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Elucidation of Residue-Level Structure and Dynamics of Polypeptides via Isotope-Edited Infrared Spectroscopy

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ABSTRACT
Infrared spectroscopy is a powerful tool for analyzing the structure of proteins and peptides. The amide I band is particularly sensitive to the strength and position of the hydrogen bonds that define secondary structure as well as dipole−dipole interactions that are affected by the geometry of the peptide backbone. The introduction of a single ^{13}C-labeled carbonyl into a peptide backbone results in a resolvable shoulder to the main amide I band, which can be analyzed as a separate peak. Thus, site-specific structural information can be obtained by sequential, systematic labeling of the backbone. This method of isotope-edited infrared spectroscopy is a tool for obtaining medium-resolution information about the backbone confromation and dynamics. This tool has been used to dissect the conformation and dynamics of α helices and amyloid aggregates, where the versatility of possible sampling with infrared spectroscopy is well-suited for studies of large-protein aggregates.

Introduction

For nearly 50 years, the infrared (IR) spectra of proteins have been recorded and interpreted in terms of the fold and conformation of the polypeptide. The amide I band, arising primarily from the backbone carbonyl stretch mode, is particularly sensitive to peptide secondary structure, because of hydrogen-bonding and transition-dipole coupling effects.[1] From deconvolution and band fitting of the amide I spectra, the secondary-structure content of a peptide can be deduced, analogous to the quantitative analysis of ultraviolet circular dichroism (CD) spectra.[2] Moreover, time-resolved infrared measurements can be used to probe changes in the secondary-structure content on time scales ranging from nanoseconds to hours.[3] The ability to measure spectra under a broad range of sample conditions (including in crystals, amorphous solids, fibrous aggregates, adsorbed to surfaces, dried protein films, inserted within lipid membranes, and in aqueous solution) makes this a particularly versatile tool for probing the protein structure.[1,4]

This use of infrared spectroscopy produces "low resolution" data on the protein structure; the secondary structure content may be elucidated, but the conformations of specific residues within the polypeptide cannot be assigned. However, recent experiments have demonstrated that more detailed information can be obtained from IR studies of polypeptides upon specific isotope labeling. The isotope-shifted amide bands can be resolved from the bands arising from the unlabeled residues and report on the conformation and environment of the specific labeled residues. The combination of site-specific labeling of the polypeptide backbone with vibrational spectroscopy results in the elucidation of residue-level structural information, including details on backbone geometry and solvent accessibility. This Account describes how the technique of isotope-edited infrared spectroscopy can be used to obtain "medium-resolution" information about polypeptide conformation and dynamics.

Transition Dipole Coupling and the Relationship between the Amide I Band and the Protein Secondary Structure

The amide moiety of the peptide linkage gives rise to a series of unique bands in the IR spectra; these amide bands are numbered I−VII (in order from high to low frequency). In addition, a high-frequency band associated with the N−H stretch is labeled amide A.[1,5,6] In polypeptides, the frequencies of the amide band (especially amide

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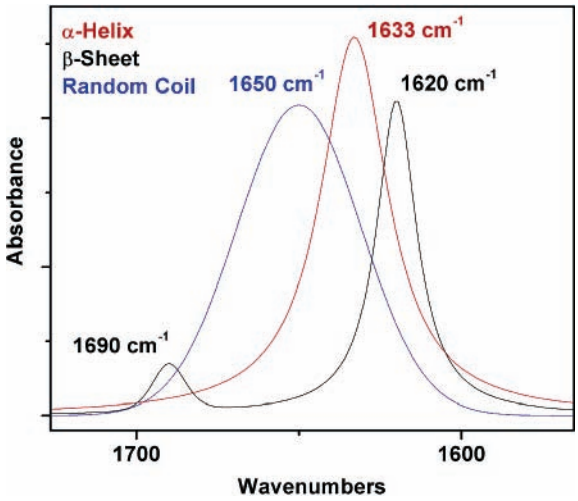

FIGURE 1. Examples of model amide I′ bands of polypeptides in different secondary structures. The band for an α helix at 1633 cm^{-1} corresponds to a fully solvated helix, with hydrogen bonding between the helix backbone and the solvent. Helices buried from the solvent give an amide I′ band at higher frequency.

I) vary from protein to protein; these variations correlate with the secondary structure and backbone solvation of the polypeptides (Figure 1). The secondary-structure dependence of the amide I band arises from coupling of the amide I modes from the polypeptide. Through-bond, covalent coupling contributes to these effects, as well as couling via the hydrogen-bond network of the peptide. However, through-space transition-dipole coupling is the dominant factor in determining amide I band frequency. The theoretical understanding of the transition-dipole coupling in polypeptides has been reviewed by Diem[4] and Krimm and co-workers.[5] Briefly, the amide I mode consists primarily of C═O stretching (with some contributions from the C−N stretch and N−H deformation, which shift the transition dipole moment of the mode slightly away from the C═O bond axis). In polypeptides with a well-defined secondary structure, the peptide carbonyl groups are held at fixed geometries. For example, in an α helix, the C═O groups are all pointing in the same direction, with each hydrogen bonded to an N−H group four residues away. This constrained geometry combined with the strong transition dipole moment for the amide I mode results in transition-dipole coupling of all of the amide groups within the secondary-structure element. In a secondary-structure element consisting of n residues, transition-dipole coupling mixes the vibrational wave functions of all n carbonyl groups, creating n delocalized vibrational wave functions. The splitting between these delocalized vibrational energy states is given by the eigenvalues of the dipolar interaction energy matrix

$$\mathbf{V}_{ij} = \frac{\mu_i \mu_j}{|T_{ij}|^3} - \frac{3(\mu_i \mathbf{T}_{ij})(\mu_j \mathbf{T}_{ij})}{|T_{ij}|^5} \qquad (1)$$

where $\mathbf{T}_{ij}$ is the distance vector between dipoles μ_i and μ_j. The dominant term in the absorption process depends upon the geometry of the peptide group (in other words, the geometry between the interacting carbonyls). Different splitting terms will dominate the absorption spectrum for different secondary-structure geometries, resulting in different observed absorption wavelengths for the amide I bands of different secondary-structure conformations.

The dependence of the amide I band frequency on the backbone conformation of the polypeptide chain provides a means for estimating the secondary-structure content of proteins using IR spectroscopy. The underlying structure of the broad amide I bands of large proteins can be revealed by either deconvolution or second-derivative techniques. The bands then can be fit to a sum of Gaussian functions, and from this fitting procedure, the contribution of α-helix, β-sheet, or β-turn bands to the overall amide I absorbance is determined. For some proteins, these procedures result in secondary-structure contents that compare favorably to values determined by X-ray crystallography or CD spectroscopy.[6,7]

Principles of Isotope-Edited IR Spectroscopy of Polypeptides

While the coupling between amide I modes of adjacent residues within polypeptides gives rise to the secondary-structure dependence of the band frequency, this delocalization prevents the use of the amide I band to report on *specific* or *individual* residues within a protein. Individual amide I modes of residues within the secondary structure can be isolated using isotopic substitution. With the simplifying assumption that the amide I vibration is a carbonyl stretch (neglecting the C−N and N−H contributions to the mode), the isotope shift expected for the amide I mode can be approximated using a simple harmonic oscillator model (eq 2, where k is the force constant and μ is the reduced mass)

$$\nu = \frac{1}{2\pi}\sqrt{\frac{k}{\mu}} \qquad (2)$$

This isotope effect shifts in the amide mode frequency from the larger amide I band. When a backbone carbonyl is labeled with ^{13}C, the amide I band for that residue is shifted to a lower frequency by ~40 cm^{-1}, creating a new feature in the IR spectrum. This ^{13}C amide I band can then be used as a site-specific probe of protein conformation and dynamics. This approach (called "isotope-edited IR spectroscopy") permits the resolution of the protein structure at the residue level from the analysis of IR spectra arising from a series of labeled peptides or proteins.[7] Larger shifts can be obtained by double-labeling a carbonyl with $^{13}C/^{18}O$; the resulting isotope-shifted band is generally well-resolved from the ^{12}C envelope.

One straightforward application of isotope-edited IR spectroscopy has been in studies of protein−protein interactions. For example, in a study of caldmodulin interactions with target peptides, caldmodulin was uniformly labeled with ^{13}C, giving an isotope shift for the full protein spectrum. The labeled protein was then mixed with unlabeled target peptides. The amide I bands of the peptide ligands and the calmodulin could be readily distinguished, and changes in these bands revealed bind-

ing-induced conformational changes in both the ligand and receptor.[8] This approach has been used to observe conformational changes involved in the binding of several ligand receptor complexes, including the binding of partially folded intermediates to the chaperone α crystallin.[9]

Isotope-edited IR has also been used to discriminate between different types of secondary structure within a single polypeptide chain.[10,11] For example, in a short peptide derived from the N-terminus lung surfactant, Gordon et al. prepared a series of ^{13}C-labeled derivatives to map regions of α helix, β sheet, and random coil in the structure.[12] Using this approach, the authors could also elucidate the effects of different lipid environments on the conformational map of the peptide, data which are not easily obtainable from solution NMR studies. However, because the amide I band frequencies can vary with the solvent environment as well as the secondary structure, spectral differences that arise from labeling different sections of the polypeptide sequence must be interpreted with caution.

Using Isotope-Edited IR To Determine Residue-Level Conformation in Helical Peptides

The isotope labeling involved in the receptor−ligand studies allows for the discrimination of different polypeptide chains but does not offer an improvement in the resolution of structural detail. Recent work in our group[13−19] and by others[20,21] uses ^{13}C labeling of specific residues in model peptides to dissect the structure and dynamics of these systems at the residue level.

Site-Specific Labeling To Determine Helix Stability at the Residue Level. Short peptides (15−25 amino acids long) based on repeats of alanines and charged side chains, such as $(AAAAK)_n$, form stable α helices in aqueous solution. These alanine-rich helical peptides have become canonical models for investigating the determinants of helix stability as well as the mechanism of helix formation.[22−24] The structure and stability of α helices are not necessarily constant throughout the sequence examined. For example, residues in the center of the helix can form hydrogen bonds toward the N and C terminus, while residues at the ends of a helix can form only one set of hydrogen bonds. This should result in "fraying" of the helix termini, reflected in either reduced stability of the helix at the N and C termini and/or changes in the conformation at the ends. Differences in helix stability between the central portions and the termini of helical peptides can be measured by isotope-edited IR spectroscopy[13−15] (Figure 2). When the peptides are labeled in a central portion of the helix (parts A and B of Figure 2), a prominent ^{13}C amide I′ band[25] is observed at low temperatures; as the temperature increases (and the peptide unfolds form the helix to form a random coil), this band decreases in intensity and shifts to a higher frequency, until (in the high-temperature, random-coil spectrum) it is a poorly resolved tail on the low-frequency side of the ^{12}C amide I′ band. When the peptides are

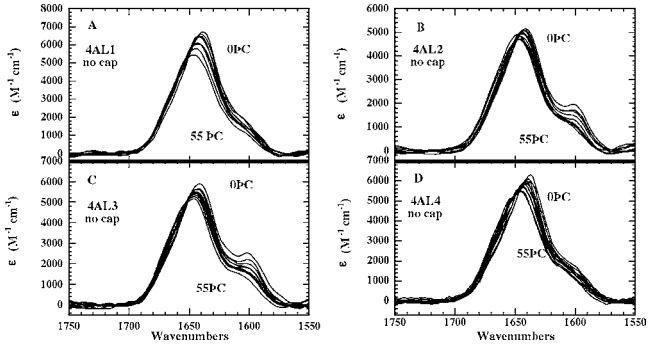

FIGURE 2. IR spectra of a 20-residue peptide with ^{13}C labels introduced into residues at the N temrnus (A), central (B and C), and C terminus (D) regions of the spectrum.

labeled at the N or C termini, the ^{13}C amide I′ band is poorly resolved even at low temperatures and does not change significantly upon an increase in the temperature (parts C and D of Figure 2). These spectra are consistent with a general model for the helical peptide, stabilized helix in the central portions and frayed helix or disordered structure at the termini. When an acetyl-capping group is added to the N terminus of the peptide, the overall helix content increases.[14] IR spectra of labeled peptides demonstrate that the primary effect of the acetyl group is on the conformation of the N-terminal residues. In comparison with the uncapped peptide, the acetylated peptides give a prominent, helical ^{13}C amide I′ band for the N-terminal residues. The C-terminal residues, however, are largely unaffected by N-acetylation.

Local conformation changes within a helix because of side-chain substitutions can also be detected by isotope-edited IR. For example, replacing alanines for bulkier leucine residues within the $(AAAAK)_n$ sequence has a small effect on the overall conformation of the peptide but a significant effect on the local conformation at the site of the substitutions. The shift in the ^{13}C amide I′ band, which occurs, is consistent with a tightening of the backbone coil and lengthening of the helix in the vicinity of the leucine residues, a backbone distortion that likely arises to the steric constraints of the bulky leucine side chains.

Using Isotope-Edited IR To Detect Local Differences in Backbone−Solvent Hydrogen Bonding. The amide I band reports on backbone−solvent hydrogen bonding as well as secondary structure. This is illustrated by the difference in the amide I frequency between small helical peptides in aqueous solution (which give rise to an amide I′ with $\nu_{max} = \sim 1633$ cm^{-1}) and either globular helical proteins (such as myoglobin) or helical peptides inserted into lipid membrane (which give rise to an amide I′ with $\nu_{max} = \sim 1650$ cm^{-1}). Hydrogen bonding between solvent water molecules and backbone carbonyls of the polypeptide decreases the amide I band frequency. Thus, the amide I frequency can be used to distinguish between helical residues, which are solvent-exposed, from those which are shielded from the solvent. DeGrado, Vanderkooi, and co-workers have used isotope-edited IR spectroscopy to demonstrate differences in solvent accessibility between residues on the outside of a coiled-coil peptide[26]

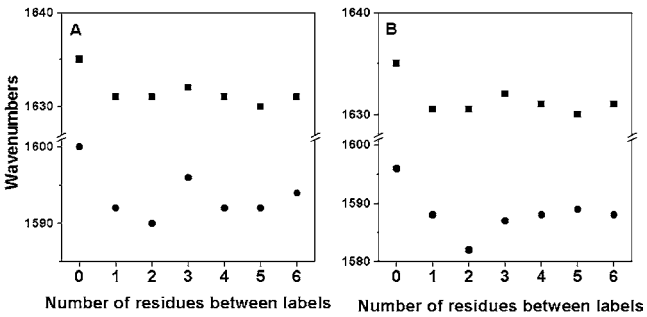

FIGURE 3. ^{13}C and ^{12}C amide I′ frequencies in doubly labeled Ac-AA(AAKAA)4AAY-NH_2 as a function of the number of residues between the ^{13}C labels. (A) Measured data. (B) Data predicted from a coupled oscillator model. On the basis of data from ref 17.

or three-helix bundle peptide[27] and those buried on the inside. This opens the way for using IR spectroscopy for obtaining site-specific information about the tertiary as well as secondary structures. In addition, IR spectra of labeled peptides can also be used to identify sites of backbone desolvation because of side-chain conformation. For example, helix-inducing cosolvents such as 2,2,2-trifluoroethanol reduce hydrogen bonding between the solvent and the helix backbone. Isotope-edited IR has been used to demonstrate that certain residue positions, such as those located four residues away from a lysine in peptides with sequence $(AAAAK)_n$, are desolvated to a greater extent than other positions in the sequence.[19]

Modeling Amide I Coupling in Labeled Helices. Infrared spectra of polypeptides have been modeled and simulated using a variety of approaches, from simple coupled oscillator models[28] to density function *ab initio* calculations;[18] these techniques have recently been reviewed.[1] These models can be modified to included effects of specific isotope labeling.[29] The effect of ^{13}C labeling on amide I coupling within α helices has been both theoretically modeled and experimentally tested using short alanine-rich helical peptides as a model system for characterizing the helical amide I band. When the ^{13}C labels are arranged at different positions on the helix, the geometry affects the coupling between amide I modes both experimentally and computationally.[17,18] For example, in peptides containing two ^{13}C backbone carbonyls, the frequency of both the ^{12}C and ^{13}C amide I′ modes varies systematically with the spacing between the labels (Figure 3). The damped sinusoidal behavior of the frequencies is consistent with simple predictions using eq 1; as the relative positions along the helix change, the projection angle between the amide I transition dipoles changes as well. This sensitivity to distance and backbone torsion angle, as well as the ability to simulate spectra of labeled peptides in fixed conformations, makes it possible to determine quantitative structural information about backbone geometry from isotope-editing experiments.

Site-Specific Folding Dynamics Using Transient IR Spectroscopy of Labeled Peptides. Changes in the amide I band during dynamic processes associated with folding and functional events have been observed on a wide range of time scales. For example, ultrafast transient infrared spectroscopy has been used to observe the folding of model α-helical peptides,[30−32] the folding of single-domain protein systems,[33−36] and protein relaxation in myoglobin upon photodissociation and recombination of diatomic ligands.[37] However, these spectral changes can be interpreted only in terms of global protein dynamics and not motions of specific residues; the observed band changes report on the protein as a whole, preventing a concrete assignment to local structural changes. However, using specifically labeled peptides in transient infrared studies allows for the observation of the dynamics of individual residues. Gai and co-workers have used a temperature jump coupled with a transient infrared absorption to study the folding of small specifically labeled helical peptides.[38,39] In their work, the relaxation kinetics upon the temperature jump of residues at the C terminus were found to be much faster than that for residues in the central or N-terminal regions of the helix, consistent with a model for helix unfolding, which begins at the frayed C terminus.[38,39] The ability to observe the dynamics of specific residues within a polypeptide may be applicable to functional and light-initiated protein dynamics, as well as folding reactions in other simple protein systems.

Using Isotope-Edited IR To Determine the Structure in β-Sheet Peptides

Vibrational Coupling in β Sheets. Transition-dipole coupling in β-sheet peptides is more complex than in α helices, resulting in more complicated spectral changes upon isotope substitution. In antiparallel β sheets, the strongest coupling occurs between carbonyls of different strands connected by a hydrogen bond. Two IR-active vibrational modes are observed: one in which the nearest neighbor carbonyls within a strand vibrate out of phase, while the carbonyls of the peptides on two different strands linked by a hydrogen bond vibrate in phase (this gives rise to a high-intensity, low-frequency band in the spectrum); and a mode in which the nearest neighbor carbonyls within a strand vibrate out of phase and the carbonyls of peptides linked by a hydrogen bond vibrate out of phase as well (this gives rise to a lower intensity, higher frequency band in the spectrum). Interstrand coupling increases as the number of strands within the β sheet increases (reaching a maximum at around six strands) and in planar (not twisted) β sheets. Thus, large and planar antiparallel β sheets give rise to amide I spectra, with the largest splitting between the low- and high-frequency bands.[2,6,40]

Brauner et al. observed that, when two site-specific ^{13}C-labeled carbonyls were introduced into the backbone of a peptide forming an antiparallel β sheet [with sequence $K_2(LA)_6$], the intensity and the frequency of the ^{13}C amide I band depended upon whether the labels were adjacent [e.g., K_2*L*A$(LA)_5$] or alternating [e.g., K_2L*AL*A$(LA)_4$] in sequence.[41] Strikingly, when the labels were placed on alternate carbonyls, the ^{13}C amide I band was the largest feature in the spectrum, although only 2 of the 14 residues were ^{13}C-labeled. Brauner et al. ascribed this "anomalous

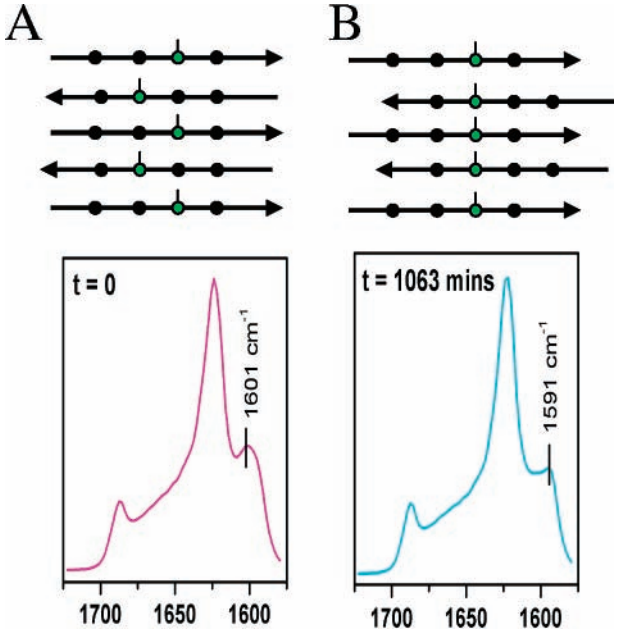

FIGURE 4. Alignment of ^{13}C-labeled carbonyls within antiparallel β sheets depends upon the strand register. When the ^{13}C labels (in green) are not aligned (A), there is only weak interstrand $^{13}C-^{13}C$ coupling, resulting in a ^{13}C amide I band at higher frequency (1601 cm^{-1}). When the ^{13}C-labeled residues are aligned (B), there is strong $^{13}C/^{13}C$ coupling and the ^{13}C amide I mode shifts to 1591 cm^{-1}.

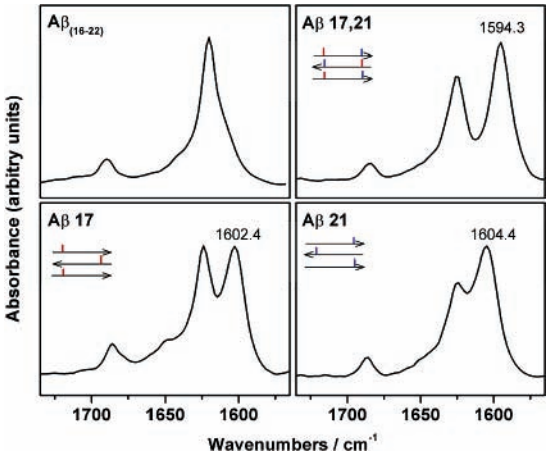

FIGURE 5. FTIR spectra of a series of singly and doubly labeled derivatives of the Aβ16–22 peptide. When the peptide is doubly labeled at residues 17 and 21, the ^{13}C amide I band shifts to 1594 cm^{-1}; this shift does not occur in any of the singly labeled derivatives, indicating that the strands must be arranged in an in-register antiparallel β sheet. Modified from ref 52.

intensity" to a mixing of ^{13}C and ^{12}C modes between strands.[41] According to this model, there is interstrand coupling between the ^{13}C and ^{12}C carbonyls whose peptide groups are linked by a hydrogen bond, and as a result, the ^{13}C modes pick up substantial intensity. This effect is strongest when ^{13}C-labeled residues are hydrogen-bonded to ^{12}C residues on adjacent strands; when the labeled groups are aligned between strands, there is less $^{13}C/^{12}C$ mixing and therefore weaker intensity enhancement of the ^{13}C modes.[42] While coupling between ^{12}C and ^{13}C modes accounts for part of the observed intensity enhancement, there may be other mechanisms at work that explain the anomalous intensity effect. In *ab initio* simulations of the IR spectra of labeled antiparallel β-sheet peptides, Kubelka and Keiderling have pointed out that the large, low-frequency band observed in the peptides studied by Brauner et al. is dominated by in-phase vibrations of interstrand-coupled ^{13}C modes, primarily from the central portions of the β sheet.[43]

Because the intensity and frequency of the isotope-shifted bands of specifically labeled β-sheet peptides are determined by interstrand coupling, isotope-edited IR spectroscopy is a powerful tool for probing the strand register within β sheets. A schematic diagram of this approach is shown in Figure 4. When the register of the strands brings the labeled groups into alignment across the strands, interstrand coupling between the ^{13}C carbonyls will shift the ^{13}C amide I band to a lower frequency, while the absence of mixing of $^{13}C/^{12}C$ modes will reduce the anomalous intensity enhancement. When the strand register of strands aligns ^{13}C groups with ^{12}C groups across strands, the resulting ^{13}C amide I band has an enhanced intensity and an increase in frequency.

Using Isotope-Edited IR To Determine the Structure of Amyloids. A number of proteins aggregate *in vivo* and *in vitro* to form large, fibrous structures; many of these aggregates are associated with diseases, including Creutfeldt–Jakob disease (prion protein), Alzheimers' (Aβ and tau proteins), Parkinson's (α synuclein), and type-II diabetes (iselet amyloid polypeptide).[44,45] Although the overall morphology of these structures have been characterized in detail and shown to have a high degree of similarity, there is very little molecular-level structural information on amyloid aggregates: their fibrous nature prevents the measurement of high-resolution, single-crystal X-ray diffraction data, and their large size is a challenge to NMR methods.[46] Isotope-edited IR spectroscopy is an ideal tool for providing medium-resolution structural information about these aggregates, which can complement other techniques (such as X-ray fiber diffraction). Moreover, the ability to measure IR spectra both in solution and in the solid-phase permits the analysis of the structure and dynamics of both oligomeric intermediates as well as stable amyloid.

A short peptide based on the sequence of residues 109–122 of the Syrian Hamster prion protein (PrP109–122) forms aggregates *in vitro*. On the basis of IR spectra, the peptides are in a β-sheet conformation in these aggregates, and fiber diffraction data indicates that the peptide strands are perpendicular to the axis of the fiber.[47,48] When single-site-specific ^{13}C labels are introduced into this sequence, an isotope-shifted ^{13}C amide I′ band appears. The frequency and intensity of this band are the same for almost every position substituted, except position 117. When this alanine is labeled, the resultant ^{13}C amide I′ band is shifted by about 10 cm^{-1} relative to the other positions and has a relatively diminished intensity. These spectral features are consistent with a specific β-sheet register, in which the hydrophobic core of the peptide (residues 111–122)

forms the β sheet, with the first three residues at the N terminus in a dangling overhang.[49,50] A similar analysis can be used to dissect the structure of the β sheet in a fragment of the Alzheimers' β peptide, Aβ_{16-22}; the ^{13}C amide I frequencies of the series of single- and double-labeled peptides are consistent only with an in-register, antiparallel β-sheet structure[51] (Figure 5).

This approach can be used to probe the dynamics of β-sheet aggregates. In the PrP109−122 peptide, the initial β-sheet aggregate formed has a disordered register, as indicated by the ^{13}C amide I′ band at 1602 cm^{-1} in the ^{13}CA117 derivative. Over time, this band shifts to its equilibrium position at 1592 cm^{-1}. Isotope-dilution experiments, in which labeled and unlabeled strands are mixed together, indicate that these rearrangements can occur through an intrasheet mechanism via strand repetition.[52] This process may be essential for the nucleation of further aggregate growth along the pathway towards amyloid formation.

Conclusions

Isotope-edited IR spectroscopy provides a "medium-resolution" window into the conformation and dynamics of polypeptides. The versatility of this approach (it can be used on samples in solution, in the solid state, or incorporated into lipid bilayers) and the ability to make measurements on a wide range of time scales (from femtoseconds to days) make it a valuable complement to traditional techniques, such as NMR spectroscopy and X-ray diffraction. Further advances in site-specific labeling technology (including semisynthesis of large polypeptides) and in the simulation of vibrational spectra will make this a broadly applicable approach to protein structure studies.

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