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Light-induced polarized Fourier transform infrared spectroscopy of bacteriorhodopsin - a study of the M_{412} intermediate by photoselection

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The orientation of the infrared transitions of bacteriorhodopsin (BR) and its M_{412} intermediate relative to the optical transition of the retinal in BR (568 nm) has been investigated by light-induced Fourier transform infrared difference spectroscopy using a photoselection technique on films of oriented purple membrane. Both the infrared beam and the visible excitation beam propagate along the normal to the film plane. The infrared beam is polarized parallel ($\Delta A_{\parallel}$) or perpendicular ($\Delta A_{\perp}$) to the polarization of the excitation beam. This geometry enables the determination of the angle θ between the projections on the film plane of a given infrared transition and of the retinal optical transition. The BR C=C and C_{14}–C_{15} retinal vibrations at 1527 cm^{-1} and 1200 cm^{-1}, respectively, as well as the M_{412} C=C vibration at 1566 cm^{-1} show a high polarization demonstrating that the θ angles are very small. The 1762 cm^{-1} C=O stretching vibration, assigned to an aspartic carboxyl group which becomes protonated in M_{412}, is not dichroic, indicating an average θ value of 45° for this transition. Together with the azimutal angles ϕ measured by infrared dichroism (Nabedryk, E. and Breton, J. (1986), FEBS Lett. 202, 356–360), this measurement reveals the relative angles between the 568-nm optical transition of the retinal and several infrared transitions in BR and M_{412}.

Introduction

In the purple membrane of the halophilic bacterium, *Halobacterium halobium*, bacteriorhodopsin (BR) transduces light energy into electrochemical energy by transferring protons across the membrane. Absorption of a photon by the retinal prosthetic group in BR triggers a cycle of photochemical reactions involving the formation of several transient intermediates in which both the conformation of the retinal moiety and the protonation state of a few amino acid residues are altered [1]. Although a wealth of structural information on this system is available (primary sequence [2], transmembrane organization of the α-helices [3–5], Schiff-base linkage of the retinal to lysine-216 residue [6], native conformation of the retinal [7]), detailed understanding at a molecular level of the light-driven proton pump mechanism is at present greatly impeded by the lack of a high-resolution three-dimensional model of BR.

Several spectroscopic techniques and more specifically resonance Raman and Fourier transform infrared (FTIR) spectroscopies, have been highly instrumental in assessing the nature of the alterations of the retinal itself and of the neighboring amino acids constituting its binding site during the photocycle of BR, and have led to various mechanistic schemes for the functioning of the proton pump [8–13]. In particular, light-induced FTIR difference spectroscopy has been extensively used to monitor the small changes in the vibrational modes of individual bonds in both the chromophore and the protein which occur upon photoconversion. By using an infrared polarized beam on a tilted film of oriented multilayers of the BR-containing purple membrane, we recently introduced an extension of the FTIR difference spectroscopy approach in order to measure the orientation of the vibrational transitions of the chromophore and protein groups involved in the photoreaction and to determine their possible changes of orientation after photoconversion [14]. This new technique was used to monitor the azimutal angle ϕ (angle between the transition dipole and the normal to the membrane plane) of

Abbreviations: BR, bacteriorhodopsin; FTIR, Fourier transform infrared.

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several vibrational transitions involved in the conversion of BR to M_{412}, an important intermediate in the photocycle. In particular, we could determine that the C=O stretching vibration at 1762 cm^{-1}, which has been assigned to a protonated side chain aspartic carboxyl group in M_{412} [11,12], is oriented at $\phi = 35 \pm 5°$ [14]. By using the same technique, this observation has been confirmed [15] and the orientation of the first stable intermediate (named K) at low temperature has also been measured (Ref. 15 and Nabedryk, E, and Breton, J., unpublished results). Although the determination of such tilt angles is important to provide a geometrical and structural basis for the mechanistic schemes already proposed for the proton pump, it is important to recognize that when two distinct transition moments are considered in the same BR molecule, the azimutal angle measured for each of them is insufficient to describe the relative orientation between the two transitions.

In this report, we describe a new photoselection FTIR difference spectroscopy approach which solves this problem. The polarization of a non-saturating actinic beam which propagates along the normal to the membrane plane is used to photoselect an anisotropic population of BR molecules and to convert it to the M_{412} intermediate. By analyzing the projections onto the plane of the purple membrane of the various vibrational transition moments, this technique allows the polar angle θ between the optical transition of the retinal and several of the vibrational transitions involved in the photoconversion of BR to M_{412} to be determined. Together with the corresponding azimutal angles, these polar angles lead to new three-dimensional information on the geometrical relationship between the retinal and those amino acid residues of its proteic cage which are involved in the proton pumping mechanism. A preliminary account of this work has been presented recently [16,17].

Experimental

FTIR spectra were recorded on a Nicolet 60SX spectrometer equipped with an MCT A detector and a KRS-5 polarizer (Eurolabo, France). The experimental conditions were similar to those used previously [14] with the exception that the purple membrane film was mounted perpendicular to the direction of propagation of the infrared beam. A Germanium mirror, inserted in the infrared beam, was tilted at 45° around a vertical axis to allow the actinic beam (cut-off point at 500 nm) to propagate also along the normal to the film of purple membrane. The actinic beam was polarized (Polaroid; HN 38) and the FTIR difference spectra $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ were obtained with the polarization of the infrared beam parallel and perpendicular to the polarization of the actinic beam, respectively. In the absence of energy transfer among the BR chromophores, these experimental conditions allow determination of the angle θ between the projections onto the membrane plane of (i) the optical BR transition and (ii) the vibrational transition detected by the FTIR measurement.

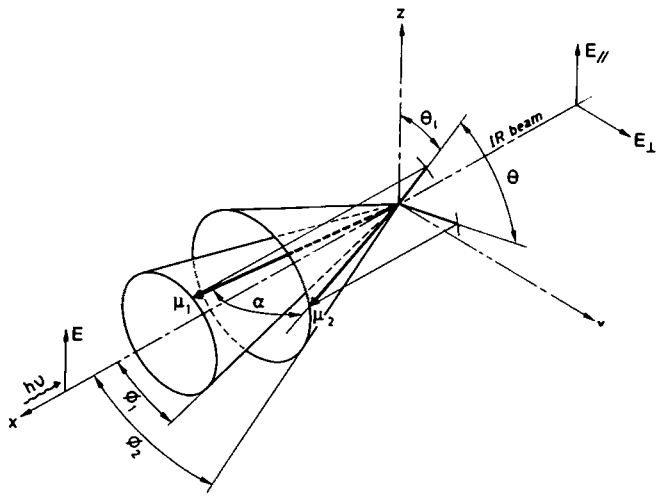

Fig. 1. Schematic representation of the photoselection FTIR measurement on membranes oriented parallel to the (Y, Z) plane. See the Experimental section in the text for a complete description.

In Fig. 1, the sheets of purple membrane are oriented parallel to the Y, Z plane and the actinic and probing beams are propagating along the X axis. The optical transition moment (μ_1) of the retinal is located on a cone forming an angle ϕ_1 with the X direction. Upon excitation of the retinal with light polarized along the Z axis the infrared dipole μ_2 is detected in the M_{412}–BR difference spectrum. Generally, this dipole would be at an angle ϕ_2 with respect to the X direction and will form an angle α with μ_1. In Fig. 1, only one set of cones of half-angle ϕ_1 and ϕ_2 is shown. There is a symmetrical set of cones on the other side of the Y, Z plane. These two sets, which correspond to the populations of top-up and bottom-up oriented membranes, lead to identical results in the photoselection experiment. The purpose of the photoselection measurement was to determine the projection (θ) of the α angle onto the (Y, Z) plane. If θ_1 is the angle between the Z axis and the projection of μ_1 on the Y, Z plane, the probability of excitation of μ_1 by the polarized actinic beam is proportional to $\cos^2 \theta_1$, while the probability of μ_2 absorbing in the measuring IR beam is proportional to $\cos^2(\theta_1 + \theta)$ and $\sin^2(\theta_1 + \theta)$ for polarization along the Z and Y axes, respectively [18,19]. Thus, the infrared absorption changes $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ along the Z and Y axes are:

$$\Delta A_{\parallel} = k \int_{\theta_1 = 0}^{\theta_1 = 2\pi} \cos^2\theta_1 \cos^2(\theta_1 + \theta)\, d\theta_1 = \tfrac{1}{4} k\pi\, (1 + 2\cos^2\theta)$$

$$\Delta A_{\perp} = k \int_{\theta_1 = 0}^{\theta_1 = 2\pi} \cos^2\theta_1 \sin^2(\theta_1 + \theta)\, d\theta_1 = \tfrac{1}{4} k\pi\, (3 - 2\cos^2\theta)$$

and the polarization $p = (\Delta A_{\parallel} - \Delta A_{\perp})/(\Delta A_{\parallel} + \Delta A_{\perp}) = 0.5 \cos 2\theta$. Thus p varies from $+0.5$ to -0.5 when θ varies from 0° to 90°. These values of p apply to ideal conditions of photoselection when the polarized actinic light excites a small subset of isotropic dipoles. However, photochemistry by polarized light can be a source of anisotropy [20]. When the rate of creation of the anisotropy is greater than the rate of relaxation to the isotropic state, then under steady-state actinic illumination, photoselection is no longer performed on an isotropic sample. Under such conditions, the measured p value (p_m) will decrease with increasing light intensity.

Air-dried films of purple membrane were prepared on CaF_2 windows. These films, assembled in a sealed microcell, were hydrated with water vapor. The water content, estimated as described in Ref. 21 was approx. 40%. At this level of humidity the mosaic spread of the purple membrane sheets was found to be negligible by controlling the linear dichroism of the amide I and II bands [4,14,22]. All measurements were performed at room temperature.

For each polarized spectrum recorded in the dark or in the light, 1024 interferograms at 4 cm^{-1} resolution were collected (accumulation time, 3 min). This dark-light sequence was repeated several times and spectra from the same series were averaged. However, the first illumination cycle was not taken into account, in order to eliminate absorbance changes occurring upon light adaptation of BR [13,14].

Results

The absorbance changes $\Delta A_{\parallel}$, $\Delta A_{\perp}$ and $\Delta A_{\parallel} - \Delta A_{\perp}$ for the M_{412}–BR difference spectra made under illumination conditions which were adjusted in order to achieve $\Delta A_{\parallel} = 2\ \Delta A_{\perp}$ at 1527 cm^{-1} are shown in Fig. 2. The calculated unpolarized spectrum (data not shown) is in good agreement with previously published M_{412}–BR difference spectra [11–15,21,23,24], thus demonstrating the absence of any contribution from dark-adapted BR. At the lowest actinic intensity which is still compatible with the measurement of $\Delta A_{\perp}$ for the disappearing 1527 cm^{-1} C=C vibration of the BR retinal in the M_{412}–BR spectrum, a p_m value of 0.35 ± 0.05 is observed. In view of the suboptimal conditions of the present photoselection experiment due to the imperfect polarization of the actinic beam, the loose parallelism of the infrared beam and the steady-state nature of the experiment, it is probable that the limiting p_m value is significantly higher. This is further demonstrated by low temperature photoselection measurements on K, in which the polarization value for the disappearing 1530 cm^{-1} C=C vibration of the BR retinal in the K–BR spectrum reaches $p_m = 0.41 \pm 0.03$ for low actinic intensity (our unpublished results). It is thus probable that a limiting value of 0.5 could be reached under ideal photoselection conditions for the C=C vibration of BR in both the K–BR and the M_{412}–BR spectra. Bearing in mind that we cannot definitively preclude from our actual measurements that the optical transition of the retinal in BR is at an angle of up to 15–20° from the C=C axis, we will nevertheless assume, in the following discussion, that under ideal photoselection conditions, a value of $+0.5$ would be measured for the polarization of the 1527 cm^{-1} band. Furthermore, in order to compromise between the opposite variations of the magnitudes of p_m and of the signal-to-noise ratio, we deliberately adjusted the actinic intensity to obtain $\Delta A_{\parallel} = 2\ \Delta A_{\perp}$ at 1527 cm^{-1}, which corresponds to $p = 1.5\ p_m$ or $p_m = 0.33 \cos 2\theta$. At this intensity of actinic light, about 30% of BR was photoconverted. In these experiments, the actinic beam was polarized vertically. As a control, we further verified that identical photoselection spectra could also be generated upon excitation with a horizontally polarized beam.

A polarization of $p_m = 0.33$ ($\theta = 0°$) was observed for several of the negative ΔA signals corresponding to the disappearing C–C stretching modes of retinal in BR, at 1167 cm^{-1} (C_{10}–C_{11}), 1200 cm^{-1} (C_{14}–C_{15}) with a shoulder at 1213 cm^{-1} (C_8–C_9). The band at 1253 cm^{-1} assigned to C_{12}–C_{13} is significantly less dichroic, probably indicating the contribution of an

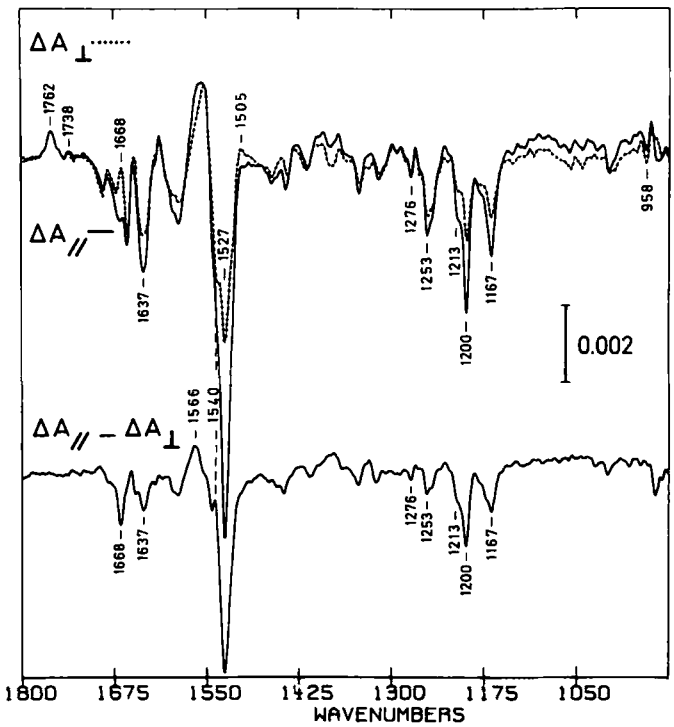

Fig. 2. Light-induced FTIR difference spectra of oriented films of purple membrane. The actinic beam was polarized and the light-induced FTIR difference spectra $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ were obtained with the polarization of the infrared beam parallel and perpendicular to the polarization of the actinic beam, respectively. $T = 21$° C. The experimental conditions are described in text. The absorbance scale is shown on the figure.

overlapping lysine mode [25] to this band. Other bands of the BR retinal include the 1637 cm^{-1} band ($p_m \approx +0.2$), which most probably corresponds to the C=N vibration of the protonated Schiff base, and the hydrogen out-of-plane wag at 958 cm^{-1} which has been assigned to the $HC_{11}=C_{12}H$ mode ($p_m \approx 0$). The band assignments are mostly those proposed in [25,26].

In the ΔA spectra, there are only very few positive bands which can be attributed to the retinal in the M_{412} species visualized. Among these, a band at approx. 1566 cm^{-1} has been assigned to the C=C stretch of the retinal [27]. Although the $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ spectra indicate that the region 1540–1570 cm^{-1} contains at least two spectral components, the shape of these signals is compatible with a large polarization ($p_m > 0.2$) of the 1566 cm^{-1} mode.

Besides the bands of the retinal in BR and M_{412}, there are several peaks which have been assigned to contributions from the protein moiety. The most conspicuous one is the 1762 cm^{-1} positive band which has been assigned to the C=O of an aspartic acid side chain residue which protonates in the M_{412} species [11,12]. A smaller signal at approx. 1738 cm^{-1} had been assigned to the C=O of a glutamic acid residue, although a recent report contends that it belongs to another aspartic acid residue [28]. Neither of these signals are seen in the $\Delta A_{\parallel} - \Delta A_{\perp}$ spectra and thus $p_m = 0$. A small signal at approx. 1276 cm^{-1}, which has been assigned to the $C-O^-$ vibration of a tyrosine group in BR [23,24], exhibits a polarization $p_m \approx 0.2$ indicating a θ angle of approx. 30° for this transition.

Discussion

Several photoselection studies in the visible spectral range have demonstrated that upon excitation of one of the three retinal chromophores present in the trimeric BR, only this initially excited chromophore was bleached [29,30]. They also show that within the patches of purple membrane, the BR molecules were immobile in the millisecond time scale of the photocycle [31,32]. Furthermore, the change occurring in the azimutal angle for the optical transition of the retinal when BR is converted to M_{412} has been reported to be small [31].

In our previously reported polarized FTIR difference spectroscopy of the orientation of BR and M_{412} [14], we have demonstrated that the tilt angle ($\phi = 66 \pm 4°$) of the ethylenic chain of retinal was the same as the tilt angle for the optical transition [31] and this observation has been further confirmed [15]. However, this information alone cannot be used to determine whether the retinal is all-*trans* or adopts a *cis*-configuration in which the plane of the bent retinal is also tilted at the same angle, ϕ, with respect to the membrane normal. The additional observations that $p_m = +0.33$ for the 1527 cm^{-1} band, which implies that the C=C stretching mode of BR is parallel to the optical transition of the retinal, thus favors a *trans*-configuration of the retinal in light-adapted BR. This property had been previously inferred from resonance Raman [33] and NMR [7] spectroscopies. The finding that p_m is close to the maximal limit for most of the bands which have been assigned to single-bond C–C stretching modes of BR [25,26] also demonstrates that the polarization of these modes is not significantly perturbed by the contributions from coupled vibrations of other nearby atoms which would move in a direction perpendicular to the ethylenic chain. Provided the assignment of these bands to individual modes, which rely on isotopic substitutions [25,26] and normal mode calculations [25,34], is correct, the strong polarization observed for these bands should allow precise measurements of the angular change of each bond direction which occurs upon isomerization. Furthermore, the observation that identical azimutal angles [14,15] and polar angles are observed for both the C=C double-bond and the C–C single-bond stretching modes is a clear demonstration of the full hyperconjugation of the retinal backbone. The polarization (Fig. 2) and the dichroism of the 1637 cm^{-1} band [14,15], the position of which is in general agreement with the C=N mode of BR [12,13,15,27], are compatible with an orientation of that vibration which is mostly along the direction of the C=C chain, as expected from the all-*trans* configuration of the retinal Schiff base [7,26,33]. However, more quantitative assessments must await polarized spectroscopy on deuterated samples and with BR specifically labelled on the Schiff base nitrogen atom [12].

From their linear dichroism study on tilted films of purple membrane, Earnest et al. have estimated a ϕ angle of 80° for the 958 cm^{-1} band of BR [15]. As this band has been assigned to the coupled hydrogen out-of-plane mode of the C_{11} and C_{12} hydrogen on the basis of isotopically induced frequency shifts [26] and because symmetry considerations require such modes to be polarized perpendicular to the plane of the polyene, these authors have inferred that the plane of the retinal was approximately perpendicular to the plane of the membrane. We have indeed confirmed their linear dichroism measurements for the 958 cm^{-1} band (our unpublished results). However, assuming their conclusions on the orientation parallel to the membrane of this hydrogen out-of-plane mode and on the orientation perpendicular to the membrane of the plane of the retinal are valid, a photoselection measurement should necessarily indicate a p_m value of -0.33 ($\theta = 90°$) for this band. As can be seen in Fig. 2, the amplitudes of the 958 cm^{-1} band for $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ are the same, implying a θ angle of approx. 45° between the projections of this transition and of the retinal chain. This result indicates that the 958 cm^{-1} band is probably not

a pure mode * and demonstrates that the dichroism of this band cannot be used to determine unambiguously the orientation of the retinal plane with respect to the purple membrane plane. We have derived an identical conclusion upon measurements of the ϕ and θ angles for various bands assigned to hydrogen out-of-plane wags of BR and K in the polarized FTIR K–BR difference spectra at 90K (our unpublished results).

In our previous study [14] we noticed that the C=C band of M_{412} appearing at approx. 1566 cm^{-1} has approximately the same dichroism as the C=C band of the disappearing BR at 1527 cm^{-1}. This observation, which has been confirmed [15], is in line with the polarized optical measurements showing that the tilt angle ϕ of the chromophore was not significantly perturbed by the isomerization [31]. Identical behavior is observed for the θ angle determined in the present study. This observation indicates that the BR to M_{412} transition does not significantly alter the direction of the C=C chain of the retinal.

While most of the absorbance changes discussed so far have a large positive polarization, indicating that they have a large component along the projection of the retinal onto the membrane plane, there are a few bands which behave differently. For the band around 1505 cm^{-1}, generally assigned to tyrosine [23,24], and that around 1540 cm^{-1}, which both exhibit negative polarization values, the linear dichroism measurements [14] indicate that both of these transitions are also preferentially oriented out of the membrane plane. At 1668 cm^{-1}, the observed signals are consistent with a band disappearing in BR in the $\Delta A_{\parallel}$ spectrum and appearing in M_{412} in the $\Delta A_{\perp}$ spectrum (Fig. 2). The wavelength position indicates that a peptidic C=O could change its orientation. Although such a putative motion has a rather large effect on the $\Delta A_{\parallel}$ and $\Delta A_{\perp}$ spectra, this observation should not be taken as an indication that extensive movement of the protein backbone is taking place. In view of the small amplitude of the $\Delta A_{\parallel}$–$\Delta A_{\perp}$ signal at 1668 cm^{-1}, which is of the same magnitude as the 1762 cm^{-1} band assigned to a change of a single bond, it seems more likely than only one or two bonds per BR molecule are involved in this motion. In Ref. 14 we show that the overall change in the azimutal angle of the α-helices was less than 2°, a value which is inconsistent with the large change in the tilt angle of the α-helices during the BR to M_{412} transition that was recently suggested [35,36]. On the other hand, the type of movement which was detected in Ref. 14 and in the present experiments (see also Ref. 21) are in very good agreement with the minor structural changes accompanying this transition which have been detected by electron diffraction techniques [37].

Besides the tyrosinate group of BR which is altered in the M_{412} transition and which is responsible for the 1276 cm^{-1} band ($p_m \approx 0.2$), the amino acid residue which is most clearly resolved in the ΔA spectra is the aspartic acid residue, the side chain of which becomes protonated in M_{412} and is responsible for the 1762 cm^{-1} positive band. The polarization value is zero leading to a θ angle of 45°. Together with the ϕ value of $35 \pm 5°$ determined in Ref. 14, this angle allows characterization of the directions of the retinal chain and of the C=O stretching vibration with respect to the plane of the purple membrane. Although with a lesser precision due to its smaller magnitude, a different geometry, with a ϕ value of 55° [14] and $\theta \approx 45°$, is observed for the 1738 cm^{-1} transition which is due to either a glutamic acid [11,12] or another aspartic acid [28] residue.

In conclusion, this study demonstrates that not only the tilt angle with respect to the plane of purple membrane [14,15], but also the relative geometrical orientations of the functional groups involved in the photocycle of BR can be investigated by polarized FTIR difference spectroscopy. The observation of very large polarization effects in the photoselection experiments reported here also demonstrates the importance of controlling the geometry of illumination in order to avoid unwanted photoselection effects in other measurements. It appears, thus, that a combination of polarized FTIR difference spectroscopy measurements of the azimutal [14,15] and of the polar angles is ideally suited to derive structural information on the active site of BR and of its various intermediates involved in the proton pumping mechanism. In the absence of a high resolution structure of BR, this technique can provide unique information on the geometrical organization of the specific groups which participate in the function. Furthermore, if a high resolution structure were available, such information would, in turn, be used to help locate these various functional groups in the structure.

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* As suggested by one referee, this composite character of the 958 cm^{-1} mode might indicate that this band is actually a mixture of $HC_{11}=C_{12}H$ and $HC_7=C_8H$ hydrogen out-of-plane modes which have different orientations. This would require some overall twist of the polyene chain.

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