat (model CF 1204, Oxford Instruments, England) which was mounted in the sample compartment of a Nicolet 1799 series FTIR spectrophotometer (Nicolet Instrument Co., Madison WI). The sample was irradiated with a 250 W optical fiber light source (Unisoku, Japan) through a ZnS window. Light of 530 ± 10 nm was used for creating LA at 273K, 530 ± 10 nm for K at 70K, 590 ± 10 nm for L at 170K, ⩾500 nm for M at 220K. A final photoequilibrium mixture resulted after 3 min of irradiation.

After irradiation to create LA, the bR sample was cooled to the temperature necessary for each intermediate, two successive 256 scans were collected. LA spectra were obtained by calculating the ratio to background which was collected for the empty sample holder in the cryostat at each temperature. Scans were repeated until two successive spectra were virtually identical. After irradiation of LA at respective temperature, the K, L, and M spectra were obtained by the same process. Difference spectra in 2 cm^{-1} resolution were obtained by subtraction of the LA spectra from other spectra.

Infrared spectra of gaseous acetic acid and phenol in CCl_4 (1 mg in 3 ml) were measured in a gas cell (10 cm path length with a ZnS window) and in a solution cell (0.1 mm path length with CaF windows), respectively.

3. RESULTS AND DISCUSSION

In our FTIR difference studies of bR containing various fluorinated retinal analogues [15], we noticed bands at 3671 cm^{-1} and 3641 cm^{-1} for M and LA, respectively (Fig. 2). Upon careful checking of the difference spectra for the same intermediates in native bR, we confirmed the presence of the same bands, however the intensity of those bands in native bR is weaker than in fluorinated analogues. The vibrations of these bands

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Abbreviations: FTIR, Fourier transform infrared; bR, bacteriorhodopsin; LA, light adapted

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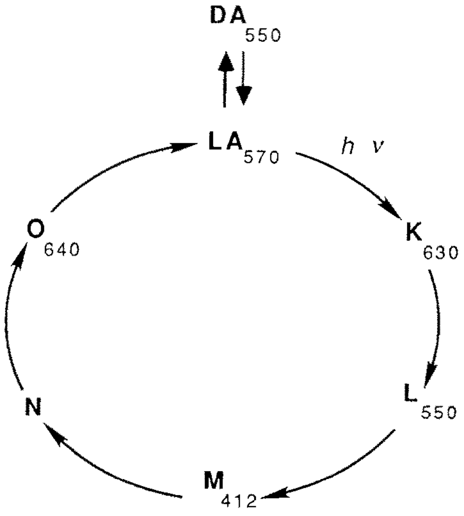

Fig. 1. Schematic diagram of bacteriorhodopsin photocycle [1].

are not sensitive to the locations of the fluoro-substituent. This suggests that the amino acid residue of concern is not hydrogen-bonded to any of the fluorine atoms and that such a perturbation is considered possible for the unusual photochemical properties of 10-fluoro rhodopsin [16]. No FTIR difference spectral studies of the retinal proteins have been reported in this region presumably due to the strong inherent absorption in the O-H and N-H stretching region of the purple membrane and water, which in turn leads to poor signal to noise ratios in the difference spectra. These difference bands are absent in the K/LA and L/LA difference spectra. The bands at 3671 cm^{-1} and 3641 cm^{-1} shifted to 2716 cm^{-1} and 2685 cm^{-1}, respectively, in D_2O. This indicates that these bands are associated with acidic side chains in bR which undergo changes between the L and M intermediates.

The high frequencies of these vibrations rule out the possibility that they are associated with the lipid moieties, since hydroxyl groups in the membrane would necessarily be hydrogen-bonded, thus, they are associated with hydroxyl groups of the bR protein that are in hydrophobic environments. Furthermore, the fact that the 3671 cm^{-1} band is associated with the M intermediate and the 3641 cm^{-1} band is associated with the LA bacteriorhodopsin indicates that the O-H group in the M intermediate is in a more hydrophobic environment.

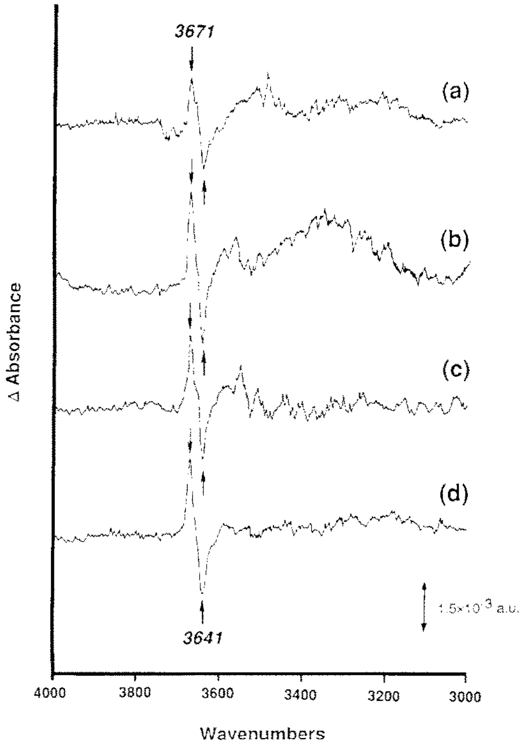

Fig. 2. Difference FTIR spectra of bR analogs containing fluorinated retinals and native bR between 4000 cm^{-1} and 3000 cm^{-1}. Positive and negative absorption bands are due to absorption of M and LA intermediates, respectively. (a) native bR, (b) 10-fluoro-bR, (c) 12-fluoro-bR, and (d) 14-fluoro-bR.

The carboxylic O-H and phenolic O-H groups of aspartic acid [3–6] and tyrosine [7–9] residues in bR have been shown to undergo changes in the bR photocycle. The protonation–deprotonation and environmental changes in aspartic acid residues have been discussed on the basis of changes in the C = O stretching bands which shift upon ^{13}C-labelling and also upon H_2O/D_2O exchange. Recently the protonation of Asp-85 and the deprotonation of Asp-96 have been reported to coincide with the Schiff base deprotonation and reprotonation, respectively [6]. The O-H (O-D) stretching vibration of the carboxylic acid moiety was observed at 3596 cm^{-1} (2648 cm^{-1}) in gaseous acetic acid; on the other hand, the O-H and O-D stretching vibrations of phenol were observed (highly diluted in CCl_4) at 3624 cm^{-1} and 2672 cm^{-1}, respectively. The evidence offered for changes in tyrosine residues were based on studies of isotopic shifts in weak difference FTIR absorptions in C = C and C-O stretching regions. Since tyrosine residues are found in very hydrophobic regions in the transmembrane α-helices that form the binding pocket of the retinal chromophore [17], it is likely that the bands we have observed are due to changes in the tyrosine residues between the M intermediate and LA bacteriorhodopsin rather than changes in the environment of aspartic acid residues. Although protonation–deprotonation changes in tyrosine residues have been reported between K/LA and L/LA, these changes could not be detected in the present FTIR difference studies in the region of O-H stretching.

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