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Collagen Fibers as a Chiral Agent: A Demonstration of Stereochemistry Effects

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Collagen is the most abundant protein in vertebrates and represents the most important structural protein in extracellular matrices and connective tissues. Its basic structure consists of left-handed, helical chains supercoiled into a right-handed triple helix.[1,2] In tissues, collagen fibers cause an anisotropy in the motion of water and other small molecules. Their dipolar and quadrupolar interactions thus do not average to zero, leading to peak splittings in NMR spectra. Such collagen-related residual interactions have been found in a variety of tissues, including tendons,[3−6] cartilage,[7−9] blood vessel walls,[10] and nerves.[11] These interactions have also been observed in other tissues containing ordered structures, such as erythrocytes[12] and muscles.[13−17] The origin of residual interactions in ordered tissues is a source of some debate. In the case of water binding to collagen,[3,4] anisotropic motion in the binding site has been suggested as an explanation for this behavior. Residual interactions in muscle, on the other hand, have been suggested to result from the confinement of small molecules by the protein fibers.[17] We have recently found that the dipolar and quadrupolar splittings due to interaction with collagen fibers in the Achilles tendon are different for the L and D enantiomers of alanine.[18] A similar result for alanine was obtained by Kobzar et al. in stretched gelatin gels.[19] These results clearly indicate that the source of these dipolar and quadrupolar splittings is a specific interaction with the collagen fibers. This could result either from differences in the number of bound molecules or from differences in the geometry of the binding of the two enantiomers. The results of the present work provide conclusive evidence that the latter mechanism predominates.

As can be seen from the single-pulse ^{2}H spectra of L- and D-alanine-d_3 in bovine Achilles tendon (Figure 1), the quadrupolar splitting of the methyl group for L enantiomer is significantly larger than that for the D enantiomer. The ^{2}H methine spectrum of the L enantiomer was measured using alanine-d_4. For this compound the methyl and methine spectra overlap, making an accurate determination of the methine quadrupolar splitting more difficult. An in-phase, double quantum filtered (IP-DQF) NMR,[5] with short creation time, was therefore used to separate the two functional groups (Figure 2). Results for the ^{1}H−^{1}H and ^{13}C−^{1}H dipolar couplings were obtained using the ^{1}H and ^{13}C single-pulse spectra, respectively. For ^{1}H spectra, however, the strong, broad water peak had to be suppressed. This was accomplished by a 1.5 s long, 10 kHz off-resonance, continuous wave (CW) presaturation. While this method also partially suppressed the alanine peaks due to the nuclear Overhauser effect (NOE; see later), the water peak was completely suppressed. Splitting of the ^{13}C peaks results from both dipolar and *J* couplings between ^{13}C and ^{1}H. The dipolar coupling was obtained by subtracting the *J* coupling value of 129 and 145 Hz for the CH_3 and CH groups, respectively, as was observed in an aqueous alanine solution with pH = 7.4. By taking into account the dependence of the dipolar interaction on the orientation of collagen fibers with respect to the external magnetic field, and the positive sign of the *J* coupling, the sign of the dipolar interaction was determined by repeating the measurement after the rotation of the sample by 90°. The results are summarized in Table 1. As can be seen from the table, the quadrupolar splitting of methyl deuterons in L-alanine-d_3 is twice as large as that in D-alanine-d_3. The opposite effect is observed for the methine deuteron, where the L-enantiomer splitting is 33% smaller than that of the D-enantiomer. This result clearly indicates that the number of bound molecules does not play a major role in the differences in the splittings; if this were the case, then the L/D splitting ratios for the methine and methyl residues would be the same. Thus we can conclude that the differences between

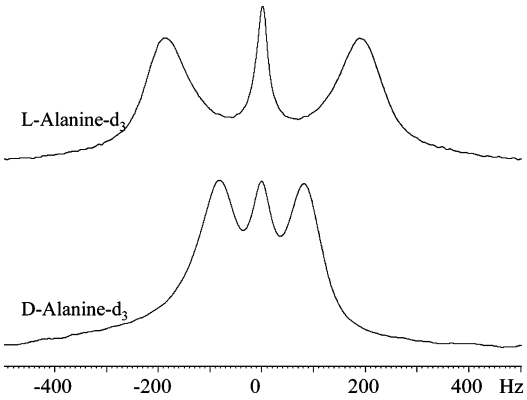

Figure 1. Single pulse ^{2}H NMR spectra of L and D-alanine-d_3 in bovine Achilles tendon after it was soaked in 300 mM solutions. The same method of sample preparation was used to obtain all the spectra in the current study.

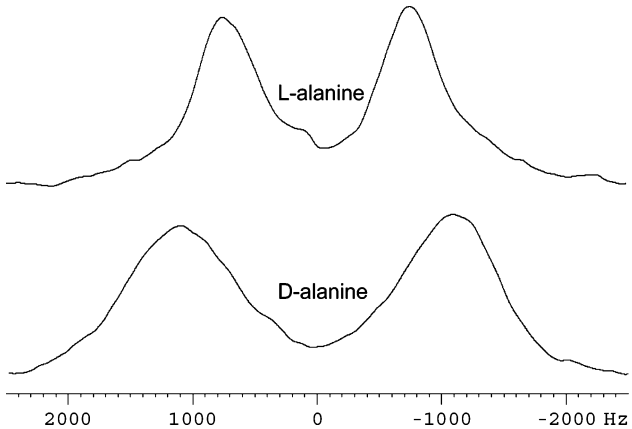

Figure 2. Methine group ^{2}H spectra of L and D alanine-d_4, obtained using an IP-DQF pulse sequence with creation time $\tau/2 = 150$ μs. To improve the suppression of the methyl group, the rf was set to the resonance frequency of the methine group and the period of the DQ evolution, t_{DQ}, was selected to be 760 μs. This gives $2\Delta t_{DQ} = 640°$, where Δ, the difference between the methine and methyl group resonance frequencies, is equal to 1150 Hz in a magnetic field of 11.75 T. The value of 640° is nearly an integral multiple of 90°, ensuring the desired suppression of the methyl group peak.

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Table 1. Contribution of the Dipolar and Quadrupolar Interactions to the Observed Splitting in the Spectra of L- and D-Alanine in the Bovine Achilles Tendon[a]

isomer	group[b]	^{2}H quadrupolar splitting (Hz)	group[b]	^{1}H–^{1}H dipolar splitting (Hz)	NOE	group[b]	contribution of the ^{13}C-^{1}H dipolar interaction to the splitting (Hz)
L	C**D**$_3$	390 ± 50	C**H**$_3$	285 ± 30	0.55	^{13}**C**−^{1}H$_3$	−45 ± 7
D		180 ± 25		150 ± 20	0.66		−25 ± 7
L	C**D**	1800 ± 200	C**H**$_3$−C**H**	90 ± 10	0.64	^{13}**C**−^{1}H	+220 ± 25
D		2700 ± 350		50 ± 7	0.7		+420 ± 40

[a] For all reported values, the tendon is parallel to the magnetic field. [b] The observed nucleus is underlined. [c] The contributions of the dipolar interactions to the splitting were obtained after subtraction of the *J* coupling as measured in liquid phase, and their signs were determined from the splitting obtained after rotating the samples by 90°.

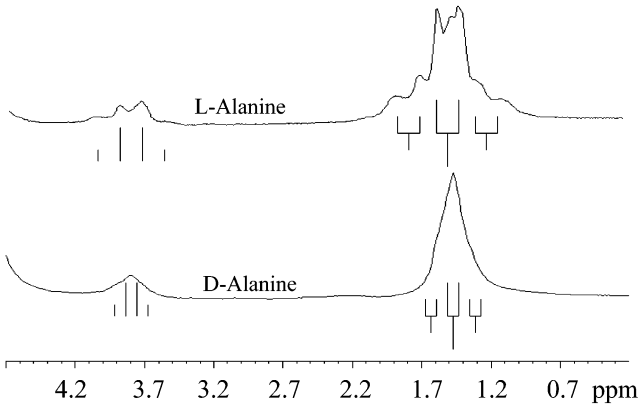

Figure 3. ^{1}H spectra of L- and D-alanine, obtained using a single 90° pulse. The tissue symmetry axis was parallel to the magnetic field and the temperature was 25 °C. The total number of accumulations is 64.

the two enantiomers stem from stereochemical effects, that is, from differences in the average angle between the molecular axes and the director determined by the collagen fibers.

If indeed stereochemistry plays an important role, then one would expect a similar trend in the ^{2}H quadrupolar interactions of the methine deuteron and the dipolar interaction of methine ^{13}C−^{1}H, since both are directed roughly along the C−H axis. Indeed, in Table 1 we see similar trends for their L/D splitting ratios which are 0.67 and 0.52 respectively. For the same reason the L/D ratios of the splitting in the deuteron spectra of the CD_3 group and the ratio of the splitting in ^{13}C spectra due to the ^{13}C−^{1}H interaction are similar (2.2 and 1.8, respectively). Again, the large spread (0.52−2.2) of the L/D ratios for the different splittings (see Table 1) clearly indicates that the main difference in the interaction of the L and D alanine enantiomers with the collagen fibers stems from differences in the stereochemistry of the binding and not in the fractions of bound molecules.

The ^{1}H−^{1}H and ^{13}C−^{1}H dipolar splittings were found to be independent of alanine concentration over a range of 0.1−0.6 M. The binding of alanine to collagen must therefore be very weak, with a dissociation constant $K \gg 0.6$ M. Still, some information regarding the nature of the binding can be obtained from a collagen−alanine NOE measurement. This measurement was performed by CW irradiation at an offset of 10 kHz from the alanine methine group resonance. The frequency of the CW irradiation was selected on the basis of our earlier DQF NMR studies, indicating that the spectral width of collagen in the Achilles tendon is about 24 kHz.[6] A significant reduction of the alanine proton signal was obtained, with a clear difference between the methine and methyl protons (Table 1). The negative sign of the NOE indicates that the modulating motion is slow ($\omega_o\tau_c > 1$), consistent with the conclusion that alanine is binding to the collagen. A significant NOE is obtained despite the weak binding and is due to transfer of NOE[20,21] between the small number of alanine molecules in close contact with the collagen fibers and the alanine in bulk solution. Since the NOE variation between the two enantiomers is small, we may conclude that there is very little difference in the number of bound L- and D-alanine molecules. In conclusion, while the number of alanine molecules bound to collagen in its natural state in intact tissue is similar for the two enantiomers, the stereochemistry of their binding is different.

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