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Infrared Dichroism of Isotope-edited α-Helices and β-Sheets

Derek Marsh

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

Isotope editing of amide infrared bands not only localises secondary structural elements within the protein but also yields conformational information that is not available from the linear dichroism of aligned samples without isotope editing. The additional information that can be derived on the orientational distribution of α-helices in membranes by the combined use of different amide bands and several positions of labelling is presented here. Also, the relationship between the azimuthal orientation of the transition moment and the protein structure is treated explicitly. A comprehensive analysis of the infrared dichroism for β-sheets and β-barrels is given here, for the first time. The orientation of the individual transition moments in a β-sheet that is essential for this analysis is derived for the different amide bands.

Keywords: α-helix; β-sheet; secondary structure; infrared; linear dichroism

Introduction

The linear dichroism of amide infrared (IR) bands from site-specifically isotope-labelled α-helical transmembrane peptides has proved to be invaluable for conformational analysis of membrane proteins.[1–4] In addition to the orientational information available from non-isotopically edited dichroism, site-specific labels also yield torsion angles and are able to discriminate local ordering from macroscopic disorder.[5] The analysis for isotopically labelled α-helices has been well developed,[5] although the relationship of transition moment orientation to the protein structure has not been fully worked out, particularly for the torsion angles. The latter is essential if the results from site-specific dichroism are to be most effectively combined with molecular modelling.[4] Also, the possibility of obtaining detailed information on the distribution of helical tilts, especially in polytopic proteins, remains to be exploited fully.

As for non-isotopically edited dichroism, the application of site-specific labelling to β-sheet proteins lags behind that for α-helical proteins. The possibility of investigating lung surfactant protein B on lipid monolayers by IR reflection–absorption spectroscopy, however, seems extremely promising.[6] An analysis of the dichroism for non-isotope-labelled (or uniformly labelled) β-sheets has been given previously.[7] In the latter case, the net IR transition moments are oriented either along or perpendicular to the β-strand axis. For isolated isotopically edited amides, however, the orientation of the individual transition moments and the relation to the structure of the β-pleated sheet should be considered. Use of several amide bands is even more necessary than for the α-helix, because only two labelling positions give non-degenerate dichroic ratios in a β-sheet.

Here, we present a comprehensive analysis of the IR dichroism of site-specifically isotope-labelled amides for α-helices, β-sheets and β-barrels. The analysis for α-helices goes beyond the previous treatment[5] in that different amide bands, and the relation between azimuthal orientation of the transition moment and the protein structure (i.e. torsion angles), are considered explicitly. Isotope edited IR dichroism of β-sheet or β-barrel structures, has not been treated before, although a limited amount of experimental data is available.[8] Further, we also consider the possibility of distributions in tilt and azimuthal orientation of the secondary structure by using explicit models. This is likely to be of considerable importance for

Abbreviation used: ATR, attenuated total reflection.

E-mail address of the corresponding author: dmarsh@gwdg.de

single membrane-spanning helices and small monomeric β-barrels.

Results and Discussion

Infrared dichroic ratios

We consider specifically aligned membranes, where the proteins are rotationally disordered about the membrane normal which is defined as the z-axis. The dichroic ratio, R_z, of the absorbances with radiation linearly polarized parallel with and perpendicular to the plane of incidence is then given by:[7]

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

where $\mathbf{E} = (E_x, E_y, E_z)$ is the radiation electric field vector in the sample, the components of which are normalized to those at incidence. The components of **E** are specific to the particular experimental set-up, e.g. attenuated total reflection (ATR) or transmission at non-zero angles of incidence.[9] The x-axis is defined as lying in the plane of incidence and the y-axis is orthogonal to the plane of incidence. Both lie within the plane of the orienting substrate. The quantity of structural interest in discussing linear dichroism is the transition moment vector, $\mathbf{M} = (M_x, M_y, M_z)$. Specifically, the quantities in angular brackets in equation (1) represent summations over the squares of the transition moment components for all isotopically labelled amide groups in the sample. Because these appear as a ratio in equation (1), the angular brackets are equivalent to taking average values. The problem, therefore, resolves itself into determining the dependence of $\langle M_z^2\rangle/\langle M_y^2\rangle$ on the angular orientation of the isotopically labelled amide. This differs between various amide bands because of different orientations of the transition moment relative to the molecular axes.

Transition moment orientation

It is assumed that the vibrations of the isotopically labelled amide are decoupled from those of the other amides in the polypeptide chain. The orientation of the transition moment then does not correspond to one of the symmetry directions, as for instance in coupled vibrations of β-sheets.[10] Further, because we are dealing with an isolated labelled residue, there is no possibility of summation over residues yielding axial symmetry, as is the case for long α-helices.[11] The orientation of the individual transition moment, **M**, relative to the peptide chain axis, therefore depends on both the tilt angle, Θ_M, and the azimuthal orientation, ϑ_M, of the transition moment, relative to the α-helix or β-strand axis.[5] The latter is, in turn, oriented at an angle γ to the z-axis (see Figure 1). The values of the tilt, Θ_M, differ between the various amide bands,[12] as do those of the azimuthal orientation, ϑ_M. Because the transition moments lie in the peptide plane for all amide vibrations considered, the azimuthal orientation (ϑ_M) is related in a predictable way to the tilt angle Θ_M. It also depends on the position of the residue in the polypeptide chain, reflecting the periodicity of the secondary structure. The origin of ϑ_M, however, is a matter of definition. It is taken in the plane containing the z-axis and the peptide chain axis, c, in the direction z to c (see Figure 1).

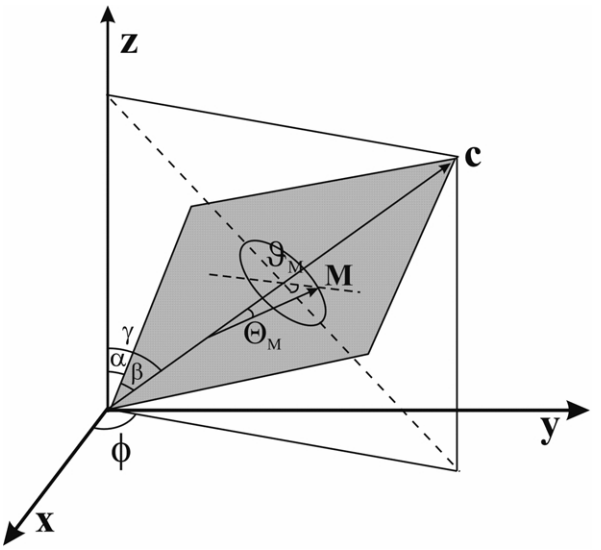

Figure 1. Orientation of the peptide chain axis, c, and the transition moment, **M**, of the isotopically labelled amide, relative to the substrate-fixed axes, x, y, z. The chain axis is tilted at an angle γ from the normal, z, to the orienting substrate and is randomly distributed in the angle ϕ, in the plane of the sample substrate. The transition moment is inclined at angle Θ_M to the chain axis with an azimuthal orientation, ϑ_M, relative to the plane containing the z and c axes. For an α-helix, the helix axis specifies the c-axis. For a β-sheet, the β-strand axis (not the sheet orientation) is the c-axis; the orientation of the sheet is indicated by the shaded plane, which is inclined at an angle α_s to the z-axis. The strand is tilted by angle β to the projection of the z-axis on the sheet.

With these definitions, the required quantity in equation (1) is given by:[7]

$$\frac{\langle M_z^2\rangle}{\langle M_y^2\rangle} = 2\frac{\cos^2\Theta_M\langle\cos^2\gamma\rangle - \sin 2\Theta_M\langle\sin\gamma\cos\gamma\rangle\langle\cos\vartheta_M\rangle + \sin^2\Theta_M\langle\sin^2\gamma\rangle\langle\cos^2\vartheta_M\rangle}{1 - \cos^2\Theta_M\langle\cos^2\gamma\rangle + \sin 2\Theta_M\langle\sin\gamma\cos\gamma\rangle\langle\cos\vartheta_M\rangle - \sin^2\Theta_M\langle\sin^2\gamma\rangle\langle\cos^2\vartheta_M\rangle} \qquad (2)$$

where $\langle\sin^2\gamma\rangle = 1 - \langle\cos^2\gamma\rangle$. Angular brackets are included in equation (2) to allow for the possibility

of a limited distribution in the values of γ and ϑ_M. Note that γ and ϑ_M are independent, and Θ_M is fixed for a given amide band.

In general, for a distribution in the angles γ and ϑ_M, several different angular averages are required to specify the values of $\langle M_z^2\rangle/\langle M_y^2\rangle$ fully, as pointed out for the case of polytopic proteins.[11] These are the four independent values: $\langle\cos^2\gamma\rangle$, $\langle\sin\gamma\cos\gamma\rangle$, $\langle\cos^2\vartheta_M\rangle$ and $\langle\cos\vartheta_M\rangle$. If γ and ϑ_M are single-valued, this number is reduced to two. Then two independent dichroic ratios are required to determine the amide orientation, similar to the case considered previously for β-sheet proteins.[7] These can be provided by measurements on a single band at two different positions of isotopic labelling, where γ and Θ_M remain fixed and the values of ϑ_M bear a fixed relation to one another that is determined by the geometry of the secondary structure.[5] Alternatively, dichroic ratios from different bands may be combined for a single site of labelling. In this latter case, γ is fixed, the different values of Θ_M, e.g. from the amide I and amide II, or amide A bands, are known,[12] and the values of ϑ_M are related to one another in a fixed way *via* those of Θ_M (see later). However, resolution of the isotopically edited amide band may not be achieved with the same isotope for different amide vibrational modes. Labelling of the $^{13}C{=}O$ has been shown to be suitable for the amide I band;[1,2,5] but $^{15}N{-}H$ labelling will be required for the amide II and amide A bands. In principle, there are 18 distinguishable azimuthal orientations for a given amide transition moment in an α-helix, but only two in a β-strand. However, it is likely that maximally seven adjacent amides in an α-helix have azimuthal angles that are sufficiently widely spaced to be practically useful.[2]

In addition to the dichroic ratios for the isotopically labelled amides, further independent measurements are provided by the dichroism of the non-isotopically labelled amides.[5] For α-helices, dichroic ratios of the latter do not depend on the azimuthal orientation, ϑ_M. For β-sheets, on the other hand, the orientations of the transition moments of the coupled modes are different, being directed either along or perpendicular to the β-strand axis.[7]

The treatment differs for α-helices and β-sheets, because of their different geometries. The former is the more straightforward and is considered first. It has been treated by Arkin *et al.*,[5] but is given here as introduction to the consideration not only of β-sheets but also of the relations between the different amide bands of an α-helix, and further as an introduction to explicit treatments of distributions in orientation of α-helix assemblies.

α-Helices: single orientations

The situation depicted in Figure 1 is related straightforwardly to the geometrical arrangement of single, bitopic transmembrane helices and of polytopic helix bundles. The angle γ is the tilt of

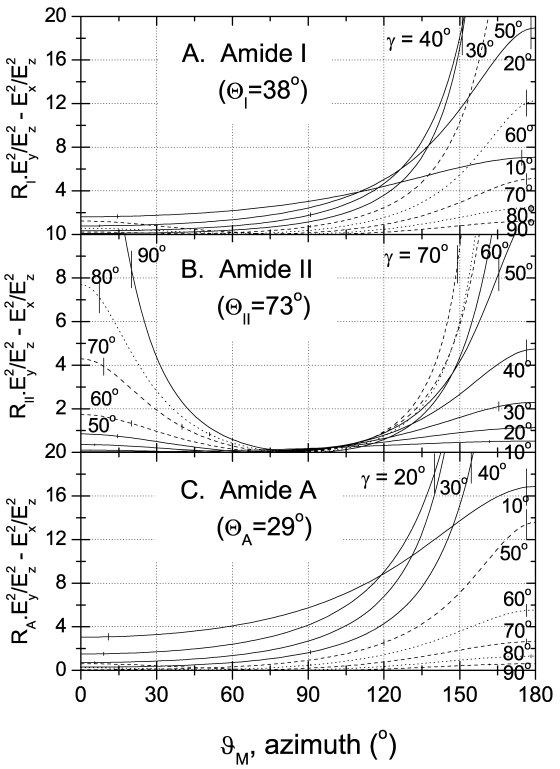

Figure 2. Dependence of the dichroic ratio, R_M, of a site-specifically labelled α-helix on the azimuthal orientation, ϑ_M, of the isotopically labelled amide for different inclinations, γ, of the helix axis to the membrane normal. Dependences are calculated from equations (1) and (2) and are given for: A, the amide I band ($\Theta_I = 38°$); B, the amide II band ($\Theta_{II} = 73°$); and C, the amide A band ($\Theta_A = 29°$). The values of γ are indicated in the Figure. The ordinate is scaled and shifted by the ratios of the components of the radiation electric field intensities, E_y^2/E_z^2 and E_x^2/E_z^2, respectively, to give values of $\langle M_z^2\rangle/\langle M_y^2\rangle$ (see equation (2)) that are independent of the particular experimental set-up. Vertical bars indicate the range of a 10% error in dichroic ratio. For a given labelled residue, the values of ϑ_M, relative to those of the amide I band, are displaced by $-4°$ to $-6°$ and $+1°$ to $+3°$ for the amide II and amide A bands, respectively.

the helix axis to the *z*-axis, and ϑ_M is the azimuthal or torsion angle of the transition moment of the isotopically labelled amide about the helix axis. The direction of the *c*-axis is that going from the N to the C terminus of the (right-handed) α-helix. A right-handed rotation, defined as positive ϑ_M, then takes $C^{\alpha,i}$ to $C^{\alpha,i+1}$. With this definition, the transition moment makes an acute angle, Θ_M, with the *c*-axis (note that this standard definition is different from the convention used by Arkin *et al.*[5]). For consecutive positions of labelling in an ideal α-helix with 3.6-residue pitch, the values of the azimuthal angle ϑ_M are related by incremental rotations of $+100°$ about the helix axis. Equation (2) is therefore applicable directly to α-helices. This case has been considered by Arkin *et al.*,[5] for fixed values of γ and ϑ_M. Equations (1) and (2) are in agreement with the previous treatment, except for an additional angle δ that was included but

put equal to zero by the latter authors. Note that the sign of the middle term in the denominator and numerator of equation (2) is determined by that of $\langle \cos \vartheta_M \rangle$. All other terms in equation (2) are positive (excluding the signs specified explicitly).

The dependence of $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ on the azimuthal orientation, ϑ_M, of the isotopically labelled amide is given in Figure 2 for various values of the tilt, γ, of the helix axis to the membrane director (z). Dependences are given for the amide I, amide II and amide A bands, by using the following values for the orientation of the transition moment: $\Theta_I = 38°$, $\Theta_{II} = 73°$ and $\Theta_A = 29°$.[12] It is clear from equation (2) that the values of $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ have a 360° periodicity in ϑ_M. They are, however, reflected about the $\vartheta_M = 180°$ axis. Values corresponding to ϑ_M and $360° - \vartheta_M$ are indistinguishable. There is a 180° periodicity in the tilt angle γ, but values of $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ for $180° - \gamma$ and $180° - \vartheta_M$ are identical with those for γ and ϑ_M. Therefore, with suitable substitutions, Figure 2 covers the full range of ϑ_M and γ.

For zero tilt (i.e. $\gamma = 0°$), the dichroic ratios are independent of the azimuthal angle ϑ_M, because of the rotational disorder in the plane of the membrane. Then $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ has the constant value: $2\cot^2 \Theta_M$. In general, the dichroic ratio is not necessarily a monotonic function of ϑ_M for a fixed value of γ. However, dichroic ratios above a certain threshold value in γ are unique within the range $\vartheta_M = 0–180°$. Only for $\gamma = 90°$ (e.g. a surface helix), is the dependence on azimuthal orientation symmetric about $\vartheta_M = 90°$. The largest dichroism is obtained with $\vartheta_M = 180°$, for a fixed helix tilt angle. Then amongst the different tilt angles, the largest dichroism is obtained for values in the region of $\gamma \sim \Theta_M$. This illustrates one of the advantages of using complementary amide bands. Comparing Figure 2A and B, it can be seen that the amide II band gives much better discrimination between high tilt angles at low values of ϑ_M than does the amide I band. The vertical bars in Figure 2, and subsequent Figures, indicate the range of a 10% error in dichroic ratio (at this level, differences between thick and thin film approximations for the evanescent field intensity or between ATR crystals are relatively unimportant). This gives some indication of the degree of discrimination that can be obtained in practice. For $\gamma = \Theta_M$, and $\vartheta_M = 180°$, the dichroic ratio diverges, because (assuming a unique orientation) the transition moment is then oriented exactly along the z-axis. In contrast to the situation for axial summation over the whole helix, very high dichroism may be achieved in principle for a single isotopically labelled amide.

Azimuthal orientation in an α-helix

In Figure 2, the values of the azimuthal angle, ϑ_M, for the different amide bands have, for simplicity, been treated as independent. As already mentioned, however, they bear a fixed relation to one another. Whenever dichroic ratios of different amide bands are combined, the relative values for ϑ_M must be included explicitly. Most importantly, such considerations are also essential if the azimuthal orientation of the transition moment is to be related to the geometry of the molecular structure (i.e. to the peptide carbonyl orientation).

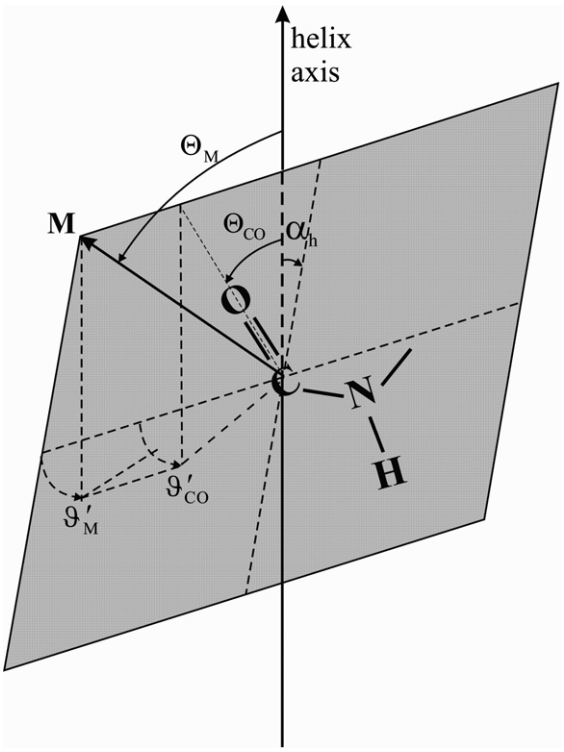

Figure 3. Azimuthal orientations, ϑ'_M and $\vartheta'_{C'O}$, of the amide transition moment (**M**) and peptide carbonyl bond (C═O), respectively, in the peptide axis system. The peptide plane is shown shaded and is inclined at an angle α_h to the helix axis. The transition moment and C═O bond are tilted at angles Θ_M and $\Theta_{C'O}$, respectively, to the helix axis. The view is from outside a right-handed α-helix, where the helix axis is directed from the N terminus to the C terminus.

The transition moments of the amide I, amide II and amide A modes lie in the peptide plane and are tilted relative to the carbonyl group in a direction away from the nitrogen atom.[13] The angular relations of the peptide group and its transition moment to the helix axis are given in Figure 3. The peptide plane is inclined at an angle α_h to the helix axis and the peptide carbonyl is tilted by an angle $\Theta_{C'O}$ to the helix axis. From the refined coordinates of α-poly-L-alanine: $\alpha_h = 6.1°$ and $\Theta_{C'O} = 14.2°$, and from an energy-refined structure of a standard right-handed α-helix: $\alpha_h = 3.3°$ and $\Theta_{C'O} = 14.8°$.[12] The azimuthal orientation, ϑ'_M, of the transition moment in the peptide axis system is defined as the angle that the plane containing the transition moment and helix axis makes with that containing the helix axis and its orthogonal within the peptide plane (see Figure 3). This is

Table 1. Tilt, Θ_M, and azimuthal orientation, ϑ'_M, of the transition moments, M, of the amide bands for α-helices and anti-parallel β-sheets

Band	α-Helix		β-Sheet	
	Θ_M (°)[a]	$\vartheta'_M - \vartheta'_{C'O}$ (°)[b]	Θ_M (°)[c]	ϑ'_M (°)[d]
Amide I	38	−17 (−8)	73 ± 3	9 ± 2
Amide II	73	−23 (−12)	29 ± 3	63 ± 10
Amide A	29	−14 (−7)	~77–78[e]	~6–7[e]

[a] Experimental values from Marsh *et al*.[12]
[b] Values relative to those of the peptide C═O bond (see Figure 3) calculated from equation (3). Values are obtained using the peptide orientation of α-poly-L-alanine and (in parentheses) an energy refined right-handed α-helix.[12]
[c] Values calculated from equation (22), using data from Appendices A and B.
[d] Values likewise calculated from equation (23) and referred to the *a*-axis joining equivalent C^α atoms of adjacent strands (see Figure 7).
[e] These values are subject to some uncertainty (see Appendix B).

given by:

$$\sin \vartheta'_M = \frac{\tan \alpha_h}{\tan \Theta_M} \tag{3}$$

and a similar equation relates the azimuthal angle, $\vartheta'_{C'O}$, of the peptide carbonyl to its orientation $\Theta_{C'O}$ to the helix axis. The azimuthal orientation of the transition moment relative to that of the peptide carbonyl bond is then given simply by $\vartheta'_M - \vartheta'_{C'O}$. This angle is negative (with *c* directed from the N to the C terminus) because the transition moment is tilted to the carbonyl bond in a direction away from the peptide nitrogen atom (see Figure 3). From equation (3) and its equivalent for the peptide C═O bond, the values of $\vartheta'_M - \vartheta'_{C'O}$ for the amide I, amide II and amide A bands are given in Table 1. The values of Θ_M given above,[12] and those of the peptide orientation from the α-poly-L-alanine (or standard α-helix) coordinates are used to obtain these data. Clearly, the choice of helix-structure coordinates introduces some uncertainty in the absolute value of the azimuthal orientation. However, the differences between amide bands, viz. −6° (−3°) and +3° (+2°) for the amide II and amide A bands, respectively, relative to the amide I band, are specified with better precision.

Finally, the azimuthal orientation, ϑ_M, defined in Figure 1 is given by: $\vartheta_M = \vartheta_{C'O} + \vartheta'_M - \vartheta'_{C'O}$, where $\vartheta_{C'O}$ is the azimuthal orientation of the peptide carbonyl bond relative to the *z*–*c* plane. The latter is the quantity of direct structural interest and can be obtained from the infrared dichroism measurements of ϑ_M to within the uncertainty in the values of $\vartheta'_M - \vartheta'_{C'O}$ mentioned above.

A quantity of ancillary interest is the tilt orientation, $\theta_{C'O}$, of the peptide carbonyl bond to the *z*-axis. This has been used in structure refinement by molecular dynamics simulations that are restrained with $\theta_{C'O}$ obtained from infrared dichroism measurements as the target value.[1,2] From the geometry in Figures 1 and 3, and the relations

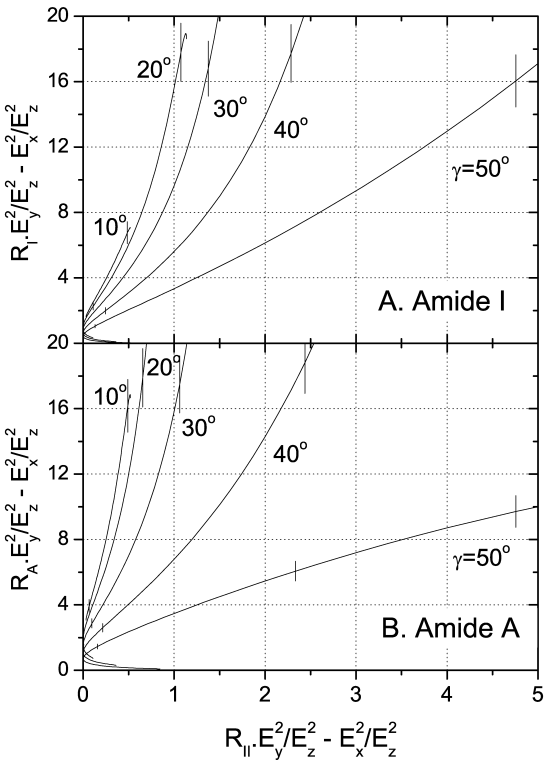

Figure 4. Relations between the dichroic ratios of: A. The amide I (R_I) and amide II (R_{II}) bands, and B. The amide A (R_A) and amide II (R_{II}) bands, for a single position of isotopic labelling in an α-helix. The dichroic ratios are scaled and shifted by E_y^2/E_z^2 and E_x^2/E_z^2, respectively, to give values of $\langle M_z^2\rangle/\langle M_y^2\rangle$ (see equation (2)) that are independent of the particular experimental set-up. Vertical bars indicate the range of a 10% error in dichroic ratio. The values of ϑ_M for the amide II band are displaced by −5° relative to the amide I band in A, and by −7° relative to the amide A band in B, in these calculations.

given above for the azimuthal orientation of the transition moment:

$$\cos \theta_{C'O} = \cos \gamma \cos \Theta_{C'O} - \sin \gamma \sin \Theta_{C'O} \times \cos(\vartheta_M - \vartheta'_M + \vartheta'_{C'O}) \tag{4}$$

where the argument of the final cosine term is the azimuthal orientation $\vartheta_{C'O}$ of the peptide carbonyl relative to the *z*–*c* plane. Equation (4) expresses the desired target angle, $\theta_{C'O}$, in terms of the variables γ and ϑ_M accessible from experiment and the fixed values $\Theta_{C'O}$, $\vartheta'_{C'O}$ and ϑ'_M (cf. equation (3)). It differs from the target angle (given by Kukol & Arkin)[2] because here the relative orientations of the transition moment and peptide carbonyl bond are treated exactly. In unfavourable circumstances, the difference can be quite appreciable.

Combination of α-helix amide bands

As already mentioned, interpretation of the helix dichroism requires at least two independent dichroic ratios, even under the assumption of

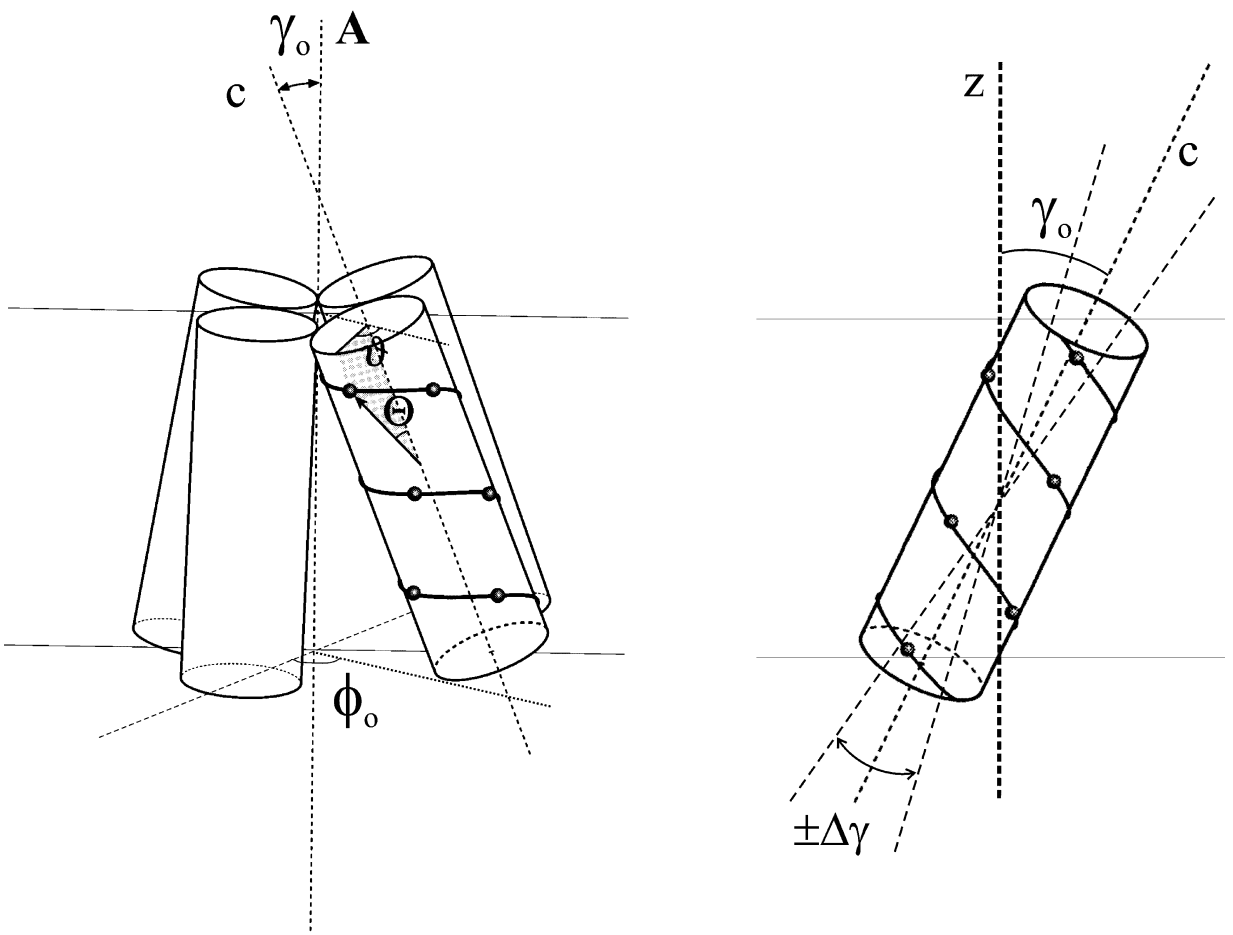

Figure 5. Left, orientation of the α-helices in a polytopic protein. The tilt, γ_o, and azimuthal orientation, ϕ_o, of an individual helix in the axis system of the α-helical bundle are indicated. In general, the symmetry axis **A** of the helix bundle is inclined at an angle α, with axial symmetry, relative to the membrane normal, *z*. The tilt, Θ, and azimuthal orientation, ϑ, of the transition moment, relative to the helix axis, are also indicated. The origin for ϑ is defined by the plane containing **A** and the helix axis. Right, orientation of a single bitopic protein, relative to the membrane normal, *z*. The helix axis has a distribution, $\pm\Delta\gamma$, of tilt angles about the mean value, γ_o.

fixed values for the angular orientations γ and ϑ_M. This is illustrated in Figure 4, by combination of the dichroic ratios of the different amide bands for a single position of isotopic labelling. The dichroic ratio of the amide I or amide A band is plotted against that of the amide II band for fixed values of the helix tilt, γ. These relations cover the whole range of azimuthal orientations of the helix from $\vartheta_M = 0°$ to $\vartheta_M = 180°$ (cf. Figure 2) and include the relative offsets in ϑ_M between the different amide bands that are obtained from Table 1. It is seen that a given combination of dichroic ratios from two amide bands is determined uniquely. This, therefore, specifies the required combination of orientations γ and ϑ_M. Note that the relation between two dichroic ratios, in general, has two limbs for a given value of γ (in the region $x < 1$), but this does not give rise to ambiguities. As stated previously, resolution of the various isotopically labelled amide bands for a single residue may require the use of more than one isotope.

For low tilt angles, $\gamma \leq 30°$, the amide A dichroism is greater than that of the amide I band and therefore could be combined advantageously with amide II dichroic measurements. As already noted in connection with Figure 2, for large tilt angles and low values of ϑ_M ($\gamma > 60°$ and $\vartheta_M < 50°$) the amide II dichroism is greater than that of the amide I band. Under the latter circumstances, combination of amide I and amide II bands for a single position of isotope labelling is likely to perform better than combination of amide I dichroic ratios for two adjacent isotopic labelling positions. Such situations cannot readily be decided *a priori*, but it is clear from Figure 4 that combination of different amide bands potentially adds flexibility to site-directed dichroism studies.

Distribution of helix orientations

In general, the orientation of the transmembrane helix, or helix assembly, may not be single-valued, but rather may have a limited angular distribution. The case of a distribution in the helix tilt, γ, is considered first. Two situations may be distinguished: that of a single bitopic transmembrane

helix, and that of a polytopic transmembrane helix bundle.

For a transmembrane helix bundle, the constituent α-helices will individually have a fixed tilt, γ_o, and a fixed azimuthal orientation, ϕ_o, relative to the symmetry axis, *A*, of the helix assembly (see Figure 5, left panel). The latter is taken to be oriented at an angle α to the membrane normal, *z*. The angle, γ, that the helix axis makes with the *z*-axis is then given by:

$$\cos\gamma = \cos\gamma_o \cos\alpha + \sin\gamma_o \sin\alpha \cos(\phi_o - \phi_\alpha) \quad (5)$$

where ϕ_o and ϕ_α are the azimuthal orientations of the helix axis and of the *z*-axis, respectively, about the axis of the helix assembly. Because the latter is axially distributed about the *z*-axis, the azimuthal angle ϕ_α must be integrated over the full range from 0 to 2π. For $\cos^2\gamma$ this gives (independent of ϕ_o):

$$\langle\cos^2\gamma\rangle = \left(\cos^2\gamma_o - \frac{1}{3}\right)\langle P_2(\cos\alpha)\rangle + \frac{1}{3} \quad (6)$$

where $\langle P_2(\cos\alpha)\rangle$ is the order parameter of the helix bundle and angular brackets indicate integration over α. Equation (6) is consistent with the addition theorem for Legendre polynomials, where $\langle P_2(\cos\alpha)\rangle = \frac{1}{2}(3\langle\cos^2\alpha\rangle - 1)$. The simplest model is to assume that α has a single fixed value. The next simplest model is one in which the symmetry axis is randomly distributed within a cone of amplitude α_o and has an order parameter given by:

$$\langle P_2(\cos\alpha)\rangle = \frac{1}{2}\cos\alpha_o(1 + \cos\alpha_o) \quad (7)$$

Both models, therefore, depend on only a single parameter, either the fixed tilt α, or the angular amplitude α_o.

The other angular average over γ that is required for equation (2) is that of sin γ cos γ which is given by:

$$\langle\sin\gamma\cos\gamma\rangle = \frac{\int_0^{\alpha_0}\int_0^{2\pi}\cos\gamma\sqrt{1-\cos^2\gamma}\,\sin\alpha\,d\phi_\alpha\,d\alpha}{2\pi(1-\cos\alpha_o)} \quad (8)$$

where cos γ is given by equation (5). This integral is independent of ϕ_o and is most conveniently evaluated numerically. The dependence on the amplitude α_o of the cone in which the symmetry axis is randomly distributed is given in Figure 6A, for different values of γ_o. For the simpler model in which α is fixed, the integral over sin α dα in equation (8) can be omitted. The resulting dependence of ⟨sin γ cos γ⟩ on the fixed value of α is given in Figure 6B.

A single bitopic transmembrane helix also may have a non-zero tilt relative to the membrane normal. This can arise because the hydrophobic span of the protein may be greater than the hydrophobic thickness of the lipid membrane. Additionally, the anchoring residues at the ends of the helix may not be situated at the same level across the diameter of the helix, which again would favour a tilted structure. In this case, it is more likely that the spread in γ will be centred about the local mean tilt γ_o, rather than about the membrane normal (see Figure 5, right panel). A possible way to model this is to consider a random distribution within cones of angles $\gamma_o \pm \Delta\gamma$, where $\Delta\gamma \ll \gamma_o$. The angular averages in equation (2) then take the form:

$$\langle\cos^2\gamma\rangle = \frac{\int_{\gamma_o-\Delta\gamma}^{\gamma_o+\Delta\gamma}\cos^2\gamma\,\sin\gamma\,d\gamma}{\int_{\gamma_o-\Delta\gamma}^{\gamma_o+\Delta\gamma}\sin\gamma\,d\gamma} \quad (9)$$

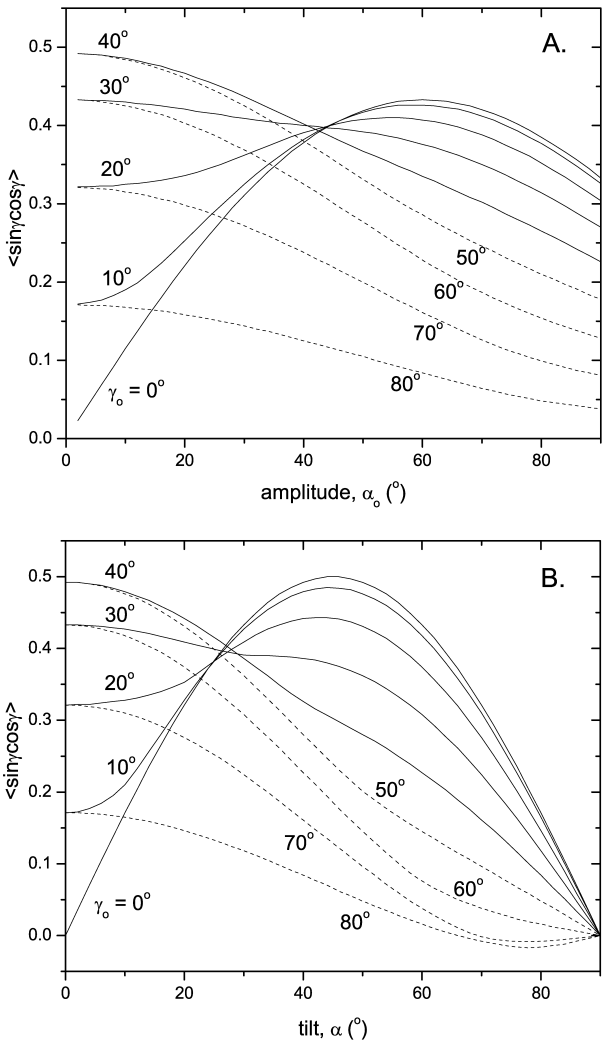

Figure 6. Dependence of the average value of ⟨sin γ cos γ⟩ on: A, the maximum angular amplitude α_o of the limited random distribution in orientation of the helix bundle axis; or on B, the fixed tilt α of the helix bundle axis, according to equations (5) and (8). Numerical integrations are given for different values of the individual helix tilt, γ_o, as indicated. Continuous lines are for $\gamma_o \leq 40°$ and broken lines for $\gamma_o > 40°$. For $\gamma_o = 90°$, the value is zero independent of α_o, or α.

for example. On performing the integrations, the following results are obtained:

$$\langle \cos^2 \gamma \rangle = \cos^2 \gamma_o \cos^2 \Delta\gamma + \frac{1}{3}\sin^2 \gamma_o \sin^2 \Delta\gamma \quad (10)$$

$$\langle \sin \gamma \cos \gamma \rangle = (\sin^2 \gamma_o \cos^2 \Delta\gamma + \frac{1}{3}\cos^2 \gamma_o \sin^2 \Delta\gamma)/\tan \gamma_o \quad (11)$$

In both cases, for either bitopic or polytopic α-helical proteins, two parameters (γ_o, and α_o or $\Delta\gamma$) are required to specify the required averages over the tilt angle in equation (2). Even for these relatively simple models, the averages $\langle \cos^2 \gamma \rangle$ and $\langle \sin \gamma \cos \gamma \rangle$ are not uniquely related by a single parameter. In consequence, at least three independent dichroic ratios (either from different amide bands or different sites of labelling) are needed in the case of a distribution in tilt angles.

The situation is slightly simpler for a single bitopic transmembrane helix that matches the hydrophobic span of the lipid membrane. A reasonable model in this case is that the helix axis is randomly distributed about the $\gamma = 0$ orientation, within a cone of semi-angle $\Delta\gamma$. The angular averages over the tilt γ are then given by:

$$\langle \cos^2 \gamma \rangle = \frac{1}{3}(\cos^2 \Delta\gamma + \cos \Delta\gamma + 1) \quad (12)$$

and:

$$\langle \sin \gamma \cos \gamma \rangle = \frac{1}{3}\sin \Delta\gamma(\cos \Delta\gamma + 1) \quad (13)$$

These depend upon a single parameter $\Delta\gamma$, viz. the amplitude of the distribution.

For a polytopic α-helical membrane protein, the azimuthal orientations of the constituent helices are fixed by the tertiary structure of the helix bundle. In this case, the azimuthal orientation, ϑ_M, is single-valued. For a single, bitopic transmembrane helix, some limited distribution in azimuthal orientation may be allowed within the membrane. The simplest model is a random distribution within the range $\vartheta_M = \vartheta_o \pm \Delta\vartheta$. The angular averages then take the form, e.g.:

$$\langle \cos^2 \vartheta_M \rangle = \frac{\int_{\vartheta_o - \Delta\vartheta}^{\vartheta_o + \Delta\vartheta} \cos^2 \vartheta \, d\vartheta}{\int_{\vartheta_o - \Delta\vartheta}^{\vartheta_o + \Delta\vartheta} d\vartheta} \quad (14)$$

and the values required in equation (2) are:

$$\langle \cos^2 \vartheta_M \rangle = \frac{1}{2}\left(1 + \frac{\cos 2\vartheta_o \sin \Delta\vartheta \cos \Delta\vartheta}{\Delta\vartheta}\right) \quad (15)$$

and:

$$\langle \cos \vartheta_M \rangle = \frac{\cos \vartheta_o \sin \Delta\vartheta}{\Delta\vartheta} \quad (16)$$

Again, two parameters, ϑ_o and $\Delta\vartheta$, are required. The total number of independent dichroic ratios needed to specify the system fully, then reaches its maximum value of four (for a perfectly ordered sample).

Contributions from membrane disorder

A further contribution to the distribution in tilt angle, γ, and in azimuthal orientation, ϑ_M, comes from the possible presence of disordered regions in the sample. For parts of the sample that are completely disordered, the membrane normal (and correspondingly the tilt of the helix axes) is randomly distributed relative to the *z*-axis, i.e. to the normal to the orienting substrate. Similarly, the origin of the azimuthal orientation, ϑ_M, which is defined by the plane containing the *z*- and *c*-axes, will be randomly distributed relative to the orienting substrate. Thus, for such non-oriented regions $\langle \cos^2 \gamma \rangle = 1/3$, $\langle \sin \gamma \cos \gamma \rangle \langle \cos \vartheta_M \rangle = 0$ and $\langle \sin^2 \gamma \rangle \langle \cos^2 \vartheta_M \rangle = 1/3$ from equations (6), (8), (15) and (16), and Figure 6. If the fraction of sample that is (completely) disordered is $(1 - f)$, the angular averages in equation (2) for the total sample become:

$$\langle \cos^2 \gamma \rangle = f\langle \cos^2 \gamma \rangle_o + (1 - f)/3 \quad (17)$$

$$\langle \sin \gamma \cos \gamma \rangle \langle \cos \vartheta_M \rangle = f\langle \sin \gamma \cos \gamma \rangle_o \langle \cos \vartheta_M \rangle_o \quad (18)$$

and:

$$\langle \sin^2 \gamma \rangle \langle \cos^2 \vartheta_M \rangle = f\langle \sin^2 \gamma \rangle_o \langle \cos^2 \vartheta_M \rangle_o + (1 - f)/3 \quad (19)$$

where the subscript o refers to the oriented part of the sample.

It follows from equations (17) and (19) that the fractions, *f*, of oriented sample act as an "order parameter" that would simply scale those of the helix axis in the case of axial symmetry.[11] For completely axial symmetry, sample disorder cannot, therefore, be separated from the orientation of the helix axis (see equation (2)). This is not the case, however, for isotopically edited amides. Combination of equations (6), (8) and equations (15), (16) with equations (17)–(19) shows that, in principle, sample disorder can readily be distinguished from an intrinsic limited distribution in the orientational angles. However, because maximally four independent angular averages can be obtained from dichroic ratio measurements (see equation (2)), this would require that one of the two angular parameters γ or ϑ_M must be fixed. From the discussion above, it seems most appropriate to assume that ϑ_M is single-valued. The case of a partially unoriented sample, in the absence of angular distributions in both γ and ϑ_M, has been considered.[5]

Practical examples

Dichroic measurements of the amide I band have been made on ^{13}C-isotopically labelled amides in the transmembrane segment of glycophorin A.[5]

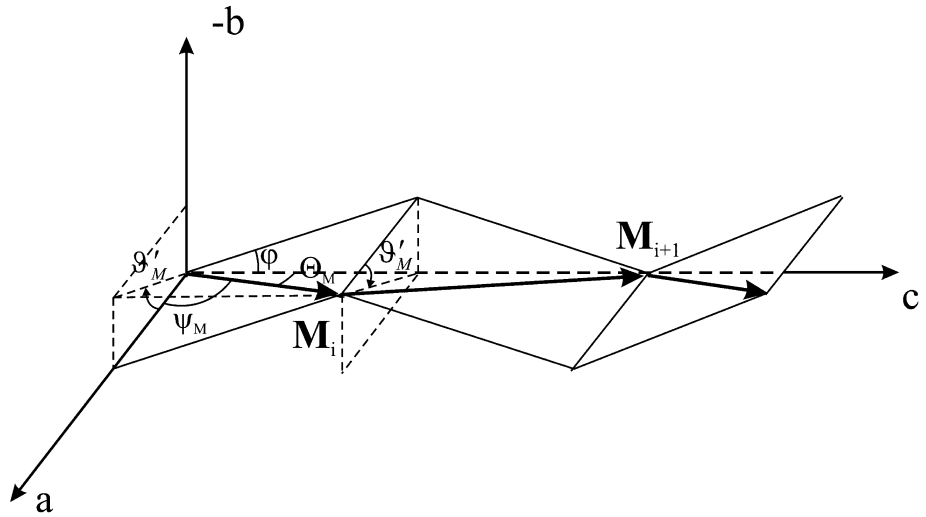

Figure 7. Orientation of the transition moments of individual amide groups, *i*, in a β-pleated sheet. The transition moment, **M**, lies in the peptide plane, which is inclined at an angle φ to the β-strand axis, *c*. The orthogonal *a*-axis lies in the plane of the sheet and is defined by the intersection of the peptide planes. The transition moment makes an angle ψ_M with the *a*-axis. Consecutive residues, *i* and $i+1$, have azimuthal orientations, ϑ'_M, of opposite sense relative to the *a*-axis.

A sufficient number of isotopic labels (G84, V85 and G86) was used such that, together with the non-isotopically labelled species, there was redundance in determination of the helix orientation, even allowing for sample disorder. A similar analysis of these data, but allowing for the possibility of a distribution in helix angles according to equations (1), (2) and (17)–(19), yields values for $\langle\cos^2\gamma\rangle$ and $\langle\sin\gamma\cos\gamma\rangle$, respectively, of 0.62 and 0.31, using $\Theta_I = 38°$ (see Table 1). The corresponding single values obtained for the azimuthal orientation, ϑ_M, and degree of alignment, *f*, are similar to those reported in the original publication. The values of the two order parameters $\langle\cos^2\gamma\rangle$ and $\langle\sin\gamma\cos\gamma\rangle$ are not entirely consistent with a single unique value for the helix tilt, γ. The dichroic ratios are fit somewhat better by a model of random distribution within a cone of semi-angle $\Delta\gamma \approx 44°$ (from equations (12) and (13), together with equations (1), (2) and (17)–(19)), than by a fixed optimum tilt of 29°. For isotopically labelled residues I41, G43 and V44 in the regular part of the transmembrane helix of CD3-ζ from the T-cell receptor,[3] the helical tilt is small and consequently no improvement in fitting the dichroic ratios is obtained by considering a distribution of tilt angles. From a practical point of view, a redundancy in positions of labelling, and use of more than one amide band, will improve the experimental statistics in deciding between fixed orientations and orientational distributions.

β-Sheets and β-barrels

β-Sheet orientation

The situation is less straightforward for transmembrane β-sheets than for α-helices, because the angular variables given in equation (2) do not correspond so directly with the β-sheet geometry. As pointed out in previous work, the tilt angle γ of the β-strand axis (i.e. the *c*-axis) is given by:[7]

$$\cos\gamma = \cos\alpha_s \cos\beta \qquad (20)$$

where α_s is the angle by which the plane of the β-sheet is inclined to the *z*-axis and β is the tilt angle of the β-strand within the sheet (see Figure 1). The origin for β is given by the projection of the *z*-axis on the plane of the sheet.

The azimuthal orientation, ϑ, of the plane of the sheet, relative to the z–c plane is as given previously:[7]

$$\cos\vartheta = \frac{\cos\alpha_s \sin\beta}{\sqrt{1-\cos^2\alpha_s\cos^2\beta}} \qquad (21)$$

where use has been made of equation (20). This is the azimuthal orientation of an axis lying in the plane of the sheet that is orthogonal to the β-strand axis (i.e. to the *c*-axis). In the local sheet coordinate system, this is defined as the *a*-axis (see Figure 7).

Transition moment orientation in a β-sheet

The transition moment, **M**, of an individual isotopically labelled amide lies in the plane of the peptide group. The latter does not coincide, however, with the plane of the β-sheet, because of its pleated structure (see Figure 7). The peptide plane is inclined at an angle φ with respect to the strand axis, *c*. The individual transition moment is tilted at an angle ψ_M to the perpendicular axis, *a*, that lies within the plane of the sheet. The inclination of the transition moment, **M**, to the strand

axis, c, is therefore given by (see Figure 7):

$$\cos \Theta_M = \cos \varphi \sin \psi_M \quad (22)$$

The pleating angle of the β-sheet is $\varphi = 25°-28°$, deduced from refined X-ray coordinates of β-poly-L-alanine and β-keratin (see Appendix A). For antiparallel β-sheets, the orientations of the transition moments of the amide I, amide II and amide A bands are in the region of $\psi_I = 19° \pm 3°, \psi_{II} = 77° \pm 5°$ and $\psi_A \sim 14°$, respectively (see Appendix B). This then yields the values of Θ_M for the orientation of the individual amide I, amide II and amide A transition moments in an antiparallel β-sheet that are listed in Table 1. The value for the amide A band is subject to some uncertainty (see Appendix B), although it must lie reasonably close to that for the amide I band.

The azimuthal orientation ϑ_M is determined by that of the transition moment relative to the a-axis, i.e. ϑ'_M (see Figure 7) and that of the a-axis, i.e. of the peptide plane, relative to the z-axis, i.e. ϑ (see Figure 1). Within the β-sheet, the azimuthal orientation of the transition moment, relative to the a-axis is given by (see Figure 7):

$$\cos \vartheta'_M = \frac{\cos \psi_M}{\sin \Theta_M} = \frac{\cos \psi_M}{\sqrt{1 - \cos^2 \varphi \sin^2 \psi_M}} \quad (23)$$

where use has been made of equation (22). With the values of φ and ψ_M quoted above, this yields the values of ϑ'_M for the amide I, amide II and amide A bands that are listed in Table 1. Again, the values for the amide A band are subject to some uncertainty.

β-Sheet dichroism

The azimuthal orientation of the transition moment, relative to the z–c plane, required in equation (2) is related to that of the β-sheet (i.e. ϑ) by $\vartheta_M = \vartheta \pm \vartheta'_M$. The alternation in sign comes from the two-residue periodicity in azimuthal orientation about the strand c-axis (see Figure 7) and corresponds to the 180° relative orientations of consecutive residues in a β-strand. From equation (21), the net azimuthal orientation of the transition moment is therefore given by:

$$\cos \vartheta_M = \frac{\cos \alpha_s \sin \beta \cos \vartheta'_M \mp \sin \alpha_s \sin \vartheta'_M}{\sqrt{1 - \cos^2 \alpha_s \cos^2 \beta}} \quad (24)$$

where ϑ'_M is obtained from equation (23). Substitution from equations (20) and (22)–(24) into equation (2) then gives the dependence of the dichroic ratio on the inclination α_s of the β-sheet, relative to the membrane normal, and on the tilt angle β of the β-strands within the β-sheet.

A compact form that gives the dependence of $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ on the orientational parameters, α_s and β, of the β-sheet explicitly is:

$$2\frac{\langle M_y^2 \rangle}{\langle M_z^2 \rangle} = \frac{1}{\langle [(\cos \varphi \sin \psi_M \cos \beta - \cos \psi_M \sin \beta)\cos \alpha_s \pm \sin \varphi \sin \psi_M \sin \alpha_s]^2 \rangle} - 1 \quad (25)$$

Even if α_s and β are single-valued, two dichroic ratios are required to specify the orientation and conformation of the β-sheet. This is already the case for non-isotopically edited β-sheets[7] except that, as already noted, here different isotopes will be required when isotopically edited data for the amide I and amide II bands are combined. A possibly better alternative, therefore, is to combine data from the same isotopic label on two consecutive residues. If there are non-singular distributions in the angles α_s and β, the dichroic ratios of isotopically edited β-sheets depend on four parameters: $\langle \cos^2 \alpha_s \rangle$, $\langle \cos^2 \beta \rangle$, $\langle \cos \alpha_s \rangle$ and $\langle \cos \beta \rangle$. This is not true in the non-isotopically edited case, where the dichroic ratios depend only on $\langle \cos^2 \alpha_s \rangle$ and $\langle \cos^2 \beta \rangle$. The distributions in α_s and β can be considered as being independent, however, because the tilt of the sheets will not be correlated with the tilt of the strands within the sheets.

It should be pointed out that this treatment for β-sheets applies equally well to isotopically edited amides in β-barrels. Just as for α-helices considered above, there is no axial symmetry imposed by the β-barrel when considering only a single isotopically labelled amide. In equations (20), (21), (24) and (25), the angle β is then the tilt of the β-strands relative to the barrel axis, and the angle α_s is the inclination of the barrel axis to the membrane normal.

β-Sheets: single orientations

For the amide I, amide II and amide A bands, the dependence of $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ on the strand tilt β within the sheet is given in Figure 8, for various values of the inclination α_s of the β-sheet to the membrane normal. Correspondingly, the functional dependence of the dichroic ratio on the tilt, α_s, of the β-sheets is given in Figure 9 for fixed values of the strand tilt, β. The values of Θ_M and ϑ'_M given for β-sheets in Table 1 are used in these calculations. For $\alpha_s = 90°$, the dichroic ratios are independent of the strand tilt β, and $\langle M_z^2 \rangle / \langle M_y^2 \rangle$ has the constant value: $2 \sin^2 \Theta_M \sin^2 \vartheta'_M / (1 - \sin^2 \Theta_M \sin^2 \vartheta'_M) = 0.046 \pm 0.022$, 0.46 ± 0.21 and $\sim 0.02 - 0.03$ for the amide I, amide II and amide A bands, respectively. Otherwise, it is clear from Figures 8 and 9 that the dependence of the dichroic ratio on the strand tilt β is very different from that on tilt α_s of the β-sheet. For each combination of α_s and β there are two possible values of dichroic ratio, corresponding to the azimuthal orientations ϑ_M and $\vartheta_M + 180°$ of successive residues. Therefore, single isotopic labelling of more than two adjacent residues is redundant for a perfect β-sheet. Practically, single isotope labels at several positions are useful for mapping out the extent of the β-strand regions and for improving statistics. In addition, multiple labelling at all-odd

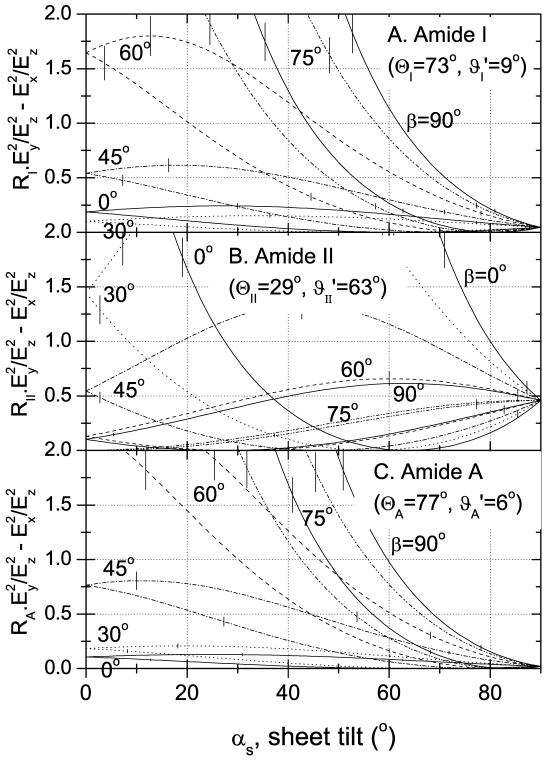

Figure 8. Dependence of: A, the amide I dichroic ratio, R_I; B, the amide II dichroic ratio, R_{II}; and C, the amide A dichroic ratio, R_A, of an isotopically edited residue on the tilt β of the β-strands in a β-sheet for various inclinations, α_s, of the β-sheets to the membrane normal. The dependences are obtained from equations (1), (2), (20) and (24) with: A, $\Theta_I = 73°$, $\vartheta'_I = 9°$; B, $\Theta_{II} = 29°$, $\vartheta'_{II} = 63°$; and C, $\Theta_A = 77°$, $\vartheta'_A = 6°$. The values of α_s are indicated in the Figure. Each pair of lines with the same pattern corresponds to a given value of α_s. In A and C, the lower line of each pair corresponds to ϑ'_M and the upper to $-\vartheta'_M$ and *vice versa* in B (cf. equation (24)). The single continuous line is for $\alpha_s = 0°$. The ordinate is scaled and shifted by the ratios of the radiation electric field intensities, E_y^2/E_z^2 and E_x^2/E_z^2, respectively, to give values of $\langle M_z^2\rangle/\langle M_y^2\rangle$ (see equation (2)) that are independent of the particular experimental set-up. Vertical bars indicate the range of a 10% error in dichroic ratio.

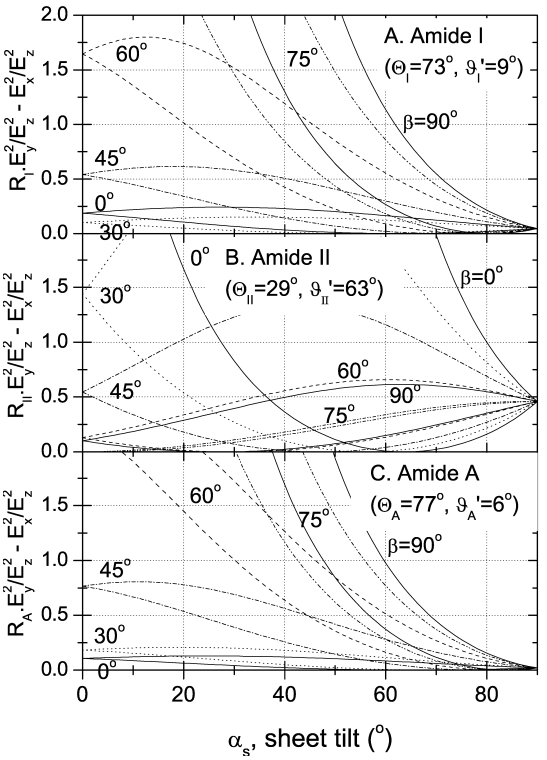

Figure 9. Dependence of: A, the amide I dichroic ratio, R_I; B, the amide II dichroic ratio, R_{II}; and C, the amide A dichroic ratio, R_A, of an isotopically edited residue on the tilt α_s of the β-sheet for various values of the strand tilt, β. The dependences are obtained from equations (1), (2), (20) and (24) with: A, $\Theta_I = 73°$, $\vartheta'_I = 9°$; B, $\Theta_{II} = 29°$, $\vartheta'_{II} = 63°$; and C, $\Theta_A = 77°$, $\vartheta'_A = 6°$. The values of β are indicated on the Figure. Each pair of lines with the same pattern corresponds to a given value of β. In A and C, the lower line of each pair corresponds to ϑ'_M and the upper to $-\vartheta'_M$, and *vice versa* in B (cf. equation (24)). The continuous lines are for $\beta = 90°$ and also for $\beta = 0°$. The ordinate is scaled and shifted by the ratios of the radiation electric field intensities, E_y^2/E_z^2 and E_x^2/E_z^2, respectively, to give values of $\langle M_z^2\rangle/\langle M_y^2\rangle$ (see equation (2)) that are independent of the particular experimental set-up. Vertical bars indicate the range of a 10% error in dichroic ratio.

or all-even residue positions would increase signal-to-noise in the isotope-edited region, provided that the labels are sufficiently well spaced in the sequence that there is no coupling between their modes. Results with α-helices double-labelled at positions seven residues apart suggest little coupling between modes.[2] In the latter case, the residues are not so rigorously equivalent as they would be in the β-sheet situation. For hydrophobic β-sheet peptides, some coupling has been detected with more closely spaced isotope labelling positions.[14]

A striking feature of Figure 8 is the very low sensitivity of the amide I (or amide A) dichroic ratio to the lower range of strand tilts, $\beta < 40°$. In this range, measurements of amide II dichroism are practically obligatory. Also, it appears from Figure 9 that the amide II dichroism has superior sensitivity at high values of the sheet tilt, $\alpha_s > 70°$. In the complementary regions of strand and sheet tilt, the double entries for the amide I dichroic ratios in Figures 8A and 9A indicate that good sensitivity can be obtained by isotopic labelling at two adjacent residue positions.

Distribution of β-sheet and β-strand orientations, and membrane disorder

Just as for α-helices, the orientation of the β-sheets or β-barrels, specified by the tilt, α_s, may have a distribution of values. A limited variation in the tilt, β, of the β-strands within the sheet or barrel may also be possible. Expansion of equation (25) shows that a maximum of six order parameters is required to allow for a general

distribution in both α_s and β. These are the distributional averages $\langle\cos^2\alpha_s\rangle$, $\langle\sin\alpha_s\cos\alpha_s\rangle$, $\langle\cos^2\beta\rangle$, $\langle\sin\beta\cos\beta\rangle$, $\langle\cos\beta\rangle$ and $\langle\sin\beta\rangle$. Only two independent dichroic ratios can be determined by isotopic labelling from a single amide band, in a β-sheet. One further independent dichroic ratio can be determined from the same band for the coupled mode of the non-isotopically labelled amides.[7] Therefore, in the general case, it would be necessary to combine measurements from minimally two modes, e.g. amide I, and amide II, both from non-isotopically labelled amides and from two (odd and even) positions of isotopic labelling. If the strand tilt, β, is assumed approximately to have a single value, then only three parameters, $\langle\cos^2\alpha_s\rangle$, $\langle\sin\alpha_s\cos\alpha_s\rangle$ and β are required to describe the orientational distribution fully. In this case, one amide band with two positions of isotopic labelling, together with the same band from the non-isotopically labelled amide, would be the minimum requirement. Alternatively, one position of isotopic labelling, e.g. with ^{13}C for the amide I band, could be combined with dichroic ratios from the amide I and amide II bands of the non-isotopic labelled sample.

Distributions in the sheet or barrel tilts, α_s, are analogous to those for the orientation of α-helical assemblies and can be described by similar models, i.e. the equivalents of equations (6)–(8) or (10) and (11). As for α-helices, however, this does not reduce the number of independent parameters required. The situation for the strand tilt, β, is analogous to that discussed already for the azimuthal orientation, ϑ_M, of an α-helix. A similar model may be applied. The tilt of the strand within the sheet is restricted to a mean angle β_o, with distribution width Δβ. Expressions equivalent to equations (15) and (16) then hold for $\langle\cos^2\beta\rangle$ and $\langle\cos\beta\rangle$, respectively. The remaining order parameters, according to this model are then given by:

$$\langle\sin\beta\cos\beta\rangle = \frac{\sin 2\beta_o \sin 2\,\Delta\beta}{4\Delta\beta} \qquad (26)$$

and:

$$\langle\sin\beta\rangle = \frac{\sin\beta_o \sin\Delta\beta}{\Delta\beta} \qquad (27)$$

This model reduces the number of parameters required to describe the distribution in β-strand tilt from four to two.

If the aligned sample contains disordered regions, then the measured dichroic ratios depend on the additional parameter, *f*, the fraction of sample that is aligned. The situation is exactly equivalent to that considered for α-helices. Combining equations (2), and (17)–(19), leads to the following version of equation (25) for β-sheets:

$$\frac{2\langle M_y^2\rangle}{\langle M_z^2\rangle} = \frac{1}{f\langle[(\cos\varphi\sin\psi_m\cos\beta - \cos\psi_m\sin\beta)\cos\alpha_s \pm \sin\varphi\sin\psi_m\sin\alpha_s]^2\rangle + (1-f)/3} - 1 \qquad (28)$$

Measurement of a further independent dichroic ratio is then required, relative to the situations considered above for ideally aligned samples, in order to eliminate *f*.

Conclusion

The principal results obtained from this study may be summarised as follows.

(1) If dichroic ratios are measured for more than one position of isotopic labelling in an α-helix, together with the non-isotopic labelled amide, information can be obtained on the distribution of helix tilts, α_h, as well as the azimuthal orientation, ϑ_M, of the transition moment. In the case of sample disorder (characterised by *f*), more than two positions of isotopic labelling are required.

(2) The azimuthal orientation, $\vartheta_{C'O}$, of the peptide carbonyl bonds in an α-helix is obtained from the dichroism measurements of ϑ_M according to: $\vartheta_{C'O} = \vartheta_M - (\vartheta'_M - \vartheta'_{C'O})$. The required values of $(\vartheta'_M - \vartheta'_{C'O})$ are given in Table 1. If dichroic ratios from different amide bands (using different isotopes) are combined, the value of ϑ_M must be incremented by the difference in values of $(\vartheta'_M - \vartheta'_{C'O})$ between the different bands. These increments in ϑ_M are $-5°$ and $+3°$ for the amide II and amide A bands, respectively, relative to the amide I band.

(3) The tilt, $\theta_{C'O}$, of the peptide carbonyl, relative to the helix axis, is given by equation (4). This may be a suitable target function for restrained molecular dynamics simulations that incorporate IR dichroic data.

(4) The dichroism of isotopically labelled β-sheets or β-barrels is given by equations (1), (2) and (17)–(19) (or equivalently equations (1) and (28)). To interpret the dichroic ratios in terms of molecular orientations, values are required for the orientation of the amide transition moment in the β-sheet peptide. These values of Θ_M and ϑ'_M are given in Table 1 (and see Appendix B). The molecular orientation is specified by the tilt, α_s, of the sheet (or barrel) and the tilt, β, of the strand in the sheet. The angles α_s and β are related to those determined directly from the dichroic ratios (viz. γ and ϑ_M in equation (2)) by equations (20) and (24).

(5) For isotopically labelled amides in β-sheets, the molecular orientation is characterised by maximally six order parameters that determine the dichroism: two for α_s and four for β. Only two (odd and even) positions of isotopic labelling generate independent dichroic ratios, for a

given amide band, in a β-sheet. These, together with the non-isotopically labelled amide, would be sufficient to determine fixed values of α_s and β, and the degree of sample alignment, *f*.

(6) Additionally, combination of dichroic ratios from different amide bands for β-sheets yields information on the orientational distribution of the sheets, and possibly of the strands within the sheets. The different orientations, Θ_M and ϑ'_M, of the transition moment that are needed for this are given for the various amide bands in Table 1.

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Appendix A: Orientation of the Peptide Plane in an Anti-parallel β-Sheet

The refined coordinates of the peptide unit in β-poly-L-alanine that were determined by Arnott *et al.*[A1] are given in Table A1. Similarly refined coordinates for β-keratin are given by Fraser *et al.*[A2] The orientation of the peptide plane is specified by the *Y* and *Z*-coordinates of successive α-carbon atoms in a strand:

$$\tan \varphi = \frac{2|Y_{C^\alpha}|}{c/2} \qquad \text{(A1)}$$

see Figure 7. From Table A1, the resulting value for the pleating angle is $\varphi = 24.6°$. The coordinates for antiparallel β-keratin, which has a shorter repeat along the strand axis, yield $\varphi = 27.6°$. For comparison, the corresponding values for antiparallel and parallel β-sheets in the standard geometry are: $\varphi = 23°$ and $\varphi = 31°$, respectively. The latter values are deduced from the coordinates (given by Fraser & MacRae).[A3] A value of $\varphi = 22°$ is obtained from the antiparallel pseudo-structures originally proposed for *Bombyx mori* and Tussah silk fibroins.[A4,A5]

The angle, $\beta_{C'O}$, that the peptide carbonyl bond makes with the *a*-axis is given by:

$$\cos \beta_{C'O} = (X_{C'} - X_O)/R_{C'O} \qquad \text{(A2)}$$

where $R_{C'O} = \sqrt{(X_{C'} - X_O)^2 + (Y_{C'} - Y_O)^2 + (Z_{C'} - Z_O)^2}$ is the length of the C═O bond. This gives

Table A1. Atomic coordinates for a peptide group in β-poly-L-alanine[A4]

Atom	*X* (nm)	*Y* (nm)	*Z* (nm)
C′	−0.049	−0.013	0.356
O	−0.170	0.013	0.353
N	0.033	0.013	0.256
C^{α}	−0.014	0.079	0.134

The *X*, *Y*, *Z* coordinates are referred to the *a*, *b*, *c* axes, respectively, in Figure 7 of the main text. The unit cell dimensions are $a = 0.473$ nm, $b = 1.053$ nm and $c = 0.689$ nm. The coordinates of the next peptide group in the strand are $-X, -Y, Z + c/2$.

inclinations of $\beta_{C'O} = 12°$ and $\beta_{C'O} = 6°$ for β-poly-L-alanine and β-keratin, respectively. Note, however, that it is directly the angle ψ_M that the transition moment makes with the *a*-axis (and not with the C═O bond) that is determined from the relative intensities of the $\nu_{\perp}(\pi,0)$ and $\nu_{\parallel}(0,\pi)$ amide modes in antiparallel β-sheets (see Appendix B).

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Appendix B: Orientation of the Transition Moments in Antiparallel β-Sheets

Information on the orientation of the individual transition moments in β-sheets is rather sparse, especially for the amide II band. At least in part, this is because, for non-isotopically edited β-sheets, the resultant transition moments are oriented either parallel or perpendicular to the strand axis and the orientations of the individual transition moments are consequently not of direct interest.[B1] For isotopically edited β-sheets, however, they are crucial.

The integrated infrared intensities resolved along the sheet axes have been determined recently for the antiparallel β-sheet conformation of a peptide with sequence Lys-(Leu-Lys)$_7$, designated K(LK)$_7$.[B2] These values are given in Table B1 for the amide I and amide II bands, and may be used to derive the orientations of the individual transition moments. Earlier intensity measurements for the amide I band of β-sheet fibrous proteins[B3,B4] are included in Table B1 for comparison. For the latter, the intensities of bands with perpendicular polarization have been multiplied by a factor of 2 because the original data refer to samples with uniaxial orientation.

From the analysis given by Miyazawa,[B5] the $\nu_{\perp}(\pi,0)$, $\nu_{\perp}(\pi,\pi)$ and $\nu_{\parallel}(0,\pi)$ infrared-active modes are oriented along the *a*, *b* and *c*-axes, respectively, as defined in Figure 7 of the main text. This information has been used to give the assignments of the modes for K(LK)$_7$ in Table B1. From the geometry in Figure 7, the transition moments of the $\nu_{\perp}(\pi,0)$, $\nu_{\perp}(\pi,\pi)$ and $\nu_{\parallel}(0,\pi)$ modes are therefore given by $|\mathbf{M}|\cos\psi_M$, $|\mathbf{M}|\sin\psi_M \sin\varphi$ and $|\mathbf{M}|\sin\psi_M\cos\varphi$, respectively, where $|\mathbf{M}|$ is the absolute value of the transition moment for a single amide. The infrared intensity is proportional to the square of the transition moment. The intensities of the two perpendicular polarized modes, relative to the parallel polarized mode, are therefore given by:

$$I[\nu_{\perp}(\pi,\pi)]/I[\nu_{\parallel}(0,\pi)] = \tan^2\varphi \qquad \text{(B1)}$$

and:

$$I[\nu_{\parallel}(0,\pi)]/I[\nu_{\perp}(\pi,0)] = \tan^2\psi_m\cos^2\varphi \qquad \text{(B2)}$$

Equation (B2) may be used to determine the orientation, ψ_M, of the transition moment if the inclination, φ, of the peptide plane is known from X-ray diffraction (see Appendix A). Otherwise, equation (B1) can be used first to determine φ, provided that the $\nu_{\perp}(\pi,\pi)$ mode is resolved.

Applying equation (B1) to the data for the amide II mode of K(LK)$_7$ yields a value of $\varphi = 30(\pm 6)°$. This is in reasonable agreement with the data from X-ray diffraction for other antiparallel β-sheets (see Appendix A). Using this value of φ, together with equation (B2), then gives a value of $\psi_I = 20(\pm 3)°$ for the orientation of the amide I transition moment of K(LK)$_7$. This is in agreement with data already established for fibrous proteins;[B3,B4] see also below. Similarly a value of $\psi_{II} = 77(\pm 5)°$

Table B1. Integrated intensities of the modes (ν) of the amide bands for antiparallel β-sheet peptides and proteins

Peptide/protein	Band	$I[\nu_{\perp}(\pi,0)]$ (cm^{-1})	$I[\nu_{\perp}(\pi,\pi)]$ (cm^{-1})	$I[\nu_{\parallel}(0,\pi)]$ (cm^{-1})	References
K(LK)$_7$	Amide I	36.5 ± 2.2	–	3.7 ± 0.5	B2
	Amide II	1.0 ± 0.6	5.0 ± 1.9	14.9 ± 1.4	
Silk fibroin	Amide I	33.93	–	3.50	B3
β-Keratin	Amide I	16.812	0.952	0.998	B4

is obtained for the orientation of the amide II transition moment of antiparallel $K(LK)_7$. The latter value is reasonably precise, in spite of the relatively low precision of the $\nu_\perp(\pi,0)$ intensity for the amide II band (see Table B1). This is important because of the paucity of data on the amide II transition moment orientation for β-sheets.

Combining the intensities of the amide I components for *Bombyx mori* silk fibroin with the value of $\varphi = 22°$ obtained from the original X-ray pseudo-structure (see Appendix A), equation (B2) yields a value of $\psi_I = 19°$.[B3] Using the value of $\varphi = 24.6°$ from refined coordinates for the closely similar structure of β-poly-L-alanine (see Appendix A), increases ψ_I only marginally to 19.5°. Applying equation (B1) to the amide I data for β-keratin yields a value of $\varphi = 44°$. This is considerably larger than the value of $\varphi = 27.6°$ obtained from the refined coordinates of β-keratin (see Appendix A). This is possibly because the $\nu_\perp(\pi,\pi)$ mode of the amide I band required for the calculation of φ is of low intensity and not well resolved. Nevertheless, taking $\varphi = 44°$ together with equation (B2) yields a value of $\psi_I = 19°$, which agrees with that originally reported.[B4] If the value of $\varphi = 27.6°$ from the refined structure of β-keratin is used, a lower value of $\psi_I = 15°$ is obtained for the orientation of the amide I transition moment.

Further information relevant to the orientation of the amide transition moments can be obtained from comparison with model compounds.[12] The amide frequencies of *N*,*N*′-diacetyl hexamethylene diamine[B6] are closest to those of β-sheet structures. For this model compound, the orientation, δ_M, of the transition moment relative to the peptide carbonyl is $\delta_I = 17°$, $\delta_{II} = 68°$, 77° and $\delta_A = 8°$ for the amide I, amide II and amide A bands, respectively. Because these transition moments lie in the peptide plane directed away from the nitrogen atom, the orientation of the transition moment to the *a*-axis is given by $\psi_M = \beta_{C'O} + \delta_M$. Here, $\beta_{C'O}$ is the inclination of the peptide carbonyl bond to the *a*-axis, as given in Appendix A. It is seen immediately that taking the smaller value of $\beta_{C'O} = 6°$ for β-keratin, rather than that for β-poly-L-alanine, gives a better agreement with measured values of ψ_M that are given above. The orientations of the transition moment predicted in this way are then: $\psi_I = 23°$, $\psi_{II} = 74°$, 83° and $\psi_A = 14°$ for the amide I, amide II and amide A bands, respectively. Corresponding predictions taking *N*-methyl acetamide,[B7] which also has amide frequencies close to those for β-sheets, as model compound are: $\psi_I = 21–31°$, $\psi_{II} = 79°$ and $\psi_A = 14°$. On the whole, these predictions are reasonably close to the direct determinations for the amide I and amide II bands that are given above. This is of some importance, because predictions from the model compounds provide the only information available on the orientation of the amide A transition moment in β-sheets.

Unlike the situation with α-helices, $^{13}C{=}O$ isotopic labelling of hydrophobic β-sheet peptides produces amide I bands at lower frequency that have anomalously high intensity, relative to that expected for an isolated oscillator.[B8] Nevertheless, a semi-empirical model that explains the anomalous intensity predicts relative intensities of those isotope-shifted bands which correspond to the $\nu_\parallel(0,\pi)$ and $\nu_\perp(\pi,0)$ modes of a homogeneous β-sheet that are similar to the relative intensities for the peptide without isotopic labels.[B9] Using equation B2, the maximum change in effective value of transition moment orientation, ψ_I, predicted for a peptide with alternating labels, is 5°.

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