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Site-Specific Examination of Secondary Structure and Orientation Determination in Membrane Proteins: The Peptidic $^{13}C{=}{=}^{18}O$ Group as a Novel Infrared Probe

Abstract: *Detailed site-specific information can be exceptionally useful in structural studies of macromolecules in general and proteins in particular. Such information is usually obtained from spectroscopic studies using a label/probe that can reflect on particular properties of the protein. A suitable probe must not modify the native properties of the protein, and should yield interpretable structural information, as is the case with isotopic labels used by Fourier transform infrared (FTIR) spectroscopy. In particular, 1-$^{13}C{=}{=}O$ labels have been shown to relay site-specific secondary structure and orientational information, although limited to small peptides. The reason for this limitation is the high natural abundance of ^{13}C and the lack of baseline resolution between the main amide I band and the isotope-edited peak. Herein, we dramatically extend the utility of isotope edited FTIR spectroscopy to proteins of virtually any size through the use of a new 1-$^{13}C{=}{=}^{18}O$ label. The double-isotope label virtually eliminates any contribution from natural abundance ^{13}C. More importantly, the isotope-edited peak is further red-shifted (in accordance with* ab initio *Hartree–Fock calculations) and is now completely baseline resolved from the main amide I band. Taken together, this new label enables determination of site specific secondary structure and orientation in proteins of virtually any size. Even in small peptides 1-$^{13}C{=}{=}^{18}O$ is far preferable as a label in comparison to 1-$^{13}C{=}{=}^{16}O$ since it enables analysis without the need for any deconvolution or peak fitting procedures. Finally, the results obtained herein represent the first stage in the application of site-directed dichroism to the structural elucidation of polytopic membrane proteins.* © 2001 John Wiley & Sons, Inc. Biopolymers 59: 396–401, 2001

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INTRODUCTION

The need for detailed chemical information in biological systems is the fuel behind the dramatic advancements in the field of structural biology. With site-specific information in hand, there is an increased possibility of better understanding numerous features of macromolecules (e.g., function, stability or structure). Detailed site-specific information can be obtained from a complete structure determination of proteins by way of diffraction experiments, or NMR. Since determining the structure of a macromolecule is time consuming and often exceedingly difficult (e.g., in the case of membrane proteins) other approaches have been developed that yield information *only* for particular sites. In such instances, a spectroscopic probe may be used to label a particular site in the macromolecule followed by observation of spectroscopic characteristics of that probe. Clearly for a probe to be useful it must satisfy several criteria:

First and foremost, the probe must not interfere, hinder or modify any of the macromolecule's native properties. As such, isotopic labels clearly are the least invasive probes. Second, the probe must generate information that can yield interpretable structural information. Third, the spectroscopic "signal" of the probe must not coincide with any signals arising from the macromolecule. Fourth, one must be able to label the macromolecule *only* at the required site. Finally, the probe should be of general usage to the majority of macromolecules. Only probes that abide by these criteria would therefore be useful in structural investigations of macromolecules.

Popular spectroscopic methods such as fluorescence and electron spin resonance (ESR) spectroscopy, while satisfying the majority of requirements, do in the majority of cases require probes that modify the properties of the macromolecule. Perhaps more importantly is the fact that the interpretation of fluorescence and ESR spectroscopic data is not straightforward. Isotopically edited infrared spectroscopy on the other hand, makes use of probes that do not modify the macromolecule in any way, and is also a powerful and straightforward method for examining local secondary structure[1] as well as obtaining orientational data.[2] The method is based upon the incorporation of an isotopic label at specific sites in the protein and examining the resulting shifted peak by polarized infrared spectroscopy.

The labels used so far were $^{13}C{=}{=}^{16}O$ carbonyl peptide bonds,[1,3] focusing on the isotope shifted amide I vibrational mode. However, this mode, while useful in small peptides is impractical in larger proteins due to large natural abundance of ^{13}C (1.11%) (a peptide larger than 90 amino acids would have a contribution as large as the site-specific label to the isotope edited shift). Furthermore, since the amide I vibrational mode peak width is roughly 30 cm^{-1},[4,5] considerable spectral overlap occurs with the $^{13}C{=}{=}^{16}O$ peak, which is shifted only by ca. -40 cm^{-1}.[1] Thus, analysis of the peak corresponding to the label, requires either peak fitting or spectral deconvolution.[6] This problem is exacerbated when the protein is larger since the relative ratio between the size of the "natural abundance" peak ($^{12}C{=}{=}^{16}O$) and the $^{13}C{=}{=}^{16}O$ increases linearly with the size of the protein.

It is for these reasons that we have set forth to design a different label that does not have these shortcomings. One possibility is the $^{13}C{=}{=}^{18}O$ pair: The natural abundance of the combined ^{13}C and ^{18}O is low, in that only a protein with 44,248 amino acids would contribute to the isotope labelled peak at equivalent intensity. Equally as important is the calculated significant shift of the vibrational band, which eliminates any need for deconvolution, since the peaks are completely baseline resolved. We have previously described the application of the above probe.[7] Herein we describe in detail all aspects regarding its synthesis, analysis, and possible utilizations.

MATERIALS AND METHODS

Label Synthesis

As doubly labeled $^{13}C{=}{=}^{18}O$ amino acids are not commercially available, a simple method for synthesizing such amino acids from ^{13}C precursors (which are widely available) was devised. Glycine labelled at 1-$^{13}C{=}{=}^{16}O$ (Cambridge Isotopes Laboratories, Andover, MA) was dissolved in $H_2{}^{18}O$ (95% ^{18}O, Isotec) : dioxane (1:1) to a final concentration of 1 g/mL. The solution was then acidified to pH 1, with gaseous HCL (obtained by adding H_2SO_4 conc. to $CaCl_2$) and kept at 100°C for 30 min. The sample was then lyophilized to recover the $H_2{}^{18}O$/dioxane solvent. Mass spectrometry was used to obtain the percentage of exchange, which was routinely above 75%. Subsequently, the amino acid was protected with an 9-fluorenylmethyoxycarbonyl (Fmoc) group using established procedures as an input for stepwise peptide synthesis.

Peptide Synthesis and Reconstitution

Three synthetic peptides encompassing the transmembrane domain of *Influenza* A virus the M2 protein (Ser22–Leu46, SSDPLVVAASIIGILHLILWILDRL), were made by standard solid-phase Fmoc synthesis. One peptide contained a 1-$^{13}C{=}{=}^{18}O$ glycine residue at position 34. A second peptide contained a 1-$^{13}C{=}{=}^{16}O$ labeled alanine residue at position

29, while the third was not labeled. Mass spectrometry of the synthetic peptides clearly detected the presence of the labels (data not shown). The peptides were purified by reverse-phase high performance liquid chromatography (HPLC), and the purity confirmed using mass spectrometry) and reconstituted into dimyristoylphosphocholine liposomes via detergent dialysis as described previously.[8] Molar protein to lipid ratios were below 1:30.

Fourier Transform Infrared Spectroscopy

Fourier transform infrared (FTIR) spectra were recorded as described previously[8] and analyzed according to the theory of site-directed dichroism as described in Ref. 2 with modifications.[8] Briefly, 200 μL of sample, ca. 0.5 mg/mL peptide, 12.5 mg/mL lipid, and 0.1 m*M* $KH_2PO_4 \cdot K_2HPO_4$, pH 7.0, were deposited under N_2 onto a Ge 25 reflections internal reflection element (50 $\times$ 20 $\times$ 2 mm, Graseby Specac, Kent UK). A total of 1000 interferograms were averaged using a Nicolet magna 560 FTIR spectrometer (Madison WI, USA) equipped with a high sensitivity MCT/A detector. A 0.25 μm wire grid ZnSe polarizer was used to obtained spectra illuminated with parallel and perpendicular polarized light. The dichroic ratio is the ratio of absorption of parallel and perpendicularly polarised light. Peak intensities were calculated using three approaches: (a) a straightforward band integration, (b) peak fitting, and (c) Fourier self-deconvolution[6] followed by band integration, all yielding indistinguishable results (indicating that simple band integration is sufficient).

Ab initio Hartree–Fock Frequency Calculations

Ab initio Hartree–Fock frequency calculations were carried out at the RHF/3-21G//3-21G level[9] using Cadpac version 6.0[10] on MeCONHMe. Hehre and co-workers report that this small basis set is as effective as more sophisticated basis sets for the calculation of vibrational frequencies of molecules containing only first row elements.[11] It is usual to scale the results, but since we are interested only in the relative frequencies for the labeled compounds, this was not required in this case.[12]

Calculation of the amide I mode as a simple harmonic oscillator was done as described previously in Ref. 1. The frequency of the amide I mode can be oversimplified as a harmonic oscillator, $\omega = \sqrt{k/\mu}$. Thus the frequency ratio between the $^{12}C{=}{=}^{16}O$ and $^{13}C{=}{=}^{16}O$ can be calculated by $\omega_{^{12}C{=}{=}^{16}O}/\omega_{^{13}C{=}{=}^{16}O} = \sqrt{(1/12) + (1/16)}/(1/13) + (1/16) = 1.023$.

RESULTS

Figure 1 shows infrared spectra in the region of the amide I and amide II bands for the three different peptides, containing either no enriched labels (top), a $^{13}C{=}{=}^{16}O$ label (middle) and a $^{13}C{=}{=}^{18}O$ label (bottom).

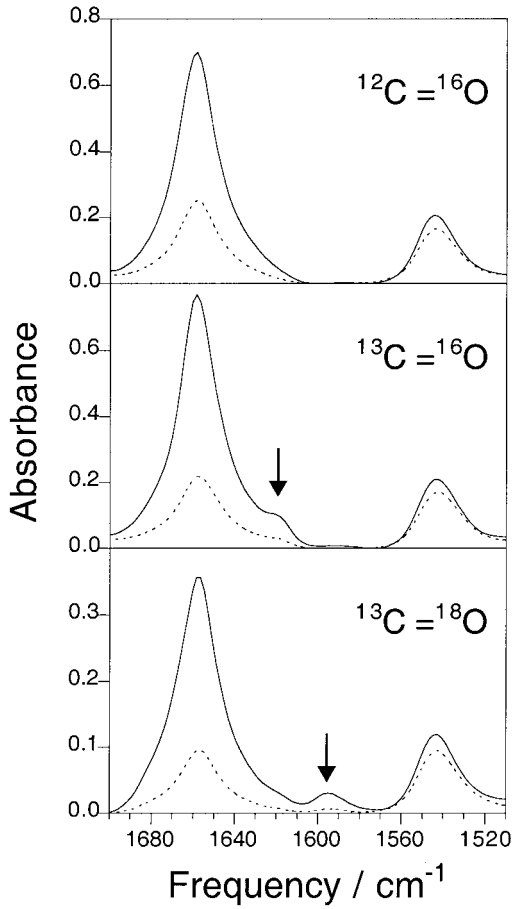

FIGURE 1 Polarized attenuated total reflectance (ATR)-FTIR spectra of the amide I and amide II regions obtained for three different peptides encompassing the transmembrane domain of *influenza* A virus M2 H^+ channel reconstituted in lipid bilayers. Spectra obtained with parallel and perpendicular polarised light are represented with solid and dashed lines, respectively. The top, middle, and bottom panels depict spectra from peptides which contain no isotopic label, a $^{13}C{=}{=}^{16}O$ at V29 and $^{13}C{=}{=}^{18}O$ at G34, respectively. The arrows indicate the position of the isotope edited peaks.

All three peptides exhibited highly symmetrical and narrow amide I bands centred at 1659 cm^{-1}, indicating a very high helical content.[4,5] This is consistent with previous CD,[13] FTIR,[8] and solid-state NMR measurements.[14,15] Furthermore, H/D exchange experiments have shown that the peptide is protected from exchange as expected from a transmembrane α-helix[5] (data not shown).

As shown in Table I, the dichroic ratios of the peptides indicate that the all peptides possess a net transmembrane orientation.[5] The differences in the

Table I FTIR Spectral Data for the Three M2 Peptides Studied Containing the Isotopic Labels as Indicated[a]

Label	$\nu(^{12}C{=}^{16}O)$	$\mathcal{R}(^{12}C{=}^{16}O)$	ν(Label)	$\mathcal{R}$(Label)	H-F [ν(Label)] $\nu(^{12}C{=}^{16}O)$]	HA [ν(Label)] $\nu(^{12}C{=}^{16}O)$]	Meas [ν(Label)] $\nu(^{12}C{=}^{16}O)$]
None	1659 cm^{-1}	3.31	N/A	N/A	1	1	1
1-^{13}C	1659 cm^{-1}	2.8 ± 0.6	1619 cm^{-1}	3.9 ± 0.7	0.975	0.978	0.976
1-$^{13}C{=}^{18}O$	1659 cm^{-1}	3.1 ± 0.3	1595 cm^{-1}	4.4 ± 1.3	0.960	0.953	0.961

[a] $\nu(^{12}C{=}^{16}O)$ is the frequency of the main amide I band and $\mathcal{R}(^{12}C{=}^{16}O)$ is its corresponding dichroic ratio. ν(Label) and $\mathcal{R}$(Label) are the frequency and dichroic ratio of the isotope shifted peak, respectively. The frequency ratios, $\frac{\nu(Label)}{\nu(^{12}C{=}^{16}O)}$ between the isotope shifted band and the main band, are obtained from *ab initio* Hartree-Fock calculations (H-F), harmonic approximation (HA) or those measured experimentally (Meas). The average values for the dichroic ratios represent the results of 1, 5, and 5 experiments for the unlabeled, ^{13}C-labeled and $^{13}C{=}^{18}O$ labeled peptides, respectively.

dichroic ratio reflect the variation of sample order in different experiments. Analysis of site directed dichroism data, which can yield explicit helix tilt angles deconvoluted from any sample disorder effect,[2] indicate that all three samples possess virtually identical tilt angles (see below).

The spectra show however, differences due to the different labels. The peptide which contains a $^{13}C{=}^{16}O$ label has an additional band present as a shoulder on the main amide I peak at 1619 cm^{-1}, as observed previously.[1,16] The peptide which contains a $^{13}C{=}^{18}O$ label has an additional peak as well but the size of the shift is even larger, -63 cm^{-1}, placing the new isotopic edited band at 1595 cm^{-1}. One can also see in the third peptide a very small but noticeable band at 1619 cm^{-1}, which is a result of the fact that the $^{13}C{=}^{16}O$ to $^{13}C{=}^{18}O$ exchange was not complete.

The frequency shift obtained for the $^{13}C{=}^{16}O$ peak is virtually identical to the calculated shift using the harmonic approximation. As the harmonic approximation does not seem to hold for the $^{13}C{=}^{18}O$ band, as shown in Table I, *ab initio* molecular orbital calculations were performed on a simple amide, MeCONHMe. These calculations have resulted in a frequency shift that correlate very well with that measured experimentally (Table I).

DISCUSSION

The aim of this study was to generate a new isotopic label for FTIR studies that can be used in larger proteins and does not require spectral deconvolution in order to resolve its contribution from that arising from the unlabeled part of the protein. As seen in Figure 1 (bottom panel), we have achieved this with the label combination of ^{13}C and ^{18}O in the carbonyl group. The $^{12}C{=}^{16}O$ amide I, peak centered at 1659 cm^{-1} is completely baseline resolved from the isotope edited peak. This is in contrast with the previously used[1,3] $^{13}C{=}^{16}O$ label (Figure 1 middle panel) that is present as a shoulder on the amide I band. We note that the $^{13}C{=}^{16}O$ is only visible in this instance due to the fact that the peptide is relatively small, hence the contribution of the label is still significant. For any sort of analysis, however, deconvolution or peak fitting procedures are needed, resulting in the possibility of incorrect interpretations. Like the $^{13}C{=}^{16}O$ label, the new label combination can provide two kinds of information: (a) site-specific secondary structure[1] and (b) site-specific orientational constraints.[2]

Secondary Structure

The correlation between the amide I vibrational frequency and secondary structure has been documented extensively. In general, bands centered around 1655 cm^{-1}, correspond to α-helices, while those centered at 1675 and 1630 correspond to β-turns and β-strands, respectively.[4,17] The frequency of the main amide I band provides information regarding the average secondary structure of the protein, and does not entail any information regarding specific residues. The use of isotope edited infrared spectroscopy can overcome this limitation by providing site-specific secondary structure assignment.[1]

The presence of the isotope changes the reduced mass of the vibrational group, and with it, its corresponding vibrational frequency. It is therefore possible to distinguish between the amide I mode of the isotopically labeled site and the amide I modes of the rest of the protein, due to the isotope edited shift. If the magnitude of the isotope shift is predictable, one can roughly estimate the frequency of the "original" mode (i.e., the frequency of the site if it were not

isotopically labeled*). This estimation can thus be used to see if the labeled residue, was in the β-turn, α-helical, or β-strand region.

Walters and co-workers,[1] have shown that the shift obtained for the $^{13}C{=}{=}^{16}O$ amide I mode is very close to that calculated assuming a simple harmonic oscillator model for the C=O stretching mode. Thus, the predictable shift of ca. -40 cm^{-1} enables determination of secondary structure using the $^{13}C{=}{=}^{16}O$ isotope combination†. In other words, all that is necessary to evaluate the secondary structure at a particular site is to determine the isotope-edited frequency at the site, and then add to it the predictable shift of ca. 40 cm^{-1}. For example, if the frequency of the isotope edited peak is 1620 cm^{-1}, then the "original" frequency‡ was 1660 cm^{-1}, which corresponds to an α-helix.[4] We note that Mendelsohn and co-workers have shown that the isotope edited shift of a residue in a β-strand may be somewhat smaller.[18] However, the amide I mode of an isotopically labeled residue in a β-strand still resonates at significantly lower frequencies ($\sim$ 1609 cm^{-1}) than an equivalent residue in an α-helix ($\sim$ 1620 cm^{-1}), enabling secondary structure assignment.[18]

The results obtained in this report for a peptide containing a 1-$^{13}C{=}{=}^{16}O$ label are entirely consistent with previous results[1] in that the isotope shift obtained is virtually the same as that calculated using a harmonic approximation (see Table I).

In the case of $^{13}C{=}{=}^{18}O$, however, the isotope edited shift of -64 cm^{-1} observed is smaller than that obtained when using a harmonic approximation (-78 cm^{-1}, see Table I). The frequency shift observed experimentally, however, is virtually identical to that obtained using a far more rigorous theory: restricted Hartree–Fock calculation. We note that the Hartree–Fock calculations predict the same frequency shift for the $^{13}C{=}{=}^{16}O$ as does the harmonic approximation. Since the shift of the $^{13}C{=}{=}^{18}O$ is predictable (similarly to the shift obtained with the $^{13}C{=}{=}^{16}O$ label), it can be used to correlate the secondary structure of the residue since one would know where nonisotopic ($^{12}C{=}{=}^{16}O$) band would be found (see above).

Since the shift measured form the $^{13}C{=}{=}^{18}O$ is roughly -64 cm^{-1}, overlap between the main amide I peak might occur if the site is in a β-turn region. Likewise on overlap with the amide II mode might occur of the site is in a β-strand region. In both of these instances, the lack of of peak in the helical shifted region would indicate one of the above possibilities and would necessitate difference spectroscopy measurements. However, the data stating that a particular amino acid is not in a helical conformation, is exceptionally valuable to membrane protein model building efforts as it delineates the transmembrane α-helices.

Orientation

The $^{13}C{=}{=}^{16}O$ isotope edited peaks have been previously used to obtain qualitative site specific orientational information.[3] More recently, site directed dichroism employing $^{13}C{=}{=}^{16}O$ isotope edited peaks has been used to obtain structures for a variety of transmembrane α-helical bundles such as *influenza* A virus M2,[8] HIV *vpu*,[19], *influenza* C virus CM2,[20] and human phospholamban.[7] The method relies on the fact that one is able to accurately measure the dichroism in two or more isotope edited peaks (each contained within a different sample). With this information in hand, it is then possible to numerically solve the coupled equations relating the measured dichroism to the helix tilt, rotational pitch angle and fractional sample order, β, ω, and f, respectively.[2]

The dichroic ratio obtained for the $^{13}C{=}{=}^{18}O$ isotope edited peak shown in Table I is significantly larger than that obtained for the main $^{12}C{=}{=}^{16}O$ natural abundance peak. This result is in accordance with previous measurements obtained for the *influenza* A virus M2,[8] and indicates that in general the $^{13}C{=}{=}^{18}O$ dichroic ratio can be used to obtain site-specific orientational information, which can later be used in site-directed dichroism analysis. The orientation properties calculated for the helix, using the new $^{13}C{=}{=}^{18}O$ probe are identical to those obtained previously using a $^{13}C{=}{=}^{18}O$ probe[8] or solid-state NMR data.[14,15] This clearly indicates that the location of the transition dipole moment of the isotope edited mode is indistinguishable from that of the semilabeled ($^{13}C{=}{=}^{16}O$) or unlabeled ($^{12}C{=}{=}^{16}O$) amide I modes. That the isotope edited amide I mode, in an α-helix, behaves as an isolated oscillator, has been demonstrated theoretically,[18] as well as by our experimental results.[2] Mendelsohn and co-workers however did note that in a β-strand the location of the transition dipole moment

* Obviously it is not possible to predict the exact frequency of the site if it were unlabeled due to the effect coupling effects between the modes. However, all that is needed in order to assign the secondary structure is a rough estimate since the frequency difference between α-helix and β-sheet amide I mode is on the order of 20–30 cm^{-1}.[4]

† This obviously assumes that it is possible to deconvolve the isotope edited peak successfully.

‡ Once again, it is not possible to predict the exact frequency of the site if it were unlabeled due to the effect coupling effect between the modes. However, all that is needed in order to assign the secondary structure is a rough estimate since the frequency difference between α-helix and β-sheet amide I mode is on the order of 20–30 cm^{-1}.[4]

of the isotope edited amide I mode may shift. We therefore qualify the applicability of the orientational analysis to α-helical regions.

CONCLUSION

In conclusion, we have shown that $^{13}C{=}{=}^{18}O$ presents a far more useful site-specific spectroscopic probe for proteins of virtually any size due to its vanishingly small natural abundance and completely resolved peak, as opposed to $^{13}C{=}{=}^{16}O$. Furthermore, even in small peptides, the $^{13}C{=}{=}^{18}O$ label is far more useful than the $^{13}C{=}{=}^{16}O$, since it does not require any curve fitting or deconvolution procedures to separate the isotope edited peak from the amide I band.

We have previously shown that site-directed dichroism, employing $^{13}C{=}{=}^{16}O$ labels, can yield spatial restraints to solve the structure of small α-helical bundles. The results of this study represent the first key step in applying spatial restraints to solve structures of polytopic membrane proteins, which are otherwise resistant to structural elucidation using conventional methods. We note that label incorporation into larger proteins is no longer a problem and can be achieved either via ligation of synthetic peptides[21] or through tRNA mediated protein engineering.[22] Currently, efforts in our laboratory are focused on this aim.

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