Multiple Site-specific Infrared Dichroism of CD3-ζ, a Transmembrane Helix Bundle

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The structure of the transmembrane domain of CD3-ζ, a component of the T-cell receptor involved in signal transduction, has been studied in its native state (a lipid bilayer) by multiple site-specific infrared dichroism. For the first time, the transmembrane domain has been labelled at multiple positions along the sequence, representing a total of 11 samples, each labelled at a different residue with an isotopically modified carbonyl group, 13C=18O. A strategy is outlined that, based on the above data, can yield the rotational orientation and the local helix tilt for each labelled residue, giving a detailed description of helix geometry. The results obtained indicate that the transmembrane segment is in an α-helical conformation throughout, with an average helix tilt of 12°. The N-terminal side of the helix is more tilted than the C-terminal.

In an accompanying paper we describe the implementation of the infrared data in a model-building study of the CD3-ζ transmembrane complex. The model obtained is entirely consistent with results based on evolutionary conservation data. Taken together, this study represents the first step towards elucidation of the backbone structure of a transmembrane α-helical bundle by infrared spectroscopy.

Introduction

Structural biology of membrane proteins is plagued by the paucity of experimental data. This is due to the known difficulties that conventional methods used for structural elucidation (i.e. X-ray crystallography and solution NMR spectroscopy) encounter when applied to membrane proteins. Since membrane proteins are genomically abundant,1 and far more biomedically important than their water-soluble counterparts,† the need to develop alternative structural methods, applicable to membrane proteins, cannot be overstated. We have recently shown that site-specific infrared dichroism (SSID) has the potential of becoming such a method.2

The method is based on the measurement of dichroism arising from site-specific isotopic labels, e.g. 1-13C=18O,3 or C-deuterated glycine,4 to yield accurate spatial restraints that can describe the structure of small membrane proteins in their native environment, a lipid bilayer. In theory, SSID can yield spatial restraints for a protein of any size, but realistically it is exceedingly difficult to insert an isotopic label selectively into a large membrane protein. We overcome the problem of deconvoluting the disorder in the sample through the use of more than one observable on the helix, the helix dichroism and the isotopic site-specific dichroism. Since both observables are present on the same molecule, they share the same disorder. With the use of one more sample, in which the isotopic label is located at a different position, it is possible to

Key words: membrane proteins; molecular dynamics; molecular modelling; site-specific infrared dichroism; CD3-ζ
obtain the average helix tilt and the rotational pitch angle of the labeled site (see Figure 5). This is possible only upon knowledge of the rotational pitch angle difference between the two labeled sites. In a canonical helix it would simply be 100° for consecutive residues.

This assumption has been employed in previous applications of SSID, where we have used only one pair of isotopically labeled carbonyls, either 1-13C-16O in Influenza M2 and CM2, or vpu from HIV5–7 or the double-isotope 1-13C-18O in phospholamban,3 to obtain the average helix tilt and rotational pitch angle of the labeled amino acid. In an effort to better characterize a transmembrane helix bundle, in the present study we have employed, for the first time, multiple 1-13C-18O labels,3,8 which allows gathering of information at different points along the helix and makes unnecessary the assumption referred to above.

We have applied this new approach to the CD3-ζ chain, one of the invariant subunits of the T-cell receptor (TCR). CD3-ζ is a glycoprotein essential for TCR expression, incorporated as the final and rate-limiting step in the assembly of the TCR complex10 and is involved in signal transduction.11 The human CD3-ζ chain is 163 residues long and spans the membrane once (residues 31–51). SDS-PAGE analysis indicates that CD3-ζ forms disulphide-linked dimers under these denaturing conditions, and that the two monomers associate through their transmembrane domains.12 Furthermore, it has been hypothesized that the transmembrane domain of CD3-ζ contains a glycine-based dimerization motif similar to that of glycoprotein A (gpA).13

We show that isotopic multiple labelling of a transmembrane α-helical bundle allows a more accurate description of the orientation of the helix, as the information is gathered at different points along its length. Thus, the current study represents the first example in which SSID is used to derive comprehensive site-specific spatial restraints.

Results and Discussion

Effect of hydration

We have used, as is usual practice,3,5 data from samples in which excess water was removed, since it is difficult to measure the site dichroism from a fully hydrated sample because both H2O and 2H2O affect the band corresponding to the 13C-16O label. The bending O-H vibration of H2O absorbs in the same region as the label 13C-18O, at 1590 cm⁻¹ (not shown), whereas H2O absorbs strongly in the amide I region, affecting the 13C-18O band. However, as described in Materials and Methods, we have monitored the effect of hydration on the structure of the protein by comparing the amide A dichroism before and after excess water removal (nine samples), as shown in Figure 1.

The average dichroisms were 4.50 ± 0.71 and 4.28 ± 0.57, for samples with or without excess water, respectively. The difference in dichroism, however, accounts for a change in helix tilt of only 1.5° (using 29° as the angle between the N-H stretching transition dipole moment and the helix axis16,17), well within the error of the measured helix tilt (see Table 2). Therefore, we can conclude that the removal of excess water does not affect the structure of CD3-ζ significantly.

Finally, dichroism of the lipid CH2 stretching modes (ca 2924 and 2852 cm⁻¹, data not shown) was not affected by the changes of hydration. Order parameters calculated from the above modes were routinely above 0.5, indicative of well-formed, parallel multi-bilayers.

Multiple site-specific infrared dichroism of CD3-ζ

Figure 2 shows representative amide I spectra for each of the 11 peptides labelled, at parallel and perpendicular polarizations. The bands arising from the 1-13C-18O-labelled residue are also shown.

All 66 spectra (six for each label) were typical of a predominantly α-helical peptide, with an amide I band with a maximum at 1657 cm⁻¹.19 Neither the original nor the deconvoluted (not shown) spectra show significant intensity around 1640–1630 cm⁻¹, indicating the absence of significant β structure.19 This result indicates that the transmembrane segments of the proteins in the regions analyzed (34L–49L) is entirely α-helical. Of these spectra, only the 33 samples (three for each label) containing less disorder, i.e., f more than 0.8, were used for the calculations of β and ω. The recorded dichroic ratios for these 33 samples, RHelix and Rspect, are listed in Table 1.
From the dichroisms in Table 1, the values for the local helix tilt $\beta$ and rotational orientation $\varphi$ for every labelled residue were calculated (see Materials and Methods). These parameters are shown in the first two columns of Table 2. From the values shown in this Table, it is clear that the tilt obtained from residues 34 to 38 is higher than the tilt obtained from residue 41 to 49, which suggests the presence of a kink or bend in the helix.

Inspection of the rotational pitch angle determined, (Figure 3, top panel) illustrates that deviations from a canonical helix (Figure 3, bottom panel) are not substantial. In line with the change in local tilt observed between residues 34L and 41I, a concomitant deviation from canonical helix increments is observed. Likewise, smaller deviations in increment are observed whenever changes in helix tilt are observed (e.g. 34-35 and 46-45).

**Comparison with previous modeling results**

We then compared these results with the calculated $\varphi$ and $\beta$ angles of the "glycophorin-like" right-handed dimeric model we reported previously (see Table 2, last two columns). Surprisingly, our experimental data are not compatible with that model.

There is, however, a general agreement with another dimeric model that is produced from global searching molecular dynamics simulations ($\varphi_{V44} \sim 230^\circ$) but was not identified as the native model. This alternative dimeric model (Figure 4,
one of the samples analysed

Table 1. Helix and site dichroisms obtained for each one of the samples analysed

<table>
<thead>
<tr>
<th>Residue</th>
<th>$R_{HELIX}$</th>
<th>$R_{C=O}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>34L</td>
<td>3.99</td>
<td>4.75</td>
</tr>
<tr>
<td>34S</td>
<td>2.76</td>
<td>3.20</td>
</tr>
<tr>
<td>35L</td>
<td>3.37</td>
<td>2.87</td>
</tr>
<tr>
<td>35S</td>
<td>3.79</td>
<td>7.66</td>
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</tr>
<tr>
<td>36S</td>
<td>3.61</td>
<td>6.65</td>
</tr>
<tr>
<td>38L</td>
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</tr>
<tr>
<td>38S</td>
<td>3.50</td>
<td>3.59</td>
</tr>
<tr>
<td>39L</td>
<td>3.48</td>
<td>4.40</td>
</tr>
<tr>
<td>39S</td>
<td>3.28</td>
<td>2.41</td>
</tr>
<tr>
<td>39I</td>
<td>3.06</td>
<td>2.24</td>
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<tr>
<td>39I</td>
<td>3.41</td>
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<tr>
<td>41I</td>
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<tr>
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<td>4.35</td>
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<td>4.17</td>
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</tr>
<tr>
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</tr>
<tr>
<td>49L</td>
<td>3.30</td>
<td>5.11</td>
</tr>
<tr>
<td>49L</td>
<td>3.25</td>
<td>3.83</td>
</tr>
</tbody>
</table>

Only three of the best measurements (see Materials and Methods) are represented for each labelled site, out of six typically measured. Variability within each sample represents variability in sample order.

Conclusion

We have, for the first time, employed multiple site-specific infrared dichroism to analyze the structure of a transmembrane $\alpha$-helical bundle. Using 11 labels in the transmembrane domain, we were able to obtain local helix tilts as well as the rotational pitch angles for each of the labels. The results indicate that there is a change in helix tilt in the helix. Thus, the work represents the first stage in employing an entirely novel method to solve the backbone structure of a transmembrane $\alpha$-helix under native conditions.
Materials and Methods

Isotopic labelling and peptide synthesis

Amino acids labelled with a double isotope, $^{13}$C-$^{18}$O, were obtained by incubating the $^{13}$C-carbonyl-containing amino acids, $^{13}$C-$^{18}$O (Cambridge Isotopes Laboratories, Andover, MA), with a mixture of H$_2^{18}$O (94.4% Promochem GmbH) and dioxane (3:1, v/v) for one hour at 100°C at acidic pH (ca 1). The extent of $^{18}$O exchange was monitored using mass spectrometry, and was typically 75%, i.e. 75% of the oxygen atoms in the carboxyl group of the amino acid. Therefore, 75% of the molecules of synthesized peptide contained a $^{13}$C-$^{18}$O-labelled residue. The fact that the exchange was not complete is not important, as the dichroism of the label is independent of its relative abundance in the sample. The mixture was lyophilized and the amino acid was derivatized with Fmoc as described.

The transmembrane segment of CD3ζ (residues 27 to 53) was synthesized by standard solid-phase Fmoc chemistry, cleaved from the resin with trifluoroacetic acid and lyophilized. As in the simulations reported previously, a transmembrane sequence with the mutation C32G was employed (see Figure 5, bottom), because it facilitates both peptide purification and molecular dynamics simulations and has no influence on TCR expression. During the synthesis of the peptide, residues with a $^{13}$C-$^{18}$O-labelled carbonyl group were introduced at positions L34, L35, I38, L39, I41, G43, V44, I45, L46, A48 or L49, so that 11 different samples were obtained, each labelled at a different residue (see Figure 5).

The lyophilized peptides were dissolved in 2 ml of trifluoroacetic acid (TFA) (final concentration ca 5 mg/ml) and immediately injected onto a 20 ml Jupiter 5 C4-300 Å column (Phenomenex, Cheshire, UK) equilibrated with 95% H$_2$O, 2% (w/v) acetonitrile and 3% (v/v) 2-propanol. Peptide elution was achieved with a linear gradient to a final solvent composition of 5% H$_2$O, 38% acetonitrile and 57% 2-propanol (Biocad Sprint, Perceptive Biosystems, Cambridge, USA). All solvents contained 0.1% (v/v) TFA. The resulting fractions were pooled and lyophilized. Peptide purity was confirmed by mass spectrometry.

Sample preparation for ATR-FTIR

Initially, 100 μl of a stock solution of DMPC in HFIP (100 mg/ml) were added to the dry peptide (typically ~3 mg). The lipid-to-protein ratio was checked using a CaF window in transmission mode, drying a 10 μl aliquot on the window surface. If the intensity of the ester band of the lipid (at 1740 cm$^{-1}$) was smaller than the amide I, additional HFIP/lipid solution was added. Then, the sample was diluted with a further 900 μl of HFIP, to a final DMPC concentration of 10 mg/ml (>15:1 DMPC/peptide molar ratio) and approximately 100 μl of this solution were deposited, in successive aliquots of 10 μl, onto a trapezoidal (50 mm x 2 mm x 20 mm) Ge internal reflection element.

At this point, immediately after evaporating the HFIP, the dry sample was oriented randomly (the amide I dichroism was ~2), but the dichroism of the amide I increased (up to typically 3 to 4) after hydrating the sample with nitrogen saturated with H$_2$O, or 2H$_2$O for 30 minutes, after which no further increase in the helix dichroism was observed. After hydration, excess water was removed using a dry N$_2$ stream through the ATR compartment and spectra were collected.

Data collection and area integration

FTIR spectra were recorded on a Nicolet Magna 560 spectrometer (Madison, USA) purged with N$_2$ and equipped with a MCT/A detector, cooled with liquid nitrogen. Attenuated total reflection (ATR) spectra were measured with a 25 reflections ATR accessory from Cemsey Specac (Kent, UK) and a wire grid polarizer (0.25 μm, Graseby Specac). A total of 1000 interferograms collected at a resolution of 4 cm$^{-1}$ were averaged for...
every sample and processed with one point zero filling and Happ-Genzel apodization.

The area corresponding to the $^{13}$C-$^{18}$O (isotope-labelled) carbonyl stretching vibration was obtained by integrating the band at 1590 cm$^{-1}$ between 1600 cm$^{-1}$ and 1580 cm$^{-1}$. The area of the amide I, corresponding to helical structure, was obtained by peak integration from 1670 to 1645 cm$^{-1}$. The area of the amide A was calculated by integrating the band centered at 3300 cm$^{-1}$ between 3200 and 3400 cm$^{-1}$.

The dichroic ratios were calculated as the ratio between the integrated absorptions of the spectra collected with parallel and perpendicular polarized light.

**Effect of hydration**

The effect of hydration on the helix dichroism was assessed by monitoring the dichroism of the amide A band (due to N-H stretching), centered at $\sim$3300 cm$^{-1}$, in different states of $^2$H$_2$O hydration. The reason for studying hydration with $^2$H$_2$O stems from the overlap of the O-H stretching modes of H$_2$O in the amide A region. The comparison of spectra before and after removal of excess $^2$H$_2$O was conducted as follows. First, the spectra of the hydrated sample were collected after flushing the sample with $^2$H$_2$O-saturated N$_2$. Then, after excess water was removed as described above.

**Multiple site-specific infrared dichroism**

The data were analyzed according to the theory of SSID presented in detail elsewhere.$^8$ Briefly, SSID is based on the fact that the measured dichroism, $R$, of a particular transition dipole moment is a function of the sample fractional order, $f$, and the spatial orientation of
Multiple Site-specific Dichroism of CD3-ζ

Figure 5. Top: Drawing representing the geometric parameters that define the orientation of a bond or a transition dipole moment in a transmembrane helix. The helix tilt \( \beta \) and the rotational orientation \( \omega \) are derived from the experimental data using the angles \( \alpha \) and \( \delta \) which are known (see the text). The helix presents axial symmetry around the \( z \)-axis, i.e. all possible \( \phi \) angles are present. The helical segment has been drawn as a perfect cylinder for simplicity. Bottom: Transmembrane sequence of CD3-ζ, where the residues modified with \(^{13}\text{C} = ^{18}\text{O}\) are indicated with a black dot.

The dipole. The latter is defined by the parameters shown in Figure 5: \( \beta \) the helix tilt, \( \alpha \) and \( \delta \) which relate the transition dipole moment to the helix director, and the rotational pitch angle, \( \omega \). The rotational pitch angle \( \omega \) is arbitrarily defined as 0° in the direction of the helix tilt when the transition dipole moment, the helix director and the \( z \)-axis all reside in the same plane. The \( \delta \) angle is defined by the angle between the transition dipole moment and the helix axis when the \( z \)-axis, the helix axis and the \( z \)-carbon atom of the residue are in the same plane (see Figure 5). The angles \( \alpha \) and \( \delta \) for the C=O and N—H transition dipole moments are known from studies using oriented fibers. The values for \( \delta \) were 0° for both transition dipole moments, whereas \( \alpha \) was 39° for C=O and 29° for N—H.

From each measurement, two different dichroisms are obtained. The first is \( R_{\text{Helix}} \), the composite dichroism that corresponds to all the \(^{12}\text{C} = ^{16}\text{O}\) dipoles, or N—H in the case of amide A, involved in the helical structure for sample 1. This dichroism arises from residues distributed around the helical axis, i.e. one every 100° for a canonical \( z \)-helix. Therefore, this dichroism is independent of \( \omega \) and dependent solely on \( \beta \) and \( f_3 \):

\[
R_{\text{Helix}}(\beta, f_3) = \frac{e_z^2(fK_x + \frac{1-f_3}{3}) + e_z^2(fK_y + \frac{1-f_3}{3})}{e_z^2(fK_z + \frac{1-f_3}{3})}
\]  
(1)

where \( K_{x,y,z}(\omega) \) are the rotationally averaged, integrated absorption coefficients and \( f_3 \) represents the fractional order of preparation \( i \). The parameter \( f_3 = 1 \) if the sample is completely ordered and zero if completely random. Finally, \( e_x, e_y \) and \( e_z \) are the electric field components for each axis given by Harrick.\(^{15} \) The values for these components were those corresponding to a thick film approximation because, whereas the thickness of the film was calculated as being more than 30 μm, the amplitude of the evanescent wave decays (1/e of its initial value) after 1 μm in a germanium plate.

Instead of using the amide I dichroism, we obtained \( R_{\text{Helix}} \) by measuring the amide A dichroism when the sample was exposed to \(^2\text{H}_2\text{O}\). The reasoning behind this experiment is twofold. (1) The sensitivity of a dichroism experiment depends on the magnitude of the \( \alpha \) angle. The range of possible dichroic ratios is a function of \( \alpha \) angle as shown in Figure 6. As an extreme example, when \( \alpha = 54.6° \) (the magic angle) the dichroic ratio is independent of the helix tilt. Clearly, the higher the range of possible dichroic ratios is, the more accurately the tilt can be determined. For example, the range of dichroic ratios for the amide I (in the Ge ATR optical configuration) is 1.4–4.6, while for the amide A it is 1.2–8.3. Thus, a helix tilt difference between \( 25 \) and \( 30 \)° will be reflected in a change of dichroism of only 9% (3.5 → 3.2) when observing the amide I mode, but a change of 16% (5.1 → 4.3) when observing the amide A mode. Incidentally, it is immediately apparent from Figure 6 why it is difficult to determine low tilts of the lipid acyl chains from the lipid CH2 stretching modes. (2) The amide A band in these conditions, i.e. when the sample is exposed to \(^2\text{H}_2\text{O} \), originates only from the regions of the peptide that did not undergo N-H/N-2H exchange, as would be expected from the transmembrane region of the protein. Thus any contributions of extramembraneous parts is removed and what one measures is the dichroism and subsequent orientation of the transmembrane region of the sample.

The second dichroism, \( R_{\text{Site}} \), corresponds to the \(^{13}\text{C} = ^{18}\text{O}\) label, which is dependent on the rotational orientation \( \omega \) for this particular residue:

\[
R_{\text{Site}}(\beta, f_3, \omega) = \frac{e_z^2(fK_x + \frac{1-f_3}{3}) + e_z^2(fK_y + \frac{1-f_3}{3})}{e_z^2(fK_z + \frac{1-f_3}{3})} \frac{e_z^2(fK_x + \frac{1-f_3}{3}) + e_z^2(fK_y + \frac{1-f_3}{3})}{e_z^2(fK_z + \frac{1-f_3}{3})}
\]  
(2)

These two equations are not sufficient to obtain \( \beta, \alpha \), and \( f_3 \) (three unknowns), therefore a second label is inserted, in a different sample, above or below the first label. As there are 3.6 residues per turn for a canonical \( z \)-helix, the increment in \( \omega \) is assumed to be 100°. Thus, with the additionally labelled sample, two additional equations can be obtained, \( R_{\text{Helix}} \), and \( R_{\text{Site}} \), dependent on \( \beta, f_3 \) and \( \beta, \alpha = 100° \), \( f_3 \), respectively.\(^2 \) Solving the four equations \( R_{\text{Helix}}, R_{\text{Helix}}, R_{\text{Site}} \), and \( R_{\text{Site}} \) for each \( i \) and \( j \) pair, will yield \( \beta_i, \alpha_i, f_3 \), and \( \beta_j, \alpha_j \) the results obtained from the combinations of sample \( i \) and sample \( j \).

These non-linear equations were solved with Newton’s method as implemented in the FindRoot function in Mathematica 3.0 (Wolfram Research, Champaign, USA). The final values of \( \beta, \alpha, \) and \( f_3 \) for a particular pair of labels \( (i, j) \) were obtained by averaging \( \beta_i \) and \( \alpha_i \) respectively:
The polymer tilt for several different \( \alpha \) angles. Thick line, \( \alpha = 29^\circ \) (amide A); thin line, \( \alpha = 39^\circ \) (amide I); and broken line, \( \alpha = 90^\circ \) (lipid CH\(_2\) stretching mode). The dichroic ratios are calculated for an ATR optical geometry using the thick film approximation and a Ge internal reflection element.

\[
\beta = \frac{1}{n^2} \sum_{i=1}^{n} \sum_{j=1}^{n} \beta_{ij}
\]
\[
\omega = \frac{1}{n^2} \sum_{i=1}^{n} \sum_{j=1}^{n} \omega_{ij}
\]

(3)

**Calculation of \( \omega \) and local tilt for each labelled residue**

The overall scheme by which the data were used to calculate the local helix tilt and rotational pitch angle is shown in Figure 7. Because the error in the assumption \( \omega_{i+1} = \omega_i + 100 \) becomes more important with the separation between residues, a first estimate of \( \omega \) for a particular residue was obtained solving the equations corresponding to helix and site dichroisms only for pairs of labels separated by one (\( i, i+1 \)) or two (\( i, i+2 \)) residues. In these first calculations it was assumed that the difference in \( \omega \) between consecutive residues is 100° although in principle this is not known.

Figure 8 shows how this was calculated. The first column in this Figure shows residues 31–51 of CD3-x. The second column (grey rectangle) indicates the pairwise combinations (\( i, i+1 \) or \( i, i+2 \)) of labels, joined by brackets, used in the initial calculation. The result of each combination is represented by one \( \omega \) angle, corresponding to the first residue in the sequence. The \( \omega \) for the other label in the pair would be obviously \( \omega + 100^\circ \) for \( i, i+1 \) or \( \omega + 200^\circ \) for \( i, i+2 \). For example, \( \omega_{43,44} \) in the first calculation in Figure 8 is the result of combining the pair 43 and 44, whereas \( \omega_{43,44} \) is the result of combining 44 and 45. The fact that the combinations (43,44), (44,45) and (45,46) produced \( \omega \) angles for residues 43, 44 and 45 that were separated by an increment of 100°, i.e. 120°, 222° and 331° (see Figure 8, grey rectangle), indicated that, at least for these three residues, the assumption \( \omega_{i+1} = \omega_i + 100 \) is a good approximation.

We then used the fact that when a good estimate for \( \omega \) is obtained for a particular residue, the \( \omega \) for a different residue can be obtained without having to assume any particular value for the increment in \( \omega \) between the two residues, because it can be treated as an unknown. For example, \( \omega \) at residue 41 was calculated fixing the known (see above) \( \omega \) at residue 43 (i.e. 120°), the four unknowns being \( \beta \), \( \omega_{41} \) and the \( f \) for the two samples, \( f_a \) and \( f_b \). In Figure 8, the \( \omega \) values that were fixed in the calculation are indicated by a box. Then, the \( \omega \) obtained for residue 41 was fixed, and \( \omega \) at residue 39 was calculated. This was propagated up and down the helix (see values to the far-right in Figure 8), so that a better estimate of \( \omega \) for each residue could be obtained.

Note however, that for certain residues, e.g. 38, 39 and 46, two possible values were obtained. These two values, ~75 and ~300 for residues 38 and 46, and ~106 and ~260 for residue 39, should give identical dichroisms due to the fact that the dichroic ratio is a function of \( \cos \omega \). However, only one of these values is obtained when it is assumed \( \omega_{i+1} = \omega_i + 100 \) (see the second column in Figure 8), which may not be a correct assumption. The other value is obtained when a good approximation for a certain residue is known, therefore we have taken the second group of \( \omega \) values (see far right values in Figure 8) as correct in the subsequent calculations.
Thus, the (i) $\omega$ angles obtained, (ii) the average value for the fractional sample order $f$, and (iii) only the site dichroism were used to calculate the local helix tilt $\beta$.

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References


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