Orientation of the Bacteriorhodopsin Chromophore Probed by Polarized Fourier Transform Infrared Difference Spectroscopy[†]

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Received September 10, 1986; Revised Manuscript Received October 3, 1986

ABSTRACT: Polarized, low-temperature Fourier transform infrared (FTIR) difference spectroscopy has been used to investigate the structure of bacteriorhodopsin (bR) as it undergoes phototransitions from the light-adapted state, bR_{570}, to the K_{630} and M_{412} intermediates. The orientations of specific retinal chromophore and protein groups relative to the membrane plane were calculated from the linear dichroism of the infrared bands, which correspond to the vibrational modes of those groups. The linear dichroism of the chromophore C=C and C—C stretching modes indicates that the long axis of the polyene chain is oriented at 20–25° from the membrane plane at 250 K and that it orients more in-plane when the temperature is reduced to 81 K. The polyene plane is found to be approximately perpendicular to the membrane plane from the linear dichroism calculations of the HOOP (hydrogen out-of-plane) wags. The orientation of the transition dipole moments of chromophore vibrations in the K_{630} and M_{412} intermediates has been probed, and the dipole moment direction of the C=O bond of an aspartic acid that is protonated in the $bR_{570} \rightarrow M_{412}$ transition has been measured.

The determination of biomolecular mechanisms requires detailed information about the structure and dynamics of specific chemical groups such as those inside the active site of a protein. X-ray crystallography provides a means of obtaining high-resolution structural information. However, unless a biomolecule can be crystallized in its transition state(s), the detailed changes that individual groups undergo during functioning are difficult to ascertain. Ideally, new techniques are required to measure the changes in the chemical state, environment, and orientation of specific groups in a protein and its active intermediates.

In this study, we demonstrate that the orientation of specific groups involved in functional changes of a macromolecule can be investigated by the method of polarized Fourier transform infrared (FTIR)[1] difference spectroscopy. This method combines the sensitivity of FTIR difference spectroscopy, which is capable of detecting alterations of single residues in a protein (Rothschild, 1986), with the ability of polarized spectroscopy to probe the orientation of chemical bonds (Fraser & MacRae, 1973). We have applied this method to study bacteriorhodopsin (bR), the light-driven, transmembrane proton pump in the purple membrane (PM) of *Halobacterium halobium* (Stoeckenius & Bogomolni, 1982). The transitions from bR_{570}, the light-adapted state of bR, to the K_{630} and M_{412} intermediates of the bR photocycle were measured at low temperature with polarized infrared light transmitted through an oriented PM film tilted relative to the incident beam. Information is obtained about the orientation of specific groups in bR relative to the membrane plane, including the angle of the polyene plane of the retinal chromophore as well as the orientations of individual C—C and C=C bonds. This approach complements previous low-temperature FTIR difference measurements on PM, which have helped identify specific groups involved in the photocycle (Rothschild & Marrero, 1982; Bagley et al., 1982; Siebert & Mäntele, 1983; Dollinger et al., 1986; Rothschild et al., 1986), and resonance Raman studies (Lewis et al., 1974; Aton et al., 1977; Stockburger et al., 1979; Braiman & Mathies, 1982; Argade & Rothschild, 1983), which have probed the configuration of the retinal chromophore at various steps in the bR photocycle.

METHODS

Oriented multilamellar films of PM on AgCl windows were formed by the isopotential spin-dry (ISD) method (Clark et al., 1980). The individual PM fragments in such films have been shown previously by electron microscopy to be oriented parallel to the sample plane with a small mosaic spread, which is due mainly to edge overlap of the fragments. Linear dichroism measurements at 570 nm of samples oriented on glass coverslips indicate the films are rotationally isotropic about the sample normal with the chromophore electronic transition moment tilted at approximately 67 ± 2° to the sample normal (Clark et al., 1980).

Measurements were made with a 60SX FTIR spectrometer (Nicolet Instruments, Madison, WI) equipped with a liquid N_2 cooled HgCdTe detector. A KRS-5 wire grid polarizer was mounted in a vertical (xy) plane[2] inside a motorized rotator such that the IR measuring beam (in the z direction) first passed through the polarizer and then entered a Helitran liquid N_2 cryostat (Air Products, Allentown, PA), equipped with KBr windows, which was capable of regulating the sample temperature from 77 to 300 K. An important feature of this cryostat is the ability to tilt the sample plane around the vertical (y) axis. This allows linear dichroism measurements to be made for several tilt angles without having to break the dewar vacuum and rewarm the sample. Polarization, illu-

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[†] Supported by grants from the National Science Foundation (DMB-8509857) and the National Institutes of Health (EY05499). M.S.B. was supported by a postdoctoral fellowship from the Helen Hay Whitney Foundation.

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[1] Abbreviations: FTIR, Fourier transform infrared; bR, bacteriorhodopsin; PM, purple membrane; HOOP, hydrogen out of plane.

[2] The following convention is used to describe the experimental geometry: the infrared measuring beam travels along the z axis, the untilted sample ($\alpha_0 = 0°$) lies in the xy plane, and the tilt angle (α_0) measures rotation about the y axis. Perpendicular polarization ($E_\perp$) is along the y axis, and parallel polarization ($E_\parallel$) is along the x axis.

0006-2960/86/0425-7793$01.50/0

mination, and temperature were controlled from the 60SX computer. Polarized FTIR difference measurements of the $bR_{570} \rightarrow K_{630}$ and $bR_{570} \rightarrow M_{412}$ transitions were made at 81 and 250 K, respectively, by procedures similar to those reported elsewhere for unpolarized measurements (Rothschild et al., 1986).

The sample was humidified and light-adapted before cooling. This procedure is important in order to avoid trapping the bR_{548} dark-adapted intermediate, which would alter the final difference spectrum obtained. The $bR_{570} \rightarrow K_{630}$ difference spectra were obtained at 81 K by (i) accumulating 4500 interferograms at 4-cm^{-1} resolution (approximately 40 min) under constant green illumination to produce the K_{630} intermediate, (ii) accumulating another 4500 interferograms under red illumination to photoreverse to the bR_{570} state, and (iii) Fourier transforming both signal-averaged interferograms and subtracting the bR_{570} spectrum from the K_{630} spectrum. This cycle was repeated up to 10 times with the incident polarization varied between perpendicular and parallel for successive cycles. The $bR_{570} \rightarrow M_{412}$ spectra were similarly obtained with cycles of constant yellow illumination followed by no illumination at 250 K. At this temperature the M_{412} intermediate decays rapidly such that spectra recorded after illumination subtracted from spectra obtained before illumination yielded no absorbance differences above the noise level.

The dichroic ratio R for a peak in the difference spectrum is defined as the ratio of the absorbance changes of parallel ($\Delta A_{\parallel}$) to perpendicular ($\Delta A_{\perp}$) polarizations. R is related to the tilt angle α_0 by the expression

$$R = 1 + \frac{\sin^2 \alpha_0}{n^2}\left(\frac{3p}{1-p}\right) = 1 + \frac{\sin^2 \alpha_0}{n^2}\left(\frac{3p_M p_T}{1 - p_M p_T}\right) \quad (1)$$

(Rothschild & Clark, 1979), where n is the film refractive index at infrared wavelengths and p, the effective order parameter of the transition dipole moment for IR absorption, is in this case the product of two order parameters p_M and p_T. The first, p_M, is the mosaic spread order parameter, which is related to the degree of orientation of the membranes relative to the sample plane and has been determined in a separate study to be between 0.9 and 1.0 (Clark et al., 1980). As an internal check on this estimate, the amide I and II linear dichroisms of the bR_{570} spectra at 250 K were calculated. Using these data and a value of $p_M = 0.95$ yields an upper limit for the average α-helical tilt of $\approx 30°$, in agreement with the three-dimensional structure from electron diffraction data (Henderson & Unwin, 1975). The second order parameter p_T is related to the orientation of the transition dipole moment relative to the membrane plane by

$$p_T = \langle (3 \cos^2 \Theta_T - 1)/2 \rangle \quad (2)$$

where Θ_T is the angle between the transition dipole moment and the membrane normal.

The orientations of the transition dipole moments for several bands were determined by plotting the dichroic ratio, R, vs. $(\sin^2 \alpha_0)/n^2$ for $\alpha_0 = 0°, 15°, 30°$, and 45°. The data were fit by a least-squares algorithm to a straight line, and the slope was used to determine Θ_T, as shown in eq 1 and 2 (Rothschild & Clark, 1979). This provides a more reliable method for the determination of Θ_T than calculations using a single tilt angle since it checks if R obeys the predicted dependence on α_0. For all calculations, we used previously estimated values of $n = 1.7$ (Henniker, 1973) and $p_M = 0.95$ (Clark et al., 1980). Standard deviations were determined by calculating Θ_T from measurements made on at least three samples. Since the overlap of bands can lead to erroneous calculations of dichroic ratios, we have calculated Θ_T only for those bands for which significant overlap does not appear to occur except where noted.

Table I: Infrared Dichroism of Major Bands[a]

frequency (cm^{-1})	assignment	Θ_T (deg)
	$bR_{570} \rightarrow K_{630}$ (81 K)	
1669 (bR)	α-helix amide I; $\parallel$ component	out of plane
1661 (K)	α-helix amide I; $\perp$ component	out of plane
1639 (bR)	C=N stretch	73
1530 (bR)	C=C stretch	73
1514 (K)	C=C stretch	79
1254 (bR)	C_{12}–C_{13} stretch	84
1215 (bR)	C_8–C_9 stretch	84
1202 (bR)	C_{14}–C_{15} stretch	80
1193 (K)	C—C stretch; predominantly C_{14}–C_{15}	86
1167 (bR)	C_{10}–C_{11} stretch	85
974 (K)	C_{15} HOOP	75
955 (K)	HOOP mode with mixed character	83
942 (K)	HC_{11}=$C_{12}H$ HOOPs	85
	$bR_{570} \rightarrow M_{412}$ (250 K)	
1762 (M)	aspartic acid COOH	43
1692 (bR)	β structure or COOH	25
1563 (M)	C=C stretch	62
1554 (M)	α-helix amide II	63
1526 (bR)	C=C stretch	69
1254 (bR)	C_{12}–C_{13} stretch	71
1213 (bR)	C_8–C_9 stretch	66
1200 (bR)	C_{14}–C_{15} stretch	70
1167 (bR)	C_{10}–C_{11} stretch	64
958 (bR)	HC_{11}=$C_{12}H$ HOOP	80
951 (M)	HOOP	83

[a] Frequency, assignment, and Θ_T (the angle between the membrane normal and the transition dipole moment) for major bands in the $bR_{570} \rightarrow K_{630}$ and $bR_{570} \rightarrow M_{412}$ difference spectra. Assignments are based on normal mode calculations and resonance Raman measurements of bR with isotopic substitutions of the retinylidene chain (Braiman, 1983; Smith et al., 1986b). The values for Θ_T represent the average from at least three samples using $n = 1.7$ and $p_M = 0.95$. Standard deviations ranged from 2° to 5°. The symbols bR, K, and M designate peaks in the difference spectra corresponding to the bR_{570}, K_{630}, and M_{412} intermediates, respectively. In the case of the 1669- and 1661-cm^{-1} peaks, Θ_T could not be accurately calculated due to the peak overlap. However, the dichroism observed is consistent with a Θ_T of approximately 0 for these bands.

RESULTS AND DISCUSSION

Figure 1 shows the polarized FTIR difference spectra for the $bR_{570} \rightarrow K_{630}$ transition measured at 81 K, and Figure 2 shows corresponding spectra for the $bR_{570} \rightarrow M_{412}$ transition measured at 250 K with $\alpha_0 = 45°$ (0°, 15°, and 30° spectra not shown). The orientation of different transition moments in the bR_{570} chromophore can be determined from the dichroism of the negative peaks in both the $bR_{570} \rightarrow K_{630}$ and $bR_{570} \rightarrow M_{412}$ difference spectra. Examples of plots of the dichroic ratio, R, vs. $(\sin^2 \alpha_0)/n^2$ (cf. Methods) are shown in Figure 3 for the 1526-cm^{-1} C=C ethylenic stretch and the 1200-cm^{-1} C—C stretch at 250 K. It is found that these plots are linear and intercept the R axis very close to 1 as expected. Table I lists the calculated Θ_T values for these peaks as well as others discussed below.

The prominent negative peaks in the difference spectra near 1530, 1254, 1215, 1200, and 1168 cm^{-1} have been assigned (cf. Table I) by resonance Raman studies to in-plane C=C and C—C stretching vibrations (Smith et al., 1985, 1986b) of the bR_{570} chromophore. While a detailed analysis using calculated normal modes is required to determine the exact orientation of the transition dipole moment of each vibration relative to the molecular axes, the predominant C=C and C—C stretching character of these modes is expected to produce transition moments that are roughly parallel to the

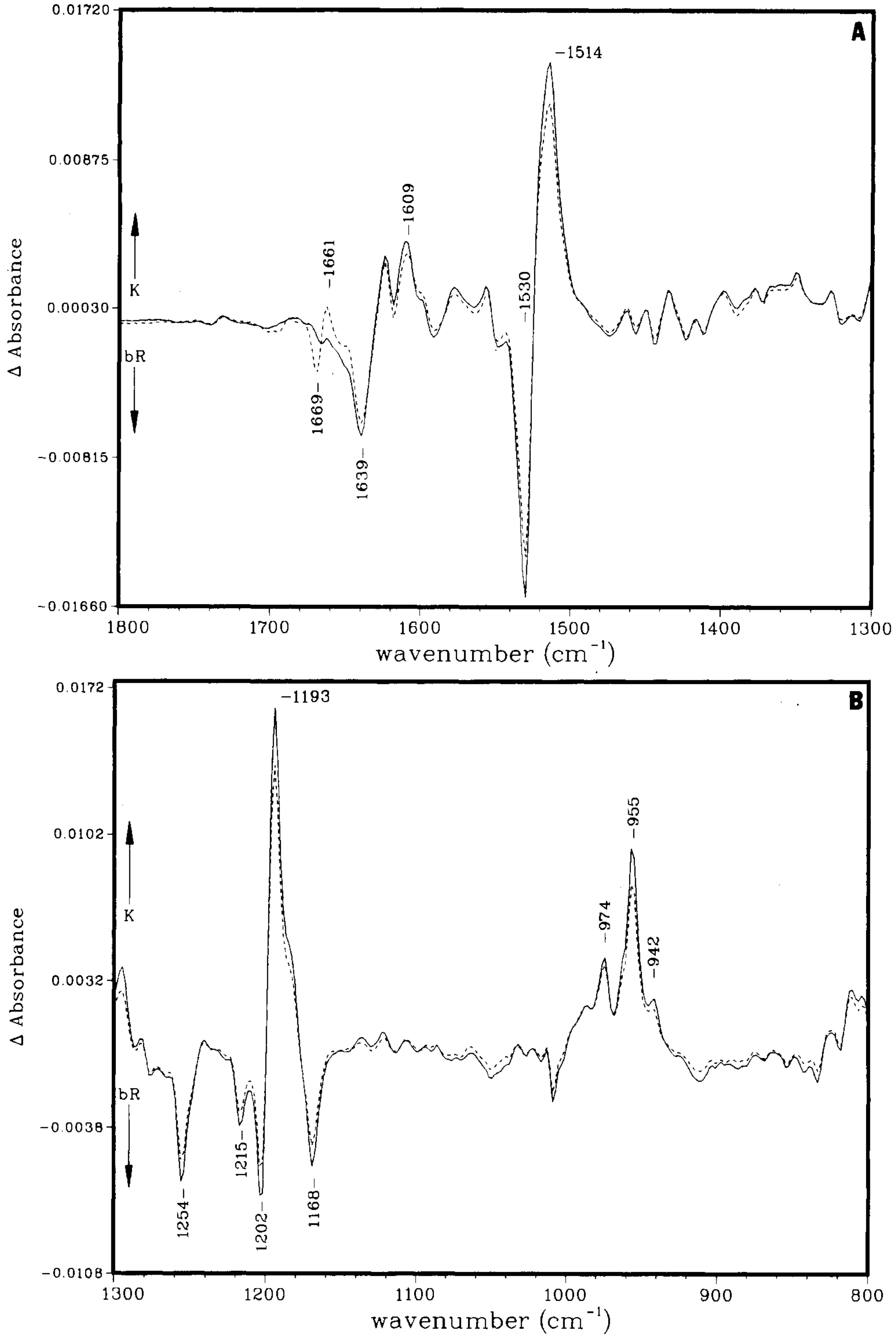

FIGURE 1: (A) $bR_{570} \rightarrow K_{630}$ FTIR difference spectra for the 1800–1300-cm^{-1} region recorded at 81 K. The solid line indicates that the spectra were recorded with an incident polarization perpendicular to the plane of incidence (parallel to the sample plane), and the dashed line indicates an incident polarization parallel to the plane of incidence (perpendicular to the sample plane). Green illumination (Ditric interference filter 15-11180, Hudson, MA) was used to drive bR_{570} into the K_{630} state, and red illumination (Ditric interference filter 15-11200) was used to photoreverse K_{630} to bR_{570}. (B) The 1300–800-cm^{-1} region.

all-trans polyene chain direction.[3] As seen in Table I for bR_{570} at 250 K, all of the C—C and C=C stretch moments for the chromophore polyene chain have very similar tilt angles in the range of 64–71°, in agreement with measurements for the electronic transition moment of the chromophore at room temperature (Heyn et al., 1977; Clark et al., 1980). In addition, the C=N stretch of the Schiff base linkage of the chromophore to lysine-216 of bR (Rothschild et al., 1982; Huang et al., 1982) at 1639 cm^{-1} exhibits a predominantly in-plane orientation in the bR_{570} state, though the overlap of other peaks in the $bR_{570} \rightarrow M_{412}$ difference spectrum precludes an accurate determination of Θ_T for this mode at 250 K. These

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[3] While the transition moment of an isolated C=C or C—C stretching vibration should be parallel to the individual bond, mixing of additional stretches and C–CH in-plane bends in the normal coordinates could change the transition moment direction. However, a normal coordinate calculation of bR_{570} (Smith et al., 1986b) shows the most intense C=C and C—C modes involve in-phase mixings. In the case of a polyene chain exhibiting charge alternation, such in-phase mixing would lead to resultant transition dipoles along the long axis of the retinal chromophore.

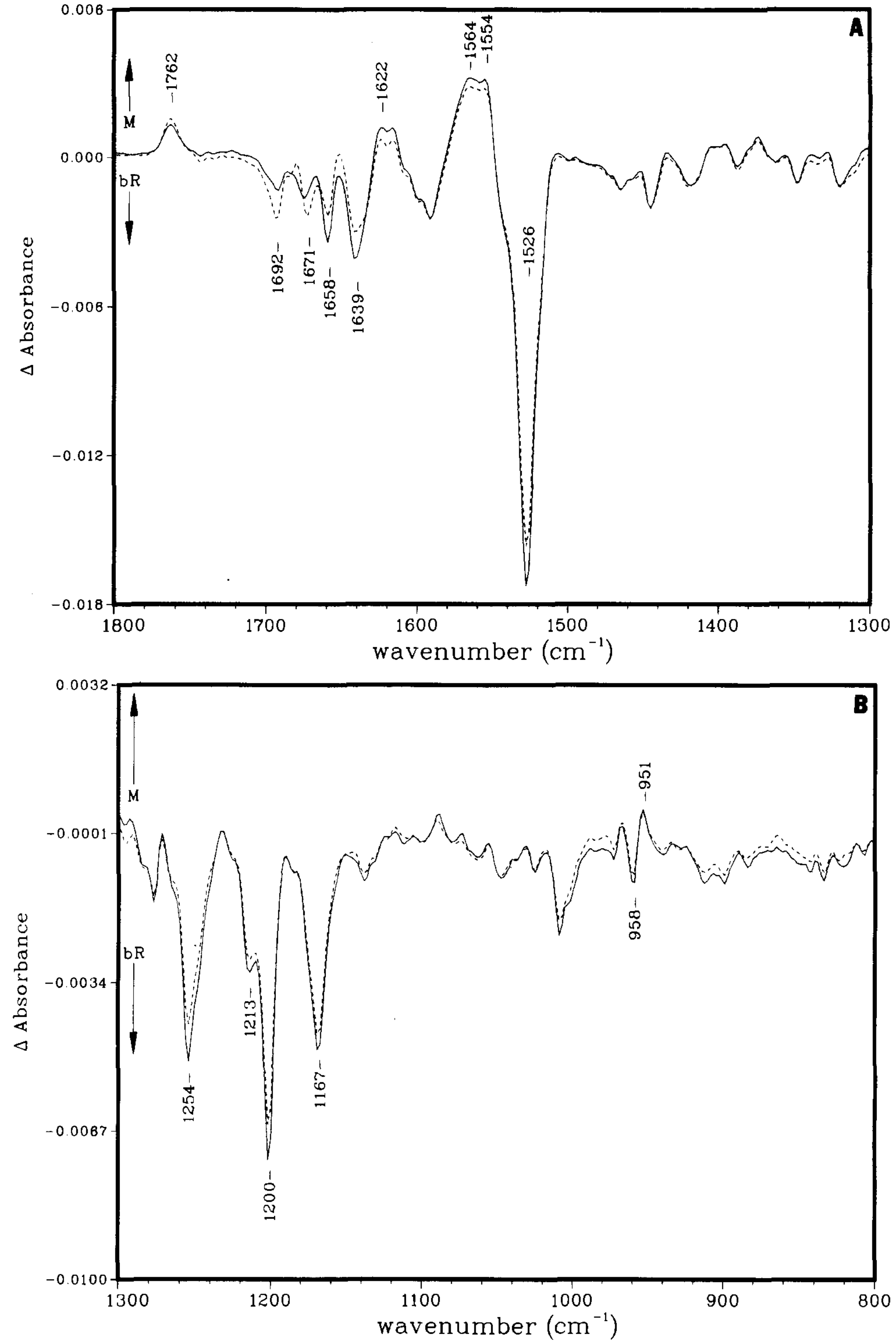

FIGURE 2: (A) $bR_{570} \rightarrow M_{412}$ FTIR difference spectra as in Figure 1, recorded at 250 K from 1800 to 1300 cm^{-1}. A long pass filter ($\lambda >$ 480 nm) was used to drive the sample into the M_{412} state. (B) The 1300–800-cm^{-1} region.

results are consistent with an all-trans configuration of retinal. In particular, rotations about single or double bonds along the polyene chain would be expected to cause a divergence in the C—C, C=C, and electronic transition moment directions. Although it is not possible without a more detailed analysis to put limits on the possible C—C and C=C bond rotations, our data do generally support the conclusion based on resonance Raman (Braiman & Mathies, 1980) and chemical extraction (Pettei et al., 1977) studies that an all-trans chromophore is present in bR_{570}.

Information about the orientation of the polyene plane with respect to the membrane can also be deduced from the dichroism of the HOOP wag vibrations. The IR transition moments of these vibrations are expected to be oriented approximately perpendicular to the polyene plane.[4] The 958-cm^{-1} peak of bR_{570}, which was assigned primarily to the coupled "A_u" HOOP mode of the C_{11} and C_{12} hydrogens on the basis of isotopically induced frequency shifts (Smith et al.,

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[4] The transition moment direction for a particular mode is determined by $\partial\bar{\mu}/\partial r$, where $\bar{\mu}$ is the dipole moment and r is the internal coordinate (Wilson et al., 1955). In the case of a HOOP coordinate of a planar polyene chain, symmetry considerations require that $\partial\bar{\mu}/\partial r$ be perpendicular to the polyene plane. "A_u" HOOP modes resulting from in-phase mixing of wags across double bonds should also be polarized out of plane.

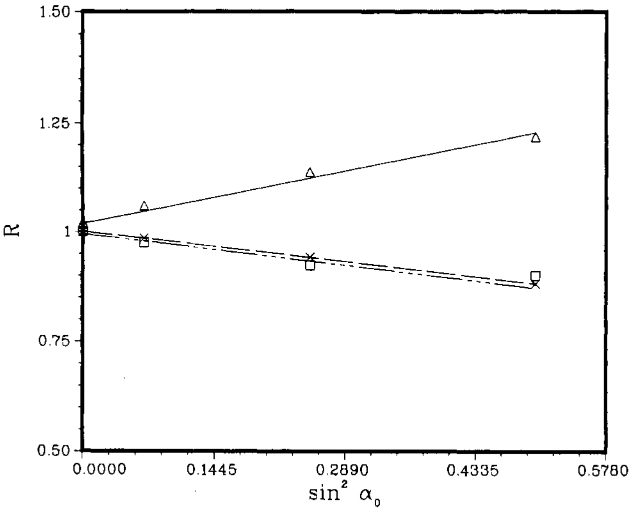

FIGURE 3: Plot of the dichroic ratio, R, vs. $\sin^2 \alpha_0$ (for $\alpha_0 = 0°, 15°, 30°$, and 45°) of the (—) 1762 (C=O), (--) 1526 (C=C), and (----) 1200 (C—C) cm^{-1} bands from the $bR_{570} \rightarrow M_{412}$ difference spectra at 250 K.

1986b), has a transition dipole moment that is only 10° from the membrane plane. Hence, the plane formed by the H-C_{11}=C_{12}H group is oriented nearly perpendicular to the membrane plane. Furthermore, if it is assumed that the retinal chromophore has an undistorted planar conformation, which is supported by the present measurements, by resonance Raman spectroscopy (Smith et al., 1986b), and by nuclear magnetic resonance spectroscopy (Harbison et al., 1985), then the chromophore (including the β-ionone ring) would be oriented with the short axis nearly perpendicular to the membrane plane.

Information can also be derived from the dichroism of the positive peaks in Figure 1 about the change in orientation of the chromophore during the transition to the early photointermediate, K_{630}. The large positive peak at 955 cm^{-1}, assigned to a K HOOP mode (Braiman, 1983), has a transition dipole moment oriented only 5–10° out of the membrane plane ($\Theta_T \approx 82.5°$). Similarly, the 942- and 974-cm^{-1} bands, assigned to the coupled "A_u" HOOP of HC_{11}=C_{12}H and to the C_{15} HOOP, respectively, have transition dipole moments oriented close to the membrane plane (see Table I). In addition, the transition moment of the 1514-cm^{-1} ethylenic C=C stretch mode of K_{630}, with $\Theta_T \approx 78 \pm 3°$, is parallel to that of bR_{570} within the range of error. The 1193-cm^{-1} peak of K_{630}, which arises from single bond stretching of carbons from 8 to 15 (Smith et al., 1986a), also has a transition moment that is nearly in-plane, although an exact estimate could not be made due to overlap with other peaks. Hence, it appears that despite the fact that the chromophore in K_{630} has undergone an isomerization to a 13-cis form (Braiman & Mathies, 1982), the dichroism of most of the vibrational bands remains similar to those in the bR_{570} chromophore. This suggests that the chromophore isomerization does not significantly alter the relative orientations of most bonds with the membrane plane, particularly in the portion of the chromophore between C_7 and C_{14}. This might occur, for example, if most of the movement was localized between C_{13} and the lysine residue including the Schiff base. The M_{412} C=C ethylenic stretch at 1564 cm^{-1} also appears to have approximately the same dichroism and orientation as the bR_{570} stretch, although overlap with another band near 1554 cm^{-1} precludes an accurate determination of Θ_T.

We also note that decreasing the temperature from 250 to 81 K causes the polyene chain to become oriented more parallel to the membrane plane. This may be related to a shortening of the bR α-helices as the temperature is decreased, as suggested by an increase in the amide II frequency (N–H in-plane bend) and a decrease in the amide A frequency (Fermi resonance of N–H stretch and first overtone of amide II) (Rothschild et al., 1985; Earnest and Rothschild, unpublished results).

The orientation of several protein-related groups can also be deduced from the data. The 1762-cm^{-1} positive peak arising from the C=O stretch mode of an aspartic acid group in M_{412} (Englehard et al., 1985) is oriented nearly 45° from the membrane plane ($\Theta_T \approx 43 \pm 4°$). This group undergoes protonation during M_{412} formation and may be part of a proton transport pathway (Nagle & Tristram-Nagle, 1983). The region between 1700 and 1630 cm^{-1} in both $bR_{570} \rightarrow K_{630}$ and $bR_{570} \rightarrow M_{412}$ contains several additional peaks that are highly dichroic. For example, the peaks at 1669 (negative) and 1661 cm^{-1} (positive) in the $bR_{570} \rightarrow K_{630}$ spectrum are seen only in the parallel polarization and thus reflect transition dipole moments that are predominantly out of plane. These peaks are likely to arise from the amide I mode of α-helical structure of the protein backbone and may reflect a change in hydrogen bonding of one or more amide carbonyl groups that are contained within the bR helices and hence oriented roughly perpendicular to the membrane plane. Alternatively, one or more carbonyl groups originally oriented parallel to the bR α-helix may switch to a predominantly in-plane orientation or there may be a very small change of the tilt angle away from the normal of one or more helices. The last interpretation is consistent with the assignment of the 1669- and 1661-cm^{-1} peaks to the out-of-plane and in-plane components of the amide I vibration in oriented bR films, respectively (Rothschild & Clark, 1979). In addition, these frequencies are close to those calculated for the out-of-plane and in-plane amide I normal mode vibrations of an α_{II} helix (Krimm & Dwivedi, 1982). The $bR_{570} \rightarrow K_{630}$ and $bR_{570} \rightarrow M_{412}$ difference spectra also display several other dichroic peaks (cf. Table I and Figures 1A and 2A), which are likely to reflect changes in the protein secondary structure. However, the small size of these peaks is inconsistent with a large change in the tilt angle of the α-helices during the $bR_{570} \rightarrow M_{412}$ transition that was recently suggested (Draheim & Cassim, 1985).

Polarized FTIR difference measurements were recently reported for the $bR_{570} \rightarrow M_{412}$ transition of PM at 20 °C (Nabedryk & Breton, 1986). The ethylenic C=C orientation in bR_{570} was estimated at 66 ± 4° and the carboxyl C=O bond orientation in M_{412} at 35 ± 5° to the membrane normal. These values are in agreement with our results, although the angles of the transition dipole moment of other bands—in particular in the HOOP mode and fingerprint regions—were not determined. In addition, several spectral features in their $bR_{570} \rightarrow M_{412}$ difference spectra disagree with previously reported low-temperature spectra as well as with recent 5-ms time-resolved FTIR measurements made at room temperature (Braiman et al., 1985). Their spectra can be reproduced more closely by reducing the degree of hydration of our samples (Braiman and Rothschild, unpublished results).

In conclusion, this study demonstrates the ability of polarized FTIR difference spectroscopy to probe the orientation of specific groups in the different functional intermediates of a protein. In the case of bR_{570} at 250 K, it is found that the polyene chain is oriented at 69 ± 3° from the membrane normal, in agreement with previous measurements of the electronic transition moment, and that the polyene plane is approximately perpendicular to the membrane plane in the

bR_{570} and K_{630} states. Further refinement of these initial results should be possible by calculating the transition dipole moment direction of the different normal modes with respect to the molecular geometry and by separating out contributions from different groups to multicomponent dichroic bands. Similar studies will be reported for the visual pigment rhodopsin.

ACKNOWLEDGMENTS

We acknowledge helpful discussions with P. Ahl, H. Marrero, R. Mathies, T. Pollard, S. Smith, and A. Warshel. D. Gray, D. Reny, and M. Tran provided technical assistance.

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