Polarized Fourier transform infrared (FTIR) difference spectroscopy of the M_{412} intermediate in the bacteriorhodopsin photocycle

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The possibility that light-induced protein conformational changes accompany the formation of the M_{412} species in the bacteriorhodopsin photocycle is investigated by polarized Fourier transform infrared (FTIR) spectroscopy on oriented films of purple membrane. From the light-induced FTIR dichroism changes, it is estimated that: (i) the C=O stretching vibration at 1762 cm^{-1}, which has been assigned to a protonated Asp carboxyl group in M_{412} [(1985) Biochemistry 24, 400–407], is oriented at $\varphi = 35 \pm 5°$ from the normal to the membrane plane; (ii) the limit for the change in the average tilt angle of the α-helices after photoconversion is less than 2°. The latter observation excludes the large variations in the protein conformation during the M_{412} formation proposed by Draheim and Cassim [(1985) Biophys. J. 47, 497–507].

Bacteriorhodopsin *M_{412}* *Polarized Fourier transform infrared spectroscopy*

1. INTRODUCTION

Bacteriorhodopsin (BR), the only protein constituting the purple membrane (PM) of *Halobacterium halobium*, functions as a light-driven proton pump [1]. This hydrophobic protein contains 7 α-helical segments which have been shown by low-dose electron microscopy [2] and polarized infrared (IR) spectroscopy [3–5] to be oriented roughly along the normal to the plane of the membrane. The chromophore retinal is bound via a protonated Schiff base linkage to a Lys residue from the protein moiety [6]. Upon absorption of light, the chromophore undergoes a cyclic reaction via the formation of several transient intermediates. Among them, the long-lived M_{412} photoproduct which contains an unprotonated Schiff base [7–9] has been the subject of extensive structural and mechanistic investigations. Moreover, one fundamental and still unsettled question is related to the extent of protein conformational changes which accompany the formation of this intermediate [10–12].

The vibrational properties of the M_{412} species have been extensively studied by resonance Raman and infrared (IR) spectroscopies [7–9,11,13–20]. Most of these investigations are devoted to the study of structural changes of the retinal itself [15] and of the neighbouring amino acid residues [16,19,20]. More specifically, light-induced Fourier transform infrared (FTIR) difference spectroscopy is ideally suited to detect, on a high but constant background absorption, small changes in the vibrational modes of single bonds in the chromophore and protein occurring upon photoconversion [11,16,18,21,22]. In addition, when using a polarized IR beam, this approach offers the potential to measure the orientation of the vibrational transitions of the chromophore and peptide groups involved in the photoreaction as well as their possible changes of orientation after photoconversion [23]. While light-induced variations in the protonation state of a few internal amino acid residues (Asp, Glu, Tyr) have been recently identified by FTIR spectroscopy [16,19,20,24,25] during the photocycle of BR,

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small conformational changes of the protein backbone at the stage of M_{412} have also been suggested. In particular, Bagley et al. [11] interpreted their FTIR spectra in terms of a minor protein rearrangement involving a slight rotation of the α-helices towards the membrane normal during the BR-M_{412} transformation. On the other hand, on investigating UV circular dichroism (CD) spectra of oriented films of PM, Draheim and Cassim [12] concluded that there occur large-scale global structural changes of the protein in the M_{412} species with a net tilting (5–15°) of the transmembrane α-helices away from the normal to the membrane plane.

In order to resolve this discrepancy between CD and FTIR data, we have developed a new approach, i.e. a combination of polarized IR spectroscopy and light-induced FTIR difference spectroscopy, to investigate conformational changes in the M_{412} intermediate of the BR photocycle on oriented multilayers of purple membrane.

2. EXPERIMENTAL

PM was a gift from Dr J.-L. Rigaud. For the FTIR spectra, films of air-dried oriented PM [4] were mounted in a closed hydration cell. These films can be tilted (usually at 45°) with respect to the IR beam. FTIR spectra were recorded at 20°C on a Nicolet 60SX spectrophotometer equipped with an MCT absorbance detector and a wire grid polarizer kindly lent by Perkin-Elmer (France). The IR beam was linearly polarized parallel (to record $A_{\parallel}$) or perpendicular (to record $A_{\perp}$) to the plane of incidence, as described in [4,5]. Polarized FTIR spectra of PM films were recorded in the dark ($A_{\parallel D}$, $A_{\perp D}$) and during illumination ($A_{\parallel L}$, $A_{\perp L}$) of the sample by a 500 W tungsten source filtered by 5 cm water and a yellow filter (cut-off point at 500 nm). Under these illumination conditions, spectra of PM films in the visible region show that about 50% of the M_{412} species can be accumulated in a photostationary state. 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. Then, spectra from the same series ($A_{\parallel D}$, $A_{\perp D}$, $A_{\parallel L}$, $A_{\perp L}$) were averaged (usually 10240 interferograms each). However, the first illumination cycle was not taken into account in order to eliminate absorbance changes occurring upon light adaptation of BR.

FTIR absorbance (A) and linear dichroism (LD) spectra were obtained from $(2A_{\perp} + A_{\parallel})/3$ and $(A_{\parallel} - A_{\perp})$ respectively, for dark and illuminated samples. Then, the difference absorbance (ΔA) and LD (ΔLD) between illuminated (M_{412}) and dark (BR) samples were calculated. The tilt angle ϕ (with respect to the normal to the membrane) for the C=C ($\phi_{C=C}$) bonds at 1527 cm^{-1} and the C=O ($\phi_{C=O}$) groups at 1762 cm^{-1} were calculated according to [4], using an average tilt angle of ϕ_{α} = 11° for the transmembrane α-helices of BR (Henderson, personal communication [4]). The values reported below are the average obtained from four different films.

3. RESULTS AND DISCUSSION

Absorbance and LD FTIR spectra of oriented multilayers of PM are depicted in fig.1. Under our experimental conditions [4,5], a positive dichroism signal is associated with the alignment of the transition at less than 55° from the membrane normal. The FTIR dichroism spectrum of oriented PM (fig.1, bottom) in the amide I (1667 cm^{-1}) and amide II (1545 cm^{-1}) regions shows the orientation of the α-helices perpendicular to the membrane plane as reported [2–5]. It should be noted that this dichroism spectrum compares very well with that previously reported with a dispersive IR spectrometer [5]. However, the signal-to-noise ratio in the FTIR dichroism spectrum is remarkably improved (fig.1, bottom).

The FTIR ΔA difference spectrum between M_{412} and BR is shown in fig.2 (top). A positive signal corresponds to vibrations ascribed to the appearing M_{412} species while a negative one corresponds to vibrations of the disappearing BR. This ΔA spectrum obtained at room temperature is in good agreement with previously reported static and kinetic spectra of M_{412} obtained at various temperatures [11,16–18,20]. In particular, the largest (negative) ΔA signal in the 1900–1100 cm^{-1} region which is due to the ethylenic C=C stretching vibrations of the retinal polyene chain is observed at 1527 cm^{-1} in BR. It has been recently proposed that the positive bands around 1760 and 1740 cm^{-1} are due to the C=O stretching vibra-

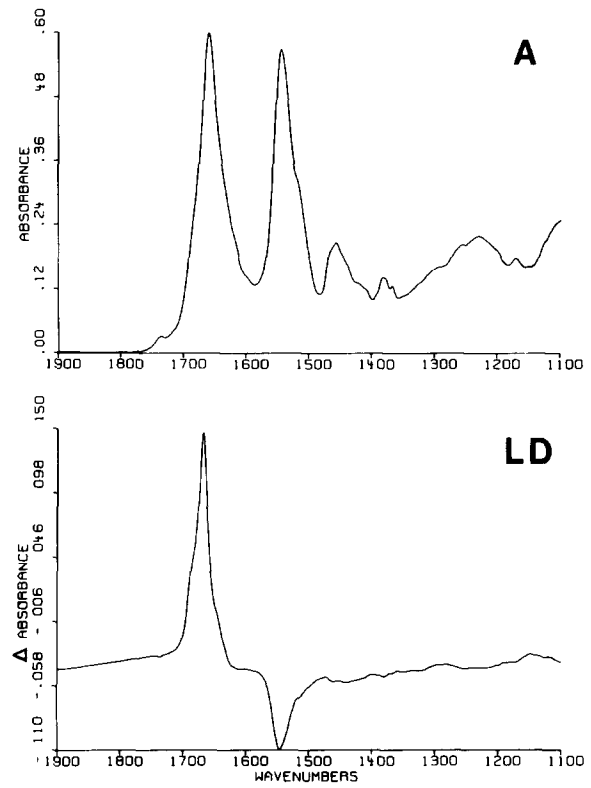

Fig.1. FTIR absorbance (top) and LD ($A_{\parallel} - A_{\perp}$) (bottom) spectra of oriented films of purple membrane from *H. halobium*.

tions of the carboxyl groups of Asp and Glu residues respectively, which are protonated in the M_{412} species [20]. More specifically, the protonation of two Asp groups was observed at 1765 and 1755 cm^{-1} (shoulder) during the BR photocycle [19,20]. However, only one C=O group (at 1765 cm^{-1}) is protonated simultaneously with the formation of M_{412} while the time course of the other (at 1755 cm^{-1}) is slower [19,20]. Under our experimental conditions, at 20°C, the 1755 cm^{-1} shoulder is neither clearly detected in the ΔA spectrum (fig.2, top), nor in the ΔLD spectrum (see below and fig.2, bottom). Thus, it seems justifiable to interpret our polarized FTIR data at 1762 cm^{-1} (fig.2, bottom) in terms of the orientation of just one Asp C=O group. Further ΔLD studies using low temperature for which the M_{412} intermediate can be trapped in a better defined state [11,17–20] would be necessary to determine the orientation of the different C=O Asp groups involved in the BR photocycle.

Fig.2, bottom shows the ΔLD spectrum corresponding to the difference between an LD spectrum of PM taken under illumination and that taken in the dark (fig.1, bottom). The actual noise level is seen in the 1900–1800 cm^{-1} region. The frequency and the sign of these ΔLD signals are highly reproducible from sample to sample. Table 1 compares the respective signs for ΔA and ΔLD bands that show identical frequencies. Under our experimental conditions [4,5], a positive (negative) ΔLD signal associated with a positive (negative) ΔA signal is due to a transition tilted at $\phi \leqslant 55°$ from the membrane normal. This is clearly observed for the stretching mode of the carbonyl group of Asp at 1762 cm^{-1}. Using the LD/absor-

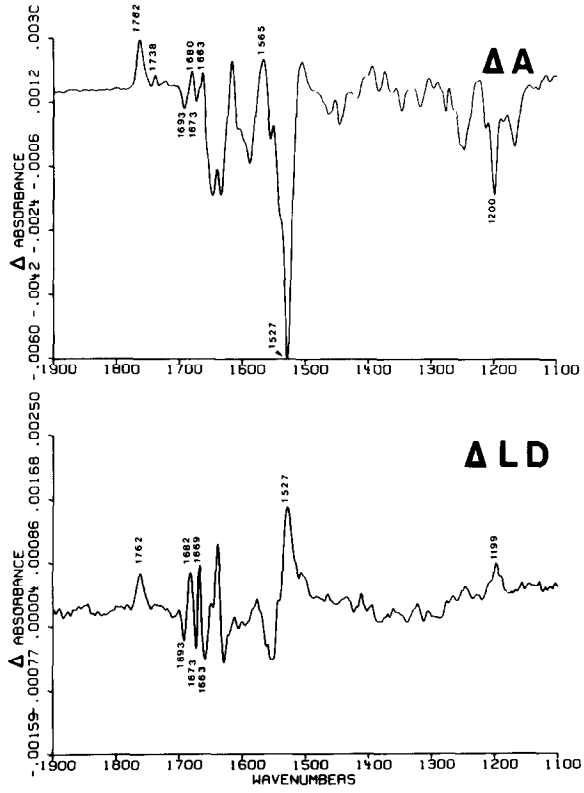

Fig.2. (Top) Light-induced FTIR difference (ΔA) spectrum obtained from a spectrum recorded with actinic light (leading to the formation of M_{412}) and a spectrum without. Bands of the appearing M_{412} species appear positive. (Bottom) FTIR difference spectrum (ΔLD) between LD spectra of M_{412} and BR. See section 2 for measuring conditions.

Table 1

Comparison of the sign of the signals for ΔA and ΔLD bands that show identical frequencies ($\pm$ 1 cm^{-1})

Frequency (cm^{-1})	ΔA	ΔLD
1762	>0	>0
1693	<0	<0
1681	>0	>0
1673	<0	<0
1663	>0	<0
1527	<0	>0
1200	<0	>0

bance values for the α-helices to calibrate our measurements, we calculated that the C=O transition of the protonated carboxyl group of Asp in M_{412} is oriented at $\phi_{C=O} = 35° \pm 5°$ from the normal to the membrane plane. In contrast, the small positive band at 1738 cm^{-1} in the ΔA spectrum, which has been assigned to the C=O stretching vibration of the protonated carboxyl group of a Glu residue [20], has almost no corresponding ΔLD signal. Thus, its orientation is close to the magic angle ($\phi = 55°$ with respect to the normal to the membrane). The positive ΔLD signal at 1527 cm^{-1} associated with the corresponding negative ΔA signal at the same frequency corresponds to C=C vibrations of the retinal oriented preferentially parallel to the membrane plane ($\phi_{C=C} = 66° \pm 4°$). This value agrees with the well-established orientation of the optical transition (parallel to the polyene chain) at about 23° from the membrane plane [26]. Moreover, the shape of the ΔLD signal in the 1550–1575 cm^{-1} region also suggests that the positive ΔA signal at 1565 cm^{-1} assigned to C=C vibrations of the polyene chain in the M_{412} species exhibits a negative ΔLD signal. This is consistent with the reported absence of orientation change of the optical transitions during the BR to M_{412} photoconversion [26,27]. In the 1300–1100 cm^{-1} fingerprint region, the most prominent feature in the ΔLD spectrum is a positive band at 1200 cm^{-1} to which corresponds a negative ΔA signal. Thus, this transition, that has been assigned to the C_{14}–C_{15} single bond stretching vibration of the retinal in BR [15], is oriented at more than 55° from the normal to the membrane plane. However, the present signal-to-noise ratio in the ΔA and ΔLD spectra does not allow a quantitative comparison of the tilt angle for the 1527 cm^{-1} C=C transitions and the C_{14}–C_{15} single bond.

Most of the other ΔLD signals are localized in the 1600–1700 cm^{-1} spectral region (fig.2, bottom and table 1). While their frequencies are highly reproducible (from sample to sample and between successive dark-light cycles on the same sample), their relative amplitude can vary. This is notably observed for the 1663, 1669, 1673, 1682 and 1693 cm^{-1} bands. This could likely be due to small variations of temperature in the sample during accumulation of-interferograms. In this spectral domain, the C=O peptide groups are known to absorb (fig.1, top). The LD spectrum of the amide I region (fig.1, bottom) shows a positive signal at 1667 cm^{-1} assigned to the transmembrane α-helices [3–5] and a small shoulder at 1685 cm^{-1} which has been tentatively attributed to a small amount of oriented β-structure [5]. Thus, the observed ΔLD signals in the 1650–1700 cm^{-1} region could be due to changes in the orientation of the protein secondary structure. In this frequency range, the maximum amplitude of the ΔA bands is less than 1% of the amide I band absorbance which excludes large variations in the conformation of the protein, as previously noted by Bagley et al. [11]. More precisely, assuming that the 1663–1669 or 1669–1673 cm^{-1} ΔLD signal is only due to a change in the tilt of the α-helices and taking into account the approx. 50% yield of M_{412} in our experimental conditions, it can be calculated that this tilt angle is less than 2°. This result excludes the large global α-helices' orientation changes of 5–15° inferred by Draheim and Cassim [12] from UV CD data on PM films. Although time-resolved X-ray diffraction studies of photostimulated PM have been interpreted to suggest some disorder of the BR packing [28], recent X-ray and electron diffraction patterns of PM in the M_{412} state show that there is no apparent change in the degree of crystalline order and that only minor structural changes (involving the movement of only a few non-hydrogen atoms) occur during the M_{412} formation (Glaeser, R.M., personal communication). This interpretation, which also excludes large BR conformational changes, is in good agreement with the conclusion drawn from our light-induced FTIR dichroism spectra.

Several authors have recently proposed functional models of the BR photocycle depicting possible deprotonation and protonation changes of the Schiff base and of a counterion such as Asp, Glu or Tyr [20,24,25,29,30]. Our polarized FTIR data demonstrate that the internal Asp residue which protonates during the M_{412} formation has its C=O side chain group oriented preferentially perpendicular to the membrane plane ($\phi_{C=O} = 35° \pm 5°$). Such information obtained by the new approach described here should help to build more precise models of the 'active sites' of BR, the binding pocket of the retinal and the changes of conformation of the chromophore during the BR photocycle. These observations should probably be taken into account in the description at the molecular level of the very efficient light-driven proton conduction mechanism occurring in the purple membrane of *H. halobium*.

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REFERENCES

[1] Stoeckenius, W. and Bogomolni, R.A. (1982) Annu. Rev. Biochem. 51, 587–616.
[2] Henderson, R. and Unwin, P.N.T. (1975) Nature 257, 28–32.
[3] Rothschild, K.J. and Clark, N.A. (1979) Biophys. J. 25, 473–488.
[4] Nabedryk, E. and Breton, J. (1981) Biochim. Biophys. Acta 635, 515–524.
[5] Nabedryk, E., Bardin, A.M. and Breton, J. (1985) Biophys. J. 48, 873–876.
[6] Bayley, H., Huang, K.S., Radhakrishnan, R., Ross, A.H., Takagaki, Y. and Khorana, H.G. (1981) Proc. Natl. Acad. Sci. USA 78, 2225–2229.
[7] Lewis, A., Spoonhower, J., Bogomolni, R.A., Lozier, R.H. and Stoeckenius, W. (1974) Proc. Natl. Acad. Sci. USA 71, 4462–4466.
[8] Aton, B., Doukas, A.G., Callender, R.H., Becher, B. and Ebrey, T.G. (1977) Biochemistry 16, 2995–2999.
[9] Braiman, M. and Mathies, R. (1980) Biochemistry 19, 5421–5428.
[10] Becher, B., Tokunaga, F. and Ebrey, T.G. (1978) Biochemistry 17, 2293–2300.
[11] Bagley, K., Dollinger, G., Eisenstein, L., Singh, A.K. and Zimanyi, L. (1982) Proc. Natl. Acad. Sci. USA 79, 4972–4976.
[12] Draheim, J.E. and Cassim, J.Y. (1985) Biophys. J. 47, 497–507.
[13] Stockburger, M., Klusmann, W., Gattermann, H., Massig, G. and Peters, R. (1979) Biochemistry 18, 4886–4900.
[14] Deng, H., Pande, C., Callender, R.H. and Ebrey, T.G. (1985) Photochem. Photobiol. 41, 467–470.
[15] Smith, S.O., Lugtenburg, J. and Mathies, R.A. (1985) J. Membrane Biol. 85, 95–109.
[16] Rothschild, K.J., Zagaeski, M. and Cantore, W.A. (1981) Biochem. Biophys. Res. Commun. 103, 483–489.
[17] Siebert, F., Mäntele, W. and Kreutz, W. (1981) Can. J. Spectrosc. 26, 119–125.
[18] Siebert, F. and Mäntele, W. (1983) Eur. J. Biochem. 130, 565–573.
[19] Siebert, F., Mäntele, W. and Kreutz, W. (1982) FEBS Lett. 141, 82–87.
[20] Engelhard, M., Gerwert, K., Hess, B., Kreutz, W. and Siebert, F. (1985) Biochemistry 24, 400–407.
[21] Mäntele, W., Nabedryk, E., Tavitian, B.A. and Breton, J. (1985) FEBS Lett. 187, 227–232.
[22] Nabedryk, E., Mäntele, W., Tavitian, B.A. and Breton, J. (1986) Photochem. Photobiol. 43, 461–465.
[23] Nabedryk, E. and Breton, J. (1986) Biophys. J. 49, 474a.
[24] Rothschild, K.J., Roepe, P., Ahl, P.L., Earnest, T.N., Bogomolni, R.A., Das Gupta, S.K., Mulliken, C.M. and Herzfeld, J. (1986) Proc. Natl. Acad. Sci. USA 83, 347–351.
[25] Eisenstein, L., Dollinger, G., Lin, S.-L. and Vittitow, J. (1986) Biophys. J. 49, 480a.
[26] Heyn, M.P., Cherry, R.J. and Müller, U. (1977) J. Mol. Biol. 117, 607–620.
[27] Stoeckenius, W., Lozier, R.H. and Bogomolni, R.A. (1979) Biochim. Biophys. Acta 505, 215–278.
[28] Frankel, R.D. and Forsyth, J.M. (1985) Biophys. J. 47, 387–393.
[29] Rothschild, K.J., Roepe, P., Lugtenburg, J. and Pardoen, J.A. (1984) Biochemistry 23, 6103–6109.
[30] Gerwert, K. and Siebert, F. (1986) EMBO J. 5, 805–811.