Isotope-Edited Raman Spectroscopy of Proteins: A General Strategy To Probe Individual Peptide Bonds with Application to Insulin

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Considerable insight could be gained into problems in protein structure, and misfolding if individual peptide bonds could be monitored in diverse environments. Here, a general strategy is proposed and illustrated based on isotope-edited Raman difference spectroscopy. Analogous to isotope-editing methods in NMR spectroscopy,[1] this strategy enables the vibrational frequencies of specific residues in a protein to be resolved. The method is applied to insulin as a model globular protein. Insulin offers the advantages of total synthetic access[2] and well-defined structural properties. Despite its small size, the structure of insulin contains representative elements of larger proteins (α-helix, β-sheet, turns, extended strands, and specific metal binding site). The present experiments focus on single crystals of T6 (2-Zn) hexamers.[3] The results demonstrate the principle of the method, verify proposed assignments of amide I Raman bands to secondary structures, and suggest that Raman difference data from the disordered region of a peptide chain are difficult to detect. In the future this method can be extended to investigate the structure of insulin in a fibril.

By means of Raman microscope,[4] spectra were recorded for unlabeled human insulin, and for three sets of crystals containing ^{13}C in different locations in insulin's B-chain. All crystals were in the T-state.[3] The labeled samples contained ^{13}C in the peptide bonds at positions B24 and B25 in the β-strand, or at B11 and B12 in an α-helix, or at B1 and B2 at the end of an extended peptide at the N-terminus lacking regular secondary structure.[5] These positions are identified for an insulin dimer shown in Figure 1. By subtracting the spectra of the labeled and unlabeled crystals, the amide I vibrations of the two labeled bonds could be identified for the B24-25 and B11-12 samples. This shows that precise assignments can be gained for peptide bonds in rigorously defined secondary structures inside a target protein.

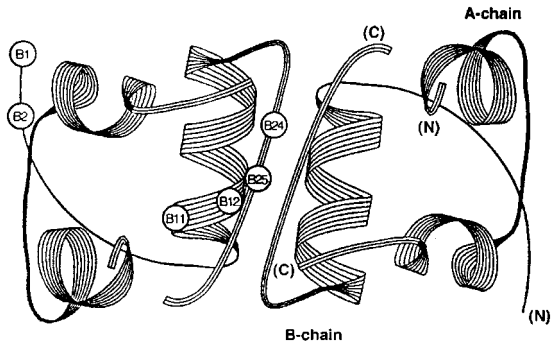

Figure 1. Cartoon of an insulin dimer within a Zn-insulin hexamer. The strands containing residues B24 and B25 form an intermolecular antiparallel β-sheet (based on Baker et al.[3]).

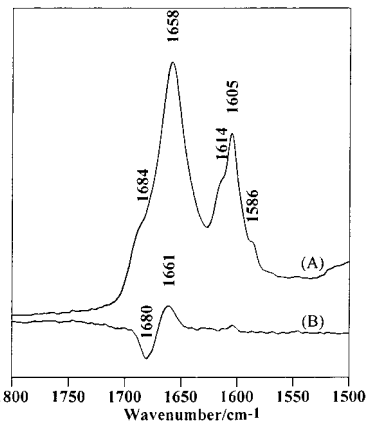

Figure 2. (A) Raman spectrum in the amide I region of B24-25 ^{13}C-labeled insulin: 647.1 nm laser excitation, 80 mW laser power, 5 min data collection. (B) Raman difference spectrum [^{13}C-labeled at B24-25] − [unlabeled]. Buffer spectrum subtracted from both crystal samples. Crystals were held in the mother liquor in a sitting drop and orientated with the rhombohedral faces perpendicular to the incident laser beam. The beam was not polarized.

In the 1970s Yu and co-workers undertook pioneering Raman spectroscopic studies of insulin crystals, normally by mounting them in air in front of a scanning Raman double monochromator.[6] Due to technical advances[4,7] it is now possible to record spectra with sensitivity improved several thousand-fold, resulting in data collection times of a few minutes. Two more crucial advantages stem from coupling an optical microscope to the Raman spectrograph. The Raman data can be collected from crystals under growth conditions in hanging or sitting drops[4] and the crystal and the impinging laser beam can be viewed through the microscope. The latter feature allows the orientation of the crystal with respect to the laser beam to be controlled. This is essential for accurate subtraction of the data from labeled and unlabeled crystals since the intensities of many Raman bands are highly dependent on the orientation of the crystal.

In Figure 2 are compared the Raman spectrum of B24-25 ^{13}C-labeled insulin in the 1500−1800 cm^{-1} region with the Raman difference spectra of ^{13}C minus ^{12}C for the corresponding samples. The Raman profile in the amide I region has a maximum at 1658

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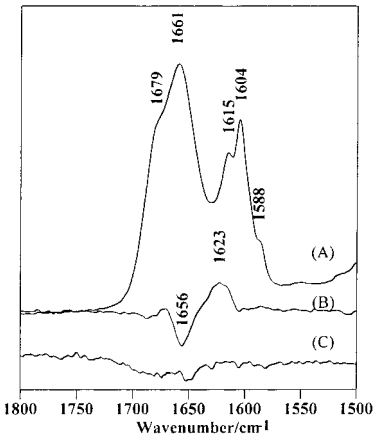

Figure 3. (A) Raman spectrum in the amide I region of B11-12 ^{13}C-labeled insulin. Conditions were the same as for Figure 2. (B) Raman difference spectrum [^{13}C-labeled at B11-12] − [unlabeled]. (C) Raman difference spectrum [^{13}C-labeled at B1-2] − [unlabeled].

cm^{-1} due to the preponderance of α-helical secondary structure in insulin whereas the shoulder near 1684 cm^{-1} has a large contribution from β-like structure. The bands at 1614, 1605, and 1586 cm^{-1} have major contributions from tyrosine and phenylalanine ring modes. In the difference spectrum of the B24-25 labeled protein the two ^{13}C labeled peptide bonds show a maximum at 1661 cm^{-1}, which is shifted 19 cm^{-1} from the corresponding feature at 1680 cm^{-1} in the unlabeled protein. A small feature at 1604 cm^{-1} is from the side-chain ring modes that have not subtracted exactly to zero. For a localized amide I mode, substituting ^{13}C amide for ^{12}C amide will cause a decrease in the amide I frequency by 35−40 cm^{-1}.[8] However, in the crystals the β-strands of two adjacent molecules make an antiparallel hydrogen bonding contact. This results in significant intermolecular vibrational coupling which, taken with intramolecular vibrational coupling, brings about a delocalization of the amide I vibration with a reduction in the isotope shift. The results in Figure 2 establish, for the first time, a quantitative spectra−structure correlation; two residues in a β-strand with respective Ramachandran angles of (−154°, 173°) and (−115°, 132°), hydrogen bonded to an adjacent β-strand, give rise to an amide I mode at 1680 cm^{-1}. The integrated intensity of either the "positive" or "negative" bands of the trace in Figure 2B is 4% that of the overall amide I profile in Figure 2A, consonant with the fact that 2 out of 55 amide bonds have been labeled. The intensity behavior seen in Figure 2 is in contrast to the findings in FTIR studies of β-form peptides where Brauner et al. noted an anomalous increase of the ^{13}C amide I band intensity compared to that of the ^{12}C isotopomer.[9] This difference is caused by the different origins of Raman and IR intensities and the different normal mode structures of the intense amide I bands in Raman and IR (the $\nu(0,0)$ mode in Raman versus $\nu_{\perp}(\pi,0)$ mode in infrared[10]). The present method will make it possible to extend our findings to catalog β-sheets, turns, etc. and to determine how sensitive the frequency is to changes in the Ramachandran angles and the hydrogen-bonding pattern.

In Figure 3A are shown the Raman spectrum for the B11-12 labeled crystal; the more prominent shoulder at 1679 cm^{-1}, compared to the 1684 cm^{-1} feature in Figure 2A, reflects the fact that ^{13}C substitution in the β-strand has downshifted significant "β-intensity" in Figure 2. The difference spectrum, Figure 3B, ^{13}C minus ^{12}C for the B11-12 sample exhibits a maximum and a minimum at 1623 and 1656 cm^{-1}, respectively. This confirms that the residues 11 and 12 in the B-chain α-helix, with respective Ramachandran angles of (−68°, −38°) and (−65°, −34°), give rise to an amide I feature at 1656 cm^{-1}. The shift upon ^{13}C substitution by 33 cm^{-1} is larger than that seen for the β-strand, demonstrating that the ^{13}C amide I vibration is more localized in the α-helix and that strong vibrational coupling to the neighboring ^{12}C (i+4) residue in the helix no longer occurs. The integrated intensity of either portion of the couplet was measured as 5% that of the complete amide I profile. The finding that the amide I modes for peptide bonds in the β-sheet and α-helix regions of conformational space occur at 1680 and 1656 cm^{-1}, respectively, is not surprising, since much effort based on model polypeptides and protein amide I profile deconvolutions has been expended in establishing amide I−secondary structure correlations and our findings are in the predicted ranges.[11] For example, a recent Raman study of a virus coat protein indicated that its helix structure shows a band position near 1611 cm^{-1} for the 37 ^{13}C═O groups labeled out of the 50-residue protein.[11c] Now, however, we have a direct and accurate means of establishing a library of Raman spectra−local structure correlations through limited labeling.

The third ^{13}C−^{12}C spectral subtraction, for substitution in B1-2, seen in Figure 3C, does not yield a clear plus/minus couplet in the amide I region. As for each of the three labeled samples, experiments were performed on 3−5 separate crystals and the results were reproducible. We believe the lack of a defined couplet for B1-2 is due to the ^{12}C and ^{13}C sample peaks being broad and as a result not being resolved above the spectral background. Possible reasons for the broadening are the presence of multiple conformations of the peptide chain near B1-2, or hydrogen-bonding interactions with water molecules, or both these factors.[12]

The present results establish that by labeling two individual peptide links with ^{13}C the precise amide I signature of those bonds, and hence the local secondary structures of those bonds, may be pinpointed selectively, provided that that part of the polypeptide chain is in an ordered environment. It is also necessary to recognize that the peptide amide I modes are not simple group frequencies and that interpretation may be complicated by the occurrence, for example, of inter-peptide vibrational coupling, whether it is interstrand or intrastrand. The method is also limited by the need to label individual bonds and to obtain very high quality Raman data. Recent experience in our laboratory with a variety of proteins has indicated that the highest quality data are usually obtained from single crystals larger than 30 μm in their minimum dimension. In contrast, aqueous solutions of the same protein are often of lower quality with interference from a broad spectral background due to protein aggregation being problematic. Solid samples, e.g. insulin fibrils, also yield low-background, high signal-to-noise Raman spectra. This suggests that the present approach can be used to study local regions of insulin fibrils or other fibril-forming proteins.

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(8) A single carbonyl stretching vibration is predicted to shift by ∼35 cm^{-1} for a ^{12}C═O to ^{13}C═O substitution when treated as a simple harmonic oscillator. More precisely, our density functional theory calculations using the Gaussian 98 program at a B3LYP/6-31G(d,p) level show that for doubly labeled trialanine both the in-phase and the anti-phase coupled amide I bands are downshifted by 41 cm^{-1}.

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