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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 ($R-COO^- \rightarrow R-COOH$). 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.

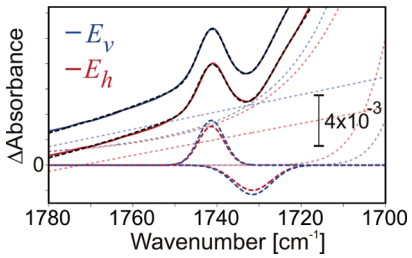

Figure 1. Polarized in situ H/D exchange measurement of the carboxylic region of D115N. The measured spectra (blue and red line), the fitted curves (dotted black lines), the individual Gaussian components of the shifted C═O stretch of D96 (thick dotted lines), and the components of the background absorbance 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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Table 1. Observed θ Angles (in degrees) between the Transition Moment of the C═O Carbonyl Group[a] and the Membrane Normal Compared to the C−$O_{\delta1}$ and C−$O_{\delta2}$ Angles (θ $O_{\delta1}$, θ $O_{\delta2}$) with Respect to the *z*-axis[b] Taken from X-ray Structure Analysis

	D96 (BR)		D115 (BR)		D115 (M)		D85 (M)	
	H	D	H	D	H	D	H	D
e_v[c]	3.5	−2.3	3.6	−2.9	2.6	−2.5	2.5	−5.1
e_h[c]	3.1	−2.0	4.0	−3.3	3.1	−2.9	1.7	−3.5
θ[d]	45 ± 4	45 ± 4	65 ± 11	71 ± 12	80 ± 12	74 ± 12	36 ± 3	36 ± 3
	(1C3W)[12]		(1C8R)[11]		(1C8S)[11]		(1C8S)[11]	
θ $O_{\delta1}$	51		39		26		**31**	
θ $O_{\delta2}$	**43**		**72**		**61**		85	

[a] The proposed C═O group is highlighted in bold. [b] The *z*-axis can be assumed to be equivalent to the membrane normal. [c] Amplitude of the fitted Gaussian component (Δabsorbance × 10^3). [d] Errors calculated via Monte Carlo ($\delta n = 0.1$, $\delta p = 0.05$, $\delta e_v = 5\%$, $\delta e_h = 5\%$).

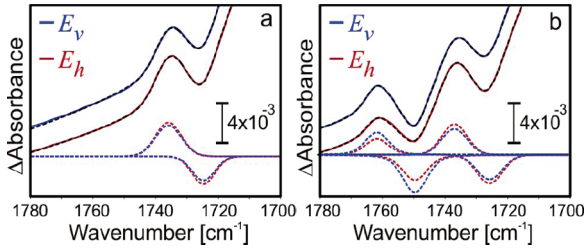

Figure 2. Polarized in situ H/D exchange measurement of D96N in BR (a) and M (b).

The orientation of the transition dipole moment of a carboxyl C═O stretch was found to be almost parallel to its bond direction (deviation < 4°),[13] so a direct comparison can be made. θ (D115) and θ (D85) closely correspond to θ $O_{\delta2}$ and θ $O_{\delta1}$, respectively. Due to the identification of the C═O bond, it is possible to determine the protonated oxygen of the carboxylic acids. Therefore, it can be concluded that $O_{\delta1}$ of D115 is protonated in BR and M and $O_{\delta2}$ of D85 is protonated in M. The latter assignment corresponds to results deduced from earlier reaction-induced polarized FTIR measurements by Kelemen and Ormos,[13,14] while the former stands in contradiction to these. Their assignment of the protonated oxygen of D115 in M was based on the investigation of the bands caused by the environmental changes of D115. In our opinion, their deconvolution of the corresponding spectral region was not unique (and thus does not fulfill requirement *b*; cf. above), and therefore, their assignments were ambiguous.

The measured θ (D96) = 45 ± 4° closely corresponds to the X-ray θ $O_{\delta2}$ = 43°. However, because the difference with θ $O_{\delta1}$ = 51° is not significant, a unique assignment of the protonated oxygen of D96 is not possible, but it is more likely $O_{\delta1}$.

It has been proposed that deprotonation of D115 at a high transmembrane pH gradient inhibits the proton pump efficiency to prevent over-acidification of the external medium.[15] This effect, known as the back-pressure effect, is probably due to an interaction of D115 and the Schiff base region, especially D85 (illustrated in Figure 3). If $O_{\delta2}$ were protonated, a deprotonation of this group would influence its vicinity merely due to electrostatic interaction. This is because $O_{\delta2}$ is not hydrogen-bonded to any further group.[16] However, as shown above, $O_{\delta1}$ binds the proton. This group is H-bonded to W511 and T90. The deprotonation of D115 converts $O_{\delta1}$ from an possible H-bond donor to an exclusive and strong (due to its negative charge) H-bond acceptor, which could force the breaking of the intrahelical H-bond between the side chain of T90 and the backbone of W86 due to a reorientation of the proton of T90 toward D115. This H-bond breaking and the associated strengthening of the other intrahelical H-bond between the backbone groups of W86 and T90 could induce conformational changes within

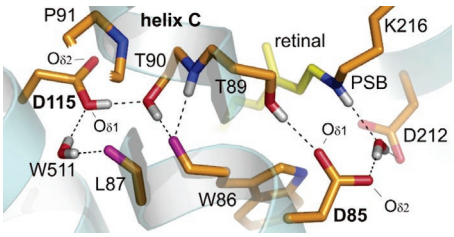

Figure 3. Structure of bR showing an possible interaction path between D115 and the Schiff base region over a distance of 10 Å. Backbone oxygens are shown in purple.

helix C, which would lead to reorientation of the side chains of T89, W86, and D85. Helix C is weakened at the position where D115 is connected, due to the loss of the interhelical backbone H-bond of L87, which causes (or is caused) by a kink in helix C at this level. The distortion of the ideal α-helix definitely benefits from a proline at position 91 but does not originate from this residuum (the kink is still present in P91A[17]). The proposed connecting link by T90 between D115 and the Schiff base region has been proven to play an important role during proton pumping by showing that the pump efficiency is reduced to 10% in the T90A mutant compared to WT.[18] An ionic D115 could cause the same impact as a replacement of T90 by an alanine, namely, the aforementioned H-bond breaking between T90 and W86.

The finding that $O_{\delta1}$ is protonated in D115 highly supports the idea of a direct interplay between D115 and D85 and provides new knowledge for a detailed analysis of the phenomenon known as the back-pressure effect on bR.

We believe that the conjunction of polarized FTIR spectroscopy with in situ H/D exchange will prove to be extremely valuable for the understanding of the structure and function of other proteins because this method can be applied to any orientable protein and is not merely restricted to bR.

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