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Polarized FTIR Spectroscopy in Conjunction with In Situ H/D Exchange Reveals the Orientation of Protein Internal Carboxylic Acids

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The seven α-helical membrane protein bacteriorhodopsin (bR) is a light-driven proton pump in *Halobacterium salinarum* with the chromophore retinal bound via a protonated Schiff base (PSB) to a lysine (Lys216) inside the protein.[1] The absorption of a photon initiates a cyclic reaction involving different intermediates (ground state (BR) → J ↔ K ↔ L ↔ M ↔ N ↔ O → BR). The first proton transfer takes place in the L → M transition from the PSB to the nearby counterion D85, roughly concomitant with a proton release to the extracellular medium. There are, in total, 18 carboxylic amino acids within bR, but only two are protonated in the ground state, namely, D115 and D96. The latter functions as the proton donor for the Schiff base in the M → N transition,[2] while the role of the former is not yet clear.

Most of what is known about the protonation state of bacteriorhodopsin's carboxylic acids and their protonation changes during the photocycle has been deduced from reaction-induced FTIR difference spectroscopy (for summary, see ref 3). By the enhanced use of a polarized FTIR beam, this technique has become a useful tool to determine the orientational change of specific groups within proteins.[4] To apply this technique, two requirements are necessary: (*a*) at least two different protein states have to be available; and (*b*) the investigated group has to undergo a significant change so that its absorbance bands become clearly distinguishable within the difference spectrum. Such a change can be the protonation of a carboxylate (R−COO$^-$ → R−COOH). The absorbance of the appearing carbonyl group (C═O) is characterized by a unique band between 1780 and 1700 cm^{-1} and therefore fulfils requirement *b*. In the case of bR, the orientation of the transition dipole moment of the C═O stretch of protonated D85 at 1762 cm^{-1} with respect to the membrane normal was measured at $\theta = 43 \pm 4°$,[4] $\theta = 35 \pm 5°$,[5] and $\theta = 36 \pm 1°$.[6] These results were deduced from measurements of the dichroic ratios (cf. below) by using M-BR difference spectra. In this case, requirement *a* was fulfilled by the use of the photocycle.

However, what do we do if both requirements are not fulfilled? In the following, we will show how to determine the orientation of a carboxylic acid which does not undergo protonation change during the photocycle, such as D115 in bR, exclusively based on the unphotolyzed protein. This was carried out by a combination of polarized FTIR difference spectroscopy, in situ H/D exchange measurements, and site-directed mutagenesis. In situ H/D exchange measurements were performed in a similar way to the process described previously.[7] Here the IR beam was polarized, and the oriented bR film was tilted with respect to the beam at $\alpha = 45°$ around a horizontal axis. The polarizer was set either in a vertical position (v), where the incident electric vector of the beam meets the sample plane with an angle of 45°, or in a horizontal position (h), where the vector is parallel.[8]

Figure 1 shows the difference spectra between bR (mutant D115N)[9] in H_2O and D_2O (H−D spectrum) in the spectral region from 1780 to 1700 cm^{-1} measured with vertical (E_v, blue line) and horizontal (E_h, red line) polarized beams.

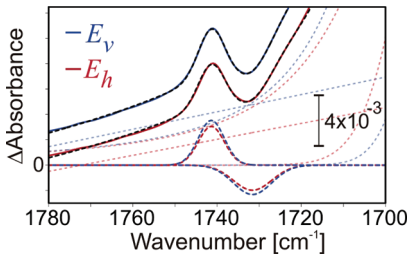

Figure 1. Polarized in situ H/D exchange measurement of the carboxylic region of D115N. The measured spectra (blue and red line), the fitted curves (dotted black lines), the individual Gaussian components of the shifted C═O stretch of D96 (thick dotted lines), and the components of the background absorbance due to the solvent, amide I, and the continuum absorbance[7] (thin dotted lines) are shown.

The observed difference band is caused by the shift of the carbonyl C═O stretch of protonated D96 (hereafter referred to as $D96_H$) due to deuteration with D_2O ($D96_D$). The positive Gaussian functions (thick dotted lines) in the deconvoluted spectrum at $1741^{(+)}$ cm^{-1} correspond to $D96_H$ and the red shifted negative functions at $1731^{(-)}$ cm^{-1} to $D96_D$. The angle θ between the C═O transition and the membrane normal can be calculated from the dichroic ratio $R = e_v/e_h$ at constant $\alpha = 45°$ by

$$\theta = \arccos\sqrt{\frac{1}{3} + \frac{4n^2(R-1)}{3+2n^2(R-1)} \times \frac{1}{3p}}$$

where $n = 1.7 \pm 0.1$[10] is the refractive index of the bR sample, $p = 0.95 \pm 0.05$[8] the mosaic spread order parameter, and e_v and e_h are the intensities of the Gaussian components. It was assumed that the relative error of the determined absorbance amplitudes e_v and e_h due to spectral noise and the deconvolution procedure was ≤5% (the upper limit was chosen). The angles θ of $D96_H$ (θ ($D96_H$)) and θ ($D96_D$) are calculated to be 45 ± 4° (Table 1).

Figure 2a shows the carbonyl region of the polarized in situ H/D difference spectra of mutant D96N in the ground state. The observed difference band at $1736^{(+)}/1725^{(-)}$ is caused by the $D115_H$ to $D115_D$ shift. In Figure 2b, the spectra are shown for the protein trapped in the M intermediate. Almost 100% of the M intermediate can be accumulated in D96N by continuous illumination of the sample at low temperature.[11] The difference band of D115 is slightly shifted to a higher wavenumber ($1737^{(+)}/1726^{(-)}$). The difference band at $1762^{(+)}/1750^{(-)}$ is due to protonated D85. The measured value of $\theta = 36 \pm 3°$ (Table 1) closely corresponds to earlier measurements based on reaction-induced polarized FTIR difference spectroscopy (cf. above).

In the following, we compare our measured θ angle values with the directions of the CO bonds obtained from X-ray structure analysis (Table 1).

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