Orientation of rhodopsin α -helices in retinal rod outer segment membranes studied by infrared linear dichroism

(transmembrane protein structure/magnetic orientation)

MICHEL MICHEL-VILLAZ, HELEN R. SAIBIL*, AND MARC CHABRE

Laboratoire de Biophysique Moléculaire et Cellulaire, Département de Recherche Fondamentale, Centre d'Etudes Nucléaires de Grenoble 85X, 38041 Grenoble, France

Communicated by John E. Dowling, May 25, 1979

ABSTRACT Frog retinal rod outer segments, oriented by a magnetic field, were shown to contain rhodopsin α -helical segments preferentially aligned perpendicular to the plane of the disc membrane, by the technique of infrared linear dichroism. Infrared absorption parallel and perpendicular to the rod axes by peptide C=O groups, whose absorption band contains α -helical and random coil components at slightly different frequencies, showed positive dichroism centered on the α -helix frequency. We conclude that the α -helical portion of the protein has an average orientation in the transmembrane direction. Furthermore, infrared spectra of rods in $^2\text{H}_2\text{O}$ Ringer's solution exhibit two distinct peptide amino group absorption bands: the unexchanged N-H band, which is dichroic, and the proton-exchanged N- ^2H band, which is nondichroic. This implies that the oriented part of the protein is in the lipid bilayer, supporting a model for rhodopsin with a hydrophobic core containing partially oriented α -helices and hydrophilic ends consisting of unoriented polypeptide.

The visual pigment protein, rhodopsin, which constitutes about 85% of the rod outer segment membrane protein is now characterized structurally in some detail. Chemical labeling experiments have shown that it is accessible from both sides of the membrane (1). Low-angle x-ray and neutron solution scattering from rhodopsin in detergent micelles have provided models for the shape of the protein, whereas comparison of the x-ray and neutron data localizes its hydrated regions, demonstrating that it is an elongated molecule with a hydrophobic middle and hydrophilic ends (2-4). Neutron diffraction from oriented rods has shown that one half or more of the protein is embedded in the hydrophobic region of the lipid bilayer (5). Peptide hydrogen-deuterium exchange measurements by infrared spectroscopy have revealed the presence of a very hydrophobic core in which peptide N-H groups are inaccessible to proton exchange except under the most severe denaturation conditions (6). It has recently been suggested that this hydrophobic core contains oriented α -helices that are responsible for the large diamagnetic anisotropy of retinal rod outer segments (7, 8).

We present direct evidence for the preferential orientation of rhodopsin α -helices by using polarized infrared spectroscopy. Infrared absorption by peptide carbonyl groups (amide I) has slightly different frequency maxima depending on protein conformation and environment (9, 10), whereas the peptide amino group (amide II) absorption from proteins in $^2\text{H}_2\text{O}$ media has two very distinct peaks for unexchanged (N-H) and proton-exchanged (N- ^2H) groups. The dichroism of amide I (C=O stretching) and amide II or II' (N-H or N- ^2H bending) is a direct measure of the average orientation in different regions of the polypeptide backbone (11-13). Rhodopsin contains around 50% α -helix, the remainder being random coil (14, 15),

and the amide I band has a peak at about 1655 cm^{-1} and a shoulder at around 1640 cm^{-1} (16), which correspond to these two conformations. In α -helices, C=O bonds are approximately parallel to the helix axis, and the C=O stretching transition moment is roughly aligned with the bond direction. Thus, the dichroism of the 1655 cm^{-1} band in oriented rod outer segments provides a direct measure of helix orientation relative to the membrane plane. The amide II dichroism is of opposite sign because it is due to an N-H bending vibration; the dichroism also gives a qualitative check on the orientation of peptide bonds.

MATERIALS AND METHODS

Sample Preparation. The major difficulty of this work is the sample preparation. Infrared spectra in the amide I and II band regions require aqueous samples to be in highly purified $^2\text{H}_2\text{O}$ and to be very thin, in order to minimize the very strong background absorption caused by H_2O and H^2HO . Rods in bulk suspension orient almost perfectly in a magnetic field (5, 17), but decreasing the sample to the $25\text{--}50\text{-}\mu\text{m}$ thickness required causes some disorientation. This could be partly avoided by allowing the rods to sediment in the field in the presence of glutaraldehyde.

Dark-adapted frog rods in H_2O or $^2\text{H}_2\text{O}$ Ringer's solution obtained by shaking the retina and pelleting gently were resuspended in Ringer's solution and oriented by a magnetic field of 15 kilogauss, with their long axes parallel to the field direction. The sample was kept slightly below room temperature. The rods were allowed to sediment onto a CaF_2 window by one of two methods:

(i) Rods from several retinas in $^2\text{H}_2\text{O}$ Ringer's solution were spun down by an air-driven centrifuge between the pole pieces to improve their sedimentation, because rod density is not much greater than that of $^2\text{H}_2\text{O}$. The apparatus for this is shown in Fig. 1 left.

(ii) Rods in H_2O Ringer's solution from two retinas were allowed to sediment in the magnetic field by gravity for 30 min in the presence of 2.5% glutaraldehyde, which fixed the rods in an oriented layer on the lower window. The H_2O Ringer's solution was then replaced by $^2\text{H}_2\text{O}$ Ringer's solution without disturbing the rods by carefully syringing off the supernatant and replacing it with $^2\text{H}_2\text{O}$ Ringer's solution.

The sample cell, with its movable upper window, is shown in Fig. 1 right. The excess Ringer's solution was squeezed out by slowly lowering the second window onto the rod layer, to a final path length of $25\text{--}50\text{ }\mu\text{m}$. The final step of the preparation was done in $^2\text{H}_2\text{O}$ Ringer's solution in a H_2O -free atmosphere, and path lengths greater than $50\text{ }\mu\text{m}$ could not be used, due to the unavoidable residual traces of H_2O , which gave

The publication costs of this article were defrayed in part by page charge payment. This article must therefore be hereby marked "advertisement" in accordance with 18 U. S. C. §1734 solely to indicate this fact.

* Present address: Department of Biophysics, University of London King's College, 26-29 Drury Lane, London WC2B 5RL, England.

FIG. 1. (Left) Electromagnet and air-driven centrifuge for sedimenting oriented rod outer segments from suspension in $^2\text{H}_2\text{O}$ Ringer's solution. The rods orient with their long axes parallel to the magnetic field direction. The field was 15 kilogauss, and rotation speed was 600 rpm. (Right) Section of cylindrical sample holder, with the two CaF_2 windows in their final positions for a 25- to 50- μm -path-length infrared cell. During sedimentation the upper window was raised to allow for a 1-ml sample volume. It was then slowly lowered to the final separation while the rods were being observed in the telescope.

an H^2HO absorption band. Method *i* had a very low success rate because of turbulence when the excess Ringer's solution was removed. The rods were kept in dim red light.

Infrared Spectrophotometer. Once the sample cell was closed, it was placed in the spectrophotometer (Beckman IR 4240) with the rod axes perpendicular to the measuring beam. The reference cell contained Ringer's solution from the final supernatant of the rod preparation, and matched polarizers (Camlab, Cambridge, U.K.) were placed behind the cells with the polarization direction either parallel or perpendicular to the rod axes. Matched silicon filters to cut off visible light from the infrared beam were placed before sample and reference cells.

Rod Orientation Measurement. To obtain an estimate of the degree of rod orientation, samples were photographed in a low-power light microscope immediately after the spectra were recorded. A photograph of an infrared sample of rod outer segments prepared by method *ii* as described above is shown in Fig. 2. Optical diffraction patterns of the samples (or of photographs of the samples, which provided increased contrast) showed that there were two components to the orientation distribution, one highly oriented ($<5^\circ$ halfwidth at half maximum) and the other totally disoriented. This corresponds to the two populations of rods: those that were isolated in suspension during sedimentation and those that stuck to impurities and other rods. The parameter characterizing the orientation function is then the percentage of rods "perfectly" oriented. This percentage was measured by cutting out and weighing the two types of areas on the photographs, allowing for the finite angular distribution in the "perfectly" oriented fraction. For 60% oriented rods, the characteristic angle used in our calculation, obtained by averaging the parameter $\cos^2\phi$ over the angular distribution of rods, is $\phi = 31^\circ$. An internal check on this measurement is provided by the phospholipid $\text{C}=\text{O}$ dichroism as described in the analysis section.

RESULTS

An infrared spectrum of oriented frog rods in $^2\text{H}_2\text{O}$ Ringer's solution between wavenumbers 1800 cm^{-1} and 1400 cm^{-1} is shown in Fig. 3. The peak at 1740 cm^{-1} can be assigned to the phospholipid ester $\text{C}=\text{O}$ stretching (18). The amide I ($\text{C}=\text{O}$) band is centered around 1650 cm^{-1} , amide II ($\text{N}-\text{H}$) at 1550 cm^{-1} , and amide II' ($\text{N}-^2\text{H}$) at 1450 cm^{-1} . The peak at 1515 cm^{-1} is probably due to tyrosine (6). The base line for the amide II band was somewhat uncertain because of H^2HO absorption centered around 1460 cm^{-1} , which was not always perfectly equilibrated between sample and reference cells. Qualitatively, the dichroism observed indicates that the phospholipid $\text{C}=\text{O}$ groups lie preferentially parallel to the membrane plane, peptide $\text{C}=\text{O}$ and $\text{N}-\text{H}$ bonds are perpendicular to the plane, and exchanged peptide $\text{N}-^2\text{H}$ bonds are randomly oriented. The amide I dichroism is maximal at 1660 cm^{-1} , at which the ratio of optical density parallel and perpendicular to the rod axes is 1.15. No difference was observed between bleached and unbleached rods at the accuracy of these experiments.

The contribution of the rod outer segment lipids to the 1650 cm^{-1} region was checked by obtaining the infrared spectrum of cattle rod outer segment lipids extracted according to ref. 19. In the lipid spectrum there is a small peak around 1650 cm^{-1} assigned to $\text{C}=\text{C}$ stretching (18), but it has only 6% of the amplitude of the amide I band, normalizing via the phospholipid $\text{C}=\text{O}$ band common to both spectra. Estimating the dichroism expected in this band (20), we find it cannot contribute significantly to the amide I dichroism observed.

ANALYSIS

To estimate the degree of orientation of the α -helices in rod outer segment membrane, a quantitative analysis of the dichroism centered on the α -helical absorption is necessary. The dichroic ratio, D , is defined as $A_{\parallel}/A_{\perp}$, where $A_{\parallel}$ and $A_{\perp}$ are

FIG. 2. A photograph of oriented frog rods in a 25- μ m-path-length infrared cell prepared by sedimentation in H_2O Ringer's solution in the presence of glutaraldehyde followed by replacement of the H_2O by $^2\text{H}_2\text{O}$.

the optical densities of the absorption band when the polarizers are, respectively, parallel and perpendicular to the rod axes. To evaluate D for the α -helical component of the amide I band, the composite band must be decomposed into α -helical and random coil peaks. We did this graphically by using bandwidths obtained from the literature (13), assuming maxima at 1660 and 1640 cm^{-1} (see figure 1 of ref. 6) for the two peaks, and fitting

FIG. 3. The infrared spectrum of oriented rod outer segments, allowed to sediment in the magnetic field in the presence of glutaraldehyde, with the polarization direction parallel (—) and perpendicular (.....) to the rod axes. Absorption bands occur at frequencies of 1740 cm^{-1} , 1650 cm^{-1} , 1550 cm^{-1} , 1515 cm^{-1} , and 1450 cm^{-1} . The base line in the 1500 cm^{-1} region is uncertain because of the H_2^{18}O absorption, which is being subtracted from the signal by the reference cell. Similar spectra were obtained with centrifuged, unfixed rods.

the observed band to the sum of two Gaussian curves of appropriate frequency and bandwidth. This yields a dichroic ratio of $D = 1.30$ for the α -helix component, and is consistent with rhodopsin's containing 50% α -helix, whereas the dichroic ratio for the composite band at 1660 cm^{-1} is 1.15. To obtain the α -helix orientation relative to the membrane plane, two additional effects must be taken into account, both of which limit the observable dichroism: (i) the angle, χ , between the transition moment of the absorption peak and the helix axis, which has been estimated as 27° (21) or 40° (22, 23); and (ii) the spread of disc membrane orientations in the sample, given by the characteristic angle ϕ and measured on our photographs. If ψ is the cosine²-averaged angle between α -helix axes and the normal to the membrane plane, the combinations of these disorientation effects can be expressed simply by the product of their order parameters:

$$S(\chi) S(\phi) S(\psi) = S(\theta)$$

where $S(\theta) = \langle (3\cos^2\theta - 1)/2 \rangle$, θ being the angle determined by the dichroic ratio, measured as $D = 2\cos^2\theta/\sin^2\theta$. By using the values $\chi = 40^\circ$ and $\phi = 31^\circ$ for the parameters obtained as described above, we find $\psi = 38^\circ$. For extreme values of the parameters, we calculate a minimum $\psi = 29^\circ$ and a maximum $\psi = 50^\circ$. The most probable value for ψ of 38° is equivalent to all the α -helices' being inclined from the membrane normal by 38°, or to 43% of the helices' being perpendicular to the plane and 57% of the remaining α -helices' being randomly oriented. It should be pointed out that, in three dimensions, ψ for a completely random orientation of α -helices would be 54.7°, so that a ψ of 38° constitutes a clear preferential orientation perpendicular to the membrane plane. By using the same infrared approach, but under different geometrical conditions, Rothschild and Clark (24) have recently obtained a value of 26° for the average tilt angle ψ of the α -helices in bacteriorhodopsin relative to the purple membrane plane. This is to be compared with the value of 11° (R. Henderson, personal communication) measured on the three-dimensional bacteriorhodopsin model determined by crystallographic methods. The apparent discrepancy may be related to uncertainties in the determination of disorientation effects in the infrared data analysis. Our analysis is subject to the same uncertainties.

By taking into account the different geometry of the N-H bending absorption, in which the vibration is parallel to the membrane plane, its negative dichroism can be used to check the orientation result. In this case the angle χ between the transition moment and the helix axes is 75° (21, 23), and we measure a dichroic ratio of $D = 0.7$, estimating the baseline from the shape of the N-²H band. This gives about the same result (40°) for ψ , consistent with the calculations for the C=O absorption. Agreement between the two results indicates that the unexchanged N-H groups correspond to the same peptide linkages as the α -helical C=O groups responsible for the amide I dichroism. The amplitude of the N-²H band was difficult to evaluate because of uncertainty in the baseline, but no dichroism was ever observed in this band, indicating that hydrophilic regions of the polypeptide chain have no preferential orientation.

The disc membrane orientation can be checked independently by using the 1740 cm^{-1} band resulting from the planar orientation of the phospholipid C=O groups. By using the angle of 40° between the phospholipid C=O transition moment and the membrane plane (25), we obtain values for the membrane orientation parameter, ϕ , in good agreement with those estimated from our micrographs.

DISCUSSION

The dichroism we observe in absorption bands from C=O and unexchanged N-H peptide bonds is direct evidence of an overall orientation of the hydrophobic part of rhodopsin in a structure similar to, but less well-oriented than, bacteriorhodopsin (26). It confirms the interpretation that oriented α -helices are the source of rod outer segment diamagnetic anisotropy (7, 8). The lack of dichroism in the exchanged N-²H absorption band shows that the hydrophilic parts of the protein, probably on both sides of the membrane, have no preferential orientation. Our results give experimental support to models recently inferred from proteolytic digestion studies on rhodopsin in native and reconstituted disc membrane vesicles (27, 28). These models, deduced from the finding that rhodopsin is a transmembrane protein, also describe the transmembrane part as an array of α -helices. Our evidence in favor of such a structure may suggest that it is a general feature of transmembrane protein, as has been found in the two best characterized membrane protein structures, bacteriorhodopsin and glycophorin.

We thank the Groupe Liaison Hydrogène of the Département de Recherche Fondamentale for the use of the infrared spectrometer and discussions; H. B. Osborne, E. Navedryk-Viala, and R. Henderson for discussions; W. R. Lieb for comments on the manuscript and C. Roche for excellent technical collaboration. H.R.S. thanks the National Research Council of Canada for a North Atlantic Treaty Organization Postdoctoral Fellowship and the Commissariat à l'Energie Atomique for a Collaborateur Temporaire Etranger grant at different stages of this work. This work was supported in part by Délégation Générale à la Recherche Scientifique et Technique, Comité des Membranes Biologiques. M. M.-V. and M. C. are members of the Equipe de Recherche 199 of the Centre National de la Recherche Scientifique.

1. Fung, B. K. K. & Hubbell, W. L. (1978) *Biochemistry* **17**, 4403-4410.
2. Sardet, C., Tardieu, A. & Luzzatti, V. (1976) *J. Mol. Biol.* **105**, 383-407.
3. Yeager, M. (1975) *Brookhaven Symp. Biol.* **27**, III-3-III-36.
4. Osborne, H. B., Sardet, C., Michel-Villaz, M. & Chabre, M. (1978) *J. Mol. Biol.* **123**, 177-206.
5. Saibil, H. R., Chabre, M. & Worcester, D. L. (1976) *Nature (London)* **262**, 266-270.
6. Osborne, H. B. & Navedryk-Viala, E. (1977) *FEBS Lett.* **84**, 217-220.
7. Worcester, D. L. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5475-5477.
8. Chabre, M. (1978) *Proc. Natl. Acad. Sci. USA* **75**, 5471-5474.
9. Susi, H., Timasheff, S. N. & Stevens, L. (1967) *J. Biol. Chem.* **242**, 5460-5466.
10. Timasheff, S. N., Susi, H. & Stevens, L. (1967) *J. Biol. Chem.* **242**, 5467-5473.
11. Ambrose, E. J. & Elliot, A. (1951) *Proc. R. Soc. London Ser A* **205**, 47-60.
12. Peer, M. (1956) *Proc. R. Soc. London Ser. A* **236**, 136-140.
13. Elliot, A. (1969) *Infrared Spectra and Structure of Organic Long-Chain Polymers* (Edward Arnold, London).
14. Shichi, H. & Shelton, E. (1974) *J. Supramol. Struct.* **2**, 7-16.
15. Stubbs, G. W., Smith, H. G. & Litman, B. J. (1976) *Biochim. Biophys. Acta* **426**, 46-56.
16. Osborne, H. B. & Navedryk-Viala, E. (1978) *Eur. J. Biochem.* **89**, 81-88.
17. Chalazonitis, N., Chagneux, R. & Arvanitaki, A. (1970) *C. R. Hebd. Seances Acad. Sci. Ser. D* **271**, 130-133.
18. Chapman, D. (1965) *The Structure of Lipids* (Methuen, London), pp. 113-131.
19. Dawson, R. M. C., Hemington, N. & Lindsay, D. B. (1960) *Biochem. J.* **77**, 226-230.
20. Fringeli, U. P. (1977) *Z. Naturforsch.* **32C**, 20-45.
21. Bamford, C. H., Elliot, A. & Hanby, W. E. (1956) *Synthetic Polypeptides* (Academic, London).
22. Bradbury, E. M., Brown, L., Downie, A. R., Elliot, A., Fraser, R. D. B. & Hanby, W. E. (1962) *J. Mol. Biol.* **5**, 230-247.
23. Tsuboi, M. (1962) *J. Polym. Sci.* **59**, 139-143.
24. Rothschild, K. J. & Clark, N. A. (1979) *Biophys. J.* **25**, 473-488.
25. Verma, S. P. & Wallach, D. F. H. (1973) *Biochim. Biophys. Acta* **30**, 122-131.
26. Henderson, R. (1975) *J. Mol. Biol.* **93**, 123-138.
27. Hubbell, W. L. & Fung, B. K. K. (1978) in *Membrane Transduction Mechanisms*, eds. Cone, R. A. & Dowling, J. (Raven, New York), pp. 17-25.
28. Albert, A. D. & Litman, B. J. (1978) *Biochemistry* **17**, 3893-3900.