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Molecular Dynamics of Collagen Side Chains in Hard and Soft Tissues. A Multinuclear Magnetic Resonance Study[†]

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ABSTRACT: We have prepared samples of (a) intact calvaria collagen (cross-linked and mineralized), (b) intact tendon collagen (cross-linked but not mineralized), and (c) reconstituted chick calvaria collagen (not cross-linked and not mineralized) containing [*methyl*-$^{2}\mathrm{H}_{3}$]methionyl, [4,4-$^{2}\mathrm{H}_{2}$]pyrrolidinyl, (4-fluorophenyl)alanyl, and [6-$^{15}\mathrm{N}$]lysyl residues. Using multinuclear magnetic resonance spectroscopy, we have investigated the molecular dynamics of the labeled amino acids. Guided by model compound studies, we reached the following conclusions regarding collagen side chain dynamics from our analysis of line shapes and relaxation rates. At 22 °C, imino residues in all samples have flexible rings with root mean square angular fluctuations in the 11–30° range. Nearly all labeled amino acid side chains reorient about at least two side chain single bonds. At temperatures below −30 °C, most of these side chain motions are absent in all the samples. Surprisingly, in contrast with results obtained for backbone motions, side chain motions are only marginally more hindered in mineralized samples as compared with nonmineralized samples, a result we discuss with reference to collagen–mineral interactions. We also discuss the possible relationship between collagen dynamics and function.

Recently, multinuclear solid-state nuclear magnetic resonance (NMR)[1] spectroscopy was used to compare the molecular dynamics of the collagen backbone in three types of collagen fibers: (a) in reconstituted fibers of chick calvaria collagen that are not cross-linked and not mineralized; (b) in intact rat tail tendon collagen fibers that are cross-linked but not mineralized; (c) in intact rat calvaria collagen fibers that are cross-linked and mineralized. These studies showed that the rms amplitude of the anisotropic backbone motion that occurs in reconstituted collagen fibers is slightly reduced by cross-links, whereas the amplitude of this motion is significantly reduced by mineral (Jelinski & Torchia, 1979; Jelinski et al., 1980; Sarkar et al., 1983, 1985; Torchia et al., 1985). In the present study we investigate the effects of cross-links and mineral upon the dynamics of several types of amino acid side chains in collagen. Although we had previously shown that a variety of side chains were flexible in reconstituted fibers (Jelinski et al., 1980; Batchelder et al., 1982; Torchia, 1984), the effect of cross-links and mineral upon side chain motions was not examined. Side chain dynamics reflect interactions that are of particular interest in the case of collagen because the side chains are at the surface of the triple helical molecule (Bornstein & Traub, 1979). Hence, it has been suggested that interactions among side chains on adjacent molecules have a major role in directing collagen fiber assembly (Bornstein & Traub, 1979; Chapman, 1984) and that interactions between collagen side chains and mineral strongly influence the structure and mechanical characteristics of bone (Glimcher, 1976; Bonar et al., 1985).

Modern solid-state NMR spectroscopy is an established technique for investigating molecular dynamics (Spiess, 1978; Mehring, 1983). The technique is applicable to biological macromolecules, provided that isotopic labeling is employed (Griffin, 1981; Opella, 1982; Keniry et al., 1984; Torchia, 1984). Herein, we report observations of $^{2}\mathrm{H}$, $^{15}\mathrm{N}$, and $^{19}\mathrm{F}$ signals of labeled Met, Pro, Lys, and Phe side chains of collagen. Because of the limited signal-to-noise ratio of the protein spectra, our analysis of NMR line shapes and relaxation times has been guided by results obtained with model compounds. Using this approach, we obtain estimates of the time scales and amplitudes of the side chain motions in collagen. This information is then discussed with reference to collagen function.

MATERIALS AND METHODS

L-[*methyl*-$^{2}\mathrm{H}_{3}$]Methionine (98 atom % $^{2}\mathrm{H}$) was purchased from Merck Isotopes; DL-[4,4-$^{2}\mathrm{H}_{2}$]proline (97 atom % $^{2}\mathrm{H}$) and DL-[3,3,4,4,5,5-$^{2}\mathrm{H}_{6}$]proline (97 atom % $^{2}\mathrm{H}$) were synthesized by the procedure of Young and Torchia (1983). DL-[6-$^{15}\mathrm{N}$]Lysine (95 atom % $^{15}\mathrm{N}$) was synthesized by the general procedure of Fields et al. (1951), starting with $^{15}\mathrm{N}$-enriched 4-(bromobutyl)phthalimide, which was synthesized by the method of Drake and Carman (1949). DL-(4-Fluorophenyl)alanine was purchased from Aldrich or Calbiochem. Each amino acid was characterized by elemental analysis and by high-resolution NMR spectroscopy before use.

Reconstituted collagen fibers were labeled with L-[*methyl*-$^{2}\mathrm{H}_{3}$]methionine, L-[4,4-$^{2}\mathrm{H}_{2}$]proline, and L-[6-$^{15}\mathrm{N}$]lysine by means of chick calvaria tissue culture (Jelinski & Torchia,

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[1] Abbreviations: NMR, nuclear magnetic resonance; rms, root mean square; ppm, parts per million; ν_i (i = X, Y, Z), principal frequencies of the $^{2}\mathrm{H}$ electric field gradient tensor in the convention $|\nu_Z| \geq |\nu_X| \geq |\nu_Y|$; σ_i (i = 1–3), principal elements of the chemical shift tensor in the convention $\sigma_3 \geq \sigma_2 \geq \sigma_1$; T_1, spin–lattice relaxation time; T_2, transverse relaxation time.

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1979). The collagen fibers, in equilibrium with 0.02 M Na_2HPO_4, were packed (unoriented) into 5-mm NMR tubes. Intact tissues of tendon and bone, containing labeled collagen fibers, were prepared by injecting rats with solutions of these three labeled amino acids as described by Sarkar et al. (1985). L-(4-Fluorophenyl)alanine was incorporated into rabbit Achilles tendon collagen fibers by the following procedure. New Zealand white rabbits were obtained from local rabbitries at approximately 2 weeks of age. They were maintained on Purina Checkers rabbit chow containing 0.3% of DL-(4-fluorophenyl)alanine by the pellet-coating procedure described previously (Gerig et al., 1983). Blood was withdrawn at approximately 2-week intervals to provide material for other studies. After 6 months, the animals had reached a body mass of approximately 2.5 kg and were then sacrificed with sodium phenobarbital. All tissues were washed with 0.15 M NaCl, defatted, and equilibrated against 0.15 M NaCl before packing the samples into NMR tubes. All ^2H-labeled samples were exchanged several times with solutions prepared with deuterium-depleted water purchased from Aldrich. Several milligrams of each protein sample was hydrolyzed in 6 N HCl (Pierce) for 24 h at 112 °C, and high-resolution NMR spectra were obtained to check for scrambling of the label. In each case, the spectrum of the hydrolyzed protein showed that scrambling was undetectable. The ^2H NMR spectrum of each sample of hydrolyzed collagen labeled with L-[4,4-$^2H_2$]proline showed the signal expected for the γ-deuteron of the 4-hydroxypropyl residue (as a consequence of a posttranslational modification, ca. 45% of the pyrrolidine residues in collagen are converted from prolyl to 4-hydroxyprolyl by prolyl hydroxylase). Previous work (Fujita et al., 1964) had shown that tritium at this position did not exchange with solvent upon hydroxylation.

The levels of ^2H and ^{15}N enrichment in the labeled collagen samples were determined either by gas chromatographic/mass spectroscopic or by ^2H NMR analysis of the protein hydrolysates. In the chick calvaria collagen samples, the levels of L-[*methyl*-$^2H_3$]methionine, L-[4,4-$^2H_2$]proline, and L-[6-^{15}N]lysine enrichment were 45%, 35%, and 65%, respectively. In the intact rat tissues, the levels of enrichment were 35%, 20%, and 12% for L-[*methyl*-$^2H_3$]methionine, L-[4,4-$^2H_2$]-proline, and L-[6-^{15}N]lysine, respectively. The levels of enrichment of proline and hydroxyproline were found to be equal. The level of enrichment of L-(4-fluorophenyl)alanine was determined to be 2% by comparing the ^{19}F NMR signal intensity of a known weight of a hydrolyzed collagen sample with the ^{19}F signal intensity of a standard solution containing a known weight of the amino acid.

^{15}N NMR spectra were obtained at 25.38 MHz on a home-built spectrometer (Sarkar et al., 1983). The nitrogen and proton radio frequency field strengths ($\gamma B_1/2\pi$) were 40–50 kHz. Free induction decay signals were detected in quadrature using 2048 points per channel. The accumulated signals were digitally filtered (50–100-Hz line broadening) to improve sensitivity, and the spectral window was 40 kHz. Sample temperature was regulated as described previously (Sarkar et al., 1983).

Solid-state ^2H NMR spectra were obtained at 38.45 MHz on a quadrupole echo pulse sequence (Davis et al., 1976), $(\pi/2)_{\pm x}$–t–$(\pi/2)_y$–t–. The duration of the $\pi/2$ pulse was 2–2.5 μs, and t was 30 μs. Free induction decay signals were accumulated in quadrature with a 2-MHz sampling rate and 2048 points per channel. Prior to Fourier transformation, the data were left shifted, so that the top of the echo was the first point in each channel, and a 2-kHz digital filter was applied to improve the signal-to-noise ratio.

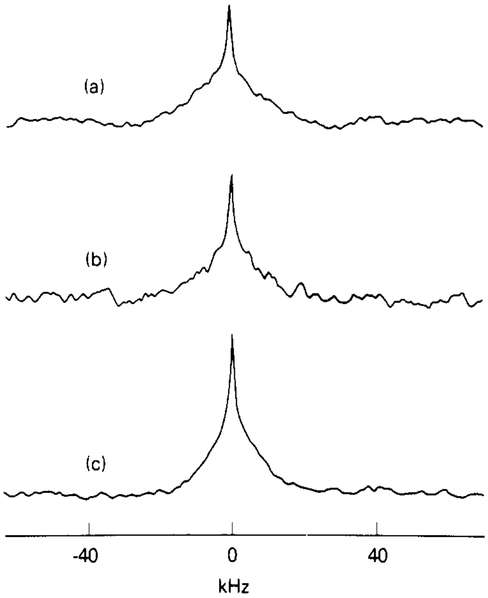

FIGURE 1: 38.45-MHz ^2H quadrupole echo NMR spectra of L-[*methyl*-$^2H_3$]methionine-labeled collagen fibers: (a) rat calvaria, 250 000 acquisitions; (b) rat tail tendon, 148 000 acquisitions; (c) reconstituted from chick calvaria collagen, 161 000 acquisitions. Each spectrum was obtained at 22 °C, with a 90° pulse of 3.2 μs, an echo delay of 30 μs, a recycle delay of 0.6 s, and digital line broadening of 1 kHz.

^{19}F NMR spectra were obtained at 470.5 MHz on a modified NIC-500 spectrometer, equipped with a home-built probe (Hiyama et al., 1986). The $\pi/2$ pulse width was 8 μs, and the EXORCYCLE pulse sequence was employed to minimize distortion of the chemical shift powder line shape resulting from finite pulse power (Rance & Byrd, 1983). Free induction decay signals were acquired in quadrature with a 1-MHz sampling rate and 2048 points per channel. A 0.5–1-kHz digital filter was applied to improve the signal-to-noise ratio.

Theoretical spectra were calculated on a DEC-10 computer and were corrected for effects of finite pulse power (Bloom et al., 1980; Rance & Byrd, 1983; Hiyama et al., 1986). No correction was made for irreversible signal loss during formation of the quadrupole echo (Spiess & Sillescu, 1981), because we did not observe evidence of short anisotropic T_2 relaxation in any of the spectra. The narrow isotropic peak observed in the room temperature ^2H spectra was not included in the calculated spectra, because this peak is primarily due to residual deuterium in the deuterium-depleted water used to hydrate the samples. As expected, this signal is absent at temperatures below 0 °C, because when water freezes, the deuterium signal broadens by several orders of magnitude and is below the level of the noise. When more than one line width component was used to simulate a spectrum (e.g., Table II or III), the minimum number of components needed to fit the spectrum was employed. The uncertainties in the principal frequencies quoted in each table indicate the listed principal frequencies could be varied without significantly affecting the agreement between the calculated and observed spectrum.

RESULTS

The 38.45-MHz spectra of collagens labeled with L-[*methyl*-$^2H_3$]methionine (Figure 1a–c) exhibit powder patterns that differ substantially, in both shape and width, from the Pake pattern (Spiess, 1978) (Figure 2f) observed in the crystalline amino acid at low temperature, where the only significant molecular motion is rotation about the S–C^ϵ bond axis. Neglecting the narrow signals in the center of Figure

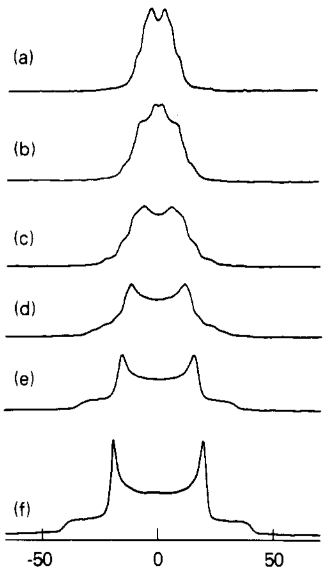

FIGURE 2: 38.45-MHz ^{2}H NMR spectra of polycrystalline L-[*methyl*-$^2H_3$]methionine obtained as a function of temperature: (a) 80 °C; (b) 45 °C; (c) 35 °C; (d) 27 °C; (e) 15 °C; (f) −67 °C.

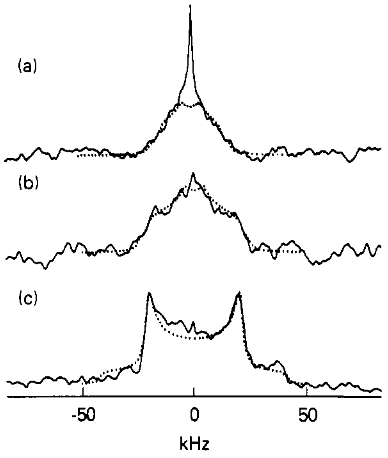

FIGURE 3: Comparison of experimental (—) and calculated (···) 38.45-MHz ^{2}H NMR spectra of L-[*methyl*-$^2H_3$]methionine-labeled rat calvaria collagen at three temperatures: (a) 22 °C, 250 000 acquisitions; (b) −35 °C, 60 000 acquisitions; (c) −135 °C, 120 000 acquisitions. Each spectrum was obtained with a 90° pulse of ca. 3.5 μs, an echo delay of 30 μs, and digital line broadening of 1–2 kHz. The recycle delay times were 0.6, 1.0, and 0.1 s, respectively, in spectra a–c.

1a–c (due, in part, to residual HDO), the collagen line shapes resemble the crystalline L-[*methyl*-$^2H_3$]methionine spectrum obtained at 80 °C (Figure 2a), where a highly averaged line shape is observed because of rotation about several side chain bonds in the amino acid.

At very low temperatures, the collagen line shapes approximate the Pake pattern in Figure 2f. As seen in Figure 3c, the spectrum of L-[*methyl*-$^2H_3$]methionine-labeled rat calvaria collagen approximates a Pake pattern at −135 °C, indicating that at this temperature the side chain motion is restricted to rotation about the S–C$^\epsilon$ bond axis. Although the line shape observed at −35 °C (Figure 3b) is significantly broader than the line shape observed at 22 °C (Figure 3a), it is clearly not a Pake pattern, showing that side chain motions, in addition to rotation about the terminal side chain S–C$^\epsilon$ bond, persist at −35 °C.

Table I: Values of the Methionine Methyl Deuteron Principal Frequencies[a] Used To Simulate Spectra in Figures 1 and 3

sample	T (°C)	ν_X (kHz)	ν_Y (kHz)	ν_Z (kHz)
RCC[b]	22	−14.0	−4.0	18.0
RTTC[c]	22	−11.5	−3.3	14.8
RCCC[d]	22	−10.3	−3.0	13.3
all	−35	−21.0	−4.0	25.0
all	−135	−21.0	−21.0	42.0

[a] Uncertainty 10–15%. [b] Rat calvaria collagen. [c] Rat tail tendon collagen. [d] Reconstituted chick calvaria collagen.

Within experimental uncertainty, the line widths observed for the reconstituted chick calvaria collagen and rat tail tendon collagen samples at −35 and −135 °C were indistinguishable from the line shapes shown for the rat calvaria collagen sample in Figure 3b,c. In order to make a quantitative comparison of the various spectra of the methionine-labeled collagen samples, motionally averaged theoretical line shapes were computer generated. In each case the motionally averaged principal frequencies were adjusted until agreement between calculated and observed spectra was attained. The dotted curves in Figure 3 are computer simulations of the rat calvaria collagen spectra. The corresponding principal frequencies, together with the principal frequencies obtained from simulations of the spectra of the other samples, are listed in Table I. Although the line shapes of the three collagen samples are the same at 22 °C, the principal frequencies of the nonmineralized collagen samples (Table I; Figure 1b,c) are 25–35% less than that of the mineralized rat calvaria sample (Figure 1a). This difference in line width ($\nu_Z - \nu_X$) among the three samples could result either from differences in side chain flexibility or from the greater backbone flexibility reported for the nonmineralized collagen samples, as compared to the mineralized collagen sample, at this temperature. Whatever the cause, the differences in line width between the mineralized and nonmineralized collagen samples at 22 °C are small compared with the much greater reduction in line width and change in line shape that occur, for each collagen sample, upon increasing temperature from −135 to +22 °C (Table I). The greatly reduced line width observed at 22 °C for all three collagen samples shows that large-amplitude rotational motions take place about at least two bonds in each methionine side chain at 22 °C in all three collagen samples. Furthermore, the fact that the side chain motions are able to average a methyl quadrupole splitting of 40 kHz implies that the rotational correlation times involved are less than ca. 10 μs. It is likely that the rotational correlation times are much less than 1 μs because we have not observed the large intensity loss expected (Spiess & Sillescu, 1981) in a quadrupole echo spectrum when the correlation time is in the 1–100-μs range.

The observation of motionally averaged ^{2}H NMR line shapes is not limited to methionine side chains in collagen. Motionally averaged line shapes are observed for collagen fibers containing $^2H^\gamma$-labeled pyrrolidinyl (prolyl and hydroxyprolyl) rings in rat calvaria (mineralized) and rat tendon (nonmineralized) samples (Figure 4). It is seen in Figure 4 that the line shape of each sample is a broad plateau covering a frequency range of 125 kHz. This line shape is also observed (but not shown) for labeled reconstituted chick calvaria collagen (not cross-linked or mineralized). It is noteworthy that the line shapes of the $^2H^\gamma$-labeled collagen samples (Figure 4a,b) do not match the line shape of DL-[4,4-$^2H_2$]proline (Sarkar et al., 1986) but are similar to the line shape of DL-[3,3,4,4,5,5-$^2H_6$]proline (Figure 4c). The line shape of the latter model compound has been analyzed in detail (Sarkar et al., 1986) and has been shown to be a superposition of three distinct motionally averaged line shapes corresponding to the β-, γ-, and δ-deuterons of the proline ring. Although the C–^{2}H bond reoriented with the same correlation time (a few pico-

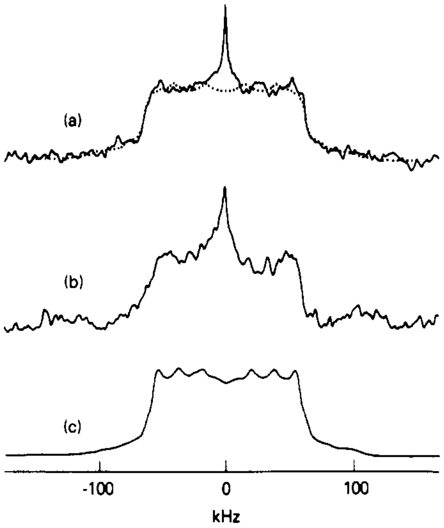

FIGURE 4: 38.45-MHz ^{2}H NMR spectra: (a) rat calvaria collagen, 89 000 acquisitions, experimental (—), calculated (···); (b) rat tail tendon collagen, 90 000 acquisitions, with each tissue containing ^{2}H$^\gamma$-labeled Pro and Hyp rings; (c) polycrystalline DL-[3,3,4,4,5,5-^{2}H$_6$]proline, 128 acquisitions. Each spectrum was obtained at 22 °C with a 90° pulse of 2.2 μs, an echo delay of 30 μs, and digital line broadening of 2 kHz. The recycle delays were 2.0, 1.5, and 30.0 s, respectively, in spectra a–c.

Table II: Values of the Principal Frequencies,[a] Generalized Order Parameters (S^2), and Θ_{rms} for the Pro and Hyp γ-Deuterons in the Collagen Samples at Two Temperatures

T (°C)	site	ν_X (kHz)	ν_Y (kHz)	ν_Z (kHz)	S^2	Θ_{rms}
22	Pro-1	−64.5	−15.2	79.7	0.43	30.3
	Pro-2	−64.5	−40.6	105.1	0.68	20.6
	Hyp-1	−64.5	−50.8	115.3	0.80	15.4
	Hyp-2	−64.5	−57.9	122.4	0.90	10.6
−30	Pro-1	−65.3	−49.3	114.6	0.78	16.6
	Pro-2	−65.3	−56.5	121.8	0.87	12.2
	Hyp-1	−65.3	−61.7	127.0	0.95	7.8
	Hyp-2	−65.3	−61.7	127.0	0.95	7.8

[a] Obtained from computer simulation of the collagen line shapes. At 22 °C; uncertainty in ν_X 5–10%; uncertainty in ν_Y 25%. Assignments are based upon a correspondence of values of S^2 obtained for the collagen samples with those obtained for model compounds. Within experimental uncertainty, the line shapes for the three types of collagen samples studied were indistinguishable, at a given temperature.

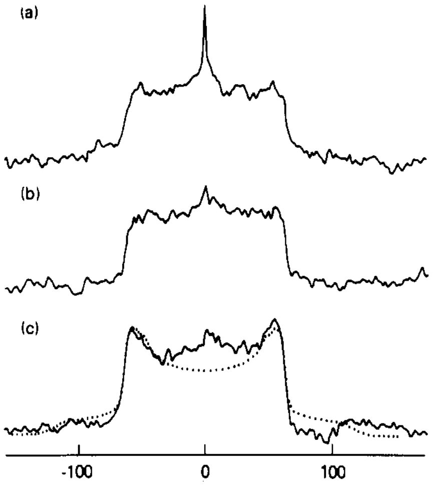

FIGURE 5: Comparison of 38.45-MHz ^{2}H NMR spectra of rat calvaria collagen, containing ^{2}H$^\gamma$-labeled Pro and Hyp rings, at three temperatures: (a) 22 °C, 89 000 acquisitions; (b) −12 °C, 74 600 acquisitions; (c) −30 °C, 82 000 acquisitions, experimental (—), calculated (···). Each spectrum was obtained with a 90° pulse of 2.2 μs, an echo delay of 30 μs, and a 2-kHz line broadening. The recycle delays were 2.0, 1.0, and 1.0 s, respectively, in spectra a–c.

seconds) at each ring position, the rms amplitude of the C–^{2}H bond motion, Θ_{rms}, varied from 12° for the δ-position to 29° for the γ-position (Sarkar et al., 1986). As a consequence of the variation in motional amplitude, the deuterons at the different ring sites in the model compound have different quadrupole splittings and line shapes, which when summed yield the plateau observed in the experimental spectrum (Figure 4c). In analogy with the model compound, we have calculated the spectrum of the [^{2}H$^\gamma$]pyrrolidinyl-labeled rat calvaria collagen sample (dotted curve in Figure 4a) assuming that the amplitudes of the motions of the pyrrolidinyl C$^\gamma$–^{2}H bond axes have a distribution of values. Specifically we assume (1) that, for a given type of pyrrolidinyl residue, there is a distribution of ring motion amplitudes and (2) that the average amplitude of the ring motion is larger in the case of the Pro residues than in the case of the Hyp residues. The first assumption is based upon the fact that the amplitude of the Pro ring motion in the solid state varies as a function of local molecular structure and packing (Sarkar et al., 1984, 1986; Torchia, 1984). The second assumption is based upon our analysis of NMR relaxation measurements (Torchia & Lyerla, 1974; Lipari & Szabo, 1982) of polypeptides containing Pro and Hyp residues. This analysis shows that the amplitude of the motion of the Pro C$^\gamma$–H bond axis is about twice as large as that of the Hyp C$^\gamma$–H bond axis in these molecules.

The parameters used to obtain the calculated spectrum of rat calvaria collagen (dotted line in Figure 4a) are listed in Table II. It is seen in Table II that two equally populated classes of Pro residues, designated Pro-1 and Pro-2 and distinguished by their different values of Θ_{rms}, were used in the calculation. Two equally populated classes of Hyp residues were also employed. We limited the calculations to two classes of residues because this assumption produced a reasonable fit to the experimental spectra while keeping the number of fitting parameters to a minimum.

The rms motional amplitudes and generalized order parameters (Lipari & Szabo, 1982; Torchia & Szabo, 1985) listed in Table II are in close agreement (typically within 15%) with values of these parameters derived from NMR studies of model amino acids in the solid state (Sarkar et al., 1986) and polypeptides in solution (Torchia & Lyerla, 1974).

Fraser and Trus (1986) have suggested that amino acid residues in the closely packed overlap domains of collagen fibers are less flexible than residues located in the less tightly packed gap regions of the fibers. The results in Table II are consistent with their suggestion when one considers that Pro and Hyp residues are about equally distributed in the gap and overlap domains of collagen fibers (Fraser & Trus, 1986).

In the crystalline amino acid, the motion of the proline ring can be suppressed, as evidenced by the appearance of a Pake pattern, by lowering the temperature (Torchia, 1984; Sarkar et al., 1986). In the case of rat calvaria collagen, the spectrum obtained at −30 °C (Figure 5c) resembles a Pake pattern. A simulation (dotted line in Figure 5c) of most of the experimental spectrum was obtained by assuming that the amplitudes of the Pro and Hyp ring motions at −30 °C were about half as large as those found at 22 °C (Table II). The fact that the simulated spectrum predicts less intensity than is observed in the low-frequency region of the spectrum indicates that 20–25% of the pyrrolidinyl rings have large-amplitude motions, Θ_{rms} ca. 20–30°, at −30 °C. We suggest that these flexible rings be assigned to Pro residues in view of the hydrogen bonding capacity of the Hyp rings.

Table III: Chemical Shift Tensor Principal Elements[a] and Relative Areas[b] of Line Shape Components Used To Obtain the Calculated ^{19}F Spectra of Rabbit Achilles Tendon Collagen in Figure 6

T (°C)	σ_1 (ppm)	σ_2 (ppm)	σ_3 (ppm)	relative area
−37	−69	6	56	1.0
−4	−69	6	56	0.63
	−32	6	36	0.21
	−18	6	29	0.11
	isotropic component			0.05
10, 26	−53	3	53	0.63,[c] 0.56[d]
	−25	3	28	0.11, 0.13
	−12	3	19	0.11, 0.13
	−6	3	16	0.11, 0.13
	isotropic component			0.05, 0.05

[a] Uncertainty: 20% for broad component; 10% for narrow components. [b] Uncertainty 20%. [c] At 10 °C. [d] At 26 °C.

Spectral changes, like those seen in Figure 5 for the rat calvaria collagen, were observed for rat tendon collagen and reconstituted chick calvaria collagen upon lowering the temperature from +22 to −30 °C.

Although rapid pyrrolidine ring motions having rms amplitudes in the range 5–30° can account for the line shapes observed for crystalline ^{2}H-labeled proline and the labeled collagen samples, ring motions on the picosecond time scale, as observed for the amino acid (Sarkar et al., 1986), cannot account for the fact that at 22 °C the T_1 values of the collagen samples are ca. 100 ms. In contrast, the T_1 values reported for the crystalline amino acid are in the 5–50-s range. The smaller T_1 values observed for the collagen samples can be accounted for if the correlation times of the pyrrolidinyl rings in collagen are ca. 100 times larger than the correlation times of the model compound. Alternatively, if the previously reported (Sarkar et al., 1985) nanosecond fluctuations of the collagen backbone cause reorientation of ring C–^{2}H bond axes through rms angles of 5–10°, the ring–deuterium T_1 values would be ca. 100 ms.

The ^{19}F spectra of rabbit Achilles tendon collagen, labeled with DL-(4-fluorophenyl)alanine, exhibit major changes in line shape as the temperature decreases from +26 to −37 °C (Figure 6a–d). At −37 °C a broad chemical shift powder pattern is observed (Figure 6d; Table III) whose line shape and width are similar to those observed for crystalline DL-(4-fluorophenyl)alanine (Hiyama et al., 1986). In contrast to the similar line shapes observed for collagen and the model compound, the fluorine T_1 value in collagen at −37 °C, ca. 2 s, is 1 order of magnitude smaller than the T_1 measured for the model amino acid at room temperature. The smaller T_1 value observed for the protein can be accounted for by small-amplitude angular fluctuations that modulate the ^{19}F chemical shift anisotropy (e.g., backbone motion or rolling motion about the C$^\beta$–C$^\gamma$ bond axis), provided that such motions have correlation times in the 0.1–1-ns range. An alternative explanation of the T_1 difference is that the phenyl rings in collagen undergo 180° ring flips at −37 °C. Such ring flipping is observed at elevated temperatures in the crystalline amino acid, and while this type of motion does not modulate the ^{19}F chemical shift tensor (and therefore does not significantly affect the line shape), it does modulate the ^{19}F–^{1}H dipolar interaction, thereby causing spin–lattice relaxation. Ring flips having correlation times in either the 0.1–1- or the 10–100-ns range can account for the ^{19}F T_1 values observed in collagen at −37 °C.

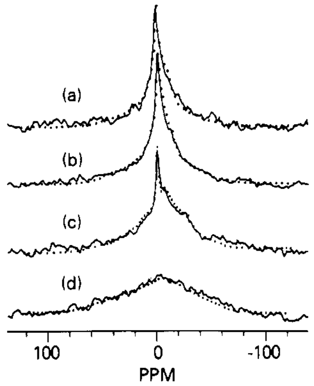

FIGURE 6: Comparison of experimental (—) and calculated (···) 470.5-MHz ^{19}F NMR spectra of rabbit Achilles tendon collagen at four temperatures: (a) 26 °C, 13 792 acquisitions; (b) 10 °C, 20 768 acquisitions; (c) −4 °C, 16 560 acquisitions; (d) −37 °C, 50 630 acquisitions. Approximately 2% of the phenylalanyl residues in the sample were fluorine labeled at the C-4 ring position. Each spectrum was obtained with an EXORCYCLE pulse sequence, with a $\pi/2$ pulse width of 8 μs, a 20-μs delay between the $\pi/2$ and π pulses, a 1000-Hz line broadening, and recycle delays of 2 s (a–c) and 4 s (d). Chemical shift scale in parts per million from the carrier radio frequency.

In contrast with the −37 °C spectrum, which contains a single broad component, the rabbit Achilles tendon fluorine spectra observed at −4 °C, and above, are composed of several components. Computer simulation shows that a minimum of four different line shape components must be coadded to simulate the −4 °C spectrum: (a) a broad component having a width of ca. 125 ppm, approximately equal to the width observed at −37 °C; (b) a narrow isotropic component, having a width of less than 2 ppm; (c) two motionally averaged components having widths of 50–65 ppm. The relative intensities and chemical shift tensor principal elements (σ_1, σ_2, σ_3) for each of the line shape components are listed in Table III. We have correlated these line shape components to particular kinds of (4-fluorophenyl)alanyl motion in collagen in the following way. We assign the broad spectral component to (4-fluorophenyl)alanyl residues whose side chain motions are confined to either ring flips or small-amplitude fluctuations. Because this component contains ca. 63% of the total signal intensity, we estimate that 25–30 of the 44 Phe residues in the collagen molecule (Fraser & Trus, 1986) undergo restricted motions of these types. The narrow isotropic component contains ca. 5% of the signal intensity, 2–3 Phe residues/molecule, and is assigned to residues whose side chains undergo essentially isotropic molecular motion. We think that the motion is isotropic, because both the chemical shift and dipolar interactions are completely averaged. It is difficult to see how side chain motion alone, which is limited to rotation about only two C–C bonds, can completely average both types of NMR interactions, and we therefore conclude that the Phe residues corresponding to the isotropic spectral component are located in regions of the collagen molecule that have substantial backbone flexibility.

The remaining signal intensity in the −4 °C powder pattern, corresponding to 12–16 Phe residues/molecule, consists of two powder patterns having widths of 50–65 ppm. In order for rotation about the Phe C$^\beta$–C$^\gamma$ bond axis to reduce the 125 ppm "static" ^{19}F line width to 60 ppm, the phenyl ring must undergo nearly free rotation about the β–γ bond axis. We think that such a motion is unlikely in view of the large activation energies reported for phenyl ring flips in solids, 5–17 kcal/mol (Hiyama et al., 1986), and therefore ascribe the observed 50–65 ppm powder line widths to motion about the Phe α–β bond axis. In a survery of Phe side chain conformations in protein crystal structures (Janin et al., 1978), it was found that the relative populations of the trans, gauche$^+$, and gauche$^-$ α–β rotomers were 0.31, 0.58, and 0.12, respectively. If we assume that these rotomers have these equilibrium populations in collagen, that there is rapid interconversion among the three rotomers, and that there is a rapid rolling motion (rms amplitude 24°), one calculates the following motionally averaged principal frequencies for the Phe ^{19}F chemical shift tensor: $\sigma_1 = -32$ ppm, $\sigma_2 = -9$ ppm, and $\sigma_3 = 32$ ppm. The line width, $\sigma_3 - \sigma_1$, of the calculated powder pattern, 64 ppm, is in qualitative agreement with the 50–65 ppm range observed at −4 °C.

When the temperature is increased from −4 to +26 °C, (a) the widths ($\sigma_3 - \sigma_1$) of the broad and intermediate components of the spectrum are observed to decrease by 30–50% (Table III), (b) three rather than two distinct intermediate components are required to fit the spectra, and (c) at 26 °C, intensity of the broad component decreases by 25%, without a concomitant increase in signal intensity of any of the narrower spectral components. The first observation is ascribed to the onset of backbone motions, above −4 °C, and to an increase in the amplitude of rolling motions about the side chain C–C bond axes, for the Phe residues corresponding to the intermediate width spectral components. The second observation indicates that the motions of the Phe side chains become more complex and heterogeneous as temperature is elevated. The third observation is explained by noting that the onset of a slow motion (correlation time ca. 10 μs) about the α–β bond axis shortens the homogeneous T_2 values of the fluorine nuclei, which contribute signal intensity to the broad component at low temperature. The reduction in T_2 will cause a substantial loss of signal intensity, because the EXORCYCLE pulse sequence (see Materials and Methods) does not refocus magnetization that dephases as a consequence of a homogeneous T_2 process.

In addition to spectra of rabbit Achilles tendon, spectra of rabbit calvaria collagen were also obtained in order to study the effect of mineral upon Phe side chain dynamics. Unfortunately the fluorine signal from collagen was completely masked by the 100-fold larger fluorine signal from the mineral component of the tissue.

The spectra of rat calvaria collagen, rat tail tendon collagen, and reconstituted chick calvaria collagen (Figure 7a–c), each labeled with [6-^{15}N]lysine, exhibit narrow nearly isotropic signals at 22 °C. The line shape is best defined in the chick collagen sample because the spectrum has a good signal-to-noise ratio (Figure 7c) as a consequence of the high level of ^{15}N enrichment in the sample. Examination of the line shape shows that, in addition to the main signal at 0 ppm, there is a smaller signal appearing as a shoulder at ca. 4 ppm. The smaller signal is assigned to ^{15}N nuclei in hydroxylysyl residues because a posttranslational modification converts about one-fourth of the lysyl residues in collagen to hydroxylysyl residues (Bornstein & Traub, 1979). Although the hydroxylysyl signal was evident in the spectra of the chick collagen sample, it was not resolved in spectra of either rat calvaria or rat tendon collagen (Figure 7a,b) because of poorer signal-to-noise ratio and resolution in the spectra of these samples. The ^{15}N line widths were 5.5, 7.5, and 8.5 ppm, respectively, for the chick, rat tail tendon, and rat calvaria collagen samples. Although cross-links and mineral produce small increases in the line width, the line widths observed in all three collagen samples at 22 °C are significantly smaller than the 15 ppm line width observed in the spectrum of polycrystalline DL-[6-^{15}N]lysine (Figure 7e). In contrast with this result, the line width of the chick calvaria collagen signal at −30 °C, 12 ppm (Figure 7d), is nearly as large as that of the crystalline amino acid. This result is strong evidence that the smaller line widths observed at 22 °C, for all three collagen samples, are caused by motions of the lysyl side chains. The observation that the lysyl line width in collagen at −30 °C is less than that of the crystalline amino acid shows that the side chain is not completely rigid at this temperature.

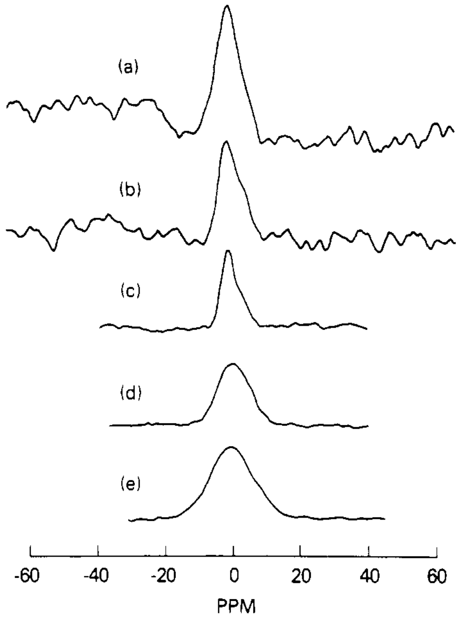

FIGURE 7: 25.38-MHz proton-enhanced ^{15}N NMR spectra: (a) rat calvaria collagen, 64 000 acquisitions, 22 °C; (b) rat tail tendon collagen, 16 384 acquisitions, 22 °C; (c) reconstituted chick calvaria collagen, 1024 acquisitions, 22 °C; (d) reconstituted chick calvaria collagen, 2048 acquisitions, −30 °C; (e) polycrystalline DL-[6-^{15}N]-lysine, 32 acquisitions, −30 °C. Each spectrum was obtained with a matched (45-kHz) Hartman–Hahn contact (2 ms) and a 50-Hz line broadening. Chemical shifts in parts per million, relative to the isotropic shift of DL-[6-^{15}N]lysine.

In the collagen samples, the ^{15}N signal intensity was observed to be extremely sensitive to the Hartmann–Hahn matching conditon (Pines et al., 1973; Mehring, 1983). This is additional evidence that the lysyl side chains are flexible. In contrast to the behavior observed for the crystalline amino acid, the ^{15}N signals in the collagen samples were reduced to unobservably low level when the mismatch of ^{1}H and ^{15}N rotating fields exceeded a few percent. This result shows that the ^{1}H–^{15}N dipolar coupling was effectively averaged by the motions of the lysyl side chains in the collagen samples. Motion about at least two side chain single-bond axes is required to average the dipolar coupling completely.

It is seen in Figure 7e that the lysine chemical shift anisotropy is only about 15 ppm. This observation implies that all motions having rates greater than a few kilohertz will average the chemical shift line shape. We can in fact conclude that most lysyl side chain motions must have rates in excess of 1 MHz, because motions having rates in the 10–100-kHz range result in small-proton rotating-frame relaxation times that short-circuit the Hartmann–Hahn cross-polarization experiment (Mehring, 1983), contrary to observation.

DISCUSSION

Previous studies showed that Glu, Lys, Met, and Pro side chains were flexible in reconstituted chick calvaria collagen (Jelinski & Torchia, 1980; Torchia, 1984). The present work shows that motions of Lys and Met side chains are slightly more hindered in the cross-linked fibers of rat tail tendon collagen than in the non-cross-linked reconstituted fibers of chick calvaria collagen. This result is in qualitative agreement with the observation that the rms amplitude of collagen backbone motions are ca. 25% smaller in rat tail tendon fibers than in reconstituted chick calvaria fibers. Unexpectedly we find that the side chain motions in mineralized rat calvaria collagen are only slightly more restricted than those observed in the nonmineralized rat tail tendon and reconstituted chick calvaria collagen samples. This conclusion contrasts with the results of studies of collagen backbone dynamics (Sarkar et al., 1983, 1985) that showed that the amplitudes of backbone motions in rat calvaria collagen were 2–4 times smaller than in the nonmineralized collagen samples.

In order to explain how mineral strongly hinders backbone, but not side chain, dynamics of collagen molecules, we note that the backbone of each polypeptide chain is buried within the ordered triple-helical collagen molecule (Bornstein & Traub, 1979). The backbone motions detected by NMR experiments have rms amplitudes ranging from 10 to 30°, depending upon the rates of reorientation. Furthermore, the backbone motions must be primarily torsions about the helix axis, because only this type of motion of the molecular backbone is compatible with X-ray diffraction data of rat tail tendon fibers (Fraser et al., 1983; Brodsky & Eikenberry, 1982). We suggest that the 10–30° rms amplitude backbone reorientation measured in the NMR experiments is the net result of rapid (picosecond), small-amplitude (a few degrees or less in rms) rotations about many backbone C–C and N–C bond axes. On the slower time scales (nano- to millisecond) to which the NMR measurements are most sensitive, the net results of a random sum of a large number of such fast, small-amplitude motions are the larger amplitude motions measured in the NMR experiments. Note that mineral needs to immobilize only a small fraction of collagen sites to reduce substantially the amplitude of these slower long-range torsional backbone motions. For example, if the motion detected in the NMR experiment is the result of small-amplitude rotations involving 100 amino acid residues in nonmineralized collagen, then, in a mineralized sample, the amplitude of the long-range backbone motion would be reduced ca. 3-fold if 1 collagen residue out of 10 is immobilized as a result of mineralization.

In contrast with the long-range nature of the collagen backbone motion, the internal motions of collagen side chains involve rotations about only a few side chain bond axes. For this reason, rare interactions between collagen molecules and mineral would be expected to immobilize only the small fraction of the collagen side chains that directly interact with mineral. Owing to the limited signal-to-noise ratio in the spectra reported herein, we could not observe a rigid spectral component, for a particular labeled residue, that accounted for less than 20% of the total signal intensity in the spectrum. Note that we would expect to see clear differences between spectra of mineralized and nonmineralized collagen samples, if mineral interacted with and immobilized most of the molecular surface, because the side chains, as noted earlier, reside at the protein surface.

We can relate the information about collagen dynamics in bone to the recent information about collagen structure in bone obtained by neutron diffraction (Bonar et al., 1985). The diffraction work indicates that collagen molecules are closely packed in bone and that mineral is located primarily outside collagen fibers. It is reasonable to suppose that close molecular packing hinders primarily long-range backbone motions, rather than short-range side chain motions, in bone collagen. Only the small fraction of side chains, located at the surface of a collagen fiber, would be expected to have strongly hindered motions because of direct interaction with mineral.

We now turn to a discussion of the possible relationship between the molecular dynamics and function of collagen. Collagen fibers, as a consequence of their tensile strength, confer connective tissues with mechanical strength. The fibers are composed of laterally aggregated, staggered molecules, which, when cross-linked, form a strong, macroscopic three-dimensional network. The molecular stagger must be precise, 234 ± 0.5 residues (Meek et al., 1979), in order to bring lysyl side chains that form intermolecular cross-links into proximity. Because the amino acid side chains are at the surface of the helical molecule, the molecular stagger undoubtedly results from favorable interactions between amino acid side chains. The side chain flexibility observed in the NMR experiments suggests that these interactions are not unique, but rather involve a myriad of conformations in fluidlike domains at the molecular surfaces.

The fluidity of the surface domains would allow collagen molecules in soft tissues some freedom to slip relative to one another when stress is applied (Mosler et al., 1985), thereby allowing the fiber network to distribute stress rapidly and uniformly. In addition, the fluid domains would permit rapid diffusion of ions within fibers, thereby facilitating mineralization. Finally the flexible molecular surfaces could adjust their shapes to accommodate for the growth of hydroxyapatite crystals in a mineralizing collagen matrix.

The backbone motions of the collagen molecule provide a means for absorbing mechanical energy that is generated when tension is applied to a network of collagen fibers. In bone, collagen flexibility, while reduced when compared with that found in soft tissues, is not eliminated and provides a means to absorb mechanical energy when a compressive load is applied to the tissue, thereby increasing the impact strength and reducing the brittleness of the tissue.

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Registry No. Met, 63-68-3; Pro, 147-85-3; Lys, 56-87-1; Phe, 63-91-2.

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