C-Deuterated Alanine: A New Label to Study Membrane Protein Structure Using Site-Specific Infrared Dichroism

Jaume Torres* and Isaiah T. Arkin†

*Cambridge Centre for Molecular Recognition, Department of Biochemistry, University of Cambridge, 80 Tennis Court Road, Cambridge, CB2 1GA, United Kingdom; and †The Alexander Silberman Institute of Life Sciences, Department of Biological Chemistry, The Hebrew University, Givat-Ram, Jerusalem, 91904, Israel

ABSTRACT The helix tilt and rotational orientation of the transmembrane segment of M2, a 97-residue protein from the *Influenza* A virus that forms H^+-selective ion channels, have been determined by attenuated total reflection site-specific infrared dichroism using a novel labeling approach. Triple C-deuteration of the methyl group of alanine in the transmembrane domain of M2 was used, as such modification shifts the asymmetric and symmetric stretching vibrations of the methyl group to a transparent region of the infrared spectrum. Structural information can then be obtained from the dichroic ratios corresponding to these two vibrations. Two consecutive alanine residues were labeled to enhance signal intensity. The results obtained herein are entirely consistent with previous site-specific infrared dichroism and solid-state nuclear magnetic resonance experiments, validating C-deuterated alanine as an infrared structural probe that can be used in membrane proteins. This new label adds to the previously reported $^{13}C{=}{=}^{18}O$ and C-deuterated glycine as a tool to analyze the structure of simple transmembrane segments and will also increase the feasibility of the study of polytopic membrane proteins with site-specific infrared dichroism.

INTRODUCTION

The use of structural data obtained from site-specific infrared dichroism (SSID) (Arkin et al., 1997) as a restraint for molecular dynamics protocols is an emerging method that has been applied to the study of the structure of various transmembrane helical bundles (Kukol and Arkin, 1999, 2000; Kukol et al., 1999; Torres et al., 2000). This technique relies upon the ability to selectively measure the infrared absorption of a particular mode in the peptide. The dichroic ratio obtained using polarized light can then be related to the orientation of the transition dipole moment and this in turn to the bond orientation of the particular chromophore.

In previous experiments we have made use of the frequency shift obtained from an isotopically labeled peptide carbonyl. In particular, $^{13}C{=}{=}^{16}O$ (Kukol and Arkin, 1999, 2000; Kukol et al., 1999) displays a carbonyl stretching frequency shifted some -40 cm^{-1} away from the main $^{12}C{=}{=}O$ amide I band (Tadesse et al., 1991), although there is still a partial overlap between the two bands. This disadvantage, compounded by the fact that ^{13}C is a relatively abundant isotope (1.1%), limits these studies to relatively small proteins (~25–30 amino acids). We have overcome these two problems through the use of $^{13}C{=}{=}^{18}O$ (Torres et al., 2000b, 2001), which increases the shift from the main amide I (-60 cm^{-1}) and also makes the natural isotope abundance issue irrelevant, as the combined abundance of ^{13}C and ^{18}O is only 0.003%.

However, whereas $^{13}C{=}{=}^{18}O$ is a significant advance over $^{13}C{=}{=}^{16}O$ as an infrared probe, it does entail a few shortcomings. One is related to the method of site-specific infrared dichroism itself, as this technique requires the labeling of at least two samples, each with a different labeled residue (Arkin et al., 1997). This is particularly important in α-helical bundles of polytopic proteins, where the introduction of a label is particularly difficult and expensive. In these cases, a label displaying more than one useful vibrational mode would be very useful. Another problem that faces $^{13}C{=}{=}^{18}O$ is that in large proteins, side chain absorption may interfere with the absorbance of the label (Kalnin et al., 1990; Venyaminov and Kalnin, 1990). Finally, to solve the series of coupled equations describing the dichroic ratios (see Materials and Methods) it is imperative that one knows the relative geometry between two labels, i.e., consecutive C=O bonds. Any deviation from an ideal helical geometry would compromise the accuracy of the method.

We have reported previously the use of C-deuterated glycine (Gly-CD_2) (Torres et al., 2000a), which allows the simultaneous measurement, and in the same residue, of two mutually perpendicular vibrational modes: the symmetric (ss) and asymmetric (as) methylene stretching vibrations of the glycine side chain. The successful use of this label allowed the determination of an α-helical bundle using a single residue (Torres et al., 2000a). The rationale behind this is that the three dichroisms (the helix dichroism R and the two dichroisms from the CD_2 group of glycine) are sufficient to obtain the three unknowns, i.e., helix tilt β, fractional order parameter f, and rotational orientation ω (Arkin et al., 1997). The problem of interference with other groups is also solved in Gly-CD_2, as the C-deuterated methylene absorbs in a transparent region where no other chromophore absorbs.

A significant advantage of the labels used in SSID is that they are noninvasive, as the only modification is an isotopic substitution and the sequence remains identical to the native one. Glycine however, is not particularly frequent in transmembrane helices (Arkin and Brunger, 1998), and the substitution of a native residue for a glycine residue in a

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Address reprint requests to Isaiah T. Arkin, The Alexander Silberman Institute of Life Sciences, Department of Biological Chemistry, The Hebrew University, Givat-Ram, Jerusalem, 91904, Israel. Tel.: 972-2-658-4329; Fax: 972-2-658-4329; E-mail: arkin@cc.huji.ac.il.

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transmembrane sequence might have unwanted structural consequences. Therefore, we have set out to find a label with the positive features of glycine and which is also more abundant in transmembrane helices.

Herein we have tested C-deuterated alanine (Ala-CD_3), which like glycine, possesses a side chain with two mutually perpendicular stretching modes with a fixed orientation relative to the helix axis. The relative geometry between the two modes does not change as a function of protein conformation and is hence far more powerful than two consecutive amide I modes. With this label we have determined the tilt and rotational orientation of the *Influenza* A H^+ channel M2, a homotetrameric α-helical bundle. The structure of this transmembrane domain has been studied previously by solid-state nuclear magnetic resonance (NMR) (Kovacs and Cross, 1997) and SSID (Kukol et al., 1999; Torres et al., 2000a), and both the helix tilt and rotational orientation are known.

Clearly, Ala-CD_3 will be useful in noninvasive studies (e.g., when a labeled alanine substitutes a nonlabeled alanine) of membrane proteins in cases where glycine is not present in the sequence of the transmembrane domain. Also, as is the case for Gly-CD_2, use of the Ala-CD_3 label facilitates experiments involving the incorporation of a single isotopically labeled residue, labeled simultaneously at the carbonyl bond ($^{13}C{=}{=}^{18}O$, (Torres et al., 2000b)) and at the side chain (C-deuterated methyl) in the transmembrane helix, the measurement of three independent site-specific dichroisms can be measured at the same time. This obviates the use of the helix dichroism, which is just a composite of all the local C=O dichroisms and therefore only indicative of the overall helix tilt, not of the local tilt where the label is located. The present study constitutes a further step in the development of SSID to study membrane protein structure.

MATERIALS AND METHODS

Label preparation and peptide synthesis

Ala-CD_3 (Cambridge Isotopes Laboratories, Andover, MA) was derivatized with 9-fluorenylmethoxycarbonyl (FMOC) as described (Wellings and Atherton, 1997). The peptide of sequence SSDPLVVAASIIGILHLILWILDRL (Kukol et al., 1999), corresponding to the transmembrane segment 22 to 46 of *Influenza* A M2 (TM-M2), was obtained by standard FMOC synthesis, cleaved from the resin with trifluoroacetic acid, and lyophilized. The sample contained two C-deuterated alanine residues Ala-CD_3, at positions A29 and A30, to increase the intensity of the bands. The peptides were then purified and reconstituted in DMPC liposomes as described previously (Kukol et al., 1999).

Fourier transform infrared spectra

Fourier transform infrared spectra were recorded on a Nicolet Magna 560 spectrometer (Madison, WI) purged with N_2 and equipped with a liquid nitrogen cooled high sensitivity MCT/A detector. Polarized attenuated total reflection (ATR) spectra were measured with an ATR accessory from Graseby Specac (Kent, UK) and a wire grid polarizer (0.25 μM, Graseby Specac). Aliquots of 200 μl ($\sim$2.5 mg/ml protein and 12.5 mg/ml lipid) were deposited onto a KRS-5 trapezoidal internal reflection element (50 $\times$ 2 $\times$ 20 mm, 45°, 25 reflections), and bulk water was removed with a stream of dry nitrogen. A total of 1000 interferograms were averaged for every sample and processed with 1 point zero filling and Happ-Genzel apodization. Amide I integration was performed on these spectra in the regions 1670 to 1645 cm^{-1} on the Fourier self-deconvoluted spectra (Kauppinen et al., 1982). Fourier self-deconvoluted was performed in the amide I region with an amplitude (full width at half height) of 15 cm^{-1} and an enhancement factor of 2.0, always below log (signal/noise) (Kauppinen et al., 1982).

The area corresponding to the as and ss CD_3 stretching vibration bands of Ala-CD_3 were measured from the original spectra without further manipulation. The dichroic ratio was calculated as the ratio between the integrated absorptions of parallel and perpendicular polarized light.

Data analysis

The data were analyzed according to the theory of site-specific dichroism presented in detail elsewhere (Arkin et al., 1997), based on the fact that the measured dichroic ratio (R) of a particular transition dipole moment are a function of the sample fractional order (f) and the spatial orientation of the dipole. This is defined by the parameters: β, the helix tilt, α and δ, which relate the transition dipole moment to the helix director, and ω, the rotational pitch angle about the helix axis.

From the sample used here, labeled with Ala-CD_3 simultaneously at two consecutive alanine residues, three different dichroic ratios were obtained. The first is R_{Helix}, the dichroic ratio of the helix that corresponds to the amide I $^{12}C{=}{=}^{16}O$ transition dipole moments distributed around the helical axis. This dichroic ratio is dependent on β and f, but independent of ω:

$$R_{Helix}(\beta, f) = \frac{e_z^2\left(fK_z + \frac{1-f}{3}\right) + e_x^2\left(fK_x + \frac{1-f}{3}\right)}{e_y^2\left(fK_y + \frac{1-f}{3}\right)} \quad (1)$$

in which K_x, K_y, and K_z are the x, y, and z components of the rotationally averaged integrated absorption coefficients (Arkin et al., 1997). The parameter f represents the fractional order, i.e., $f = 1$ if the sample is completely ordered and $f = 0$ if the sample is completely random. Finally, e_x, e_y, and e_z are the electric field components for each axis given by Harrick, assuming a thick film approximation (Harrick, 1967). For KRS-5, the material used here, e_x, e_y, and e_z are 1.16, 1.77, and 2.223, respectively. The refractive indices of the KRS-5 and the sample used were 2.37 and 1.43, respectively.

When two alanines are labeled at positions 29 (located at ω) and 30 (at $\omega + 100°$), the experimental dichroic ratios for the as and ss stretching vibrations of the CD_3 groups, R_{as} and R_{ss}, are the composite dichroisms that originate from the two alanine residues, and were obtained modifying Eq. 1 accordingly:

$$R_{site}(\beta, f, \omega) = \frac{e_z^2\left(fK_{site_z}(\omega) + \frac{1-f}{3}\right) + e_x^2\left(fK_{site_x}(\omega) + \frac{1-f}{3}\right) + e_z^2\left(fK_{site_z}(\omega + 100°) + \frac{1-f}{3}\right) + e_x^2\left(fK_{site_x}(\omega + 100°) + \frac{1-f}{3}\right)}{e_y^2\left(fK_{site_y}(\omega) + \frac{1-f}{3}\right) + e_y^2\left(fK_{site_y}(\omega + 100°) + \frac{1+f}{3}\right)} \quad (2)$$

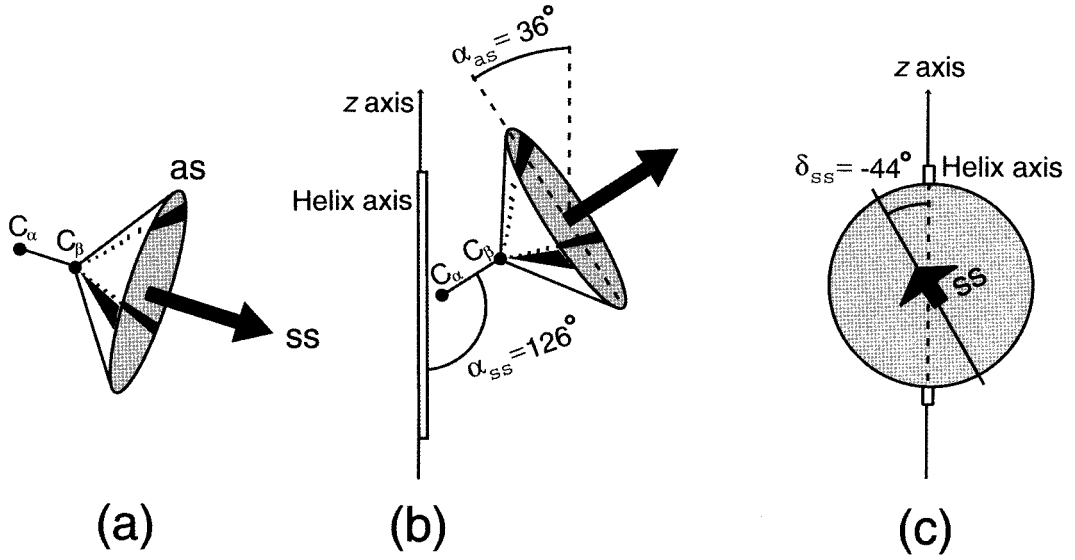

FIGURE 1 Schematic diagrams showing the orientation of symmetric (ss) and asymmetric (as) transition dipole moments (tdp) for the methyl stretching vibration in alanine. (*a*) Definition of the direction of the as and ss vibration modes. The ss-tdp is parallel to the Cα—Cβ bond, whereas the as-tdp defines the plane that includes the three methyl hydrogens and points toward any direction on this plane. (*b*) Angle α between the ss-tdp or as-tdp and the helix axis. (*c*) Angle δ between the ss-tdp and the plane formed by the helix axis and the *z* axis when the *z* axis the helix axis and the α carbon of alanine are all in the same plane and the residue is in a proximal position (this defines $\omega = 0$). The angle δ_{ss} is $-44°$, whereas δ_{as} can have any value. Other parameters used in the study, i.e., the helix tilt β, the rotational orientation ω, and ϕ have been defined previously (Arkin et al., 1997).

in which R_{site} is either R_{as} or R_{ss}. These three equations, R_{Helix}, R_{as}, and R_{ss}, are sufficient to obtain the three unknowns β, ω, and f. The nonlinear equations were solved with Newton's method as implemented in the FindRoot function in Mathematica 3.0 (Wolfram Research, Champaign).

Orientation of the transition dipole moment

The geometric parameters that describe the orientation of the stretching transition dipole moments (tdp) of the methyl group relative to the *z* axis (Fig. 1) were calculated using a model of alanine where the α carbon is located in an α-helical environment in which the helix axis coincides with the *z* axis, using CHI (CNS Helix Interaction), a program suite for CNS (Crystallography and NMR System (CNS Version 0.3) (Brunger et al., 1998)). The angle α between the C═O transition dipole moment and the helix axis is known to be 39° from oriented fiber studies (Tsuboi, 1962; Marsh et al., 2000).

As described previously (Arkin et al., 1997), the Cartesian coordinates of the vibrational transition dipole moments are given by the following axial rotation matrices:

$$R_z(\phi)\cdot R_x(-\beta)\cdot R_z(\omega)\cdot R_x(-\alpha)\cdot R_y(\delta)\cdot \vec{i}_z \qquad (3)$$

in which R_x, R_y, and R_z are the rotation matrices around the axes *x*, *y*, and *z*. The absorption coefficients were obtained integrating through all possible ϕ angles due to uniaxial symmetry of the helices around the membrane normal for either as and ss. Note that for as, integration was performed through all possible δ angles too, as the as transition dipole moment points to any direction in the plane that contains the three methyl hydrogens (see Fig. 1). This integration was done using the following relationships:

$$\langle\cos(\tau)^2\rangle = \langle\cos(\tau)^2\rangle = \frac{1}{2}, \quad \text{and}$$

$$\langle\cos(\tau)\sin(\tau\rangle = \langle\cos(\tau)\rangle = \langle\sin(\tau)\rangle = 0 \qquad (4)$$

in which τ is either ϕ (in both or ss and as) or δ in the case of the as transition dipole moment.

Analysis of two labels

Fig. 2 shows how R_{as} and R_{ss} depend on β and ω. It is clear that when the signal originates from a single residue (Fig 2, *a* and *b* for R_{as}, or *d* and *e* for R_{ss}) the range of possible dichroic ratio values R is larger than when two residues are labeled simultaneously (Fig. 2 *c* for R_{as} and Fig. 2 *f* for R_{ss}). The smaller amplitude of the changes in dichroic ratio when two alanines are labeled, as compared with the case when only one is labeled, is best appreciated on a slice of these three-dimensional plots at $\beta = 32°$ (panels on the right of each three-dimensional plot). The resolution of the method however, is not significantly affected, as the changes in dichroic ratio when two residues are labeled are still large enough to allow the determination of β and ω.

Error analysis

The effect of the uncertainty in the determination of the angles indicated in Fig. 1 on the final result was evaluated by a modification of the protocol used to solve the system of equations referred to above. For a fixed pair of experimental values for R_{as} and R_{ss}, the orientational parameters (β and ω) were calculated iteratively for tdp values up to 5% above or below the ones reported in Fig. 1 in 20 steps, i.e., from 119.7 to 132.3 for the angle α of ss-tdp and from -48.4 to -39.6 for δ_{ss} of ss-tdp, whereas the value of α as-tdp was set to be 90° smaller than α-ss-tdp during these iterative calculations. Thus, a total of 400 combinations were obtained, and the resultant distribution of the different values of ω_{Ala29} and β was calculated and plotted as a histogram.

Similarly, as the bands corresponding to the methyl ss and ss are of low intensity, the effect of variations in dichroic ratio quantification was evaluated. This was done by fixing the tdp values to those shown in Fig. 1 and iteratively solving the system of equations allowing a pair of experimental values R_{as} and

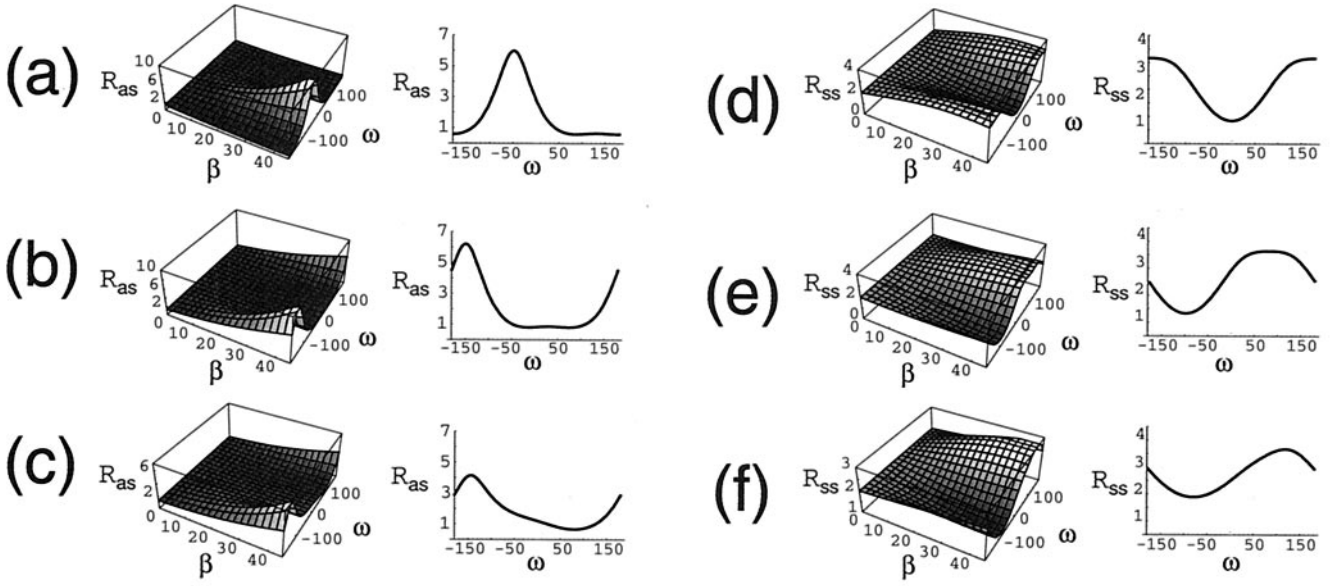

FIGURE 2 Plots showing the distribution of possible dichroic ratios of methyl as and ss vibrations (R_{as} and R_{ss}) as a function of β and ω. (*a*) Three-dimensional plot representing R_{as} as a function of β and ω for a single labeled alanine residue located at ω. (*b*) Same for a single labeled alanine at $\omega + 100°$, i.e., located in a contiguous position in the helix. (*c*) Same for the simultaneous presence of two consecutively (ω, $\omega + 100°$) labeled alanines. Obviously, *a* and *b* are exactly the same, but shifted 100°. Slices of these three-dimensional plots at $\beta = 32°$, i.e., the previously determined experimentally determined value (Kovacs and Cross, 1997; Kukol et al., 1999) are also shown on the right of *a* through *c*. Analogous plots are shown for R_{ss}, for a single alanine label (*d* and *e*) and two consecutive alanine labels (*f*). For the sake of representation, the value for *f* has been fixed at an arbitrary value, 0.8.

R_{ss} to vary ±5% in small steps during the calculations. As before, 400 combinations were calculated and the frequencies plotted as a histogram.

RESULTS AND DISCUSSION

Transition dipole orientation

Fig. 1 shows the orientation of the assymetric and symmetric transition dipole moments, as-tdp and ss-tdp, for the methyl group of alanine. The ss-tdp is aligned along with a vector that joins the α-carbon and β-carbon of alanine, whereas the as transition dipole moment (as-tdp) is found anywhere on a plane defined by the three methyl hydrogens (Fig. 1 *a*). This means that δ, defined by the angle between the ss-tdp and the helix axis when the *z* axis, the helix axis, and the α-carbon of the alanine residue are in the same plane with the residue located in the direction of the tilt, can have any value, from 0 to 360°. The angle α between the ss-tdp and the helix axis was found to be 126°. The δ angle for ss-tdp was found to be −44°. The smaller angle between the plane formed by the three methyl hydrogens and the helix axis was found to be 36°, i.e., the angle between this plane and the ss-tdp is 90°.

$AlaCD_3$ vibrational modes

Upon isotopic substitution, the methyl stretching modes of alanine are red shifted relative to the methylene CH_2 and methyl CH_3 stretching modes that are dominated by the lipid. The precise location of the bands that arise from the C-deuterated methyl in the infrared spectrum was determined using a sample of labeled TM-M2 peptide in which lipids were absent, after dissolving the peptide in chloroform/methanol and drying. The infrared spectrum of this sample (Fig. 3, *spectrum a*) shows four bands, analogous to the bands we reported for C-deuterated glycine (Torres et al., 2000a). As expected, nonlabeled TM-M2 does not display any of these bands in these conditions (Fig. 3, *spectrum*

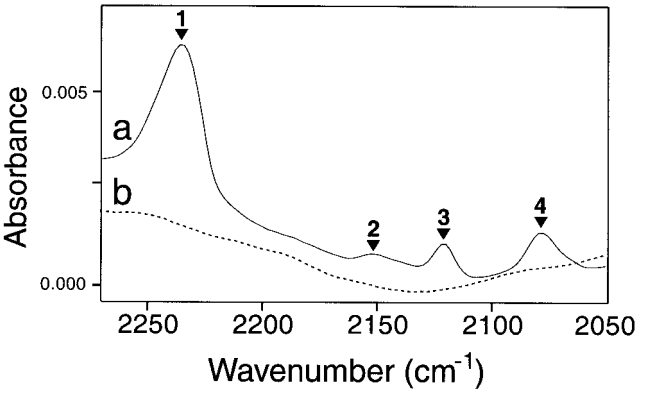

FIGURE 3 Original infrared spectra of TM-M2 in the CD_3 stretching region. Labeled TM-M2 with Ala-CD_3 at positions 29 and 30 (*spectrum a*) in the absence of lipids after drying from a chloroform:methanol solution (see text) and nonlabeled TM-M2 (*spectrum b*). The bands corresponding to CD_3 stretching are indicated by numbers from 1 to 4. Only bands 1 and 3 were used in the calculations (see Results).

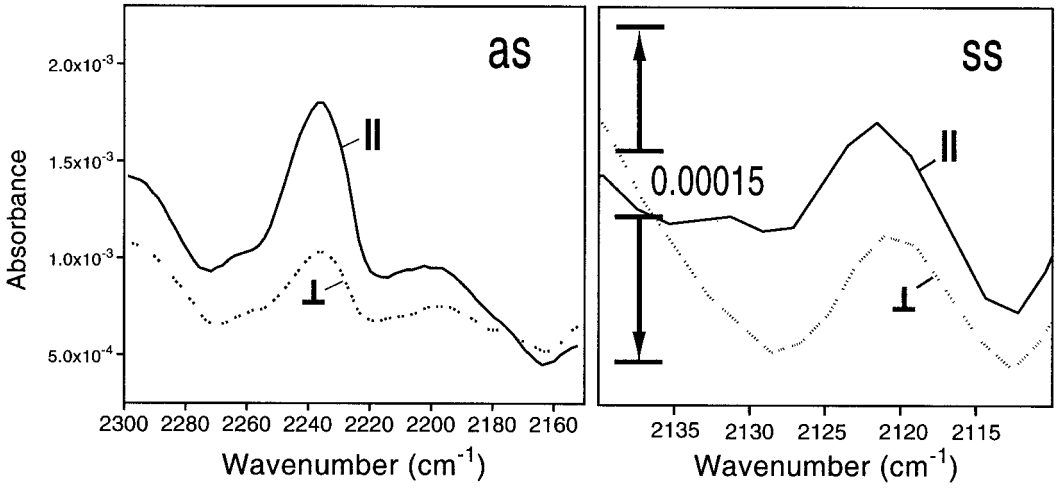

FIGURE 4 Original infrared spectra of TM-M2 in DMPC liposomes labeled with Ala-CD_3 simultaneously at positions 29 and 30 using parallel (∥, *solid line*) or perpendicular polarized (⊥, *dotted line*) light for the as stretching of CD_3 (*left panel*) or the ss CD_3 stretching band (right panel).

b), nor does any other peptide tested (data not shown). The bands in *spectrum a* appear in a region consistent with the red shift expected (~700 cm^{-1}). A rough estimate for this shift can be obtained if the CH bond is treated as an harmonic oscillator, following the equation

$$\nu = \sqrt{\frac{k}{\mu}} \tag{5}$$

in which ν is the frequency of absorption, k is the force constant of the bond, and μ is the reduced mass of the two atoms involved in the bond, either C and H or C and D. Thus, considering only one of the C—H bonds in the methyl group, the ratio between the two stretching frequencies of bonds C—H and C—D becomes

$$\frac{\nu_{C—H}}{\nu_{C—D}} = \sqrt{\frac{\mu_{C—D}}{\mu_{C—H}}} \tag{6}$$

in which $\nu_{C—D}$ and $\nu_{C—H}$ are the vibration frequencies of the C—H and C—D groups, and $\mu_{C—D}$ and $\mu_{C—H}$ are their respective reduced masses. As this ratio is 1.36, the expected stretching frequency of C—H will shift from 2883 cm^{-1} (Krimm and Bandekar, 1986) to ~2120 cm^{-1} upon deuteration. Further, as the methyl vibration is always blue shifted relative to C—H, assuming that the difference in energy between C—D and as CD_3 is the same as that between C—H (at 2883 cm^{-1}) and as CH_3 (at 2983 cm^{-1}) (Krimm and Bandekar, 1986), the predicted location of the as vibration for CH_3 band would in fact be 2226 cm^{-1}, which is close to the observed 2236 cm^{-1}. An analogous argument can be developed for the ss vibration. As in glycine, we have assigned the intense band at 2236 cm^{-1} (labeled 1) to as stretching. Also as in glycine, the lower frequency bands (at 2121 cm^{-1} and 2075 cm^{-1}, labeled 3 and 4) have been assigned to ss stretching.

Only two of the observed bands, located at 2236 cm^{-1} (as) and 2121 cm^{-1} (ss), which are labeled 1 and 3 in Fig. 3, were used to obtain the dichroic ratios. Note that these bands are very close to the bands corresponding to the as and ss vibration previously used for glycine CD_2, at 2242 cm^{-1} and 2098 cm^{-1}, respectively (Torres et al., 2000). Therefore glycine CD_2 and Ala-CD_3 cannot be used simultaneously.

Derivation of orientational parameters from Ala-CD_3-labeled TM-M2 incorporated in DMPC liposomes

The as and ss bands corresponding to the label Ala-CD_3 when the TM-M2 peptide was reconstituted in DMPC lipo-

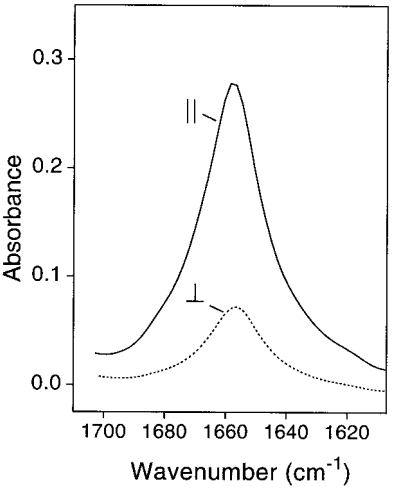

FIGURE 5 Original amide I infrared spectra of TM-M2 in DMPC liposomes labeled with Ala-CD_3 simultaneously at positions 29 and 30, collected with parallel (∥, *solid line*) or perpendicular polarization (⊥, *dotted line*).

TABLE 1 Helix dichroic ratios R_{HELIX} and site specific dichroic ratios R_{as} and R_{ss} for TM-M2 labeled with Ala-CD_3 simultaneously at positions 29 and 30

R_{HELIX}	R_{as} (2236 cm^{-1})	R_{ss} (2121 cm^{-1})
3.62	2.46	1.3
3.86	2.50	1.3
3.84	2.85	1.1

somes are shown in Fig. 4, whereas the corresponding band in the amide I region of the same sample is shown in Fig. 5.

The results of three separate measurements, from three different preparations, are given in Table 1, which shows the dichroic ratios for the amide I band (helix dichroism) and for the methyl as and ss stretching modes. The solutions to the system of equations described in Materials and Methods were averaged using the three experiments, yielding $\omega_{Ala\text{-}29}$ of $-60° \pm 5°$ and helix tilt β of $30° \pm 3°$, whereas f ranged from 0.8 to 1, depending on the sample. These results are almost identical to the values reported previously using SSID (Kukol et al., 1999; Torres et al., 2000) ($\omega_{Ala\text{-}29} = -60° \pm 11°$ and $\beta = 33° \pm 6°$ (Kukol et al., 1999)) where the labels were the carbonyl $^{13}C{=}{=}^{16}O$ (Kukol et al., 1999) or Gly-CD_2 (Torres et al., 2000a). The orientation is also consistent with a model obtained using solid-state NMR data (Kovacs and Cross, 1997).

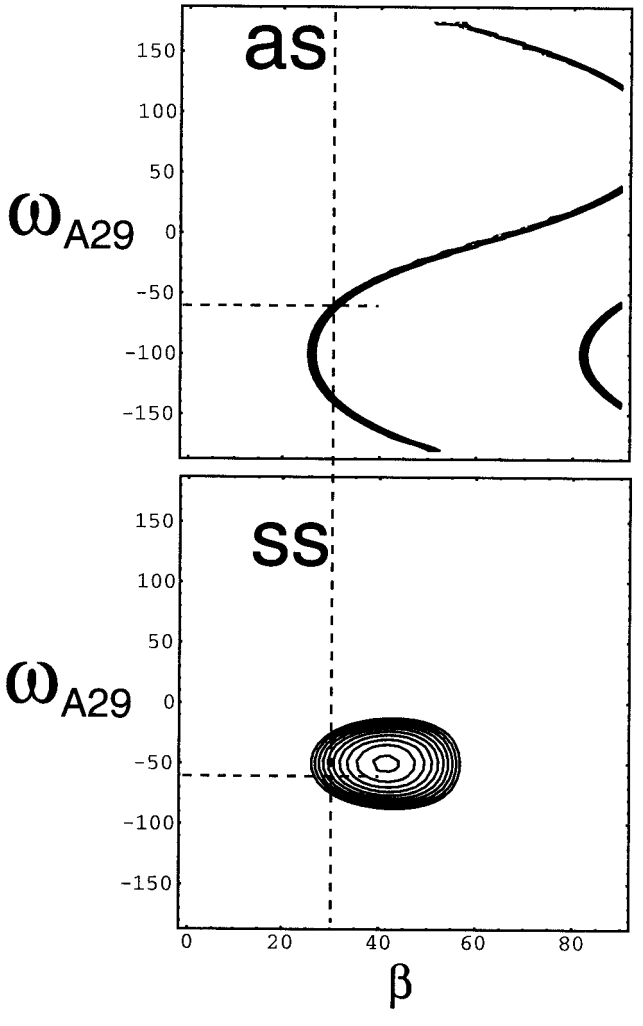

FIGURE 6 Contour plots showing the allowed values of $\omega_{Ala\text{-}29}$ and β for a limited range of fractional sample order f values (from 0.82 to 1) for the pair of experimental values R_{as} 2.5 (*top*) and R_{ss} 1.3 (*bottom*) shown in Table 1. The values obtained here for $\omega_{Ala\text{-}29}$ and β are indicated by a broken line.

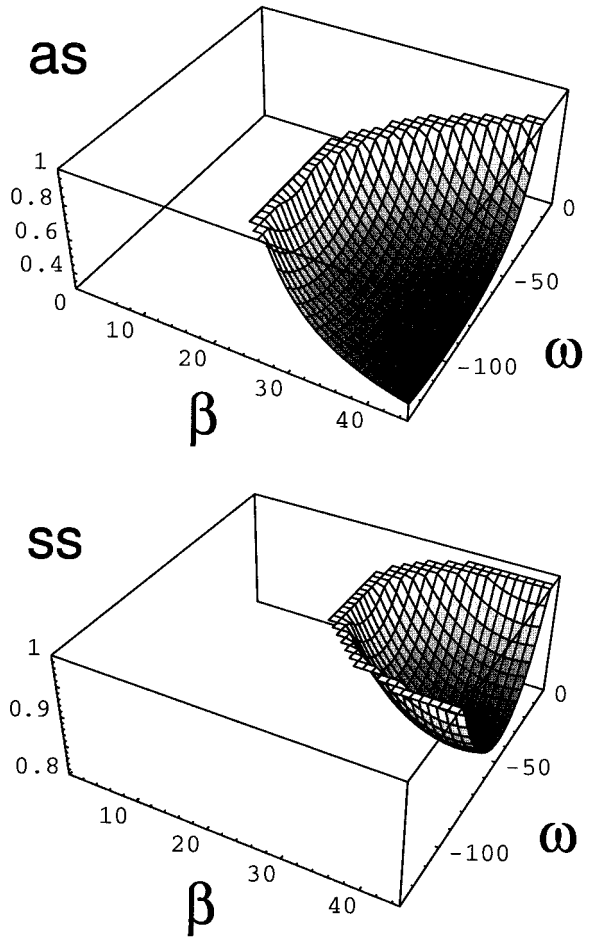

FIGURE 7 Three-dimensional plots showing the allowed values (i.e., f values between 0 and 1) of $\omega_{Ala\text{-}29}$ and helix tilt β for the experimental values of R_{as} of 2.5 (*top*) and R_{as} of 1.3 (*bottom*).

A visual representation of the possible range of $\omega_{Ala\text{-}29}$ and helix tilt β values for the range of f values encountered, i.e., from 0.8 to 1, is given in Fig. 6 when the dichroic ratios are those in the second row in Table 1 (2.5 and 1.3). Also, a three-dimensional representation of theoretically possible $\omega_{Ala\text{-}29}$ and β (i.e., for f between 0 and 1) is shown in Fig. 7. The intersection between these two surfaces and the plane formed by the dependence of the helix dichroism (data not shown) on β and f is the solution to the system of equations.

Last, as the estimate of the orientation of the tdp (Fig. 1) is subjected to a certain degree of error, we have represented

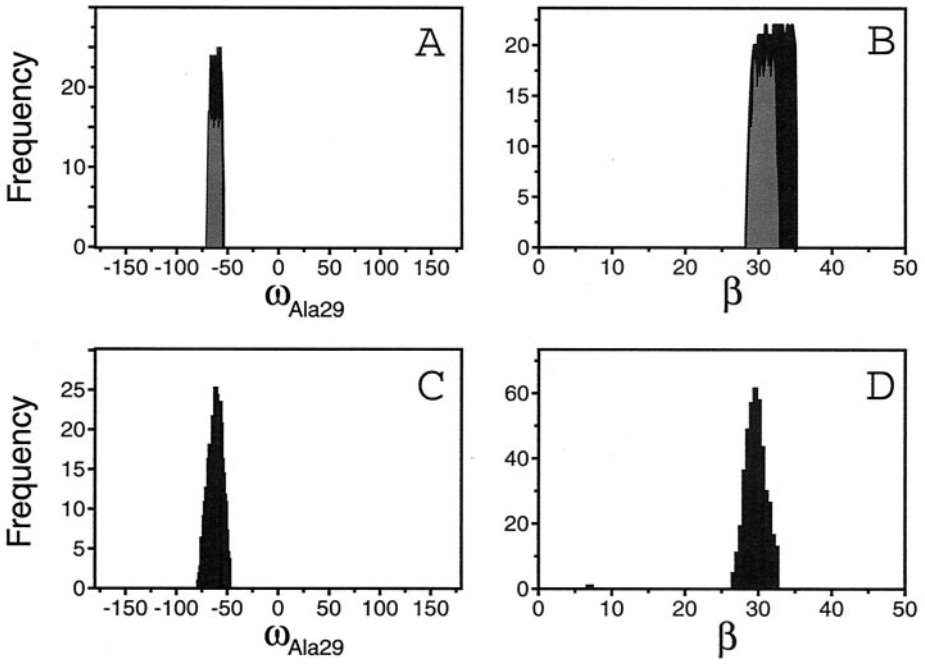

FIGURE 8 Frequency distribution of $\omega_{Ala\text{-}29}$ and β values. (*First row*) Frequency distribution of $\omega_{Ala\text{-}29}$ (*A*) and β (*B*) obtained when the transition dipole moments were allowed to change ±5% (see Materials and Methods). The total distribution obtained is shown in black. The combinations that produced meaningful values for *f*, i.e., $0 < f < 1$, are indicated in gray. (*Second row*) Frequency distribution of $\omega_{Ala\text{-}29}$ (iC) and β (*D*) obtained when the dichroic ratios in the second row in Table 1 (2.5 and 1.3) were allowed to change ±5% (see Materials and Methods) for a fixed set of tdp orientations (those indicated in Fig. 1).

in Fig. 8 the distribution of values for ω and β and their frequency of occurrence (see Materials and Methods) when the transition dipole moments were allowed to vary ±5% (Fig. 8, *A* and *B*). These values obtained range from −70° to −55° for $\omega_{Ala\text{-}29}$ and from 28° to 32° for β.

Also, as the bands corresponding to Ala-CD_3 are not very intense, this figure also provides the same representation if an uncertainty of ±5% (Fig. 8, *C* and *D*) was present in the quantification of the dichroic ratios. The values range from −77° to −45° for $\omega_{Ala\text{-}29}$ and from 27° to 32° for β. These extreme values are of comparable magnitude to the experimental error reported here obtained by taking three different measurements ($\omega_{Ala\text{-}29}$ of −60° ± 5° and helix tilt β of 30° ± 3°). The values of −60° for $\omega_{Ala\text{-}29}$ and 30° for β are located near the middle of the histograms.

Use of Ala-CD_3 as a spectroscopic probe

To summarize, the increased amount of information to be obtained from the Ala-CD_3 label will be particularly valuable in the study of polytopic membrane proteins in which the dichroic ratio of a particular helix cannot be obtained, especially when glycine is not present in the transmembrane domain. The complementary use of Ala-CD_3, Gly-CD_2, and $^{13}C{=}{=}^{18}O$ will be extremely useful in the analysis of large proteins. These C-deuterated labels have virtually no natural abundance and their signal is completely isolated from the bands that originate from either lipid or protein.

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