Isotope-Edited Infrared Linear Dichroism: Determination of Amide Orientational Relationships[⊥]

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Abstract: A new approach for the determination of local amide orientation in ordered insoluble proteins using linearly-polarized infrared radiation is described. The method was applied to the crystalline peptide cleromyrine (cyclo-(GlyProLeuProGlyTyr)). Labeling of individual amide carbonyl carbons with ^{13}C resulted in the frequency shift of the affected vibrational mode. ^{13}C labeling allowed the amide I modes to by systematically assigned ("isotope editing"). Subsequently, the absorption of linearly-polarized infrared radiation was measured in order to determine the orientation of the individual amide carbonyl relative to the incident radiation (infrared linear dichroism). The relative orientations of four cleromyrine amide carbonyls determined in this way were within 15° of those measured from the crystal structure. This method may be useful for the determination of amide orientation in peptides and proteins that form oriented insoluble aggregates that are resistant to crystallization.

Introduction

Current methods for protein structure determination require the sample to be soluble and/or crystalline. However, many ordered protein aggregates are neither. For example, several proteins form ordered, yet noncrystalline, insoluble amyloid fibrils; one type (derived from β protein) may be involved in the etiology of Alzheimer's disease (AD),[1] another (islet amyloid polypeptide, IAPP) in type II diabetes.[2] We are in the process of developing a general approach for the determination of amyloid fibrillar structure involving solid-state NMR (SSNMR) and Fourier-transform infrared spectroscopy (FTIR).[3,4] The rotational resonance SSNMR method measures intramolecular intercarbon distances which can be used to determine backbone structure.[4] However, there are often two, and sometimes more, low-energy structures that are consistent with the measured distances.[4] Accordingly, we have made use of ^{13}C chemical shifts and amide I vibrational frequency data to distinguish between possible structures.[4] The method discussed herein is being developed as a third method to be used for this purpose.

Infrared spectroscopy is currently used in protein structure analysis as a means of determining global structural information.[5,6] The amide I absorption band is particularly well studied because its vibrational frequency is sensitive to local secondary structure.[5] Unfortunately, the inherent breadth of individual amide I absorption bands in most peptides and proteins makes their separation and assignment impossible. In addition, most common secondary structures, especially the antiparallel β sheet which dominates amyloid fibrillar structure, are characterized by extensive dipole coupling interactions so absorption bands correspond to groups of proximal amide carbonyls vibrating as a unit. One solution to this problem is to incorporate ^{13}C into a single amide, thus shifting its vibrational frequency by a sufficient amount to decouple it from the neighboring ^{12}C amide modes and allow its selective observation.[7−9] This approach, which we call isotope editing, has been used to determine local structure in amyloid fibrils comprising peptide fragments of the β protein[8] and IAPP.[9] In the latter case, intermolecular dipole coupling was also observed, allowing the determination of intermolecular alignment in the amyloid β sheet.

Isotope-edited FTIR anlysis provides information about local secondary structure, but no long-range information regarding the relative orientations of amide carbonyls. Infrared linear dichroism spectroscopy (IRLD), which relies on the ability of an oriented sample to differentially absorb plane-polarized infrared radiation, provides information about the relative orientation of IR absorbing vibrational modes.[10,11] Thin films of A-, B-, and C-form DNA oligonucleotides have been studied by IRLD, demonstrating that the base pairs are highly inclined from perpendicular to the helix axis.[12] Fibrillar polyaminoacid samples have also been studied by IRLD. In these studies, the average orientation of the amide groups relative to the fibril axis has been determined.[13] Finally, helical peptides have been oriented by incorporation into lipid bilayers and have been studied by polarized attenuated total internal reflectance techniques.[11] The limitation of IRLD analysis, like traditional FTIR analysis, is that the method does not provide information about specific IR-active functional groups. By combining isotope-editing with IRLD, we sought to solve this problem and thus

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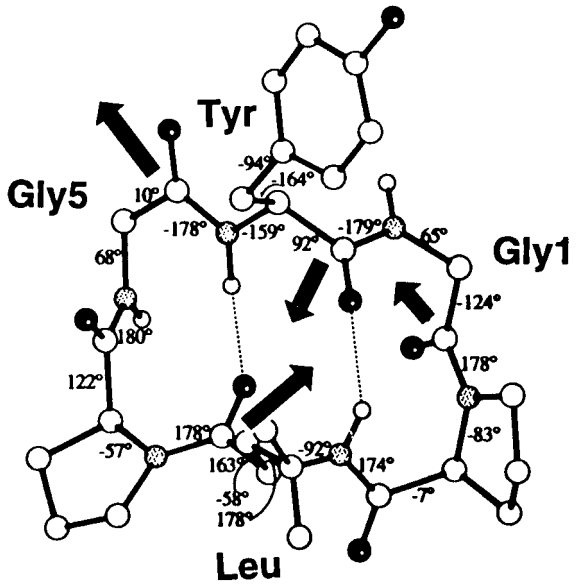

Figure 1. Crystal structure of cleromyrine, based on coordinates given in ref 15 (representation from *Peptide Science (Biopolymers)* **1996**, *40* (1), 59). The amides discussed herein are labeled, and the approximate amide I absorption dipoles are indicated. The plane of the page is approximately the plane of polarization depicted in Figure 3. The orientations of these projected dipoles are measured herein. The lengths of these dipoles are related to the azimuthal angle described in Figure 3. Longer dipoles should have a smaller azimuthal angle and a larger maximum absorption.

allow the determination of the relative orientations of specific amides in oriented peptide and protein samples.[14]

The peptide cleromyrine (cyclo(GlyProLeuProGlyTyr)) was chosen for the initial studies since its crystal structure had been determined (Figure 1).[15] Cleromyrine analogs with one, two, and three ^{13}C-labeled amide carbonyl carbons were synthesized and crystallized. Individual cleromyrine crystals were analyzed by isotope edited IRLD microscopy. These studies allowed the assignment of individual, uncoupled absorption bands to particular peptide amides. The linear dichroism of each absorption band was then determined and compared to that predicted by analysis of the amide orientation in the crystal structure. Good agreement between experimentally-determined orientation and that measured in the crystal structure was found, suggesting that this method could be applied to crystalline or oriented fibrillar peptides of unknown structure.

Experimental Section

Synthesis and Purification of Cleromyrine (cyclo(GlyProLeuProGlyTyr)). The peptide cyclo(GlyProLeuProGlyTyr) (amino acids numbered from 1 to 6, *e.g.*, Gly1, Pro2, *etc*., in the order shown) was synthesized on the Kaiser oxime resin according to standard procedures[16−20] with a few alterations that are described below. All reagents used in this synthesis were purchased from commercial sources. 1-^{13}C amino acids were purchased from Cambridge Isotope Laboratories (Cambridge, MA, 99% 1-^{13}C). ^{13}C-Labeled amino acids were protected as *tert*-butoxycarbonyl (*t*-Boc) carbamates in accordance with a published procedure.[21] The syntheses were designed so that tyrosine was added in the final step, avoiding the need to protect its hydroxyl group.

All peptides were synthesized on the glycine-functionalized Kaiser oxime resin (0.6 mmol/g, Nova Biochem). Based on amino acid analysis, the proline and leucine couplings went to 60% completion using standard conditions. To improve the substitution level, the coupling reaction was repeated, the time was extended from 1 to 2 h, and 5 equivalents of amino acid instead of 3 were used to give an approximate coupling yield of >85%. Under this regimen, the glycine and tyrosine couplings went essentially to completion. After each coupling reaction, unreacted amines were acetylated (10 equiv of acetic anhydride/diisopropylamine (DIEA) in methylene chloride for 30 min). For synthesis of the labeled cleromyrine analogs, only 1.5 equivalents of 1-^{13}C proline or 1-^{13}C tyrosine were added, and couplings were extended to 3 h. These couplings proceeded to 40−90% and were followed by acetylation. Final substitution levels of resin bound cyclo-(GlyProLeuProGlyTyr) and ^{13}C-analogs were between 0.04 and 0.10 mmol/g (7−17% overall, based on Gly-resin).

The resin-bound peptide was treated with 25% trifluoroacetic acid in methylene chloride to remove the N-terminal *t*-BOC carbamate and then with diisopropylethylamine (10 equivalents in methylene chloride at 23°) to promote cyclization/cleavage. The progress of the reaction was followed by two methods: analysis of resin by hydrolysis followed by amino acid analysis (Waters Pico-Tag) and analysis of the released material by reversed-phase HPLC (C4, 3.9 mm × 300 mm, linear gradient conditions of 95% H_2O/0.1% trifluoroacetic acid (TFA): 5% CH_3CN/0.1% TFA to 5% H_2O/0.1% TFA: 95% CH_3CN/0.1% TFA over 20 minutes). Both methods indicated that the reaction was *ca*. 90% complete after 24 h and essentially complete after 48 h. Two side products (each ≤15% of total product by weight) were formed as a result of the cyclization reaction: the linear dimer (GlyProLeuProGlyTyr)$_2$ and the cyclic dimer cyclo(GlyProLeuProGlyTyr)$_2$. Under the RPHPLC gradient conditions (see above), both peaks were cleanly separated from the desired product. Assignment of these products is based on FABMS analysis (MW: 584-CM, 1168-CD, 1186-LD), amino acid analysis (each product has identical amino acid ratios).

Purification of cyclo(GlyProLeuProGlyTyr) was accomplished using RPHPLC (Waters Delta-Pak C4 column, 5 min at 95% H_2O/0.1% TFA: 5% CH_3CN/0.1% TFA followed by a 20 min linear gradient to 5% H_2O/0.1% TFA:95% CH_3CN/0.1% TFA). The desired product was obtained as a white powder in a yield of 10−25% based upon initial resin-amino acid substitution levels. This white powder was dissolved in wet ethanol and stored at 4 °C for 2 months. By that time, crystals had formed, which were used as seeds for future experiments. The crystalline sample was washed in cold absolute ethanol and allowed to air dry. Crystalline samples were not pumped dry under vacuum to prevent crystal dehydration and subsequent degradation. The purity of crystalline samples was assessed by redissolving crystals and analyzing the solution by analytical RPHPLC under isocratic conditions. The yield of crystallization was 33%. Thus, the final yields from the initial resin-amino acid varied between 3% and 10%.

Characterization of Cleromyrine. Purified cyclo(GlyProLeuProGlyTyr) was analyzed by hydrolysis (1:1 proprionic acid-HCl, 3 h at 130 °C) followed by derivatization with PITC and amino acid analysis (Waters Pico-Tag). The amino acid ratios were 2.3 Gly:1.8 Pro:1 Leu (standard):0.8 Tyr. Proton and ^{13}C NMR spectra were obtained on a Varian XL-300 in CD_3OD using trimethylsilane as an internal standard. These spectra matched the published data.[15] ^{1}H NMR (distinctive peaks only) δ 1.00 and 1.03 [2d, J = 6 Hz $CH(CH_3)_2$], 6.68 and 7.03 (AA′XX′syst.); ^{13}C NMR δ 21.5, 23.9, and 26.5 [$CD(CH_3)_2$]; 25.2, 26.8, 30.8, 40.7, 43.3, 44.4, 48.2, 48.8, 50.6, 57.6, 62.4, 63.2, 116.1, 129.6, 131.5, 157.2, 170.6, 171.8, 172.5, 172.9, 174.3, and 175.3. Each peptide was also analyzed by FABMS (nitrobenzyl alcohol matrix). The molecular weights of unlabeled, singly, and doubly labeled compounds were 584, 585, and 586, respectively. Mass spectrometry fragments were used to verify ^{13}C location. Crystals grown in wet ethanol over a 2 month period were submitted for X-ray analysis. The space group, P321, is uncommon and is isomorphous to cleromyrine crystals grown from naturally-derived peptide in another laboratory (Figure 1).[15]

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Preparation of Cleromyrine Crystals for Analysis. Crystals formed from the above procedure tended to be too thick for direct infrared analysis via transmission methods. This problem was circumvented by redissolving crystals in wet ethanol and transferring the solution to a barium fluoride plate (13 mm diameter × 2 mm BaF_2 plates, Spectra Tech Inc., Shelton, CT). The ethanol was air evaporated over a 10−15 min period, and crystals of appropriate thickness were obtained. It was found that crystals between 10−20 μm in length by 5−10 μm wide yielded the best spectroscopic results. The cross section of a crystal is an ellipsoid with an eccentricity of about 0.8. These crystals are similar in shape to the larger crystals grown over a 2 month period for X-ray analysis, which suggests the crystals are isomorphic. Crystals were rejected as too thick if the maximum absorption between 1700−1600 cm^{-1} exceeded 1.5 AU and as too thin if the maximum absorption was less than 0.4 AU. Occasionally, residual water would result in H_2O peaks in the 1700−1600 cm^{-1} region; these crystals were rejected if noise peaks altered overall absorptions by greater than 5% of the absorption for any individual peak.

For polarization studies, crystals were oriented with the major axis perpendicular to the plane of observation. Linearly polarized light was defined as being oriented at 0° to the sample if it was parallel to the major axis. In most samples, the stage was rotated rather than the polarizer so that the polarizer could be set to maximize throughput energy. Reference spectra were taken with each new rotation in order to ensure proper spectral subtractions. Low purge conditions were used because crystals lost structural integrity under higher purge rates due to dehydration (three water molecules are associated with a unit cell[15]). Data sets were rejected if the baseline-subtracted, maximal absorption was reduced by more than 5% during the course of the measurements (*ca.* 3 h).

Acquisition of Infrared Spectra. Infrared spectra of proper size crystals were acquired with either a NicPlan IR microscope coupled to a Magna 550 FT-IR spectrophotometer (Nicolet Instruments Corporation, Madison, WI) or an IRμs scanning infrared microprobe (SpectraTech, Shelton, CT). Single crystals of cyclo(GlyProLeuProGlyTyr) were examined in transmission mode after apertures at image planes above and below the specimen were set to 10−20 μm diameter inspection zones at the sample plane. Spectra of all species were obtained at 2 cm^{-1} resolution with Happ Genzel apodization and no zero filling of the data sets. Excellent signal-to-noise ratios were attained with coaddition of either 256 or 1024 scans. Absorbance band assignments for labeled and unlabeled peptides were determined through spectral subtractions of appropriate reference spectra.[22]

Polarization studies were conducted with a gold wire grid polarizer attachment placed in the beam path of the microscope. Spectra were gathered at 2 cm^{-1} resolution after at least 256 scans. The polarizer was rotated in 10° increments, and data was taken for orientations between 0°−180° whenever possible. Identical results were obtained by fixing the polarizer at maximum throughput and rotating the specimen on an indexed circular stage. For either method, the angular estimate was accurate to ±2°. The orientation is defined as 0° when the major axis of the ellipsoidal crystal is aligned parallel to the polarized light plane.

Analysis of Infrared Spectra. Because unambiguous results were derived from the high signal-to-noise spectral data, modest data manipulation was required. Spectra presented herein have not been smoothed or deconvolved. Subtraction of the natural abundance spectrum from the single ^{13}C spectrum was used to determine the exact locations of the ^{12}C and ^{13}C bands. The difference of the natural abundance spectrum from the double ^{13}C spectrum was used to confirm band assignment and the decoupled nature of the carbonyls. In addition, analysis of the second derivative spectra corroborated band assignment. These spectral operations were performed using OMNIC software (Nicolet Instruments, Madison, WI).

Infrared linear dichroism spectra were analyzed using the PeakFit v3.10 program (Jandel Scientific, NH). The curve fit analysis employed in this paper is commonly used.[23] In a review of curve fitting procedures and their inherent limitations, Maddams has pointed out that the evidence in favor of the general applicability of the Lorentzian peak shape is strong.[23] The Voigt function is the convolution of a Lorentzian with a Gaussian. However, the convolution integral describing the Voigt function cannot be evaluated analytically. The PeakFit program employs an approximation method in which the Voigt function is approximated by four generalized Lorentzians.[24]

Before conducting a fit, a baseline was established at the minimum absorption value, which occurs at 1680 cm^{-1}. Initial peak placement in the 1680−1580 cm^{-1} region was done using the estimates determined from spectral subtractions. To confirm that this placement procedure leads to the best fit, initial peak placement was also done using Fourier self-deconvolved and second derivative data, and the same results were observed. Six Voigt peaks generally fit the data to R values > 0.995. The actual fit in this region was done on 204 data points. Lorentzian peaks were also used, and their fits were found to yield R values of between 0.985 and 0.998. Similar placement results were obtained for the Lorentzian fits, and the Voigt peaks were found to have a_3 widths indicating a highly Lorentzian character. The number of iterations typically needed to achieve a satisfactory fit was between 7 and 20. If after 20 iterations a fit of R >0.995 had not resulted, which is evidence of a local but not global minima, the calculations were halted, the refined data was manually refit, and the iterations resumed. This process was repeated until R values of at least 0.995 were achieved. In cases where random noise lead to fits of less than 0.995, the spectra were rejected.

The angular dependence of each amide I absorption (see Figure 5) was determined by three methods. First, the amplitude of the absorption band in question at a particular angle was compared to the maximum amplitude of that band at any angle. The relative amplitude varies between one and, in a hypothetical case where no absorption was observed, zero. This value is useful for comparing relative angles of two projected dipoles in the plane of polarization (Figure 3), but not for comparing the out-of-plane (azimuthal) angles of two dipoles. For this purpose, the amplitude of one band at a given angle was divided by the sum of the amplitudes of all six bands at that same angle (percentage amplitude). Alternatively, the area under a band was divided by the total area (percentage area). All areas are determined from spectra which have been baseline subtracted to establish the 0 value at 1680 cm^{-1}. This subtraction was employed in order to normalize the values determined for crystals of different thickness. This subtraction was also necessary due to the relatively small field of study and the, therefore, critical dependence on aperture placement. Different baselines are, thus, partially a result of the mechanical placement of the apertures and not reflective of any orientation effect. The percentage amplitude and percentage area approaches are only applicable to samples in which there is a distribution of amide orientations, this was determined to be the case for cleromyrine, where the total amplitude and areas showed little sensitivity to polarizer angle. For the ^{13}C-labeled cleromyrine samples analyzed herein, all three methods of determining relative orientations were in agreement, although only the percentage area data is shown in Figure 5.

Results and Discussion

The cleromyrine structure contains a ProGly turn and a GlyPro turn, connected by a region of antiparallel β sheet-like structure (Figure 1).[15] The amide carbonyls are oriented in different directions and are clearly not coplanar. Thus, cleromyrine is an ideal model compound with which to test the methodology proposed herein.

The Amide I Mode Is Predominantly a C−O Stretch and Is Sensitive to Isotope Labeling. The amide functional group has several IR-active normal modes. The amide I vibrational mode is largely a carbonyl stretching vibration.[5] In order to reduce the amide I stretch to a two body Hooke's law problem, it is necessary to rotate the vector 20° in the plane of the amide in a direction away from the N to account for the minor motions of the amide N and H. The exact amount of the rotation is derived from *ab initio* normal mode calculations of the amide system and estimates vary from 15 to 23° (Figures 1 and 3).[5,11]

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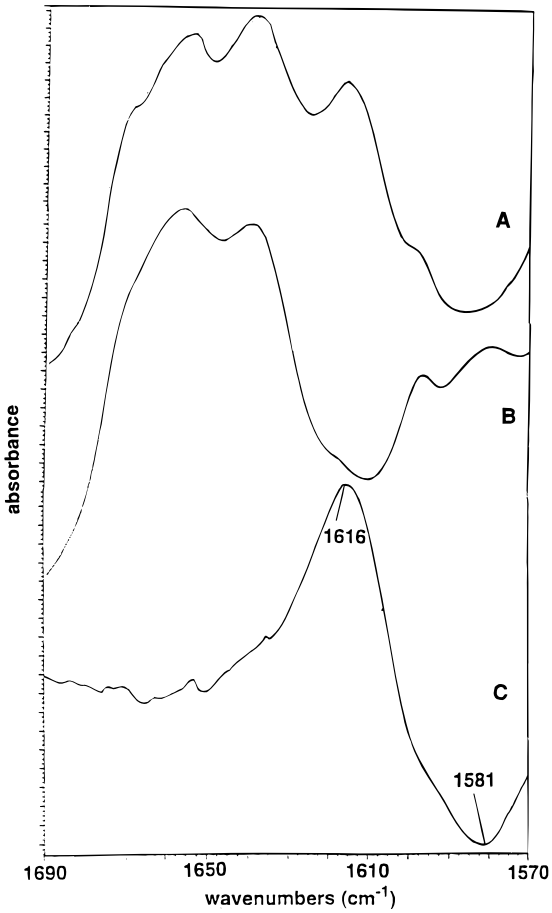

Figure 2. ^{13}C isotope-editing allows assignment of amide I bands. Infrared spectra of cleromyrine and labeled analog. Spectrum **A** (top) is the natural product cyclo(GlyProLeuProGlyTyr), showing maximum absorbance at 1638 cm^{-1} and discrete peaks at 1669, 1655, and 1616 cm^{-1}. Spectrum B (middle) is the spectrum of the singly ^{13}C-labeled analog cyclo(GlyPro***Leu***ProGlyTyr), showing a maximum absorbance at 1656 cm^{-1}. The bottom trace (**C**) results from the subtraction of spectrum **B** from spectrum **A**. It shows the position of the shifted Leu carbonyl peak, which shifts from 1616 to 1581 cm^{-1}. The magnitude of the ^{13}C-induced shift is 35 cm^{-1}, indicating that the Leu amide is not coupled.[8]

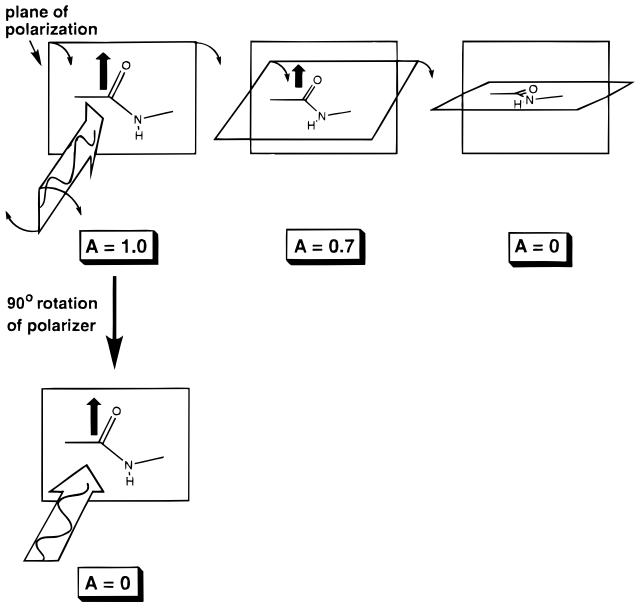

Figure 3. A schematic diagram illustrating the two central principles of infrared linear dichroism (IRLD). The orientation of the amide I dipole relative to the direction of polarization of the incident IR radiation and the plane of polarization (azimuthal angle) determines the absorption of polarized IR radiation. The arrows represent the amide I vibrational dipoles discussed herein.[5,11] Absorption will be maximal in the case where the direction of polarization is parallel to that dipole (top left). When the incident radiation is perpendicular to the dipole (bottom left), no absorption will occur (at right). For a given orientation, the magnitude of the maximal absorption depends on the azimuthal angle between the dipole and the plane of polarization. Maximal absorption will occur when the azimuthal angle is 0° (at top left, note that the amide plane need not be as drawn). Intermediate absorption will occur when the azimuthal angle is 45° (top center). No absorption will occur when the dipole is perpendicular to the plane of polarization (azimuthal angle = 90°, projection = 0, at top right). The projected dipoles are indicated by the solid arrows (see Figure 1).

However, since one is comparing relative orientations and each carbonyl is rotated by the same amount provided each is decoupled, this manipulation will not appreciably affect angle estimates (see Figure 3).

In an uncoupled system such as cleromyrine, replacement of the amide ^{12}C-carbonyl by ^{13}C should result in a shift to lower frequency of the amide I absorption band by *ca*. 37 cm^{-1} (*e.g.*, *ca*. 1650 to 1613 cm^{-1}, due to an increase in the reduced mass of the system).[7,8] The residual unlabelled absorption bands will not shift at all. The area under the ^{13}C absorption band will be equal to a fraction of the total amide I area, $1/n$, where n is equal to the total number of amides in the molecule.

Cleromyrine Contains Localized, Uncoupled Amide I Modes. In contrast to the regular, idealized β sheet peptides, cleromyrine produces an infrared spectrum characterized by multiple peaks in the amide region. These peaks correspond to individual, at least partially uncoupled, amide carbonyl groups (Figure 2A). For five of the six singly-labeled cleromyrine compounds, introduction of the ^{13}C label at the C-1 position resulted in a new amide band shifted 37 cm^{-1} from its original position, and unshifted residual ^{12}C bands indicating the uncoupled nature of the carbonyls (Table 1).[25] The area of each ^{13}C-labeled band was roughly 1/5 of the total ^{12}C bands' area, which is further indication of decoupling.[14] Because these carbonyls are decoupled, they can be studied individually and can be treated as separate harmonic oscillators. Thus, any angular dependence observed in the absorption of a ^{13}C carbonyl band using linearly polarized infrared radiation can be attributed to the labeled amide functionality.

Assignment of Cleromyrine Amide I Bands by Isotope-Editing. The IR spectrum of unlabeled cleromyrine is characterized by a broad absorption between 1680 and 1600 cm^{-1} comprising four major bands (Figure 2A). Singly and multiply labeled ^{13}C compounds were used to assign individual amide I stretching bands and totally decouple the labeled stretch mode from the unlabeled stretch modes. For example, the leucine carbonyl has an unusually low unlabeled absorption at 1615 cm^{-1}. This conclusion is based upon the singly labeled spectrum for cyclo(GlyPro***Leu***ProGlyTyr). (Bold italicizing of an amino acid indicates the presence of a ^{13}C carbonyl in the C-1 position.) In this spectrum, the peak at 1615 cm^{-1} disappears completely, and a peak at 1580 cm^{-1} appears (Figure 2B). The exact location of these peaks (Table 1) can clearly be seen if one subtracts the two spectra (Figure 2C).

The Leu absorption at 1616 cm^{-1} is at an unusually low frequency for an amide I vibration. This result could be due to intermolecular Leu-Leu coupling in the crystal or to intrinsic electronic factors. Isotope dilution studies distinguished between the two possibilities. If the Leu amide is intermolecularly coupled, then crystals formed from mixtures of labeled cyclo(GlyPro***Leu***ProGlyTyr) (L, see below)) and unlabeled cler-

Table 1. Assignment of Amide I Bands[a]

label	lost ^{12}C band(s) (cm^{-1})	new ^{13}C band(s) (cm^{-1})
cyclo(***Gly***ProLeuProGlyTyr)	1634	1597
cyclo(Gly***Pro***LeuProGlyTyr)	1654	1620
cyclo(GlyPro***Leu***ProGlyTyr)	1616	1580
cyclo(GlyProLeu***Pro***GlyTyr)	1672	1660
cyclo(GlyProLeuPro***Gly***Tyr)	1637	1601
cyclo(GlyProLeuProGly***Tyr***)	1658	1619
cyclo(GlyPro***Leu***ProGly***Tyr***)	1653, 1616	1618, 1581
cyclo(***Gly***Pro***Leu***ProGlyTyr)	1633, 1616	1597, 1581
cyclo(GlyPro***Leu***Pro***Gly***Tyr)	1636, 1616	1601, 1580
cyclo(***Gly***Pro***Leu***Pro***Gly***Tyr)	1635, 1616	1602, 1580

[a] Band assignments based upon subtraction of an unlabeled spectrum from a labeled spectrum (see Experimental Section, Figure 2 for example spectra). The standard deviation of these assignments was found to be ± 1 cm^{-1}. For the triply labeled compound (bottom line), the band disappearing at 1635 cm^{-1} is a result of the two labeled glycine carbonyls shifting to 1602 cm^{-1}. The 1602 cm^{-1} can be effectively modeled with two Voigt or Lorentzian peaks.

omyrine (U) should show ^{13}C amide bands which absorb at a frequency intermediate between the pure samples. If the Leu amide is not intermolecularly coupled, then dilution of the singly labeled compound with unlabeled material should result in no shift in the peak position of the ^{13}C-labeled band, and the spectra will look like a sum of the isotopically pure spectra. When these cocrystallization experiments were conducted at label dilution levels (L:U) of 3:1, 1:1, and 1:3, no intermediate spectra were observed, indicating that intermolecular coupling is not a factor. Thus, the low frequency absorption of the Leu amide I band must be due to intrinsic factors which are unclear at this time.

In order to selectively observe ^{13}C amide I modes from carbonyls other than Leu, the Leu amide was also labeled, in order to clear the spectral region around 1616 cm^{-1}. The spectra of the doubly-labeled compounds in the ^{13}C amide I region were indistinguishable from the sum of the two singly-^{13}C-labeled compounds. As expected, the remaining ^{12}C absorptions retained their position. This approach was particularly useful in assigning the Tyr ^{13}C amide I band which, in the singly-labeled compound, could not be resolved from the Leu ^{12}C amide band.

The non-Lorentzian shaped absorption band around 1635 cm^{-1} in the unlabeled spectrum (Figure 2A) was assigned to overlapping Gly amide I absorption bands. This assignment was based on the spectrum of the triply labeled analog cyclo(***Gly***Pro***Leu***Pro***Gly***Tyr), in which that band disappeared. The single Gly labeled analogs cyclo(***Gly***ProLeuProGlyTyr) and cyclo(GlyProLeuPro***Gly***Tyr), together with the doubly labeled compounds in which one Gly was labeled: cyclo(***Gly***Pro***Leu***ProGlyTyr) and cyclo (GlyPro***Leu***Pro***Gly***Tyr) confirmed this assignment and separated the two Gly amide I bands for subsequent IRLD studies (*vide infra*).

The proline amide I bands proved difficult to analyze because both their ^{12}C and ^{13}C bands overlapped with the other ^{12}C amide I stretches (Table 1). The 12 cm^{-1} shift of the Pro amide I band in cyclo(GlyProLeu***Pro***GlyTyr) indicated that the ^{13}C stretch is highly coupled to the other ^{12}C vibrations.[25] In theory, this problem could be circumvented by analysis of the multiply ^{13}C-labeled compounds cyclo(***GlyProLeu***Pro***GlyTyr***) and cyclo(***Gly***Pro***Leu***Pro***GlyTyr***). However, the prohibitive expense precluded preparation of these compounds, preventing analysis of the Pro amide I bands.

IRLD Provides Information about Amide Orientation. Infrared linear dichroism spectroscopy is based on the fact that the absorption of plane-polarized infrared radiation is dependent upon the orientation of the vibration relative to the direction of polarization, as seen in the schematic in Figure 3. The cleromyrine crystal structure is drawn in Figure 1 to highlight the amide I orientations, projected onto the plane of the page. Absorption is maximized when the projection of the orientation onto the plane of polarization is parallel to the direction of polarization.[10] Figure 4 shows two typical spectra taken of cleromyrine at 0° and 90° plane polarized IR radiation. Minimal absorption should be observed when the incident IR radiation is perpendicular to the projection of the vibration (Figure 3). Nonzero minimum absorptions (Table 3) are due to disorder, overlap with weakly absorbing peaks, and imperfect polarization of the incident radiation.

Amide orientation determines the polarization angle at which IR absorption will be maximized. The magnitude of the maximal absorption (Table 3) is related to the azimuthal angle which describes the projection of the amide I dipole; when that angle is equal to 0°, the absorption is maximal (Figure 3). The two-dimensional representation of the cleromyrine crystal structure shown in Figure 1 is a projection of that structure onto the plane of the page. In that representation, the projected length of the amide carbonyl bonds is related to the azimuthal angle. The longer the bond, the closer the azimuthal is to 0°; the shorter the bond, the closer the azimuthal is to 90° (Figure 3). A large maximum absorption is indicative of a dipole which is nearly coincident with the plane of polarization. Since the cleromyrine amides are not coplanar, large deviations with respect to the difference between maximal and minimal amide I absorption were expected and observed (Figures 4 and 5). Table 3 reports the absorbance maxima and minima for the analyzed carbonyls. Differences in the minimal absorptions of each amide may arise from local disorder in the cleromyrine backbone.

Table 2. Assessment of Relative Amide I Orientation[a]

carbonyl	X-ray rel angle	polarization estimate
Leu	0°	0° (standard)
Tyr	−24.4°	−20°
Gly1	98.3°	100°
Gly5	86.7°	100°

[a] Orientations determined by isotope-edited IRLD as compared to those measured from the crystal structure of cleromyrine (Figure 1).[15] All reported angles are $\pm 180°$. With the exception of Tyr, assignments are based on at least two labeled systems. For Tyr, only the compound cyclo(GlyPro***Leu***ProGly***Tyr***) was used to determine relative orientation. The Leu amide I was set to be 0°, and all other angle assignments are reported relative to this value (see Experimental Section).

IRLD Analysis of Crystalline Cleromyrine and ^{13}C-Labeled Analogs. Cleromyrine crystals were analyzed by IRLD, and the absorption was followed as a function of the angle of polarization. The absorption was measured by both area and peak height; the results were identical (Table 2). For each ^{13}C-labeled sample, five crystals were studied, and all of the measured angles were within 10%. Each amide was studied in the context of all analogs in which its absorption was clearly resolved, including the unlabeled analog, if applicable. The angle at which the absorption of the Leu amide carbonyl was maximal was arbitrarily chosen to be 0° (Figure 3). The orientation of the tyrosine could only be assessed using the multiply labeled compound cyclo(GlyPro***Leu***ProGly***Tyr***). The singly labeled compound cyclo(GlyProLeuProGly***Tyr***) could not be used to determine tyrosine orientation because the tyrosine ^{13}C-amide I band at 1619 cm^{-1} overlapped with the highly dichroic ^{12}C leucine band at 1616 cm^{-1}. Because these carbonyls are only 20° ($\pm 180°$) apart in orientation, the amide I bands need to be separated in order to observe the distinctive angular dependence of the Tyr amide. Furthermore, the

(25) The coupling of ^{13}C-labeled Pro4 to neighboring amides was predicted by simple calculations.[14]

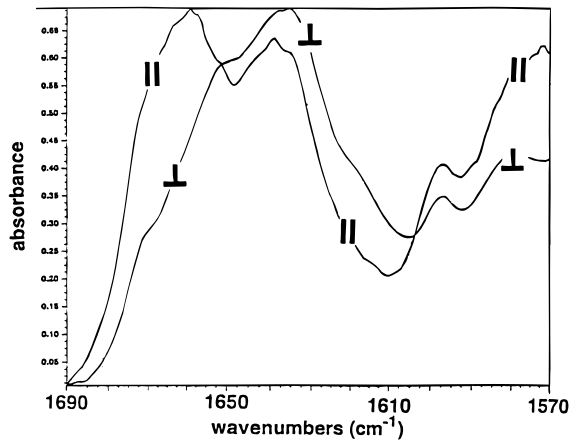

Figure 4. Parallel and perpendicular IR spectra demonstrate IRLD effect. The spectra shown were measured for the sample cyclo(***Gly***ProLeuProGlyTyr). A complete plot of the absorbance vs polarizer angle for the ^{13}C-Gly1 absorption band is shown in Figure 5.

Table 3. Linear Polarization Absorption Data[a]

carbonyl	max. Abs.	min. Abs.
Leu	0.310	0.185
Tyr	0.242	0.223
Gly1	0.176	0.093
Gly5	0.366	0.238

[a] Absorbance values for each amide I band are reported as fraction of total amide I absorption at that polarizer angle (see Experimental Section). Maximum absorption is related to the azimuthal angle of the amide plane relative to the plane of polarization (Gly5 is the closest to parallel, see Figures 1 and 3). Minimum absorption is related to the degree of local order (Gly1 is the most ordered). For tyrosine, the estimate is based on cyclo(GlyPro***Leu***ProGly***Tyr***) rather than cyclo(GlyProLeuProGly***Tyr***) due to overlap of the ^{13}C tyrosine band with the ^{12}C band of leucine. Uncertainty in these measurements was on the order of ±0.01 absorbance units.[14]

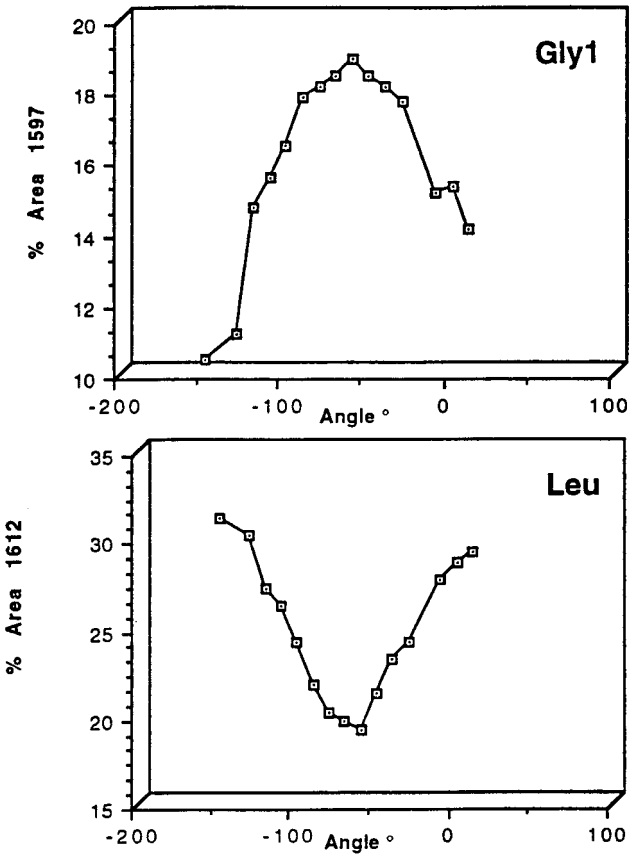

Figure 5. Plot of amide I absorption, as measured by percent of total amide I absorption vs polarizer angle. Amide I absorption is reported as a fraction of the total amide I absorption intensity at that polarizer angle, as in Table 3. Other methods to quantify absorption provided identical results.[14] These two plots demonstrate that the Gly1 (top) and Leu (bottom) amide I dipoles are close to perpendicular to one another (see Figure 1).

polarization effect observed for Tyr is much weaker than for Leu, which further obscures the angular information (Table 3). The small polarization effect of the Tyr band compared to the Leu band is due to its larger azimuthal angle (leading to a smaller maximum absorption, Figure 3) and its higher degree of disorder (leading to a larger minimum absorption).[14,26]

In addition to providing information about the relative orientations of projected amides, this method allows the approximation of the projection (azimuthal) angle. Leu and Gly5 have the two largest maximal absorptions (as predicted by examination of the two-dimensional representation of clermyrine, Figure 4), with relative areas of 0.310 and 0.366, respectively, and also have very large minima, 0.185 and 0.238 (Table 3). Because both these maxima and minima are large, it can be concluded that these two carbonyls have small azimuthal angles relative to the plane of polarized light but have a relatively large distribution of planar projection orientations. The Gly1 absorbance band has a maximum of 0.176 and minimum of 0.093 indicating it is more ordered, and its azimuthal angle is larger than Gly5 (Figure 1). Because tyrosine has such a weak polarization effect, the maximum and minimum angles for tyrosine are reproducible to only ±20.0° due to difficulties resolving extrema. Because the Tyr maximal absorption is larger than Gly1 but smaller than Gly5, tyrosine's azimuthal angle must be smaller than that of Gly1 but greater than that of Gly5. Finally, the relatively large minimal absorption of the Tyr amide indicates local disorder, which has also been suggested by SSNMR measurements of the Tyr ring flipping rate.[26]

Extension of this Method to More Complex Systems. The method presented herein allows the determination of relative amide orientations in the ordered solid state. Suitable substrates include protein crystals, amyloid fibrils, and membrane-bound proteins. Relative orientations of amide I dipoles can be determined by measuring IRLD effects; azimuthal angles can be estimated according to the maximal absorption. This method could be used to distinguish between two or more structures which each fit constraints derived from SSNMR methods for structure determination.[4,27] However, the sample to be analyzed must meet four criteria. **(1) All of the molecules in the ordered solid must have identical, or very similar, structures and must have the same relative orientation.** Thus, small peptide crystals are not suitable substrates, since they often contain several molecules with different orientations relative to the crystal axis per unit cell. These molecules are often structurally distinct. However, peptide amyloid fibrils that we have analyzed seem to comprise a single group of closely related structures.[4] Extension of this method to the analysis of amyloid fibrils requires orientation of fibrils relative to each other, since IR microscope cannot focus on a single fibril. **(2) The amide I absorption bands must be localized to single amide carbonyls. This can be accomplished in most systems by isotope**

(26) Griffiths, J. M.; Anderson, T. S.; Lansbury, P. T., Jr.; Griffin, R. G. unpublished data.

(27) Spencer, R. G. S.; Halverson, K. J.; Auger, M.; McDermott, A. E.; Griffin, R. G.; Lansbury, P. T., Jr. *Biochemistry* **1991**, *30*, 10382−10387.

editing. Cleromyrine is an ideal system for this method, since no dipolar coupling is observed and the amide I bands can be assigned to individual amides. However, in many protein secondary structures, amide I vibrational modes of similar energy couple to one another, provided that they are close in space (the magnitude of the effect is inversely proportional to r^3) and are properly oriented.[5] Idealized antiparallel β-sheets (polyamino acids) have highly-coupled amide I modes. Thus the resulting spectra in the amide I region have two absorption peaks; a low intensity band at high frequency (*ca*. 1680 cm^{-1}) and a characteristic intense band at low frequency (*ca*. 1620 cm^{-1}) which is the focus of these studies. Incorporation of ^{13}C-amides into β sheet structures has been shown to decouple amide I modes, resulting in an apparent ^{13}C-induced shift of less than 37 cm^{-1} and a small shift in the residual ^{12}C band to higher frequency due to lost coupling.[8] The magnitude of these shifts has been used to place an upper limit on amide−amide distances.[9] In the case of the highly coupled β sheet, some coupling between the ^{13}C- and ^{12}C-amides persists, as indicated by the fact that the area of the ^{13}C absorption band is far greater than would be anticipated.[14] Complete elimination of this residual coupling could be achieved by incorporation of ^{18}O into the ^{13}C-labeled amide, further shifting its absorption frequency. **(3) The sample must be oriented on the scale of the resolution of the IR microscope ($5 \times 2\ \mu$m).** The method is ideally suited for the analysis of crystalline samples, such as cleromyrine, which can be oriented on the IR microscope stage. However, crystalline samples can be analyzed by single crystal X-ray diffraction, which provides complete structural information and does not require isotope labeling. The original motivation for developing this technique was to solve structural problems which cannot be approached using traditional methods, specifically amyloid structure.[3] The applicability of this method to amyloid depends on the ability to orient and analyze single fibrils. In addition to amyloid fibrils, other noncrystalline ordered systems could be analyzed using this method. For example, peptides imbedded in lipid bilayers have been analyzed by attenuated total reflectance IRLD, providing information regarding the relationship between secondary structural elements and the plane of the bilayer.[11] Isotope editing of these spectra would allow the relative orientation of local structural elements to be determined. **(4) Specific isotope labeling must be practical.** Systematic labeling of amide carbonyls is not practical for systems that are much more complex than cleromyrine. Therefore, this method is not to be used for total structure determination but rather as a complement to another method. For example, there are many situations which arise when analyzing SSNMR distance information where two local structures are consistent with the measured distances and similar with respect to local conformational energy.[27] In these cases, isotope-edited IRLD could be critical for determining which structure is actually present in the amyloid fibril. We envision this technique as a complement to the approach that we have described elsewhere,[4] to be used in the second stage of structure determination to distinguish between possible structures. Significantly, the amount of material required for IRLD experiments is less than 1% that required for SSNMR analysis. Finally, this approach could also be used in the Raman mode, since the amide I absorption bands af cleromyrine are better resolved in the Raman as compared to the FTIR.[14] Isotope-edited Raman spectroscopy could utilize ^{15}N labeling to analyze the amide III mode, which is not IR-active.

Conclusions

We report herein a new approach to the structure determination of insoluble proteins using infrared spectroscopy. The method, a hybrid of two known methods, isotope-edited FTIR and infrared linear dichroism, is capable of measuring relative orientations of peptide backbone amides, as demonstrated on the crystalline cyclic peptide cleromyrine. Amide orientations determined by isotope-edited infrared linear dichroism were within 15° of those measured from the known crystal structure, suggesting that this approach could be applied to peptide and protein samples of unknown structure. In addition, absorption magnitudes are also related to amide orientation, and this information could be used to distinguish alternative structures. This method is intended as a complement to SSNMR methods which generate distance constraints that, in some cases, fail to distinguish between two possible structures.

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