

## Nonaxiality in Infrared Dichroic Ratios of Polytopic Transmembrane Proteins

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**ABSTRACT** In polytopic  $\alpha$ -helical transmembrane proteins, the distribution of amide vibrational transition moments can be nonaxial, if the helix axes are tilted relative to the symmetry axis of the helix bundle. The infrared dichroic ratios from oriented samples then contain nonaxial terms and, in the most general case, require a second-order parameter for the axis of the helix bundle. The extent of nonaxiality depends on the summation over the individual amide transition moments along the helix. Because this is strongly oscillatory, with a 3.6-residue periodicity, complete axial symmetry is not achieved rapidly on progressive summation. Expressions for the contributions of residual nonaxiality to the dichroic ratios are derived. A similar situation arises for oligomers of transmembrane  $\beta$ -barrel proteins, e.g., the porin trimer. In this case, the extent of nonaxiality depends not only on the number of residues in the  $\beta$ -barrel, but also on the tilt of the  $\beta$ -strands relative to the barrel axis and the characteristic dimensions of a  $\beta$ -sheet, which together determine the axial periodicity. The nonaxial contributions to the dichroic ratios of  $\beta$ -barrel oligomers are also derived. Estimates are given of the likely size of the nonaxial contributions for the different  $\alpha$ -helical and  $\beta$ -sheet systems.

### INTRODUCTION

In a recent paper, I explored the consequences of the inevitably nonaxial distribution of the amide transition moments in  $\beta$ -sheet proteins on the infrared dichroic ratios obtained from oriented samples, including biological membranes (Marsh, 1997). This is an issue that had already been raised in connection with attenuated total reflection studies on the  $\beta$ -barrel outer membrane protein OmpA (Rodionova et al., 1995). In the appendix to my previous paper, I presented equivalent results for the dichroic ratios of an  $\alpha$ -helical protein that corresponded to the expressions normally used in analyzing polarized infrared experiments on such proteins. In the derivation, complete axial symmetry of the distribution of amide transition moments about the helix axis was assumed. This is certainly true to a good approximation for a bitopic  $\alpha$ -helical membrane protein, for which, in fluid membranes, there is complete rotational disorder about the axis of the single transmembrane helix. However, this is not necessarily the case for polytopic  $\alpha$ -helical proteins, in which the helix axes are tilted relative to the axis of the whole molecule, or, more generally, with respect to the membrane normal (see Fig. 1, *left*). In the latter situation, the transition moments are fixed within the protein molecule and are distributed azimuthally with a 3.6-residue periodicity about the helix axes. The strongly oscillatory nature of this periodicity means that complete axial symmetry is not achieved rapidly on progressive summation over the peptide

residues along the helix. (Rotational disorder about the molecular axis alone is insufficient to produce axial symmetry for the transition moment of an individual residue because the axis of an individual moment will not, in general, intersect that of the whole molecule.)

It is therefore of interest to explore the consequences of residual nonaxiality about the helix axes of polytopic membrane proteins for the dichroic ratios of the amide bands in their infrared spectra. Such considerations will be of importance to short peptide stretches whose spectra are specifically resolved by isotopic editing (Halverson et al., 1991; Tadesse et al., 1991). They will certainly also be of relevance to any detailed analysis of experimental dichroic ratios from polytopic proteins of known structure. Of possibly greater importance is to take into account such effects in testing proposed molecular models against experimental data from infrared dichroism. The most stringent tests in the latter case would come specifically from isotopic editing. More generally, such a treatment sheds further light on the origins of the effects of nonaxial symmetry on the infrared dichroic spectra from  $\beta$ -sheets and, in particular, clarifies the issue of infrared order parameters for the  $\beta$ -barrels of porin-like membrane proteins.

The possibility of a nonaxial distribution of transition moments for a polytopic  $\alpha$ -helical protein has been considered previously (Rothschild and Clark, 1979). It was concluded that experimental uncertainties were too great, however, to specify the distribution in its most general form. Here I concentrate on the specific contributions to nonaxiality from protein segments with perfect  $\alpha$ -helical structure.

### METHODS

The nonaxial situation is illustrated schematically in Fig. 1 (*left*). Although the scheme is quite general, for concreteness the example given is that of

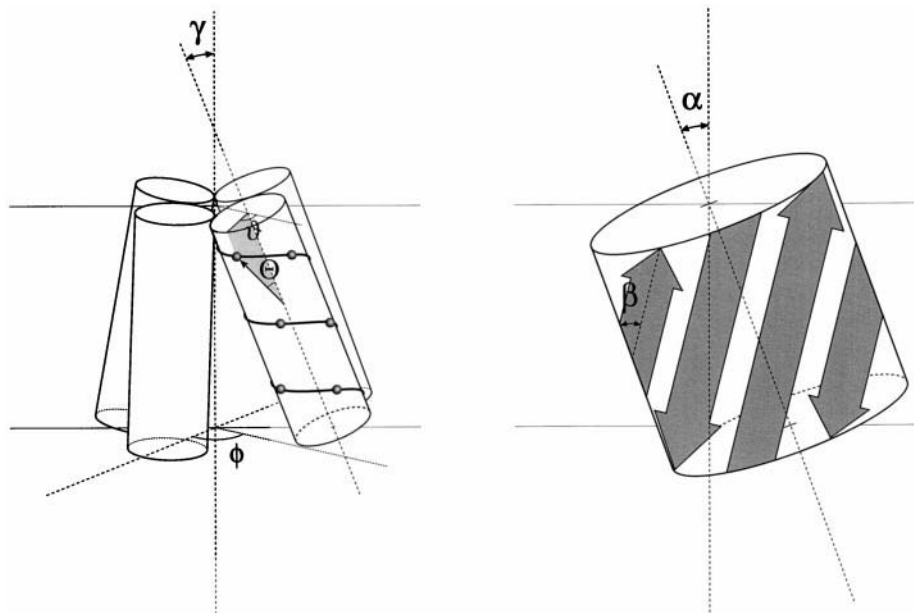
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FIGURE 1 (*Left*) Angular orientations of the  $\alpha$ -helices in a polytopic transmembrane protein. The azimuthal angle  $\phi$  of the helical axis, and the tilt angle  $\gamma$  of the helix axis, relative to the longitudinal axis of the protein, are indicated. The transition moments of the amide groups are oriented at an angle  $\Theta$  to the helical axis, and the azimuthal orientation  $\vartheta$  of one of the residues about the helical axis is indicated. The origin for  $\vartheta$  is defined by the plane containing the helical axis and the longitudinal axis of the protein. (*Right*) Orientations of the  $\beta$ -strands in a  $\beta$ -barrel protein. The axis of the  $\beta$ -barrel is tilted at an angle  $\alpha$  to the membrane normal, and the  $\beta$ -strands are tilted by an angle  $\beta$  within the  $\beta$ -sheets.



an  $\alpha$ -helical protein. The axis of the helix is tilted at an angle  $\gamma$  with respect to the molecular axis. This defines an azimuthal angle  $\phi$  of the helix axis in the plane of the membrane. Axial symmetry is established for the whole protein by complete summation over the angle  $\phi$ , because the polytopic protein itself is rotationally disordered about the membrane normal in fluid membranes. The transition moment of any residue within a given helix is inclined at an angle  $\Theta$  to the helical axis. The orientation of the transition moment of a given residue has a fixed azimuthal angle,  $\vartheta$ , about the helical axis. The origin for this angle  $\vartheta$  is defined by the plane containing the helix axis and the molecular axis. Summation is performed over all azimuthal angles  $\vartheta$  for residues within a helix of given tilt,  $\gamma$ . In this sense, the orientational angles  $\vartheta$  and  $\gamma$  for a particular residue are correlated, i.e., in that the origin for measuring  $\vartheta$  may change when the tilt  $\gamma$  of the helix changes (see Fig. 1, *left*). For such cases, independent summations are required for the differently tilted helical segments.

Given the geometric relations for the polarizations of the incident beam in an attenuated total reflection or transmission infrared experiment that were defined in the previous paper, the infrared dichroic ratio is given by (Marsh, 1997)

$$R = \frac{E_x^2}{E_y^2} + \frac{\langle M_z^2 \rangle}{\langle M_y^2 \rangle} \frac{E_z^2}{E_y^2} \quad (1)$$

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. In Eq. 1, summation has already been performed over the azimuthal angle  $\phi$  within the membrane plane. However, summation over the other angular variables,  $\vartheta$  and  $\gamma$ , must also be performed, and this is indicated by the angular brackets about the squares of the  $M_z$  and  $M_y$  components of the transition moment. The definitions of the angles  $\vartheta$ ,  $\gamma$ , and  $\Theta$  that are given in Fig. 1 (*left*) correspond exactly to the general scheme used previously in the analysis of nonaxiality for  $\beta$ -sheet structures. The expression required for  $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ , after rotational summation over the azimuthal angle ( $\phi$ ) about the  $z$  axis, was given in the previous publication (Marsh, 1997). This may be rewritten in the following form, to make explicit the effects of nonaxiality, and to express the orientational distribution of the helix axis in

terms of the order parameter formalism that is frequently used:

$$\begin{aligned} & \frac{1}{2} + P_2^0(\cos \Theta) \langle P_2^0(\cos \gamma) \rangle \\ & + \frac{1}{3} [1 - P_2^0(\cos \Theta)] [1 - \langle P_2^0(\cos \gamma) \rangle] (2 \cos^2 \vartheta - 1) \\ \frac{\langle M_z^2 \rangle}{\langle M_y^2 \rangle} = 2 & \frac{-\frac{1}{3} P_2^1(\cos \Theta) \langle P_2^1(\cos \gamma) \rangle \langle \cos \vartheta \rangle}{1 - P_2^0(\cos \Theta) \langle P_2^0(\cos \gamma) \rangle} \\ & - \frac{1}{3} [1 - P_2^0(\cos \Theta)] [1 - \langle P_2^0(\cos \gamma) \rangle] (2 \cos^2 \vartheta - 1) \\ & + \frac{1}{3} P_2^1(\cos \Theta) \langle P_2^1(\cos \gamma) \rangle \langle \cos \vartheta \rangle \end{aligned} \quad (2)$$

where  $P_2^0(\cos \gamma) = \frac{1}{2}(3\cos^2 \gamma - 1)$  and  $P_2^1(\cos \gamma) = 3\sin \gamma \cos \gamma$ , etc. A generalization of the angular order parameters in terms of the associated Legendre polynomials,  $P_l^m(x)$ , is required here to take into account completely the terms arising from nonaxiality. The single order parameter that is conventionally used for a rotationally disordered  $\alpha$ -helix is  $\langle P_2^0(\cos \gamma) \rangle = \langle P_2^0(\cos \gamma) \rangle$ . For rotation around the helix axis, the angular ordering is expressed simply in terms of  $\langle \cos^2 \vartheta \rangle$  and  $\langle \cos \vartheta \rangle$ , to emphasize the uniaxial character. In Eq. 2, the last two terms in the numerator and denominator completely determine the nonaxiality. For a fully axial system,  $\langle \cos^2 \vartheta \rangle = \frac{1}{2}$  and  $\langle \cos \vartheta \rangle = 0$ , and the last two terms disappear. The resulting expression for  $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ , and consequently for the infrared dichroic ratio, is then identical to that usually employed for  $\alpha$ -helical transmembrane proteins and contains only the product  $P_2^0(\cos \Theta) \langle P_2^0(\cos \gamma) \rangle$  as the sole angular-dependent term (see, e.g., appendix in Marsh, 1997). When  $\langle P_2^0(\cos \gamma) \rangle = 1$ , i.e., there is perfect alignment of the helical axes along the membrane normal, then  $\langle P_2^1(\cos \gamma) \rangle = 0$ , and all terms in Eq. 2 that involve nonaxiality about the helical axis again disappear, as expected.

## RESULTS AND DISCUSSION

### Polytopic $\alpha$ -helical proteins

It is seen from Eq. 2 that, for the general nonaxial case, two different order parameters for the helical axis,  $\langle P_2^0(\cos \gamma) \rangle$  and  $\langle P_2^1(\cos \gamma) \rangle$ , are required to describe the dichroic ratios

from polytopic  $\alpha$ -helical membrane proteins. In addition, two further order parameters,  $\langle 2 \cos^2 \vartheta - 1 \rangle$  and  $\langle \cos \vartheta \rangle$ , are required, which are specified by the degree of nonaxiality. The latter are determined by the azimuthal orientation,  $\vartheta_o$ , of the first residue in the tilted helix and the sum over all  $N_r - 1$  subsequent residues in the tilted helix. The required expressions are

$$\langle 2 \cos^2 \vartheta - 1 \rangle = \frac{1}{N_r} \left[ 1 + \sum_{i=1}^{N_r-1} \cos \left( \frac{4\pi i}{p} \right) \right] \cdot \cos 2\vartheta_o - \frac{1}{N_r} \left[ \sum_{i=1}^{N_r-1} \sin \left( \frac{4\pi i}{p} \right) \right] \cdot \sin 2\vartheta_o \quad (3)$$

and

$$\langle \cos \vartheta \rangle = \frac{1}{N_r} \left[ 1 + \sum_{i=1}^{N_r-1} \cos \left( \frac{2\pi i}{p} \right) \right] \cdot \cos \vartheta_o - \frac{1}{N_r} \left[ \sum_{i=1}^{N_r-1} \sin \left( \frac{2\pi i}{p} \right) \right] \cdot \sin \vartheta_o \quad (4)$$

where  $p = 3.6$  is the number of residues per turn in an  $\alpha$ -helix, and the summation index,  $i$ , represents the position

of a residue in the helix. The dependence of the four helical averages that are needed on the number of residues,  $N_r$ , per helix is given in Fig. 2. It is seen that, although these averages lie considerably below unity for  $N_r \geq 4$ , they are highly oscillatory in nature, with phase-shifted components, and do not decay rapidly to zero with increasing  $N_r$ . Even for  $N_r \approx 19$ –20, corresponding to a full transmembrane  $\alpha$ -helical segment, the angular averages may still have appreciable residual values.

The values for the azimuthal orientation of the first residue can be restricted as a result of the helical periodicity to the range  $-\pi/p < \vartheta_o < \pi/p$ . The maximum value of the initial phase-shift term in Eq. 3 is  $\cos 2\vartheta_o = 1$ , which occurs for  $\vartheta_o = 0$ . This corresponds to values of  $\langle 2 \cos^2 \vartheta - 1 \rangle = 0.12$  and  $0.05$  for  $N_r = 8$  and  $19$ , respectively. If the nonaxial terms involving the order parameter  $\langle P_2^1(\cos \gamma) \rangle$  are neglected, it is possible to make some estimate of the effect that this nonaxiality may have on the dichroic ratio. For a value of  $\langle P_2^0(\cos \gamma) \rangle = 0$ , at which  $R$  is most sensitive to nonaxiality, the latter would contribute maximum discrepancies in the dichroic ratio, relative to a purely axial system, of  $\Delta R \approx 0.07(E_z^2/E_y^2)$  and  $0.03(E_z^2/E_y^2)$  for  $N_r = 8$  and  $N_r = 19$ , respectively (taking  $\Theta = 39^\circ$ , for an  $\alpha$ -helix; Tsuboi, 1962). It is therefore unlikely, depending on the experimental set-up, that nonaxiality will be of overwhelm-

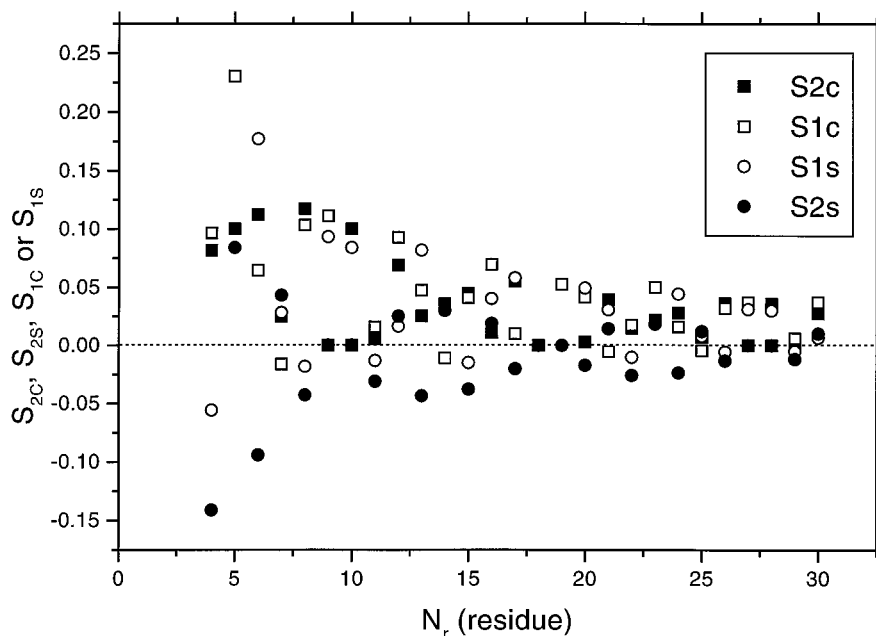


FIGURE 2 Dependence on the number of residues,  $N_r$ , in the  $\alpha$ -helix of the helical rotational sums,

$$S_{2c} = (1/N_r) \left[ 1 + \sum_{i=1}^{N_r-1} \cos(4\pi i/p) \right] \text{ (filled squares)}, S_{2s} = (1/N_r) \sum_{i=1}^{N_r-1} \sin(4\pi i/p) \text{ (filled circles)},$$

$$S_{1c} = (1/N_r) \left[ 1 + \sum_{i=1}^{N_r-1} \cos(2\pi i/p) \right] \text{ (open squares)}, S_{1s} = (1/N_r) \sum_{i=1}^{N_r-1} \sin(2\pi i/p) \text{ (open circles)},$$

that appear in Eqs. 3, 4 and are involved in determining the infrared dichroic ratios. The position of a residue in the helix is specified by  $i$ , the summation index.

ing practical importance for long helices. For shorter helical segments, demarcated by kinks in a transmembrane helix, the effects of nonaxiality might be significant. In addition, there may be supplementary contributions from the second nonaxial term, involving  $\langle P_2^1(\cos \gamma) \rangle$ , that are difficult to estimate unambiguously.

Measurements of the dichroism of the amide I and amide II bands of bacteriorhodopsin were not entirely consistent with a unique helix orientation when analyzed with an axial model (Rothschild and Clark, 1979). This might be evidence for residual nonaxiality, although other possible reasons for this were advanced. In addition, such a comparison also depends on the values taken for the orientation of the transition moments, which has been the subject of recent discussion (Axelsen et al., 1995). Some estimate of the possible contribution from nonaxiality to the dichroic ratios of the amide I and amide II bands of bacteriorhodopsin can be made if the terms involving  $\langle \cos \vartheta \rangle$  in Eq. 2 are ignored. Using the orientations of the transition moments given by the authors, physically realistic solutions (i.e.,  $\langle \cos^2 \vartheta \rangle \leq 1$ ) can only be obtained with dichroic ratios close to the low end of the range determined by Rothschild and Clark (1979), for both the amide I and amide II bands. At the lower limit, values of  $\langle \cos^2 \vartheta \rangle = 0.95$  and  $\langle P_2^0(\cos \vartheta) \rangle = 0.63$  are obtained. For dichroic ratios higher in the experimental range, the values of  $\langle \cos^2 \vartheta \rangle$  obtained are even larger. This therefore indicates the possibility of quite considerable contributions from nonaxiality to the dichroic ratios of bacteriorhodopsin. For comparison, the fully axial approximation yields a mean helix order parameter of  $\langle P_2^0(\cos \gamma) \rangle = 0.68$  for the same value of the dichroic ratio of the amide I band. The value obtained from both dichroic ratios, with partial allowance for nonaxiality, is smaller and corresponds to a larger effective helix tilt angle of  $\gamma_{\text{eff}} \approx 30^\circ$ . The maximum tilt between adjacent  $\alpha$ -helices in the bacteriorhodopsin structure is on the order of  $25^\circ$  (Grigorieff et al., 1996).

### $\beta$ -Barrel assemblies

A situation similar to that for polytopic  $\alpha$ -helical proteins also arises for oligomeric assemblies of axially symmetrical  $\beta$ -barrels, e.g., in the porin trimer. For this case, however, the order parameters associated with the azimuthal orientations,  $\vartheta$ , of the residues depend in detail on the structure of the  $\beta$ -barrel. In particular, they depend on the number of strands,  $n_\beta$ , in the barrel, and on the tilt,  $\beta_j$ , of the individual strands relative to the barrel axis (see Fig. 1, right), as well as on the characteristic dimensions of the  $\beta$ -sheet. The fundamental repeat in the azimuthal angle  $\vartheta$  is  $(2\pi/n_\beta)$ , between adjacent  $\beta$ -strands in the barrel. Within the  $(j + 1)$ th strand, the decrement in  $\vartheta$  between successive residues is  $\delta_j = (d_\beta/D_\beta)\sin \beta_j \cos \beta_j$ , expressed as a fraction of the fundamental interstrand repeat, where  $d_\beta (= 3.47 \text{ \AA})$  and  $D_\beta (= 4.72 \text{ \AA})$  are the interresidue separation within a strand and the separation of the strands, respectively. The expres-

sions that correspond to Eqs. 3 and 4 for the nonaxial order parameters for an  $\alpha$ -helix are then the following, for a  $\beta$ -barrel of circular cross section:

$$\langle 2 \cos^2 \vartheta - 1 \rangle = \frac{1}{\sum_{j=0}^{n_\beta-1} N_{r,j}} \left\{ \cos 2\vartheta_0 \sum_{j=0}^{n_\beta-1} \sum_{i=0}^{N_{r,j}-1} \cos \left[ \frac{4\pi(i\delta_j + j)}{n_\beta} \right] - \sin 2\vartheta_0 \sum_{j=0}^{n_\beta-1} \sum_{i=0}^{N_{r,j}-1} \sin \left[ \frac{4\pi(i\delta_j + j)}{n_\beta} \right] \right\} \quad (5)$$

$$\langle \cos \vartheta \rangle = \frac{1}{\sum_{j=0}^{n_\beta-1} N_{r,j}} \left\{ \cos \vartheta_0 \sum_{j=0}^{n_\beta-1} \sum_{i=0}^{N_{r,j}-1} \cos \left[ \frac{2\pi(i\delta_j + j)}{n_\beta} \right] - \sin \vartheta_0 \sum_{j=0}^{n_\beta-1} \sum_{i=0}^{N_{r,j}-1} \sin \left[ \frac{2\pi(i\delta_j + j)}{n_\beta} \right] \right\} \quad (6)$$

where  $N_{r,j}$  is the number of residues in the  $(j + 1)$ th strand. The summations in Eqs. 5 and 6 are over each residue in a strand (index,  $i$ ) and over all strands (index,  $j$ ). For staggering between adjacent strands by an integral number of residues,  $\Delta N_{r,j}$ , the tilt angle of the  $(j + 1)$ th strand is given by  $\tan \beta_j = (d_\beta/D_\beta)\Delta N_{r,j}$ , and the corresponding azimuthal decrement becomes  $\delta_j = [1 + (D_\beta/\Delta N_{r,j}d_\beta)^2]^{-1/2}$ . The degree of nonaxiality therefore depends in detail on the properties of the individual strands. However, for barrels with even numbers of strands, the tilt angles and numbers of residues of which are all equal, the high degree of symmetry results in perfect cancellation for the sums given in Eqs. 5 and 6. This degree of symmetry is not realized in any of the known  $\beta$ -barrel structures of membrane porins (Cowan, 1993).

Some idea of the overall size of the effects of nonaxial contributions in  $\beta$ -sheets can be obtained by comparing the  $\beta$ -sheet system having the greatest nonaxiality, viz. a planar sheet, with that having full axial symmetry, viz. an isolated  $\beta$ -barrel of circular cross section. Expressions for the former were given in the previous paper (Marsh, 1997). For the latter, in analogy to the axial expression for an  $\alpha$ -helix (cf. Eqs. 1 and 2), the order parameter of the barrel axis is given in terms of the dichroic ratio  $R(\Theta)$  by (Tamm and Tatulian, 1997)

$$\langle P_2^0(\cos \alpha) \rangle = \frac{E_x^2/E_y^2 - R(\Theta) + E_z^2/E_y^2}{\langle P_2^0(\cos \Theta) \rangle [E_x^2/E_y^2 - R(\Theta) - 2E_z^2/E_y^2]} \quad (7)$$

where  $\alpha$  is the tilt of the barrel axis relative to the membrane normal (see Fig. 1, right), and  $\Theta$  is the orientation of the resultant transition moment of the  $\beta$ -strand relative to the barrel axis. For the amide I band, the resultant transition moment is oriented perpendicular to the  $\beta$ -strand axis, i.e.,  $\Theta = \pi/2 - \beta$ , and for the amide II band, the resultant transition moment is parallel to the  $\beta$ -strand axis, i.e.,  $\Theta = \beta$ , where  $\beta$  is the angle that the strand axis makes with the barrel axis (see Fig. 1, right). Applying Eq. 7 to dichroic data for both the amide I and amide II bands therefore

allows both the order parameter of the barrel and the tilt of the strands to be determined. Using data for the OmpF porin from Nabadryk et al. (1988) yields values of  $\langle P_2^o(\cos \alpha) \rangle = 0.69$  and  $\langle P_2^o(\cos \beta) \rangle = 0.28$  for the order parameter of the  $\beta$ -barrel and the orientation of the  $\beta$ -strands within the barrel, respectively (see Fig. 1, *right*), assuming axial symmetry. These may be compared with the corresponding values that are obtained by using the nonaxial planar sheet model:  $\langle P_2^o(\cos \alpha) \rangle = 0.85$  and  $\langle P_2^o(\cos \beta) \rangle = 0.26$  (Marsh, 1997). The order parameter deduced for the  $\beta$ -sheet is changed considerably in the nonaxial model, as expected, but the orientation deduced for the strands within the sheet remains approximately the same.

## CONCLUSIONS

Although present, nonaxial contributions to the infrared dichroism of polytopic  $\alpha$ -helical proteins may be relatively small, in the case of long helices. This would justify the use of the common axial approximation for whole proteins, but becomes an important issue in the case of isotopic editing. For  $\beta$ -sheet proteins, nonaxial contributions to the dichroic ratios can arise for tilted  $\beta$ -barrels in aggregates, particularly if there is heterogeneity among strands, or the barrels are not of circular cross section. The limiting case of nonaxiality is the planar/pleated  $\beta$ -sheet. Nonaxiality may be detected experimentally for both  $\alpha$ -helical and  $\beta$ -sheet proteins by measurement of the dichroic ratio of two bands with independent polarizations, e.g., the amide I and amide II bands. For proteins of known structure, the contribution of nonaxiality can be calculated directly and might aid determination of the orientation of the assembly in the membrane by infrared dichroism measurements.

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