# Infrared Dichroism from the X-Ray Structure of Bacteriorhodopsin

Derek Marsh and Tibor Páli

Abteilung Spektroskopie, Max-Planck-Institut für biophysikalische Chemie, 37070 Göttingen, Germany

ABSTRACT A detailed comparison with the three-dimensional protein structure provides a stringent test of the models and parameters commonly used in determining the orientation of the $\alpha$-helices from the linear dichroism of the infrared amide bands, particularly in membranes. The order parameters of the amide vibrational transition moments are calculated for the transmembrane $\alpha$-helices of bacteriorhodopsin by using the crystal structure determined at a resolution of 1.55 Å (PDB accession number 1C3W). The dependence on the angle $\delta_M$ that the transition moment makes with the peptide carbonyl bond is fit by the expression $(\tfrac{3}{2} S_\alpha \cos^2 \alpha)\cos^2(\delta_M + \beta) - \tfrac{1}{2} S_\alpha$, where $S_\alpha$ (0.91) is the order parameter of the $\alpha$-helices, $\alpha$ (13°) is the angle that the peptide plane makes with the helix axis, and $\beta$ (11°) is the angle that the peptide carbonyl bond makes with the projection of the helix axis on the peptide plane. This result is fully consistent with the model of nested axial distributions commonly used in interpreting infrared linear dichroism of proteins. Comparison with experimental infrared dichroic ratios for bacteriorhodopsin yields values of $\Theta_A = 33 \pm 1°$, $\Theta_I = 39.5 \pm 1°$, and $\Theta_{II} = 70 \pm 2°$ for the orientation of the transition moments of the amide A, amide I, and amide II bands, respectively, relative to the helix axis. These estimates are close to those found for model $\alpha$-helical polypeptides, indicating that side-chain heterogeneity and slight helix imperfections are unlikely to affect the reliability of infrared measurements of helix orientations.

## INTRODUCTION

The dichroic ratios of the amide infrared bands obtained from proteins by using linearly polarized radiation can be used to determine the average orientation of the $\alpha$-helices in aligned samples (see, e.g., Tamm and Tatulian, 1997). Such structural information is especially useful in the case of membrane-bound proteins, for which aligned samples can readily be prepared. However, determination of the helix orientation requires knowledge of the orientation of the amide vibrational transition moments relative to the helix axis, and relies on a model involving independent nested axial distributions to characterize the various angular relations. Currently, there is by no means universal accord in the values adopted for the orientations of the transition moments (see, e.g., Axelsen et al., 1995; Bechinger et al., 1999), and a possible cause for concern is whether the values usually obtained from highly helical homopolypeptides may be applied reliably to membrane proteins. In addition, the model used for interpreting the data on dichroism has never been tested critically against a high-resolution protein structure.

The recent availability of the three-dimensional structure of bacteriorhodopsin at a resolution of 1.55 Å (Luecke et al., 1999) now allows these problems to be addressed. Direct calculation of the order parameters associated with the amide transition moments of the seven transmembrane helices provides a test of the orientational model, and comparison with dichroic ratios determined experimentally for bacteriorhodopsin (Rothschild and Clark, 1979; Nabedryk and Breton, 1981; Draheim et al., 1991) allows estimation of the orientation of the transition moments relative to the helix axis. We find that the calculated dependence of the amide order parameters on the angle that the transition moment makes with the peptide carbonyl bond can be described by a simple expression that is fully consistent with the nested axial model. In addition, the values that we derive for the orientation of the transition moments of the amide A, amide I, and amide II bands are close to those found for model polypeptides.

## DICHROIC RATIOS AND ORDER PARAMETERS

The protein assemblies are rotationally disordered within the plane of the membrane. (Note that, in itself, this rotational disorder is insufficient to establish axial symmetry in the distribution of transition moments; see Marsh, 1998.) With conventionally defined axes, the dichroic ratio, $R_z$, of the absorbances with parallel and perpendicular polarized radiation is then (Marsh, 1997):

$$R_z = \frac{E_x^2}{E_y^2} + \frac{E_z^2}{E_y^2} \cdot \frac{\langle M_z^2 \rangle}{\langle M_y^2 \rangle} \qquad (1)$$

where $\mathbf{E} = (E_x, E_y, E_z)$ is the radiation electric field vector with the $z$-axis along the membrane normal, and with the $x$- and $y$-axes in the membrane plane and within or orthogonal to the plane of incidence, respectively. It is understood that the electric field components in the sample are normalized to those at incidence. The transition moment vector, $\mathbf{M} = (M_x, M_y, M_z)$, is determined by the protein structure and its orientation relative to the membrane normal. The angle brackets in Eq. 1 indicate that summation must be per-

*Received for publication 10 April 2000 and in final form 23 June 2000.*

Address reprint requests to Dr. Derek Marsh, Abt. 010 Spektroskopie, MPI fuer Biophysikalische Chemie, Am Fassberg 11, D-37077 Gottingen, Germany. Tel.: 49-551-201-1285; Fax: 49-551-201-1501; E-mail: dmarsh@gwdg.de.

Tibor Páli's permanent address is Institute of Biophysics, Biological Research Center, H-6701 Szeged, Hungary.



0006-3495/01/01/305/08 $2.00

formed over the components of the transition moment of all amide groups. The electric field vector is specific to the particular infrared (IR) experiment, either transmission at non-zero angles of incidence, or attenuated total reflection.

The structure-specific quantity required is $\langle M_z^2\rangle/\langle M_y^2\rangle$. Because this is a ratio, the angle brackets can be taken as equivalent to average values. Axial symmetry is assumed for the distribution of transition moment orientations because the transmembrane helices of bacteriorhodopsin are relatively long ($\geq$21 residues) and summation is performed over all helices (166 residues) (see Marsh, 1998). This point is considered further in the Appendix. Under these conditions, the relation to the amide transition moment orientational distribution is given by (Marsh, 1997):

$$\frac{\langle M_z^2\rangle}{\langle M_y^2\rangle} = \frac{1 + 2\langle P_2(\cos\theta_M)\rangle}{1 - \langle P_2(\cos\theta_M)\rangle} \tag{2}$$

where $\theta_M$ is the orientation of an individual transition moment relative to the membrane normal, and the second-order Legendre polynomial is $P_2(x) = \frac{1}{2}(3x^2 - 1)$. The average $\langle P_2(\cos\theta_M)\rangle$ is therefore the order parameter of the amide transition moments, relative to the membrane normal.

## AMIDE ORDER PARAMETERS FROM X-RAY STRUCTURE

The amide order parameter can be calculated from the molecular coordinates according to:

$$\langle P_2(\cos\theta_M)\rangle_o = \frac{3}{2N_r}\sum_{i=1}^{N_r}\cos^2\theta_{M,i} - \frac{1}{2} \tag{3}$$

where $\theta_{M,i}$ is the orientation of the amide transition moment of peptide $i$, relative to the ordering axis (i.e., the membrane normal), and $N_r$ is the number of peptide units over which the summation is made. The summation may be over the whole protein, or simply over all helices, depending on the dichroic ratio measured (see below).

The transition moment for an individual amide lies within the peptide plane and is tilted by an angle $\delta_M$ to the C′—O bond, in a direction away from the C′—N bond (Fraser and MacRae, 1973). It is assumed that $\delta_M$ is the same for all peptide units, at least for a given secondary structure, because it is determined only by the peptide unit and its H-bonding. A unit vector in the direction of the transition moment is given by:

$$\hat{\mathbf{r}}_M = \left(\cos\delta_M + \frac{\sin\delta_M}{\tan\angle OC'N}\right)\frac{\overrightarrow{C'O}}{|\overrightarrow{C'O}|} - \frac{\sin\delta_M}{\sin\angle OC'N}\frac{\overrightarrow{C'N}}{|\overrightarrow{C'N}|} \tag{4}$$

where the peptide plane is defined by the amide bond vectors $\overrightarrow{C'O}$ and $\overrightarrow{C'N}$ for each amide, $i$. The value of $\cos\theta_{M,i}$ is then given by the scalar product of this vector with a unit vector parallel to the ordering axis (i.e., the membrane normal). Both bond vectors, $\overrightarrow{C'O}$ and $\overrightarrow{C'N}$, are obtained from the molecular coordinates, as is the angle $\angle OC'N$ between these bonds.

It is useful to note that, for a given $\alpha$-helix, the orientation $\Theta_M$ of the amide transition moment relative to the helix axis is given by (Marsh et al., 2000):

$$\cos\Theta_M = \cos\alpha\cdot\cos(\beta + \delta_M) \tag{5}$$

where $\alpha$ is the angle that the peptide plane makes with the helix axis and $\beta$ is the angle (away from C′—N) that the C′—O bond makes with the projection of the helix axis on the peptide plane. From the refined coordinates of $\alpha$-poly-L-alanine, $\alpha = 6.1°$ and $\beta = 12.9°$; from an energy-refined structure of a standard right-handed $\alpha$-helix, $\alpha = 3.3°$ and $\beta = 14.5°$ (Marsh et al., 2000). As for $\delta_M$, the value of $\Theta_M$ is assumed to be effectively constant, and it is this orientation of the amide transition moment that is normally used in interpreting IR dichroism measurements on $\alpha$-helical proteins (see, e.g., Tamm and Tatulian, 1997).

The amide order parameter for the whole protein given in Eq. 3 may be related to the orientation of the transmembrane helices via the transition moment orientation $\Theta_M$ of Eq. 5. This requires the approximation of independent axial distributions that is normally assumed in interpreting the amide IR dichroism of proteins. For independent nested axial distributions, $j$, with order parameters $\langle P_2(\cos\theta_j)\rangle_j$, the net order parameter is given by:

$$\langle P_2(\cos\theta_M)\rangle_o = \prod_j\langle P_2(\cos\theta_j)\rangle_j \tag{6}$$

which follows from the spherical harmonic addition theorem. If $\langle P_2(\cos\gamma_\alpha)\rangle$ is the order parameter of the helix axes relative to the membrane normal, then (see, e.g., Rothschild and Clark, 1979; Rothschild et al., 1980):

$$\langle P_2(\cos\theta_M)\rangle_o = P_2(\cos\Theta_M)\langle P_2(\cos\gamma_\alpha)\rangle f_\alpha \tag{7}$$

where $f_\alpha$ is the fraction of peptides that are in $\alpha$-helices. It is assumed that the remainder of the peptides are randomly oriented on average, i.e., that their order parameter is zero. If summation is made only over the transmembrane helices in Eq. 3, then $f_\alpha = 1$. Equation 7 is the expression routinely used to extract the mean helix orientation from IR dichroism measurements (see, e.g., Tamm and Tatulian, 1997). Because Eq. 7 depends directly on Eq. 6, it requires axial symmetry for the distribution of amide transition moments about the axis of each helix (see Appendix).

## AMIDE ORDER PARAMETERS FROM DICHROIC RATIOS

Experimental measurements of the amide order parameter, $\langle P_2(\cos\theta_M)\rangle_{exp}$, can differ from the ideal structural quantity, $\langle P_2(\cos\theta_M)\rangle_o$, calculated above, for various technical reasons. The membrane sample may not be perfectly aligned and the method of measurement of the dichroic ratio from the amide band may differ. Again using Eq. 6, the relation between the experimental and structural order parameters may be written from Eq. 7 as:

$$\langle P_2(\cos\theta_M)\rangle_{exp} = \langle P_2(\cos\theta_M)\rangle_o \langle P_2(\cos\gamma_{ms})\rangle f'_\alpha/f_\alpha \quad (8)$$

where $\gamma_{ms}$ is the angle of mosaic spread in sample alignment, and $f'_\alpha$ is the fraction of α-helix contributing to the absorbance of the band position at which the dichroic ratio is measured. Generally, the degree of sample alignment is rather high and $\langle P_2(\cos\gamma_{ms})\rangle \sim 0.95$ (Rothschild and Clark, 1979; Clark et al., 1980).

In Eq. 8, the factor $f'_\alpha/f_\alpha$ allows for the possibility that the band position characteristic of an α-helix (or band maximum) at which the dichroic ratio is measured may contain an admixture from overlap with disordered band components. Whenever, measurement is made at the α-helix position, $f'_\alpha > f_\alpha$, where $f_\alpha$ is the true fraction of α-helix in the structure. Alternatively, when integration is performed over the entire amide band, $f'_\alpha = f_\alpha$, where again $f_\alpha$ is the true fraction of α-helix in the protein, assuming the remainder of the protein to be disordered on average. If, however, measurement of the dichroic ratio is made just on the α-helical component, without admixture from other components, then $f_\alpha = 1$ (cf. previous section) and $f'_\alpha = 1$. For a highly helical protein, such as bacteriorhodopsin, measurements at the amide band maximum are likely to approximate to the last situation, especially for the amide II band. A possible exception is the amide A band, for which the different components remain unresolved.

## BACTERIORHODOPSIN 1.55 Å STRUCTURE

The x-ray structure of bacteriorhodopsin at 1.55 Å resolution of Luecke et al. (1999) is used (PDB accession number 1C3W). A view of the protein backbone along the crystallographic *c*-axis is given in Fig. 1. The latter axis is parallel to the sixfold symmetry axis, and is taken to be equivalent to the ordering axis or membrane normal (Luecke et al., 1998, 1999). The orientations of the individual transmembrane helices relative to the membrane normal are given by $\gamma_\alpha$ = 23.7°, 4.7°, 10.6°, 7.9°, 12.2°, 14.3°, and 16.0° for helices A–G, respectively. These values were calculated by fitting cylinders to the backbone atoms of the individual helices (see Fig. 1) with the program MOLMOL (Koradi et al., 1996). This corresponds to an orientational order parameter for the assembly of transmembrane helices of $\langle P_2(\cos\gamma_\alpha)\rangle = 0.915$, relative to the membrane normal.

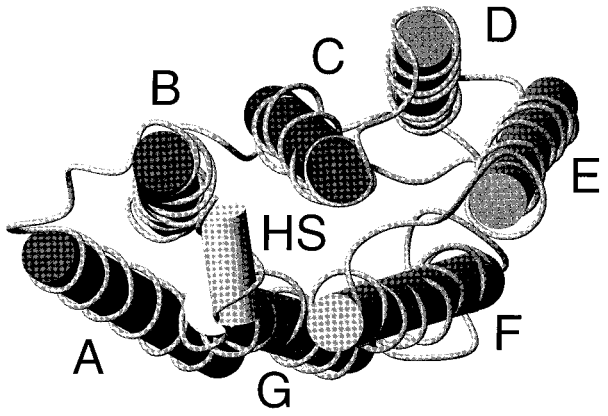


FIGURE 1 Projection of the backbone structure of bacteriorhodopsin (Luecke et al., 1999; PDB accession number 1C3W), viewed along the bilayer normal. Visualization is with the program MOLMOL (Koradi et al., 1996). The individual transmembrane helices, A–G, are fitted by cylinders according to the MOLMOL algorithm. HS is a short surface helix.

The angle the C′—O bond of each peptide unit makes with the crystallographic *c*-axis is given in Fig. 2. These values are obtained from the x-ray coordinates by using Eq. 4 with $\delta_M = 0$. The transmembrane helices (A–G) are clearly discerned, with alternating polarity. Helices B and D are most closely oriented to the bilayer normal. This is seen by the relatively small amplitude of the periodic variation in their orientation along the helix. Furthermore, the mean orientation of these two helices corresponds most closely with the orientation $\Theta_{C'O}$ = 14.2–14.8° of the carbonyl bond to the axis of an α-helix (Marsh et al., 2000). The mean angle of the carbonyl bond is larger for the more tilted helices, and also the amplitude of their helical periodicity is greater. Distortions in the orientation arising from proline residues Pro-50, Pro-91, and Pro-186 in helices B, C, and E, respectively, and from the π-bulge in helix G at Ala-215, appear to be rather localized.

The values for the orientations of the peptide carbonyls, $\theta_{C'O}$, in Fig. 2 give a qualitative impression of the effects of the helix orientations on the dichroic ratios. For quantitative results, the direction of the transition moment specified according to Eq. 4 must be obtained with a specific value of the angle $\delta_M$. The values of $\cos^2\theta_M$ for $\delta_M = 20°$, which corresponds to the region between the amide I and amide A vibrations of an α-helix (Marsh et al., 2000), are given in Fig. 3 as a function of peptide position in the sequence. Data of this type can be used to calculate order parameters for specific sections of the protein, as desired, by summation according to Eq. 3. Values of the order parameter were obtained from the x-ray coordinates in this way by using Eqs. 3 and 4. These are given as a function of the amide transition moment orientation, $\delta_M$ (see Eq. 4), in Fig. 4. Results are presented for summation over the entire protein and for summation over the transmembrane helices only (see Eq. 3). The former corresponds to dichroic ratios mea-

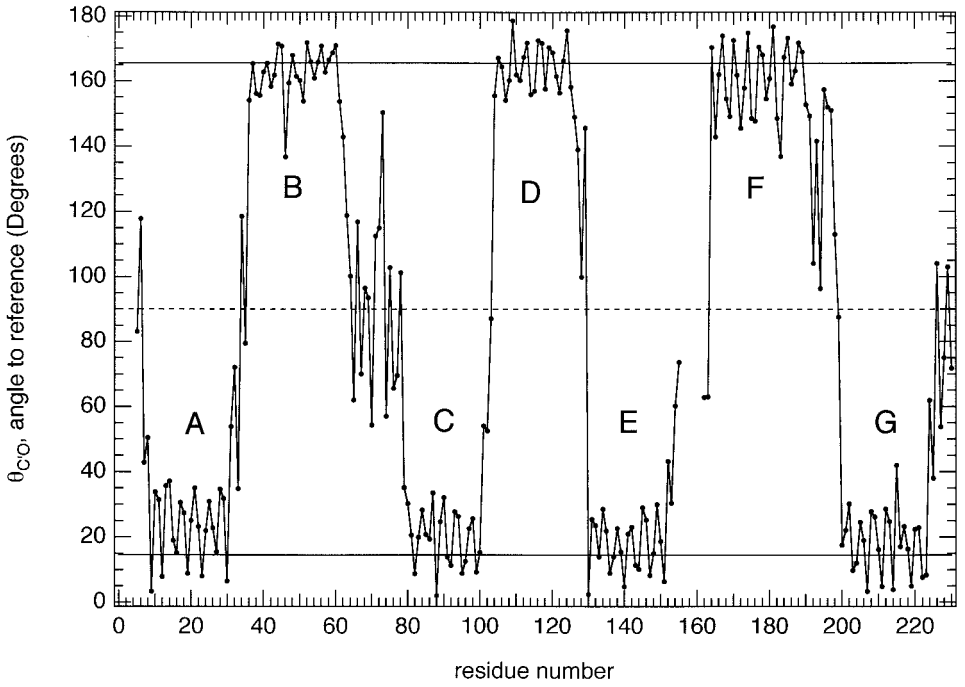


FIGURE 2 Angle, $\theta_{C'O}$, which the individual peptide carbonyl bonds make with the crystallographic *c*-axis from the structure of bacteriorhodopsin (PDB:1C3W) of Luecke et al. (1999). Transmembrane helices are labeled A–G. Residues 156–162 of the E–F loop are disordered. Full horizontal lines represent the orientation of the C′—O bond relative to the axis of an $\alpha$-helix.

sured from absorbances integrated over the whole amide band, and the latter to dichroic ratios measured solely from the $\alpha$-helical component of the amide band. The transmembrane $\alpha$-helices are defined, according to Swiss-PDB viewer v.3.5 (Guex and Peitsch, 1997), as residues 9–30, 37–62, 81–101, 105–127, 131–153, 165–191, and 201–224. The short three-residue surface $3_{10}$-helix is neglected.

By combining Eqs. 5 and 7, the dependence of the amide order parameter on the transition moment orientation $\delta_M$ should be given by:

$$\langle P_2(\cos\theta_M)\rangle_o = [3/2\,\langle P_2(\cos\gamma_\alpha)\rangle f_\alpha \cos^2\alpha] \times \cos^2(\delta_M + \beta) - 1/2\,\langle P_2(\cos\gamma_\alpha)\rangle f_\alpha \quad (9)$$

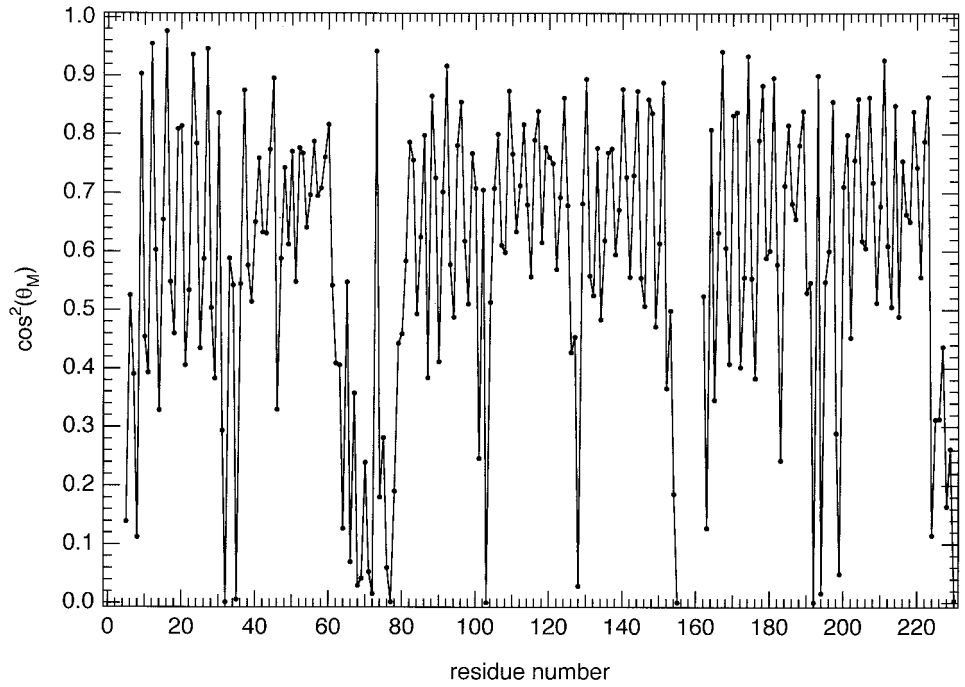


FIGURE 3 Values of $\cos^2\theta_M$ as a function of sequence position, where $\theta_M$ is the orientation of the transition moment of an individual peptide group relative to the crystallographic *c*-axis for the bacteriorhodopsin structure of Luecke et al. (1999). Data are calculated from the PDB:1C3W coordinates by using Eq. 4 for $\delta_M$ = 20°.

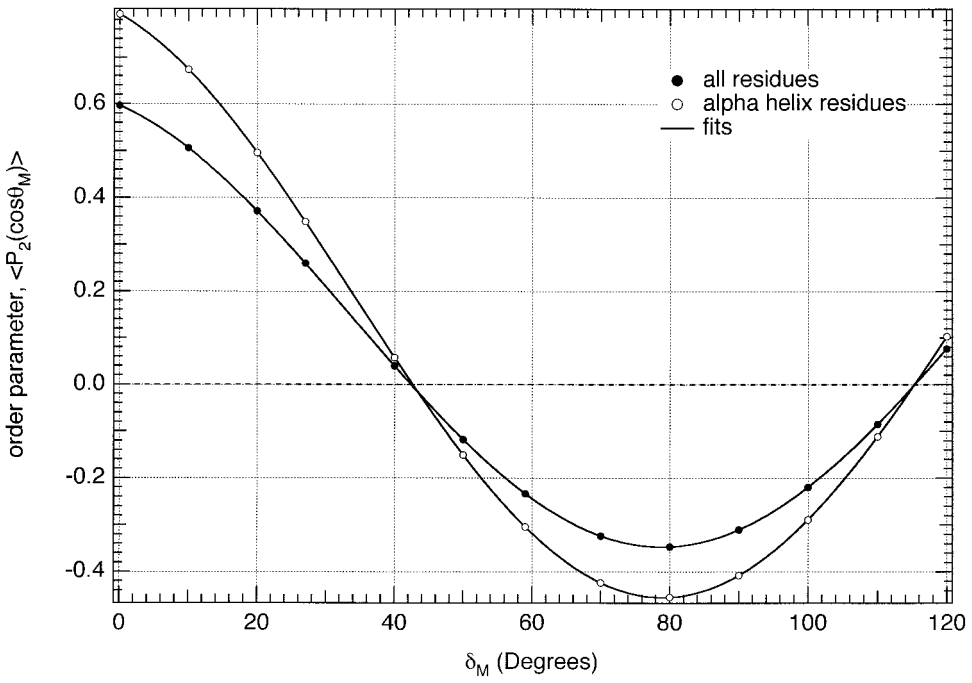


FIGURE 4 Orientational order parameters, $\langle P_2(\cos\ \theta_M)\rangle_o$, for the amide transition moments relative to the crystallographic *c*-axis for the bacteriorhodopsin structure of Luecke et al. (1999). Order parameters are given as a function of the orientation $\delta_M$ of the peptide transition moment relative to the C′—O bond by using Eqs. 3 and 4. *Filled circles*: summation according to Eq. 3 over all peptides in the structure; *open circles*: summation over the transmembrane helices only. Continuous lines represent nonlinear least-squares fits of Eq. 9, with the following optimized fitting parameters: $\langle P_2(\cos\gamma_\alpha)\rangle\cos^2\alpha = 0.864$, $\langle P_2(\cos\gamma_\alpha)\rangle = 0.911$, $\beta = 11.0°$ (*open circles*) and $f_\alpha\langle P_2(\cos\gamma_\alpha)\rangle\cos^2\alpha = 0.653$, $f_\alpha\langle P_2(\cos\gamma_\alpha)\rangle = 0.694$, $\beta = 11.1°$ (*filled circles*).

if the model conventionally used for interpreting amide dichroism data is to hold. Here $f_\alpha = 1$ when only the transmembrane helices are included in the summation over the individual amides. It is seen from Fig. 4 that the dependence calculated from the x-ray structure can be fit almost perfectly with Eq. 9, where the fitting parameters are $\beta$, the factor multiplying the cosine-squared term, and the constant offset. This consistency, especially for $f_\alpha = 1$, demonstrates that the $\alpha$-helices of bacteriorhodopsin are rather regular and can be described by a single effective configuration for the peptide groups. It also supports the assumption of axial symmetry about the helix axes that was made in Eq. 7 (also see Appendix). The good fit is highly significant because there is nothing inherent in the data (which are obtained from x-ray atomic coordinates) that requires Eq. 9 to hold exactly (see also Marsh, 1998). Agreement with this functional form therefore implies that the model used for interpreting IR dichroism measurements is an adequate representation of the molecular structure when the appropriate summation is made over the individual amide coordinates. Further consistency is found in the parameters of Eq. 9 that may be deduced from the fits.

Both fits in Fig. 4 yield an essentially identical value $\beta = 11°$ for the orientation of the amide carbonyl relative to the projection of the helix axis on the peptide plane. This can be compared with values of $\beta = 12.9°$ or 14.5° that were given above for $\alpha$-poly-L-alanine or a standard $\alpha$-helix, respectively. From the fit with $f_\alpha = 1$, it can be deduced that the orientation of the peptide plane to the helix axis is $\alpha = 13°$, as compared with values of $\alpha = 6.1°$ or 3.3° for $\alpha$-poly-L-alanine or a standard $\alpha$-helix, respectively. The values obtained for $\alpha$ and $\beta$ from Fig. 4 therefore illustrate that only limited structural deviations from a standard $\alpha$-helix are found in the transmembrane helices of bacteriorhodopsin. Note that these deviations are not very much greater in size than those between the standard $\alpha$-helix reference systems that were obtained from refined x-ray coordinates and an energy-minimized structure, respectively.

The order parameter of the helix axis is given by $\langle P_2(\cos\gamma_\alpha)\rangle = 0.91$, from the fit for $f_\alpha = 1$ in Fig. 4. This compares well with the value of $\langle P_2(\cos\gamma_\alpha)\rangle = 0.92$ deduced above, directly from the molecular structure (see Fig. 1). By combining the coefficients of the $\delta_M$-dependent (or $\delta_M$-independent) term from the two fits, a consistent value of $f_\alpha = 0.76$ is obtained for the fraction of $\alpha$-helical peptides. This can be compared with the ratio of the respective numbers of peptides used in the two calculations, which is $f_\alpha = 166/220 = 0.75$. The closeness of these two values indicates that the assumption that the nonhelical residues have a vanishing order parameter (cf. above), is substantially correct.

## COMPARISON WITH EXPERIMENT

Several measurements have been made of the dichroic ratios from highly oriented purple membranes by using transmission IR spectroscopy at non-zero angles of incidence (Rothschild and Clark, 1979; Nabedryk and Breton, 1981; Draheim et al., 1991). For this experimental configuration, the ratios of the components of the electric field vectors appearing in Eq. 1 are given by Snell's law:

$$\frac{E_z^2}{E_y^2} = 1 - \frac{E_x^2}{E_y^2} = \frac{\sin^2 i}{n^2} \tag{10}$$

where $i$ is the angle of incidence and $n$ is the refractive index of the sample. The various experimental measurements of order parameter differ in the value assumed for $n$, and also in the way that allowance has been made for possible band overlap characterized by the value of $f'_\alpha$ in Eq. 8.

To account for anomalous dispersion at the amide IR frequencies, a value of $n = 1.7$ has been used (Rothschild and Clark, 1979). This corresponds to a mean of the local maximum values in the frequency-dependence of $n$ (Henniker, 1973). Other workers have assumed a value of $n = 1.5$ (Nabedryk and Breton, 1981; Draheim et al., 1991), corresponding to the frequency-independent contribution. Because the anomalous dispersion goes through zero at the center of an absorption band, and also because even lower values of $n = 1.4$ have been reported for the amide I and amide II bands (Fringeli et al., 1989), an average value of $n = 1.5$ is used here. Also, because inconsistent methods of accounting for disordered and other structures have been used (Nabedryk and Breton, 1981; Draheim et al., 1991), dichroic ratios have been recalculated from the original spectral maxima according to the convention of Nabedryk and Breton (1981) without correction for spectral overlap, which is considered subsequently.

The various measurements standardized in the above ways, i.e., for $n = 1.5$ and without corrections for $f'_\alpha$, are then expressed as amide order parameters corresponding to the band maxima. For the amide I, the values of $\langle P_2(\cos \theta_I)\rangle_{exp}$ are 0.43 ± 0.01, 0.33, and 0.31 from the data of Rothschild and Clark (1979), Nabedryk and Breton (1981), and Draheim et al. (1991), respectively. For the amide II, the values of $\langle P_2(\cos \theta_{II})\rangle_{exp}$ are −0.29 and −0.31 from the data of Nabedryk and Breton (1981) and Draheim et al. (1991), and for the amide A, $\langle P_2(\cos \theta_A)\rangle_{exp} = 0.51 \pm 0.01$, 0.50, 0.50 from the data of Rothschild and Clark (1979), Nabedryk and Breton (1981), and Draheim et al. (1991), respectively.

If it is assumed that $\langle P_2(\cos \gamma_{ms})\rangle f'_\alpha/f_\alpha = 1$ in Eq. 8, i.e., perfect sample alignment and no overlap from disordered components, then least-squares fitting of $\langle P_2(\cos \theta_M)\rangle_o$ (i.e., Eq. 9) for the transmembrane helices alone to the experimental data yields values of $\delta_A = 20 \pm 1°$, $\delta_I = 27 \pm 1°$, and $\delta_{II} = 59 \pm 2°$ for the orientation of the transition moment of the amide A, amide I, and amide II bands, respectively, relative to the amide carbonyl. Using Eq. 5 together with the values of $\alpha$ and $\beta$ established by the fit in Fig. 4 then yields values of $\Theta_A = 33 \pm 1°$, $\Theta_I = 39.5 \pm 1°$, and $\Theta_{II} = 70 \pm 2°$ for the orientation relative to the helix axis of the transition moment of the amide A, amide I, and amide II bands, respectively. For comparison, values of $\Theta_M$ obtained recently from mid-IR measurements on an $\alpha$-helical modified polyglutamate copolymer are $\Theta_A = 29°$, $\Theta_I = 38°$, and $\Theta_{II} = 73°$ (Marsh et al., 2000). This comparison suggests both that the samples are reasonably well aligned with relatively little band component overlap, and that the orientations of the amide transition moments in bacteriorhodopsin transmembrane helices are similar to those determined from model polypeptides. The latter is especially the case when it is considered that neglect of sample misalignment and possible band overlap yields maximum values for $\Theta_A$ and $\Theta_I$, and a minimum value for $\Theta_{II}$. Assuming a sample misalignment characterized by $\langle P_2(\cos \gamma_{ms})\rangle = 0.95$ changes the values determined for $\Theta_M$ by ~1°, i.e., within the error range. Correspondingly, overlap with non-dichroic bands of 10–15% relative intensity (e.g., from side chains) would change $\Theta_M$ by 2–3° or 3–4°, depending on the band (the smaller differences being for the amide I and the larger ones for the amide A, with the amide II intermediate).

It should also be noted that the choice of the refractive index of the sample (see above) is relatively important for these calculations. Increasing the refractive index from $n = 1.5$, which we have argued is appropriate, to the extreme value of $n = 1.7$ decreases the calculated orientation of the amide A and amide I transition moments by almost 3°, and increases that of the amide II by 11°. Taking a value of $n = 1.43$ that is frequently adopted in attenuated total reflection studies (Tamm and Tatulian, 1997), however, changes these values by only +1° and −2°, respectively.

The possible degree of misalignment and band component overlap also can be estimated by assuming the values of $\delta_M$ that were determined for the modified polyglutamate copolymer (Marsh et al., 2000). By using the results of Fig. 4 together with Eq. 8, the values of $\langle P_2(\cos \gamma_{ms})\rangle f'_\alpha/f_\alpha$ are estimated to be: 0.84–0.88, 0.87–0.93, and 0.93–1.0 for the amide A, amide I, and amide II bands, respectively. These estimates confirm the expectation (cf. above) that the effects of band component overlap are greatest for the amide A band and least for the amide II band. In addition, the high value estimated for the amide II band implies that the samples are well aligned, consistent with direct observation (Clark et al., 1980). The total fraction of $\alpha$-helix in the bacteriorhodopsin structure is given by the ratio of the respective numbers of amide residues as $f_\alpha = 166/247 = 0.67$. Comparison with the above therefore confirms, as expected, that $f'_\alpha$ is considerably greater than $f_\alpha$ when measurement is performed at the amide band maxima.

Finally, the values of the orientations $\Theta_M$ of the amide transition moments, relative to the helix axis that were obtained from Fig. 4, can be used to estimate the order parameters, $\langle P_2(\cos \gamma_\alpha)\rangle$, of the transmembrane helices from the experimentally measured amide order parameters, $\langle P_2(\cos \theta_M)\rangle_{exp}$. This is the method normally used for analyzing data from IR dichroism. By combining Eqs. 8 and 9, with $f_\alpha = 1$ and $\langle P_2(\cos \gamma_{ms})\rangle f'_\alpha/f_\alpha = 1$ that are appropriate for $\Theta_A = 33 \pm 1°$, $\Theta_I = 39.5 \pm 1°$, and $\Theta_{II} = 70 \pm 2°$, values of $\langle P_2(\cos \gamma_\alpha)\rangle = 0.91 \pm 0.04$, $0.91 \pm 0.06$, and $0.91 \pm 0.09$ are obtained from the experimental data for the amide A, amide I, and amide II bands, respectively. These can be compared with the values of $\langle P_2(\cos \gamma_\alpha)\rangle = 0.92$ and 0.91 that were deduced directly from the structure and from

fitting the data of Fig. 4, respectively. The good agreement illustrates the consistency of the conventional determinations of helix order parameters from IR dichroism with calculations directly from the molecular structure. The precision obtained with dichroic ratios from the amide II band, the transition moment of which is oriented preferentially perpendicular to the helix axis, however, is not as high as that with the amide I and amide A bands, whose transition moments are oriented preferentially along the helix axis.

## CONCLUSIONS

Analysis of the orientation of the peptide groups in the high-resolution x-ray structure of bacteriorhodopsin (Luecke et al., 1999) demonstrates that the average orientation, $\Theta_M$, of the amide transition moments of the transmembrane helices are close to those found in model $\alpha$-helical polypeptides (cf. Marsh et al., 2000). The latter, therefore, may be used with some confidence in analyzing data from IR dichroism for $\alpha$-helical proteins of which the detailed three-dimensional structure is not known. Furthermore, a detailed comparison of direct calculations from the molecular structure fully supports the analysis in terms of nested axial distributions (see, e.g., Rothschild and Clark, 1979) that is conventionally applied to IR dichroism data from $\alpha$-helical proteins. Finally, the methods in this paper, particularly the parametrization in terms of Eq. 9, can be applied quite generally to the calculation of the amide order parameters of other proteins for which the three-dimensional structure is known. Comparison with IR dichroism will then be especially informative in cases for which the symmetry axis of the crystal structure does not coincide with the ordering or director axis in the membrane (e.g., Silvestro and Axelsen, 1999).

## APPENDIX

### Axiality of the helical sums

To check on the assumption of axiality for the sums over individual helices (cf. Marsh, 1998), the following further calculations were performed. The tensor corresponding to the average pairwise products of the direction cosines of the transition moments $\langle \cos\theta_i \cos\theta_j \rangle$ for $i, j \equiv x, y, z$ was first evaluated in the membrane coordinate system for each transmembrane helix by using Eq. 4. Each of these tensors was then diagonalized to yield the elements $\langle \cos^2 \Theta_i \rangle$ in the helix axis system, i.e., for $i \equiv 1, 2, 3$ where the 3-axis corresponds to the helix axis. From the resulting eigenvectors, the orientation $\gamma_3$ ($\equiv\gamma_\alpha$) of each helix axis relative to the membrane normal (i.e., the $z$-axis) was also calculated. Results for the seven transmembrane helices are given, for different values of $\delta_M$, in Table 1. The values of $\delta_M$ = 20°, 27°, and 59° were chosen to correspond approximately to those appropriate to the amide A, amide I, and amide II bands, respectively (cf. above).

From Table 1 it is seen that the ordering tensors of the amide moments relative to the helix axis are approximately axial, i.e., $\langle \cos^2 \Theta_1 \rangle \approx \langle \cos^2 \Theta_2 \rangle$, for each transmembrane helix and for the different orientations $\delta_M$ of the transition moment. This result is required for the assumption of axial symmetry in Eq. 2 (Marsh, 1998), and further for Eq. 7, and hence Eq. 9, to hold. Additionally, for a given helix, the values of $\gamma_\alpha$ calculated for the orientation of the helix axis are reasonably consistent between the different values of $\delta_M$. With the exception of helix C, the values of $\gamma_\alpha$ are also reasonably consistent with those calculated by an entirely different method, viz., the fitting of cylinders to the helix backbones that is given in Fig. 1.

**TABLE 1 Mean square values of the direction cosines, $\langle \cos^2 \Theta_i \rangle$, of the transition moment, relative to the axes ($i \equiv 1$ and 2) orthogonal to the helix axis and the orientation, $\gamma_\alpha$, of the helix axis relative to the membrane normal, for each of the transmembrane helices (A–G) of bacteriorhodopsin and various values of $\delta_M$**

| Helix | No. of amides | $\delta_M$ | $\gamma_\alpha$ | $\langle \cos^2 \Theta_1 \rangle$ | $\langle \cos^2 \Theta_2 \rangle$ |
|---|---|---|---|---|---|
| A | 22 | 20° | 20.3° | 0.15 | 0.14 |
| | | 27° | 20.2° | 0.20 | 0.19 |
| | | 59° | 21.1° | 0.45 | 0.43 |
| B | 26 | 20° | 3.5° | 0.18 | 0.15 |
| | | 27° | 3.8° | 0.23 | 0.20 |
| | | 59° | 7.1° | 0.46 | 0.43 |
| C | 21 | 20° | 5.1° | 0.17 | 0.18 |
| | | 27° | 4.0° | 0.21 | 0.24 |
| | | 59° | 8.0° | 0.43 | 0.45 |
| D | 23 | 20° | 7.8° | 0.17 | 0.13 |
| | | 27° | 7.8° | 0.18 | 0.22 |
| | | 59° | 8.7° | 0.41 | 0.47 |
| E | 23 | 20° | 15.0° | 0.15 | 0.15 |
| | | 27° | 16.8° | 0.20 | 0.21 |
| | | 59° | 7.2° | 0.42 | 0.47 |
| F | 27 | 20° | 15.8° | 0.15 | 0.16 |
| | | 27° | 17.0° | 0.20 | 0.21 |
| | | 59° | 12.2° | 0.43 | 0.46 |
| G | 24 | 20° | 14.1° | 0.13 | 0.16 |
| | | 27° | 14.5° | 0.18 | 0.21 |
| | | 59° | 11.1° | 0.42 | 0.44 |

The mean square of direction cosines, relative to the helix axis (3-axis), is given by the relation $\langle \cos^2 \Theta_1 \rangle + \langle \cos^2 \Theta_2 \rangle + \cos^2\langle \Theta_3 \rangle = 1$.

In a previous treatment of $\alpha$-helical clusters the nonaxiality was expressed in terms of the azimuthal angle, $\vartheta$, about the helix axis (Marsh, 1998). In this formulation $\langle \cos\vartheta \rangle = 0$, because the tensor is diagonal in the present 1, 2, 3-axis system. The remaining order parameter that characterizes the nonaxiality in the previous analysis is given by:

$$\langle 2\cos^2\vartheta - 1 \rangle = 2\langle \cos^2\Theta_1 \rangle / (1 - \langle \cos^2\Theta_3 \rangle) - 1 \qquad \text{(A.1)}$$

where the term on the right refers to the present 1, 2, 3-axis system. From the data in Table 1, the weighted sums over all seven transmembrane helices yield values of $\langle 2\cos^2\vartheta - 1 \rangle = 0.02$, $-0.02$ and $-0.02$ for $\delta_M$ = 20°, 27°, and 59°, respectively. This confirms that the amide order parameters for the whole protein are almost axial.

When averaged over all transmembrane helices, the results on the near-axiality of the amide transition moment distributions for the individual helices therefore account for the good agreement with Eq. 9 of the directly calculated results that are given in Fig. 4. The order parameter of the helix axes, relative to the bilayer normal, that is calculated from the weighted means over all transmembrane helices in Table 1 is given by $\langle P_2(\cos\gamma_\alpha) \rangle = 0.93$, 0.92, 0.92, and 0.94, for $\delta_M$ = 0°, 20°, 27°, and 59°, respectively. These values are quite close to those of $\langle P_2(\cos\gamma_\alpha) \rangle = 0.92$ and 0.91 deduced from Figs. 1 and 4, respectively, consistent with the axial approximation. Similarly, the effective orientations of the transition moment, relative to the helix axis, calculated from the weighted means of

$\langle \cos^2 \Theta_3 \rangle$ over all transmembrane helices are 18°, 34°, 40°, and 70° for $\delta_M = 0°$, 20°, 27°, and 59°, respectively. These can be compared with values of $\Theta_{C'O} = 17°$, $\Theta_A = 33 \pm 1°$, $\Theta_I = 39.5 \pm 1°$, and $\Theta_{II} = 70 \pm 2°$ for the orientation of the carbonyl and of the amide A, amide I, and amide II transition moments, respectively, that were deduced above by using the axial approximation. From these comparisons it can again be deduced that the axial approximation is reasonable for bacteriorhodopsin.

T.P. was partially supported by the Volkswagen-Stiftung (Germany).

## REFERENCES

Axelsen, P. H., B. K. Kaufman, R. N. McElhaney, and R. N. A. H. Lewis. 1995. The infrared dichroism of transmembrane helical polypeptides. *Biophys. J.* 69:2770–2781.

Bechinger, B., J.-M. Ruysschaert, and E. Goormaghtigh. 1999. Membrane helix orientation from linear dichroism of infrared attenuated total reflection spectra. *Biophys. J.* 76:552–563.

Clark, N. A., K. J. Rothschild, D. A. Luippold, and B. A. Simon. 1980. Surface-induced lamellar orientation of multilayer membrane arrays. Theoretical analysis and a new method with application to purple membrane fragments. *Biophys. J.* 31:65–96.

Draheim, J. E., N. J. Gibson, and J. Y. Cassim. 1991. Dramatic in situ conformational dynamics of the transmembrane protein bacteriorhodopsin. *Biophys. J.* 60:89–100.

Fraser, R. D. B., and T. P. MacRae. 1973. Conformation in Fibrous Proteins and Related Synthetic Peptides. Academic Press, New York.

Fringeli, U. P., H. J. Apell, M. Fringeli, and P. Lauger. 1989. Polarized infrared absorption of $Na^+/K^+$-ATPase studied by attenuated total reflection spectroscopy. *Biochim. Biophys. Acta.* 984:301–312.

Guex, N., and M. C. Peitsch. 1997. Swiss model and the Swiss-Pdb viewer: an environment for comparative protein modeling. *Electrophoresis.* 18: 2714–2723.

Henniker, C. J. 1973. Infrared refractive indices of some oriented polymers. *Macromolecules.* 6:514–515.

Koradi, R., M. Billeter, and K. Wüthrich. 1996. MOLMOL: a program for display and analysis of macromolecular structures. *J. Mol. Graphics.* 14:51–55.

Luecke, H., H.-T. Richter, and J. K. Lanyi. 1998. Proton transfer pathways in bacteriorhodopsin at 2.3 Å resolution. *Science.* 280:1934–1937.

Luecke, H., B. Schobert, H.-T. Richter, J.-P. Cartailler, and J. K. Lanyi. 1999. Structure of bacteriorhodopsin at 1.55 Å resolution. *J. Mol. Biol.* 291:899–911.

Marsh, D. 1997. Dichroic ratios in polarized Fourier transform infrared for nonaxial symmetry of $\beta$-sheet structures. *Biophys. J.* 72:2710–2718.

Marsh, D. 1998. Nonaxiality in infrared dichroic ratios of polytopic transmembrane proteins. *Biophys. J.* 75:354–358.

Marsh, D., M. Müller, and F.-J. Schmitt. 2000. Orientation of the infrared transition moments for an $\alpha$-helix. *Biophys. J.* 78:2499–2510.

Nabedryk, E., and J. Breton. 1981. Orientation of intrinsic proteins in photosynthetic membranes—polarized infrared-spectroscopy of chloroplasts and chromatophores. *Biochim. Biophys. Acta.* 635:515–524.

Rothschild, K. J., and N. A. Clark. 1979. Polarized infrared spectroscopy of oriented purple membrane. *Biophys. J.* 25:473–488.

Rothschild, K. J., R. Sanches, T. L. Hsiao, and N. A. Clark. 1980. A spectroscopic study of rhodopsin alpha-helix orientation. *Biophys. J.* 31:53–64.

Silvestro, L., and P. H. Axelsen. 1999. Fourier transform infrared linked analysis of conformational changes in annexin V upon membrane binding. *Biochemistry.* 38:113–121.

Tamm, L. K., and S. A. Tatulian. 1997. Infrared spectroscopy of proteins and peptides in lipid bilayers. *Q. Rev. Biophys.* 30:365–429.