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Observation of the Glycines in Elastin Using ^{13}C and ^{15}N Solid-State NMR Spectroscopy and Isotopic Labeling

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We report the solid-state ^{13}C and ^{15}N NMR of insoluble elastin which has been synthesized in vitro with isotopically enriched glycine. Elastin is a fibrous protein found only in vertebrates and imparts elasticity to key connective tissue.[1] This work represents one of very few studies which have successfully labeled and characterized an insoluble biopolymer with a large monomer size. We show that many of the glycines reside in a domain with good cross-polarization (CP) efficiencies, although surprisingly, a portion resides in an environment that is not detectable using CP. Our data indicate that much of the ^{13}C population resides in regions of significant conformational flexibility. To support these conclusions, we present ^{13}C and ^{15}N cross-polarization with magic-angle-spinning (CPMAS) data in conjunction with "direct-polarization", nonspinning CP, and T_1 measurements.

Elastin's structure and organization is controversial, as high-resolution methods such as solution NMR and X-ray crystallography are not applicable. However, current consensus rests with a description of elastin having two prominent "domains", namely hydrophobic and cross-linking. Glycine is the most abundant amino acid in this protein, comprising approximately 38% of the total amino acids in rat elastin.[2] Most of the glycines tend to reside in the largely hydrophobic regions of elastin. A smaller population, however, reside in or near the alanine-rich cross-linking regions.

Our laboratory and others have previously reported NMR studies of insoluble elastin and elastin peptides;[3−9] ^{13}C and ^{2}D NMR yielded information on the overall structure of insoluble elastin but provided limited insight into the role of specific residues.[3−5,8,9] Torchia and co-workers focused on an enriched ^{13}C-labeled elastin sample, but this work utilized only static experiments.[6] Studies of smaller elastin peptides were often conducted using organic solvents, limiting their physiological relevance.[10−12] We have also reported results of variable-temperature ^{13}C CPMAS NMR studies on hydrated insoluble elastin;[9] summarily, hydrated insoluble elastin is heterogeneous and is characterized by an unusual degree of mobility.

The initial hurdle to solid-state NMR studies on labeled proteins, particularly those of high molecular weight, is the lack of a suitable expression system. However, the production of labeled elastin is feasible with the use of a mammalian cell type used in cardiovascular research. Specifically, primary cultures of neonatal rat smooth muscle cells (NRSMC) provide abundant quantities of insoluble elastin and elastic fibers.[13,14] Smooth muscle cells are isolated from the aortae of newborn Sprague−Dawley rats and are grown in a mixture which includes fetal bovine serum and other standard growth medium components, including Dulbecco's modified Eagle's medium (DMEM) and nonessential amino acids. After about one week, the cells reach confluency and initiate abundant elastin synthesis.

The primary source of glycine is contained in the DMEM. Therefore, DMEM is purchased "without Gly", and the isotopically enriched glycine (1-^{13}C-Gly, 99%; 2-^{13}C-Gly, 99%; or ^{15}N-Gly, 98%) is supplemented into the medium at the appropriate concentration (30 mg Gly/L media) via a prepared nonessential amino acid mixture. The label is introduced immediately after seeding of the cells to ensure maximal incorporation of the label. After 6−7 weeks of growth, the mixture of cells, elastin, growth medium, and other components of the matrix is harvested. Insoluble elastin is purified using the cyanogen bromide method, as described previously.[9,15]

The level of incorporation was determined using acid hydrolysis and solution ^{13}C NMR. The purified elastin (15−30 mg) was heated at 110 °C for 24 h in 6 M HCl. The solvent was removed under a stream of nitrogen, and the hydrolysis products were dissolved in 0.5 mL of D_2O. The relative heights of $^{13}C_\alpha$-Gly peaks were used to determine the incorporation of 1-^{13}C-Gly; that is, the unlabeled glycines are represented by the singlet at 40.9 ppm, whereas a doublet ($^1J_{CC}$=58.9 Hz) results from the $^{13}C_{C=O}-^{13}C_\alpha$ spin pair in the labeled residues. A similar approach was used for 2-^{13}C-Gly and ^{15}N-Gly, and the level of incorporation in each of the three samples was ca. 40%. Also, isotopic enrichment was not found in other amino acids. A similar method for measuring incorporation of enriched alanine and glycine into spider silk was reported by Meier and co-workers.[16]

Data were acquired on a Varian Unity Inova WB 400 spectrometer, equipped with a 4-mm double-resonance MAS probe (Chemagnetics, Fort Collins, CO). Rotors were sealed from moisture with a top spacer machined from Kel-F and fitted with fluorosilicone micro O-rings (Apple, Lancaster, NY).[17] Fluorinated polymers were used for reduced ^{13}C background in CPMAS experiments. Typical size of the fully hydrated elastin sample is 60−70 mg, which is ca. 60% water by weight. Data were acquired at 37 °C. ^{13}C chemical shifts are referenced to the TMS scale, using HMB as an external standard. ^{15}N chemical shifts are externally referenced to ^{15}N-glycine (32 ppm).

For CP, a 5.0 μs ^1H 90° pulse was followed by a 1−2 ms contact time with a 5 s recycle delay. For direct polarization, a 5.0 μs ^{13}C 90° pulse was used with a 20 s recycle delay. Typical applied field strengths for high-power decoupling during acquisition were $\gamma B_1/2\pi = 70-80$ kHz. The spinning speed used in MAS experiments was 8 kHz. ^{13}C T_1 values were obtained by the method of Torchia.[18]

Figure 1A shows the ^{13}C CPMAS spectrum of 1-^{13}C-Gly elastin. The major feature has a chemical shift of 171.9 ppm and a width (fwhm) of ca. 200 Hz. Interestingly, the line width observed is relatively narrow for such a large biopolymer. The ^{13}C T_1 of this population is 5.1 (±0.5) s. Previously we reported similar T_1's for the natural-abundance $^{13}C_{C=O}$ population in hydrated elastin.[9]

Direct-polarization magic-angle-spinning (DPMAS) experiments reveal an additional spectral feature; Figure 1B shows the difference spectrum obtained by subtracting the DPMAS spectrum of the

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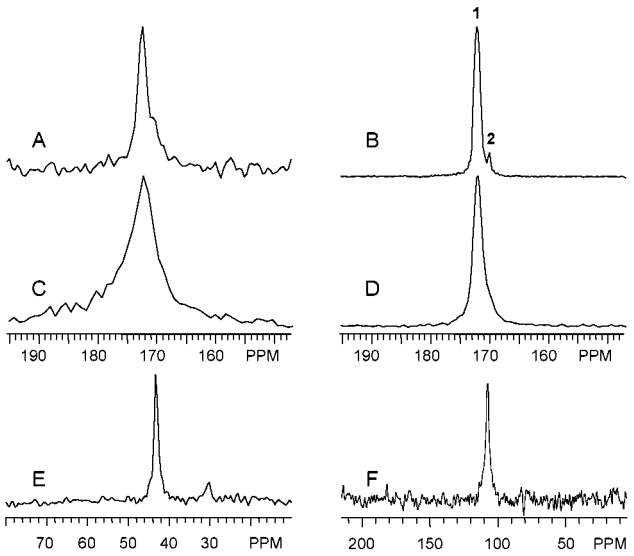

Figure 1. Solid-state ^{13}C and ^{15}N NMR spectra of hydrated NRSMC elastin, with incorporation of 1-^{13}C-Gly, 2-^{13}C-Gly, or ^{15}N-Gly. (a) ^{13}C CPMAS of 1-^{13}C-Gly elastin, 4000 scans; (b)^{13}C DPMAS difference spectrum of 1-^{13}C-Gly (1000 scans) and unlabeled elastin; (c) static ^{13}C CP of 1-^{13}C-Gly elastin, 19560 scans; (d) static ^{13}C DP difference spectrum for 1-^{13}C-Gly elastin, 1000 scans; (e) ^{13}C CPMAS of 2-^{13}C-Gly elastin, 1000 scans; (f) ^{15}N CPMAS of ^{15}N-Gly elastin, 2 ms CP, 1500 scans.

1-^{13}C-Gly elastin with that of the unlabeled NRSMC elastin. Most of the carbonyl peak intensity (~90%) lies in the peak **1** at 172.0 ppm, which has a line width of ~120 Hz and is not purely Lorentzian. In addition, this difference spectrum clearly shows a smaller peak **2** at 170.0 ppm, which has a width of ~70 Hz. The ratio of populations seen in the DPMAS analysis corresponds to a distribution of glycines seen in the amino acid sequence of rat tropoelastin;[2] that is, relative peak intensities of 90:10 correspond roughly with the number of glycines in hydrophobic and cross-linking regions, respectively. Finally, it is noted that the signal-to-noise ratio is actually much greater than that observed for the CPMAS in 1A; this result is surprising, as the reverse is expected for most typical solids. These results are consistent with previous solid-state NMR studies of elastin,[9] in which portions of the backbone were not observable with CP.

Narrow, Lorentzian line widths observed in biopolymers is often indicative of structural homogeneity.[19] However, our data indicate that motional narrowing may be a more likely scenario. Figure 1C is a ^{13}C CP spectrum of a nonspinning sample. Although this peak is broader than those seen in the MAS spectra of Figures 1A and 1B, it has line width (fwhm) of <5 ppm, which is atypical for a carbonyl in a rigid environment. The difference spectrum of a static ^{13}C DP experiment is shown in Figure 1D, and again, the narrow line width (<2 ppm) provides further support for the dynamic nature of the Gly-rich areas. As with the other experiments, a comparable result was seen in our previous studies,[9] whereby an unusually narrow component was observed in this chemical shift region of the nonspinning spectrum of hydrated elastin.

The ^{13}C CPMAS spectrum of the 2-^{13}C-Gly elastin is shown in Figure 1E. The major feature has a chemical shift of 43.2 ppm (fwhm $\approx$ 120 Hz), with a ^{13}C T_1 of 0.32 ($\pm$0.01) s. DPMAS data show no significant differences. The line shape, whether obtained by CPMAS or DPMAS, is Lorentzian.

Finally, Figure 1F shows the ^{15}N CPMAS of the ^{15}N-Gly elastin. A feature centered at 107.5 ppm is observed. This line shape has a width of 140 Hz. Contact times of 0.5 to 3.0 ms yield spectra with different intensities, but no additional, distinguishable features are observed.

As a final note, isotropic chemical shifts have often been used to determine secondary structure. Some approaches to structural analysis of proteins and peptides typically cite the ca. 4 ppm difference between α-helical and β-sheetlike residues in the solid-state.[20] There are limitations to this approach, particularly with regard to glycine. First, Gly-rich biopolymers tend not to form α-helices in nature; solid-state ^{13}C CPMAS NMR has been used to correlate chemical shift information to the β-sheet and triple helical structures in *Samia cynthia ricini* silk and collagens, respectively.[21] Furthermore, ambiguity exists, because the resolved sites of glycine in several secondary structures, such as the 3_1-helix, α-helix (in nonnatural glycine derivatives), and the β-turn, have nearly identical ^{13}C and ^{15}N chemical shifts.[20,22,23]

In conclusion this study demonstrates the suitability of the NRSMC system for producing abundant quantities of a nativelike elastin for NMR characterization. This isotopic labeling strategy is appropriate for observation of the glycines, in addition to the other major amino acids of elastin. The NMR data indicate that the glycines are found in at least two distinguishable environments and have high relative mobility. A more precise definition of the role of glycines in hydrated, insoluble elastin requires more extensive study by solid-state NMR and is the theme of ongoing work.

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