DEUTERIUM NUCLEAR MAGNETIC RESONANCE OF SPECIFICALLY LABELED NATIVE COLLAGEN

Investigation of Protein Molecular Dynamics Using the Quadrupolar Echo Technique

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ABSTRACT Collagen was labeled with [3,3,3-d_3]alanine and with [d_{10}]leucine via tissue culture. ^{2}H nuclear magnetic resonance (NMR) spectra were obtained of collagen in solution and as fibrils using the quadrupolar echo technique. The ^{2}H NMR data for [3,3,3-d_3]alanine-labeled collagen fibrils were analyzed in terms of a model for motion in which the molecule is considered to jump between two sites, separated azimuthally by an angle 2δ, in a time which is rapid compared with the residence time in both sites. The data suggest that the molecule undergoes reorientation over an angle, 2δ, of $\sim 30°$ in the fibrils, and that the average angle between the alanine C^α–C^β bond axis and the long axis of the helix is $\sim 75°$. Reorientation is possibly segmental. The T_2 for [3,3,3-d_3]alanine-labeled collagen fibrils was estimated to be 105 μs. The ^{2}H NMR data for the methyl groups of [d_{10}]leucine-labeled collagen were analyzed qualitatively. These data established that for collagen in solution and as fibrils, rotation occurs about the leucine side-chain bonds, in addition to threefold methyl rotation and reorientation of the peptide backbone. The T_2 for the methyl groups of leucine-labeled collagen is estimated to be ~ 130 μs. Taken together, these data provide strong evidence that both polypeptide backbone reorientation and amino acid side-chain motion occur in collagen molecules in the fibrils. Stabilizing interactions that determine fibril structure must therefore depend upon at least two sets of contacts in any given local region.

INTRODUCTION

The type I collagen molecule is a $\sim 1.5 \times 300$ nm rod-like triple helix of molecular weight 285,000 (1). It is composed of two α1(I) chains and one homologous α2 chain. Except for short regions at the ends of the molecule, the polypeptide chains contain the Gly-X-Y triplet repeat, where the amino acids X and Y are often proline and hydroxyproline, respectively (2). The triple-helical collagen molecules associate in a highly regular manner to form fibrils (3). These fibrils give rise to a characteristic band pattern by electron microscopy (4), and to characteristic reflections by low angle x-ray fiber diffraction (5–6). Although these techniques suggest that there is overall long-range order in the collagen fiber, they do not by their nature provide information about the local dynamic fluctuations of amino acid side-chains, or about overall motion of the peptide backbone.

^{13}C nuclear magnetic resonance (NMR) of specifically labeled collagen has been used previously to provide information about the local motions of various amino acids (7–9). Analysis of ^{13}C NMR relaxation data for amino acids comprising the protein backbone ([1-^{13}C]glycine and [2-^{13}C]glycine) (7) indicated that in solution, collagen undergoes reorien-

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tation about its long axis with a correlation time, τ_c, of $\sim 10^{-7}$ s. Comparison of the ^{13}C NMR relaxation data for collagen in solution with that for collagen fibrils indicated that in the fibrils motion remains rapid ($\tau_c \sim 10^{-7}$ s), although the lineshapes indicated that the motion occurs over a limited range in azimuthal angle. A lower limit of 30° for this angle was estimated from the T_1 data (9). These results indicate that the peptide backbone in collagen undergoes rapid motion about its long axis, and that contacts between the collagen molecules must be fluid.

That amino acid side-chains on surfaces of the collagen molecules are not fixed was independently established by ^{13}C NMR investigation of the terminal carbons of certain amino acids ([3-^{13}C]alanine, [methyl-^{13}C]methionine, [6-^{13}C]lysine, and [5-^{13}C]glutamic acid) (8). The ^{13}C NMR lineshapes showed that the rate of isotropic reorientation was $\sim 10^3$ s^{-1} for the labeled side-chains. The ^{13}C NMR relaxation parameters for these labeled collagen samples indicated that the rate of anisotropic motion for these amino acids is rapid ($\sim 10^9$ s^{-1}). The rates of reorientation were similar for collagen as fibrils and for collagen in solution, which suggested that there is not a unique set of interactions between amino acid side-chains in collagen fibrils. Rather, the data suggested that the collagen amino acid side-chains undergo rapid fluctuations in the fibril.

We report here the application of ^2H NMR to the study of molecular motion in collagen. The quadrupolar echo technique for solids was employed, which preserves the inhomogeneously broadened portion of the signal (10–12). Because the deuterium electric field gradient tensor is generally axially symmetric along the C–D bond axis (13), the interpretation of lineshapes in the presence of motion is more straightforward than for ^{13}C NMR. Therefore, a ^2H NMR study affords the possibility of a more precise determination of the range in azimuthal angle about which reorientation of the collagen peptide backbone occurs. In addition, the quadrupolar relaxation mechanism is dominant for deuterium (14). Therefore, information obtained from ^2H NMR of collagen serves as an independent confirmation of the previous ^{13}C NMR results in which dipolar relaxation was assumed.

METHODS

Chemicals

L-[3,3,3-d_3]alanine (98 atom % ^2H) and D,L-[d_{10}]leucine (98 atom % ^2H) were obtained from Merck Isotopes (Merck & Co., Inc., Rahway, N. J.) and were characterized by ^{13}C NMR (15.09 MHz), mass spectroscopy, and by elemental and amino acid analyses. L-[2,3-^3H]alanine was purchased from New England Nuclear (Boston, Mass.), and L-[U-^{14}C]alanine, L-[U-^{14}C]leucine, and L-[4,5-^3H]leucine were obtained from Schwartz/Mann Div., Becton, Dickinson & Co. (Orangeburg, N. Y.). Pronase R (B grade) was obtained from Calbiochem (San Diego, Calif.), and aminopeptidase M (amino acid arylamidase) from Boehringer-Mannheim Biochemicals (Indianapolis, Ind.). Deuterium-depleted water was obtained from Aldrich Chemical Company (Milwaukee, Wis.).

Collagen Preparation

Lathyritic [3,3,3-d_3]alanine-labeled collagen and [d_{10}]leucine-labeled collagen were obtained from chick calvaria culture using previously described methods (7–9). For these preparations, 10 mg of L-[3,3,3-d_3]alanine or 12.5 mg of D,L-[d_{10}]leucine was added per 100 ml of incubation medium. (The incubation medium was devoid of the unlabeled isomer of the amino acid interest.) Both ^3H-labeled alanine (30 μCi/100 ml of medium) and ^{14}C-labeled alanine (15 μCi/100 ml of medium) were used as radiotracers for the alanine experiments. ^3H-labeled leucine (50 μCi/100 ml of medium) and ^{14}C-labeled leucine (25 μCi/100 ml of medium) were used as radiotracers for the leucine experiments. Preincubation, incubation, and purification procedures were identical to those described previously (7–8).

Collagen Characterization

Collagen concentrations were determined at 313 nm in 0.5 M acetic acid with a Cary 60 spectropolarimeter (Cary Instruments, Fairfield, N. J.), using −2,300 as the specific rotation for collagen (1, 5). Amino acid analyses were performed on protein hydrolyzates (6 N HCl for 24 h at 110 °C) with both Beckman (Beckman Instruments, Inc., Fullerton, Calif.) and Durrum (Durrum Instrument Corp., Sunnyvale, Calif.) automatic amino acid analyzers. The Beckman analyzer was equipped with a radioactivity flow counter for monitoring ^{14}C. Fractions were collected for ^{3}H counting. Amino acid analyses for all samples were correct for pure collagen. In addition, enzymatically hydrolyzed collagen was analyzed for both ^{14}C and ^{3}H.

Measurement of Percent Incorporation of ^{2}H

A small amount of proton exchange occurs at alanine and leucine during acid hydrolysis of some proteins (16). An enzymatic hydrolysis procedure was developed for collagen to avoid artifacts in the determination of the percent incorporation of ^{2}H which may be introduced by this exchange. Thus, 2 mg of native collagen was heated for 10 min at 60 °C in 2 ml of 0.5 M acetic acid. The denatured collagen was lyophilized and dissolved in 0.5 ml of 0.05 M $NaHCO_3$ (pH 7.2). 25 μg of pronase R was added and the solution was incubated for 48 h at 25 °C. The hydrolyzed solution was lyophilized and derivatives were prepared for gas chromatography-mass spectroscopy (gc-ms) analysis of the percent incorporation of ^{2}H. Amino acid analyses of enzymatically hydrolyzed collagen were similar to those for acid hydrolyzed collagen, indicating that a representative portion of the protein was enzymatically hydrolyzed.

N-acetyl methyl ester derivatives of the amino acids in the enzyme hydrolyzate were prepared as described previously (7, 8). The derivatized collagen hydrolyzate was analyzed using chemical ionization gc-ms as described previously (7, 8). No deuterium isotope effect was observed for gas chromatography of the *N*-acetyl methyl ester of [3,3,3-d_3]alanine. However, an isotope effect was observed for the gas chromatographic separation of the deuterated and nondeuterated *N*-acetyl methyl esters of leucine.

^{2}H NMR Instrumentation

The home-built ^{2}H NMR spectrometer employed a Varian 220 superconducting magnet (Varian Associates, Palo Alto, Calif.) (5.2 Tesla), operating at 33.775 MHz for ^{2}H. The dewared sample probe contained a single tuned coil accepting 8 mm sample vials which had 0.4-ml capacity. Pulse programming for the solid echo experiment ($90°_{\pm x}$ - t_1 - $90°_{y}$ - t_2 - T) (10–12) was achieved using a Nicolet 293 I/O controller (Nicolet Instrument Corp., Madison, Wis.). The relative phases of the 90° pulses were controlled by a Palindi Associates model 7801 transceiver (Palindi Associates, Gaithersburg, Md.). A Heath model SB-200 amplifier (Heath Co., Benton Harbor, Mich.) provided final amplification of the rf pulses, and an ENI 3100L amplifier (Electronic Navigation Industries, Inc., Rochester, N. Y.) provided primary amplification. The ^{2}H NMR signal was amplified and phase-detected by the transceiver, digitized by a Nicolet Explorer II fast oscilloscope, and transferred to a Nicolet 1080 computer for accumulation and subsequent manipulation by Nicolet software.

^{2}H NMR Sample Preparation and Data Acquisition

Collagen fibrils, obtained by dialysis against 0.02 M Na_2HPO_4, were washed with a total of 50 ml of 0.02 M Na_2HPO_4 in deuterium-depleted water by repeated suspension and centrifugation. All subsequent operations were performed in a dry nitrogen atmosphere with predried equipment. The fibrils were packed into 8-mm diam sample vials, and excess 0.02 M Na_2HPO_4 was removed by pipet.

Samples of collagen in solution were prepared by dissolving salt-free, lyophilized collagen in 0.1 M acetic acid in deuterium-depleted water so that the final collagen concentration was ~10 mg/ml. All operations were performed under nitrogen using predried equipment.

A control spectrum of unlabeled collagen was obtained under conditions which were identical to the conditions for each spectrum of labeled collagen reported here. This ensured that the portions of the spectrum arising from the labeled sample and from water in natural abundance could be assigned with confidence.

Data were collected in quadrature using 2 K points/channel and a sampling rate of 5 μs/pt. The value of t_2 in the quadrupolar echo pulse sequence was adjusted by observing the maximum free induction decay for a sample of [3,3,3-d_3]alanine. The optimum length of the 90° pulse (~5 μs) was determined from the best null obtained with a 180° pulse on a 10% D_2O in H_2O sample. Generally, 200,000 accumulations were obtained for each protein spectrum. Spectra were obtained at 18 °C unless otherwise noted.

RESULTS

[3,3,3-d_3]Alanine-labeled Collagen

Collagen was labeled with L-[3,3,3-d_3]alanine via chick calvaria culture. Amino acid analyses for these samples were consistent with pure collagen (17). Radiotracer analysis established that alanine was the only labeled amino acid. Mass spectroscopy indicated that 19% of the alanine residues in collagen contained deuterium. Of the labeled alanine residues, approximately half contained three deuterons, one-quarter contained two deuterons, and one-quarter contained one deuteron. Because ~95% of both the starting material and the alanine control for the enzymatic hydrolysis contained three deuterons, partial loss of deuterium from the alanine methyl group must occur during biosynthesis.

As a powdered amino acid, L-[3,3,3-d_3]alanine gives rise to the ^{2}H NMR powder pattern shown in Fig. 1 *a*. The quadrupolar splitting, $\Delta\nu_q$, is 38.8 kHz. Lineshape distortion, estimated from a computer simulation of this lineshape (Fig. 1 *e*), is <10% over the range ±40 kHz.

The ^{2}H NMR spectrum of [3,3,3-d_3]alanine-labeled collagen as frozen fibrils has a lineshape (Fig. 1 *b*) and quadrupolar splitting (Table I) similar to that of the powdered amino acid. The quadrupolar splitting for the alanine methyl is slightly smaller for the frozen collagen fibrils than for the powdered amino acid. This may be due to a difference in the quadrupolar coupling constants, or may reflect a small amount of molecular motion in the frozen fibrils.

As fibrils at +18 °C, the alanine-labeled collagen sample gives rise to a spectrum in which approximately two-thirds of the signal intensity is lost (Fig. 1 *c*), and in which the quadrupolar coupling constant has collapsed to an apparent value of 30 kHz (Table I). A spectrum obtained at the shortest quadrupolar echo delay times consistent with recovery of the spectrometer (30 and 39 μs) contained more intensity, but was otherwise identical in shape to the spectrum in Fig. 1 *c* (within the uncertainties due to baseline distortion). Therefore, the lineshape in Fig. 1 *c* is representative of the entire sample. The sharp peak in the center of Fig. 1 *c* is due to deuterated water in natural abundance. Spectra of [3,3,3-d_3]alanine-labeled collagen fibrils were obtained at various quadrupolar echo delay times, and the natural logarithms of their relative intensities were plotted against the sum of the delay times (18). When extrapolated back to zero delay time, this straight line predicted an intensity which was approximately equal to the intensity obtained from the spectrum of the frozen fibrils at −18 °C (Fig. 2). The T_2 determined from this plot is 105 μs.

The ^{2}H NMR spectrum for [3,3,3-d_3]alanine-labeled collagen in solution shows further reduction of the linewidth to an apparent quadrupolar splitting of ~10 kHz (Fig. 1 *d*), Table I). Comparison of the spectrum in Fig. 1 *d* with a control spectrum of unlabeled collagen shows that approximately one-third of the sharp peak in the center of the spectrum in Fig. 1 *d* is due to deuterated water in natural abundance; the rest arises from [3,3,3-d_3]alanine-labeled collagen. This peak may be due to the 3% of the alanine residues which reside in the nonhelical termini of the molecule, or to certain of the alanine residues which make the magic angle (54.7°) with the long axis of the molecule.

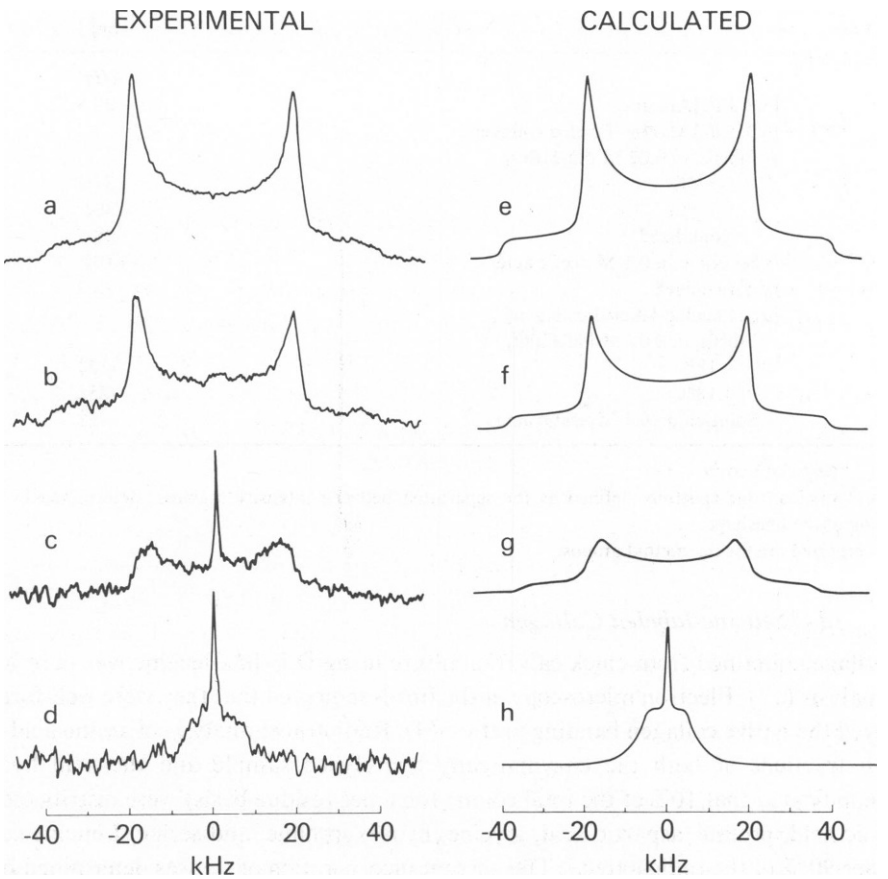

Figure 1 (*a–d*) Experimental ^2H NMR spectra for [3,3,3-d_3] alanine and [3,3,3-d_3]alanine-labeled collagen. These spectra were obtained in quadrature using the solid echo pulse sequence ($90°_{x}$ - t_1 - $90°_{y}$ - t_2 - $T)_m$, with t_1 and t_2 set at 50 and 59 μs, respectively. (*a*) [3,3,3-d_3]Alanine as a polycrystalline amino acid; 256 accumulations, 0.25-s repetition rate, 18 °C. (*b*) [3,3,3-d_3]Alanine-labeled collagen fibrils, 20 mg, in equilibrium with excess Na_2HPO_4; 2×10^5 accumulations, 0.25-s repetition rate, −18 °C. (*c*) [3,3,3-d_3]Alanine-labeled collagen fibrils, ~15 mg, in equilibrium with excess Na_2HPO_4; 1.67×10^5 accumulations, 0.25-s repetition rate, 18 °C. (*d*) [3,3,3-d_3]Alanine-labeled collagen in solution, ~10 mg/ml in ~0.4 ml 0.1 M acetic acid in deuterium-depleted water; 2.5×10^5 accumulations, 0.1-s repetition rate, 18 °C.

(*e–h*) Calculated spectra for [3,3,3-d_3]alanine and for [3,3,3-d_3]alanine-labeled collagen. Lorentzian line broadening was employed in all cases. (*e*) Calculated spectrum of polycrystalline [3,3,3-d_3]alanine; $\Delta\nu_q$ = 38.8 kHz, line broadening = 1.3 kHz. (*f*) Calculated spectrum of frozen [3,3,3-d_3]alanine-labeled collagen fibrils: $\Delta\nu_q$ = 37.3 kHz, line broadening = 1.5 kHz. (*g*) Calculated spectrum of [3,3,3-d_3]alanine-labeled collagen fibrils at 18 °C. The alanine C^α–C^β bond axes are assumed to undergo jump diffusion between two equally populated sites. The angles θ between the C^α–C^β bond axes and the helix axis are 69° and 85° (Figs. 2 (*a, b*). The angle 2δ through which each alanyl C^α–C^β bond axis is carried by reorientation of the peptide backbone is 30°. The spectra calculated for θ = 69° and θ = 85° were summed with equal weight; $\Delta\nu_q$ = 37.3 kHz, line broadening = 2.5 kHz. (*h*) Calculated spectrum of [3,3,3-d_3]alanine-labeled collagen in solution. Reorientation of the alanyl C^α - C^β bond axis about the long axis of the helix has averaged the 37.3 kHz quadrupolar splitting to ~10 kHz. Line broadening = 4.1 kHz.

TABLE I
QUADRUPOLAR SPLITTING ($\Delta\nu_q$) FOR VARIOUS ^{2}H-LABELED AMINO ACIDS AND ^{2}H-LABELED COLLAGEN

	$\Delta\nu_q$*
	(kHz)
[3,3,3-d_3]Alanine	38.8
[3,3,3-d_3]Alanine-labeled collagen	
Fibrils: in 0.02 M Na_2HPO_4	
−18°C	37.3
+18°C	~30‡
lyophilized	38
Solution: in 0.1 M acetic acid	~10‡
[d_{10}]Leucine§	35.2
[d_{10}]Leucine-labeled collagen§	
Fibrils: in 0.02 M Na_2HPO_4	
−18°C	~32‡
+18°C	~25‡
Solution: in 0.1 M acetic acid	~9‡

*$\Delta\nu_q = (3e^2Qq/4h)(3\cos^2\theta - 1)$.
‡Apparent quadrupolar splitting, defined as the separation between intensity maxima, determined by computer simulation of the lineshape.
§Values reported are for the methyl groups.

[d_{10}]Leucine-labeled Collagen

The collagen obtained from chick calvaria culture using D,L-[d_{10}]leucine was pure by amino acid analysis (17). Electron microscopy of the fibrils indicated that they were well-formed and displayed the native collagen banding pattern (4). Radiotracer analysis of amino acid analysis column fractions of both the enzymatically hydrolyzed sample and the acid hydrolyzed sample indicated that 10% of the total counts (on a per residue basis) were distributed among glutamic acid, proline, aspartic acid, glycine, hydroxyproline, and serine. Leucine contained the other 90 % of the radioactivity. The percent incorporation of ^{2}H was determined by gc-ms of the *N*-acetyl methyl ester of leucine. Although the *N*-acetyl methyl ester of deuterated

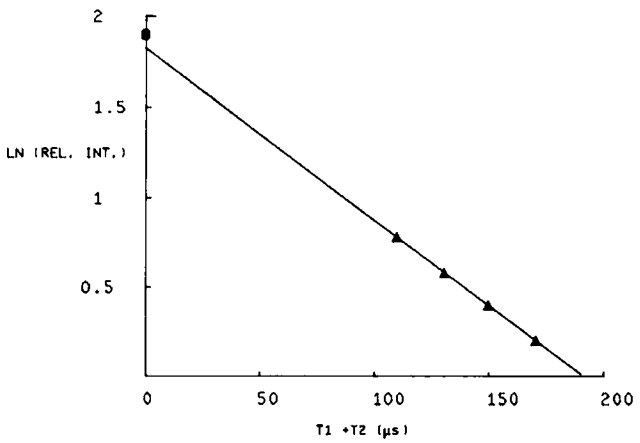

Figure 2 Plot of the relative intensity of the ^{2}H spectra for [3,3,3-d_3]alanine-labeled fibrils versus the sum of the delay times in the quadrupolar echo pulse sequence ($90°_{\pm x} - t_1 - 90°_y - t_2 - T$). The point at $t_1 + t_2 = 0$ represents the intensity obtained from the spectrum of frozen fibrils. A T_2 of 105 μs was obtained from the slope of the least squares fit of these data.

leucine is partially separated from its nondeuterated counterpart, the nondeuterated leucine and isoleucine derivatives do not separate under the conditions used for gas chromatography. Since radiotracer analysis indicated that isoleucine was not labeled during biosynthesis, and since the relative amounts of leucine and isoleucine were known from amino acid analysis of this enzymatically hydrolyzed sample, the percent incorporation of deuterated leucine into collagen was determined by straightforward calculation. Thus, of the leucine in the labeled collagen sample, 35% contained deuterium. Of the leucine which was deuterated, 83% contained nine deuterons. ~65% of the starting leucine and ~65% of the control leucine for the enzymatic hydrolysis and derivatization procedure contained 10 deuterons. Therefore, the partial loss of one deuteron must occur during biosynthesis. Biosynthetic loss of the α-proton of leucine has been observed in other systems (19), and a mechanism for the loss of α-protons on amino acids has been shown to occur through a pyridoxyl-phosphate-mediated transamination exchange (20, 21).

The deuterated amino acid used for the sample preparation, D,L-[d_{10}]leucine, gives rise to the ^{2}H NMR powder pattern shown in Fig. 3 *a*. The portion of the spectrum which arises from deuterons in positions other than the methyl groups (i.e., the α, β, and γ deuterons) shows that these deuterons all have approximately the same quadrupolar splitting ($\Delta\nu_q$ = 117 kHz) and are outside the region of the methyl pattern. (In this spectrum, only the maxima of these powder patterns are observed; the wings are outside the spectral window.) The methyl quadrupolar splitting, $\Delta\nu_q$, for this sample is 35.2 kHz (Table I).

As frozen fibrils, [^{2}H]leucine-labeled collagen displays a lineshape (Fig. 3 *b*) which is similar to that observed for the powdered amino acid. The apparent quadrupolar splitting for the methyl groups ($\Delta\nu_q$ = ~32 kHz, Table I) is 10% smaller than for the powdered amino acid, and may reflect a small degree of motion which occurs in the frozen fibrils at −18 °C. The intensity maxima due to the three nonmethyl deuterons appear just above the noise level.

As fibrils at +18 °C, [^{2}H]leucine-labeled collagen gives rise to a spectrum in which the splittings of the methyl groups have collapsed to an apparent quadrupolar splitting of ~25 kHz (Fig. 3 *c*, Table I) and in which approximately half of the signal is lost. A spectrum at the shortest echo delay times consistent with recovery of the spectrometer (30 and 39 μs) contains more intensity but is otherwise identical in shape, within the uncertainties of baseline distortion. Therefore, the lineshape observed is representative of the true lineshape for the sample. Signals due to the singularities of the nonmethyl deuterons are not observed for several reasons. First, the splittings are larger than those of the methyl groups, so that the intensities are affected more greatly by pulse power fall-off than are the methyl signals. Second, because of their large splittings, the T_2 for these side-chain deuterons is expected to be much smaller than the T_2 of the methyl groups. The sharp line in the center of the spectrum in Fig. 3 *c* is due to deuterated water in natural abundance.

In solution, where collagen is free to undergo rotation about the long axis of the molecule, the apparent quadrupolar splitting is further reduced to ~9 kHz (Fig. 3 *d*, Table I). The sharp peak in the center of the spectrum is due to deuterated water in natural abundance.

DISCUSSION

Two-Site Jump Model for Motion

Motion of the C^α–C^β bond axis of alanine in collagen requires reorientation of the peptide backbone, because alanine has no other side-chain bonds about which motion can occur. In the simplest model for motion, the molecule is considered to jump between two orientations in

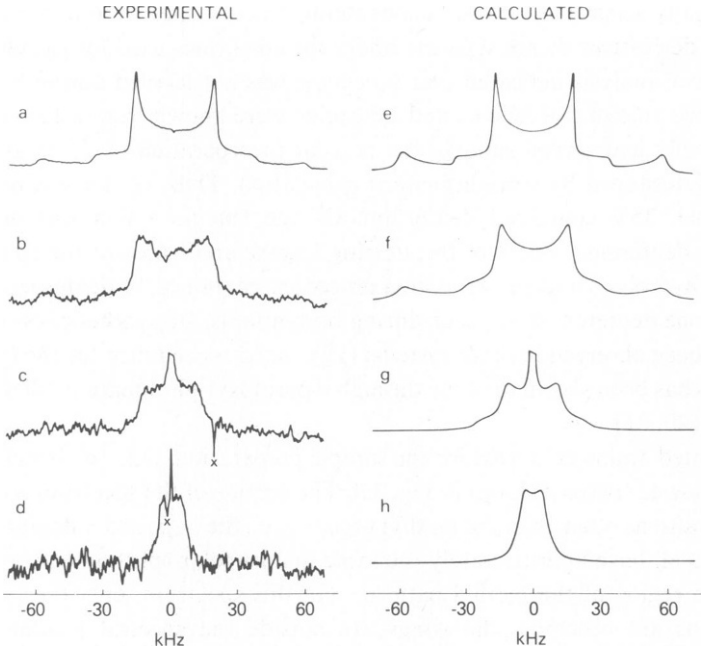

Figure 3 (*a–d*): Experimental ^{2}H NMR spectra for [d_{10}]leucine and [^{2}H]leucine-labeled collagen. These spectra were obtained in quadrature using the solid echo pulse sequence ($90°_{\pm x}$ - t_1-$90°_y$ - t_2 - T), with t_1 and t_2 set at 50 and 59 μs, respectively. The spectral window for all spectra was ±100 kHz. (*a*) [d_{10}]Leucine as a polycrystalline amino acid; 1,024 accumulations; 0.1-s repetition rate; 18 °C. (*b*) [^{2}H]Leucine-labeled collagen fibrils; 15 mg; in equilibrium with excess Na_2HPO_4; −18 °C. (*c*) The same sample in *b* under identical conditions, except 2.12 × 10^5 accumulations, +18 °C. (*d*) [^{2}H]Leucine-labeled collagen in solution, 4.5 mg in 0.2 ml 0.1 M acetic acid in deuterium-depleted water; 2.07 × 10^5 accumulations; 0.25-s repetition rate; 18 °C.

(*e–h*): Calculated spectra for [d_{10}]leucine and for [^{2}H]leucine-labeled collagen. Lorentzian line broadening was employed in all cases. (*e*) Calculated spectrum of polycrystalline [d_{10}]leucine; $\Delta\nu_q$ = 35.2 kHz for the methyl groups; $\Delta\nu_q$ = 117 kHz for the nonmethyl deuterons; line broadening = 1.1 kHz for the methyl groups; line broadening = 4.4 kHz for the nonmethyl deuterons. (*f*) Calculated spectrum of frozen [^{2}H]leucine-labeled collagen fibrils. $\Delta\nu_q$ = 32 kHz;line broadening = 3.6 kHz. (*g*) Calculated spectrum of [^{2}H]leucine-labeled collagen fibrils at +18 °C. $\Delta\nu_q$ = 25 kHz; line broadening = 4.4 kHz. A Lorentzian signal with 670-Hz broadening was superimposed in the center of the spectrum to simulate the signal from deuterated water in natural abundance. (*h*) Calculated spectrum of [^{2}H]leucine-labeled collagen in solution; $\Delta\nu_q$ = 9 kHz; line broadening = 3.5 kHz.

a time which is much less than the residence time in each of the two sites and much less than $(\Delta\nu_q)^{-1}$ (22). The assumption that the jump rate is much less than $(\Delta\nu_q)^{-1}$ is consistent with the reorientation rate of $\sim 10^7$ s^{-1} derived from previous ^{13}C NMR studies (7,8).

As a consequence of motion, the observed ^{2}H NMR powder pattern will not, in general, be axially symmetric. The principal axis system (X, Y, Z) and the frequencies corresponding to the principal values of the motionally averaged field gradient tensor (ω_X, ω_Y, ω_Z) can be determined from the geometrical construction shown in Fig. 4 *a*. For clarity, we consider the powder pattern corresponding to the transition for which $\omega_\perp \leq \omega \leq \omega_\parallel$, and we employ the convention that $\omega_X \leq \omega_Y \leq \omega_Z$.

Motion of the collagen backbone makes the alanine C^α–C^β bond axis jump between two orientations which are labeled 1 and 2 in Fig. 4 *a*. The alanine C^α–C^β bond axis forms an angle θ with the long axis of the collagen molecule (the z axis in Fig. 4 *a* represents the long axis of the collagen molecule). The axes 1 and 2, when projected onto the x,y plane, make an angle 2δ.

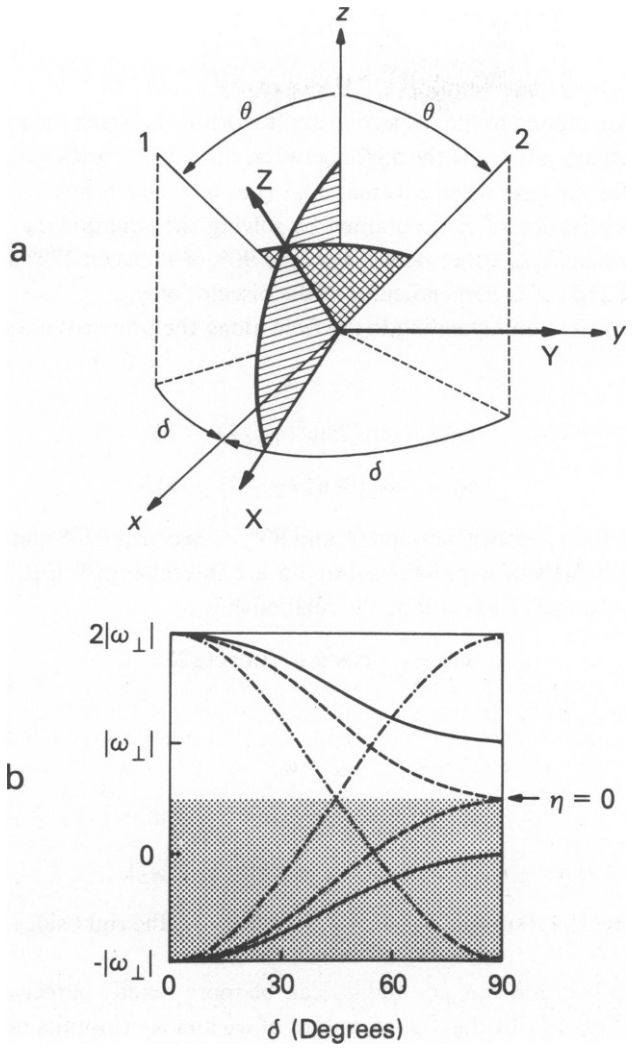

Figure 4 (*a*) Geometrical construction used to obtain ω_X, ω_Y, and ω_Z in the presence of two-site jump motion. Axes 1 and 2 refer to the two orientations between which the alanine C^{α}–C^{β} bond axis is considered to jump. θ is the angle between both axes (1 and 2) and the *z* axis. δ, half the angle through which the helix rotates, is the angle made by the projections of 1 and 2 in the *x,y* plane. (*b*) Graphical representation of the relationship of ω_Y and ω_Z (in units of $|\omega_\perp|$) with the jump angle, δ, for various values of θ. — corresponds to $\theta = 35.3°$; - - -corresponds to $\theta = 45°$, and -●- corresponds to $\theta = 90°$. The shaded portion of the graph represents the correspondence between δ and ω_Y; the nonshaded part represents the correspondence between δ and ω_Z. $\eta = 0$ when $\omega_Y = \omega_Z$. Otherwise, an axially asymmetric pattern is obtained, and $\eta = 1$ when $\omega_Z = |\omega_\perp|$ and $\omega_Y = 0$.

We first consider the case for which ω has its minimum value. When the applied field, B_o, is normal to the 1,2 plane, ω has a value, $\omega_\perp$, when the alanine C^{α}–C^{β} bond axis is oriented along either 1 or 2. This value is the minimum value for ω, and by convention, is equal to ω_X. Since the principal axes are mutually perpendicular, the other principal axes, *Y* and *Z*, must be in the 1,2 plane.

When B_o is in the 1,2 plane, the observed frequency is:

$$\omega = 0.5[\omega_\parallel(\cos^2\chi_{1B} + \cos^2\chi_{2B}) + \omega_\perp(\sin^2\chi_{1B} + \sin^2\chi_{2B})] , \quad (1)$$

which reduces to:

$$\omega = -0.5\omega_{\perp}[3\cos^2\chi_{1B} + 3\cos^2(\gamma_{12} - \chi_{1B}) - 2]. \quad (2)$$

Here, χ_{1B} and χ_{2B} correspond to the respective angles formed between the applied field, B_o, and the 1 and 2 orientations, and γ_{12} is the angle between the 1 and 2 orientations.

Last, we consider the case when ω is maximal (i.e., $\omega = \omega_Z$). Since ω is maximal when B_o lies along Z, the orientation of Z is obtained by solving the equation $d\omega/d\chi_{1B} = 0$. We find that Z bisects γ_{12} when γ_{12} is either between 0° and 90°, or between 270° and 360°. When γ_{12} is between 90° and 270°, Z is perpendicular to the bisector of γ_{12}.

Thus, the frequencies corresponding to the field along the principal axes are:

$$\omega_X = \omega_{\perp} \quad (3)$$

$$\omega_Y = -\omega_{\perp}[3\sin^2(\gamma_{12}/2) - 1] \quad (4)$$

$$\omega_Z = -\omega_{\perp}[3\cos^2(\gamma_{12}/2) - 1]. \quad (5)$$

These results apply for γ_{12} either between 0° and 90°, or between 270° and 360°. When $90° \leq \gamma_{12} \leq 270°$, the right sides of expressions 4 and 5 are interchanged. Eqs. 3–5 can be cast in terms of the angles θ and δ of Fig. 4 *a* by the relationship:

$$\cos\gamma_{12} = \cos^2\theta + \sin^2\theta\cos 2\delta. \quad (6)$$

Therefore:

$$\omega_X = \omega_{\perp} \quad (7)$$

$$\omega_Y = 3|\omega_{\perp}|\sin^2\theta\sin^2\delta - |\omega_{\perp}| \quad (8)$$

$$\omega_Z = 3|\omega_{\perp}|(1 - \sin^2\theta\sin^2\delta) - |\omega_{\perp}|. \quad (9)$$

Eqs. 7–9 apply when $3|\omega_{\perp}|\sin^2\theta\sin^2\delta \geq 1.5|\omega_{\perp}|$. Otherwise, the right sides of Eqs. 8 and 9 are interchanged.

The relationship between θ, δ, ω_Y, and ω_Z can be more readily perceived in the graphical form presented in Fig. 4 *b*. In this figure, values of ω_Y and ω_Z (in units of $|\omega_{\perp}|$) are plotted against δ for certain values of θ (90°, 45°, and 35.3°). The shaded portion of Fig. 4 *b* corresponds to the relationship of the frequency ω_Y to δ; the nonshaded portion corresponds to the relationship between ω_Z and δ. This figure shows that in the presence of motion, an axially asymmetric pattern is generally obtained, and that $\eta = 1$ when $\omega_Z = |\omega_{\perp}|$ and $\omega_Y = 0$.

Application of Motional Model to [3,3,3-d_3]Alanine-labeled Collagen Fibrils

The C^{α}–C^{β} bond axis of the alanine methyl group makes an angle θ with the long axis of the collagen helix (the *z* axis in Fig. 4 *a*). The angle δ is half of the angle through which the alanine C^{α}–C^{β} bond axis is carried by reorientation of the peptide backbone (Fig. 4 *a*).

Collagen in solution behaves as a rod-like monomer of dimensions ~1.5 × 300 nm (23), and is free to undergo 360° reorientation about its long axis. The rate of this rotation is ~10^7 s^{-1} (7, 8). End-over-end rotation is slow (~10^2 s^{-1}) and is restricted at the concentrations used for these ^2H NMR experiments due to occupied volume considerations (7). Rapid (10^9–10^{10} s^{-1} (8) internal threefold rotation of the alanine methyl group has already narrowed the ~165 kHz methyl quadrupole coupling constant to the observed ~37 kHz quadrupolar splitting. Therefore, the quadrupolar splitting observed for the frozen [3,3,3-d_3]alanine-labeled fibrils would be expected to collapse by a factor of $(1 - 3\cos^2\theta)/2$ in the presence of free rotation

about the long axis of the molecule. The observed quadrupolar splitting for alanine-labeled collagen in solution is ~10 kHz, which requires that θ be ~70°. An angle θ of 70° is in good agreement with various models for collagen structure, which predict that θ is between 60° and 90° (Table II) (1, 6, 24). These models also show that the angle which the C^{α}–C^{β} bond makes with the helix axis depends upon whether the considered amino acid occurs in the X or Y position of the Gly-X-Y triplet repeat sequence (Table II). Representative values of θ, 85°, and 69° were obtained from an average of the values presented in Table II. (The values for the Ramachandran 2-bond model (6) were not included in this average.) These values of θ were used in the calculation of the lineshape for collagen fibrils in the presence of two-site jump motion. The lineshape patterns from these two values of θ were summed with equal weight because alanine is distributed equally in positons X and Y in the collagen sequence (2). The angle δ and the line broadening were varied until the best match between the experimental and calculated lineshape for [3,3,3-d_3]alanine-labeled collagen fibrils was obtained (Fig. 1 *c* and *g*). The quadrupolar splitting used for these calculations was 37.3 kHz.

The best fit of the data indicates that the collagen molecule in the fibril undergoes reorientation over a ~30° range in azimuthal angle (i.e., 2δ = 30°). This angle is in good agreement with an estimate obtained from previous ^{13}C NMR studies, in which the minimum angle, 2δ, consistent with the T_1 data, was ~30° (9). A line broadening of ~2.5 kHz is also required to fit these data. This line broadening is attributed, in part, to the short T_2 (~105 μs) (18), and in part to the D-H dipolar interactions between residual protons and deuterium on the methyl group (see Results).

Qualitative Analysis of [^{2}H]Leucine-labeled Collagen

A quantitative analysis of the motion of the methyl groups of [^{2}H]leucine-labeled collagen fibrils in terms of a simple two-site jump model is not possible because of multiple reorientations which may occur about the side-chain bonds of leucine. However, the data for [^{2}H]leucine-labeled collagen can be analyzed qualitatively by comparison with the data for [3,3,3-d_3]alanine-labeled collagen.

Apparent values for the quadrupolar splitting for labeled collagen samples were determined from the frequency difference between maxima from the best match between calculated and experimental spectra (Table I). The ^{2}H NMR spectra for [^{2}H]leucine-labeled collagen in solution and as fibrils have smaller quadrupolar splittings than do the respective [3,3,3-d_3]alanine-labeled collagen spectra (Table I). This indicates that motion other than peptide backbone reorientation and threefold methyl rotation must be responsible for the

TABLE II
ANGLE MADE BY THE C^{α}—C^{β} BOND AND THE HELIX AXIS FOR VARIOUS MODELS OF COLLAGEN STRUCTURE

Model	C^{α}—C^{β} angle in position X*	C^{α}—C^{β} angle in position Y*	Reference
	(degrees)	*(degrees)*	
(Gly-Pro-Hyp)$_n$	81	67	24
(Gly-Pro-Pro)$_n$	89	73	1
Ramachandran 2-bond	74	62	6
Ramachandran 1-bond	86	70	6
Rich-Crick 1-bond	82	65	6

*Refers to the position in the Gly-X-Y triplet.

apparent collapse of the quadrupolar splittings. This motion is attributed to reorientation which occurs about the side-chain bonds of leucine. Reorientation about the leucine side-chain bonds is consistent with ^{13}C NMR data for other amino acid side-chains of collagen, in which multiple side-chain bond reorientations were observed for methionine, glutamic acid, and lysine in collagen fibrils (8).

The estimated T_2 for [^{2}H]leucine-labeled collagen fibrils (~130 μs) provides information about the motion of the methyl groups of leucine (other than threefold methyl rotation). The observed collapse of the quadrupolar splitting for leucine-labeled collagen fibrils to about two-thirds of its static value suggests that the leucine side-chain motions in the fibrils are on the fast side of the ^{2}H NMR T_2 minimum. The ~130 μs T_2 for leucine-labeled collagen fibrils therefore suggests that the motion for the leucine side chain is faster than that of the alanine C^α–C^β, for which the methyl T_2 is 105 μs.

GENERAL SUMMARY

Taken together, these ^{2}H NMR data for [3,3,3-d_3]alanine- and [^{2}H]leucine-labeled collagen require a motionally dynamic model for the collagen molecule in the fibril. Analysis of the data in terms of a model in which the alanine C^α–C^β bond axis is considered to jump between two sites separated by an angle 2δ, in a time which is fast compared with the residence time in both sites, indicates that the molecular reorientation occurs over an azimuthal angle of ~30°. This reorientation may be segmental. The average angle between the C^α– C^β bond axis and the long axis of the collagen helix is estimated to be ~75°. The ^{2}H NMR data for the methyl groups of leucine indicate that rotation occurs about the leucine side-chain bonds in the fibrils, in addition to threefold methyl rotation and reorientation of the peptide backbone. Stabilizing interactions that determine fibril structure must therefore depend upon at least two sets of contacts in any given local region.

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Note added in proof: After completing this work we were informed by Dr. Eric Oldfield that equations for the field gradient tensor averaged by 180° flips have been published by G. Soda and T. Chiba. 1969. *J. Chem. Phys.* **50**:439–455. Since any two site jump can be viewed as a 180° flip these authors were the first to derive Eqs 3–5.

DISCUSSION

Session Chairman: Hans Frauenfelder *Scribe*: William A. Wegener

LUMRY: Did you use Redfield's solution for a methyl group spinning on a larger body in your analysis?

JELINSKI: We used an ellipsoid undergoing a jump between two sites and an attached rapidly spinning methyl group represented as a CD bond vector. On our timescale the alanine methyl group undergoes rapid three site jump diffusion. This averaging motion accounts for the ~40 kHz quadrupolar coupling for the methyl group.

NÉMETHY: What accounts for the ±15° azimuthal rotation in the fibrils?

JELINSKI: It's probably a segmental motion and not a concerted process.

NÉMETHY: About the angle θ between the C^{α}–C^{β} bond and the helix axis which you said is 70°, how well does your measurement determine θ and is there any wagging?

JELINSKI: The angle of 70° can be calculated from the collapse of the quadrupolar splitting of the alanine-labeled collagen sample in solution. Average values for this angle, based on an average of 5 models from fiber diffraction studies, suggest that there are two values for θ. These depend upon whether the considered amino acid is in the X or Y position of the Gly-X-Y triplet. The angle θ is 69° in the Y position and 85° in the X position. The computer-simulated spectrum used an equally weighted sum as justified because half of the alanine residues are in the X and half in the Y position. In the fibrils, constraints will probably not permit substantial fluctuations in θ. In addition, the calculated spectra are not strongly dependent upon θ.

WÜTHRICH: Internal mobility is clearly established in many macromolecules. Perhaps it is time to correlate this mobility with functional properties. Have you compared normal and pathogenic collagen?

JELINSKI: We have obtained detailed information about internal motions in collagen through the use of specifically labeled collagen. Using natural abundance ^{13}C NMR we have found little difference between tendon collagen and keloid collagen. A culture system would be needed which could produce labeled pathological collagen in order to do more detailed studies.

BERGET: Does your chick calvaria system produce cross-linked collagen?

JELINSKI: No. We inhibit crosslinking to allow preferential extraction of newly synthesized, i.e. highly labeled, material. We are currently developing a system to produce crosslinked collagen. However, using natural abundance ^{13}C spectra, a comparison was made of steer Achilles tendon collagen and calf Achilles tendon collagen. We found very little difference in the natural abundance ^{13}C NOE values, T_1 values, lineshapes, and cross-polarization enhancements between tendon collagen and calvaria collagen in natural abundance.

DOBSON: In the discussion of your results you comment only briefly on the significance of the T_2 values. Can these values be interpreted quantitatively on the basis of your model? Would you comment on the deuterium T_2 minimum? Is it possible by looking at the T_2 values to pick up different types of motion from those you have described?

JELINSKI: The quadrupolar echo sequence ($90°_x - t_1 - 90°_y - t_2$) preserves the inhomogeneously broadened portion of the line. What we observe is a homogeneous broadening caused by molecular motion. The homogenous T_2 is the unrefocused portion of the deuterium spectrum; it exhibits the same frequency dependence as the T_1. Fig. 2 shows the natural log of the spectral intensity plotted against the sum of the delay times $t_1 + t_2$. The homogeneous T_2 can be obtained from the slope of the line.

It is premature to interpret this in terms of the model, but a minimal value for the T_2 would indicate that the motions are on the order of the quadrupolar splitting.

DOBSON: The spectra of collagen fibrils at 18°C and frozen at −18°C are very different. Have you looked at this temperature dependence in more detail? Do you think that the molecular motion of the collagen is simply related to the freezing of the surrounding water, or is there a gradual increase in molecular motion with temperature as seen, for example, for specific motions in globular proteins (e.g. Campbell et al., *F.E.B.S. Letters* **70**:96).

JELINSKI: Those are good questions. The experiments have not yet been done.

EIKENBERRY: Why did you choose a model with a rapid transition between two sites? How would other models influence your conclusions?

JELINSKI: The model is simple and gives the least discrepancy with what is currently known about collagen structure. It also gives rise to the smallest variation in azimuthal angle.

LEHRER: Do you have any information about the motions involved in fluctuations in chain separation?

JELINSKI: No. I assume those are very slow motions which we can't detect.

F. RICHARDS: To repeat the question, you would not suggest collagen as a good model for tropomyosin?

LEHRER: The question is, are you looking at the right motions?

JELINSKI: Do you mean by interpreting what we are seeing as backbone reorientations? I think we are. Analysis of the ^{13}C NMR relaxation data for these labeled samples, both as fibrils and in solutions, suggests that we are looking at these azimuthal motions.

LEHRER: I meant, are you looking at the relevant motions?

STERNLICHT: I got the impression that the study was based on polycrystalline samples, i.e., a random orientation of collagen fibers. Is that correct?

JELINSKI: Yes. The samples are like collagen in the skin in the sense that they were randomly oriented and fully hydrated.

STERNLICHT: Are there any advantages to using oriented fibrils?

JELINSKI: Yes. You could use oriented samples to obtain orientations of specific groups with respect to the fiber axis. Also, collagen fibers in x-ray studies are stretched to remove a crimp in the structure. It would be interesting to compare stretched and unstretched samples by NMR.

EIKENBERRY: To correct any misimpressions, fibers don't have to be stretched to get diffraction patterns, but stretching improves the pattern.