faster for calcium than for magnesium.

In conclusion, X-14885A behaves like calcimycin as a selective calcium ionophore. The selectivity differences between the ionophores is predominantly of kinetic origin, with faster formation and dissociation rates for calcium complexes than for magnesium. Replacement of the -NHMe group in calcimycin by an -OH group in X-14885A has a drastic effect on the acid-base properties of X-14885A and on the proton-assisted dissociation pathway of its complexes.

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Solid-State ^2H NMR Study of Thymidine. Base Rigidity and Ribose Ring Flexibility in Deoxynucleosides

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Abstract: We have investigated the internal motions of the nucleoside thymidine in the solid state by deuterium NMR spectroscopy. The base position was found to be essentially rigid, even at elevated temperatures. On the other hand, T_1 measurements on 2',2''-dideuteriothymidine indicated the presence of small-amplitude motions on the nanosecond time scale. In addition, spin alignment and distorted echo experiments revealed the presence of large-amplitude motion on millisecond to microsecond time scales. This motion is hypothesized to be 2'-endo ↔ 3'-endo interconversion. It was also shown that the large-amplitude motion may be very sensitive to the crystal packing forces.

It has been clear for a while that the three-dimensional conformation of biological macromolecules is the determinant of biological function.[1] A molecule may have more than one such stable conformation available to it. If transitions between such conformations are kinetically possible, then more than one conformation can be utilized for more than one biological task. Thus, a measure of dynamic flexibility (i.e., transitions between different conformations) can provide information about accessible conformations of a biomolecule.

A number of techniques have been employed to measure dynamical properties of molecules.[2] Nuclear magnetic resonance spectroscopy has been widely used to measure motional properties in solution and in the solid state.[3-6] Several types of nuclei can be utilized as probes for measuring dynamics in the solid state. One of the most useful nuclei is ^2H. This quadrupolar nucleus has been used to detect motions in the 10^{-5}-10^{-11}-s range.[7] Recently, spin alignment experiments[8,9] were used to detect motions on very slow time scales. DNA in the cell nucleus spends most of its time in a solidlike environment. There it undergoes many conformational changes, some of which involve a change in conformation of the sugar residues (e.g., B to Z, B to A, etc). Energetics of such transitions have been reported in solution, but very little is known in solid or solidlike states.

We have focused our attention on characterization of the motions of sugar rings of nucleosides in the solid state. In solution, nucleosides are known to interconvert rapidly between 2'-endo and 3'-endo conformations. Such information about ring mobility of nucleosides in the solid state was not available before. Previously we have reported a preliminary study of sugar ring mobility in thymidine and 2'-deoxyguanosine.[10] Here we present a further characterization of such motion in the solid state.

Experimental Section

Thymine-*methyl*-d_3 was purchased from Merck, Sharp and Dohme Isotopes. 2-Deoxyribose 1-phosphate and thymidine phosphorylase were obtained from Sigma Chemical Co. Thymidine phosphorylase was dialyzed twice against 2 L of 0.01 M Tris-*d* and 0.05 M NaCl (pH 7.5) for 2 days, before use. A 10-mL portion of 2-deoxyribose 1-phosphate in water was added to 500 mL of 0.2 M Tris-*d* (pH 7.5) containing 0.05 M NaCl. A 10-mL portion of 0.2 M thymine-*methyl*-d_3 in 0.05 M NaOH was added. This was followed by addition of approximately 300 units of thymidine phosphorylase. Progress of the reaction was monitored by the change in optical density at 295 nm. After the reaction was completed, the reaction mixture was lyophilized, redissolved in water, and purified according to Roy et al.[10] Purity of the sample was checked by UV and ^1H NMR spectra. Since recrystallization of thymidine-*2'-C,2'-C*-d_2 was difficult because of the small amount, all samples for the solid-state NMR work were lyophilized from H_2O or D_2O. Thymidine-*3,3'-O,5'-O*-d_3 was obtained by dissolving thymidine in D_2O and then lyophilizing the solution to remove excess D_2O.

Solid-state ^2H NMR spectra were observed at 38.45 and 76.76 MHz by a home-built[11] and a modified[12] NIC-500 spectrometer, respectively. The 90° pulse length was 2.2 and 2.5 μs at 38.45 and 76.76 MHz. Anisotropic T_2 distortion was measured by changing delay time between the two pulses. Spin alignment echo experiments were performed at 76.76 MHz. The delay time between the first pulse and second pulse, τ_1, was 30 μs, and the delay time between the second pulse and third pulse, τ_2, varied from 500 μs to 100 ms.

Results

Base Rigidity. Figure 1 shows ^2H NMR spectra of thymidine-*methyl*-d_3 at +75 °C (a) and +22 °C (b), which have typical ^2H NMR line shapes for the reorientating methyl group. However,

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(1) Anfinsen, C. B.; Haber, E.; Sela, M.; White, F. H., Jr. *Proc. Natl. Acad. Sci. U.S.A.* **1961**, *47*, 1309-1314.
(2) Bayley, P. M., Dale, R. E., Eds. *Spectroscopy and the Dynamics of Molecular Biological Systems*; Academic Press: London, 1985.
(3) *Mobility and function in proteins and nucleic acids*; Ciba Foundation Symposium 93; Pitman Books: London, 1983.
(4) Griffin, R. G. *Methods Enzymol.* **1981**, *72*, 108-174.
(5) Opella, S. J. *Annu. Rev. Phys. Chem.* **1982**, *33*, 533-562.
(6) Torchia, D. A. *Annu. Rev. Biophys. Bioeng.* **1984**, *13*, 125-144.
(7) Spiess, H. W.; Sillescu, H. *J. Magn. Reson.* **1981**, *42*, 381-389.
(8) Spiess, H. W. *J. Chem. Phys.* **1980**, *72*, 6755-6762.
(9) Fujara, F.; Wefing, S.; Spiess, H. W. *J. Chem. Phys.* **1986**, *84*, 4579-4584.
(10) Roy, S.; Hiyama, Y.; Torchia, D. A.; Cohen, J. S. *J. Am. Chem. Soc.* **1986**, *108*, 1675-1678.
(11) Sarkar, S. K.; Sullivan, C. E.; Torchia, D. A. *J. Biol. Chem.* **1983**, *258*, 9762-9767.
(12) Hiyama, Y.; Silverton, J. V.; Torchia, D. A.; Gerig, J. T.; Hammond, S. J. *J. Am. Chem. Soc.* **1986**, *108*, 2715-2723.

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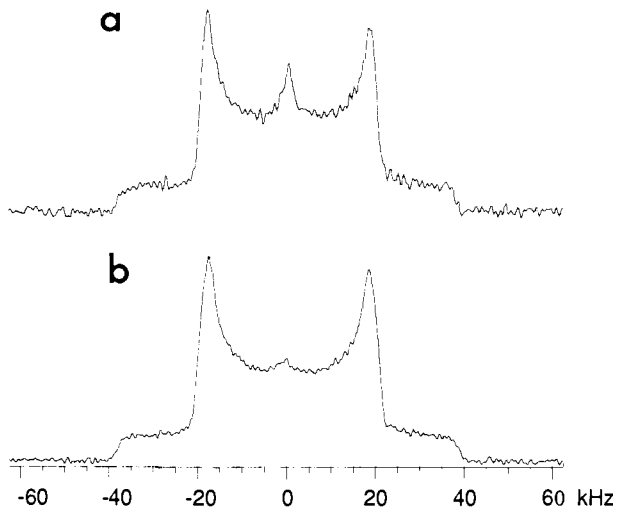

Figure 1. 76.76-MHz ^2H NMR of thymidine-*methyl*-d_3 (a) at +75 °C with 160 scans and (b) at +22 °C with 640 scans.

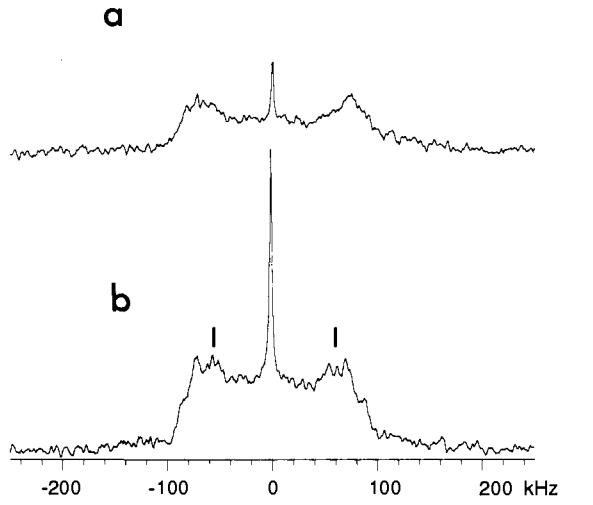

Figure 2. ^2H NMR of thymidine-*3,3′-O,5′-O*-d_3 (a) at +75 °C with a cycle delay time of 450 s and (b) at +22 °C with a cycle delay time of 600 s.

it should be noted that (a) the asymmetry parameter of the spectra is about 6%, unusually high for a methyl group powder pattern, and (b) quadrupole splitting (between the two singularities) is only 35 kHz. These features are caused by an electronic effect on the deuterium electric field gradient (EFG) tensor of the methyl deuterons of thymine[13] rather than by further motional averaging proposed by a recent study.[14]

There was no substantial change in the methyl ^2H line shape from −125 to +95 °C. No T_2 distortion was observed in this temperature range, which indicates that there is no slow (correlation time, τ_c, range 10^{-4}–10^{-8} s) motion. Above −50 °C, the spin–lattice relaxation time (T_1) was not field dependent (at 38.45 and 76.76 MHz) so the correlation time of methyl reorientation is on the fast side ($\omega_0 \tau \ll 1$) of the T_1 minimum. The ^2H T_1 at +22 °C was 860 ms. Using the formalism of Torchia and Szabo,[15] and ignoring the electronic effect on the ^2H EFG, correlation times of the methyl reorientation assuming a 3-fold jump model were calculated. An Arrhenius plot of the correlation times against $1/T$ gives a straight line, yielding an apparent activation energy of 1.65 kcal/mol and a preexponential factor of 2.0×10^{-13} s, which are identical with values found for thymine. Echo heights of the spin alignment experiments showed no decay up to τ_2 = 50 ms, whih suggests the absence of ultraslow motion at the methyl position.

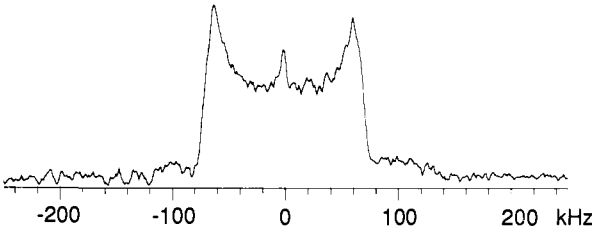

Figure 3. ^2H NMR spectrum of deoxyguanosine-*8-d*. Conditions: 240 acquistions; cycle delay time 10 s.

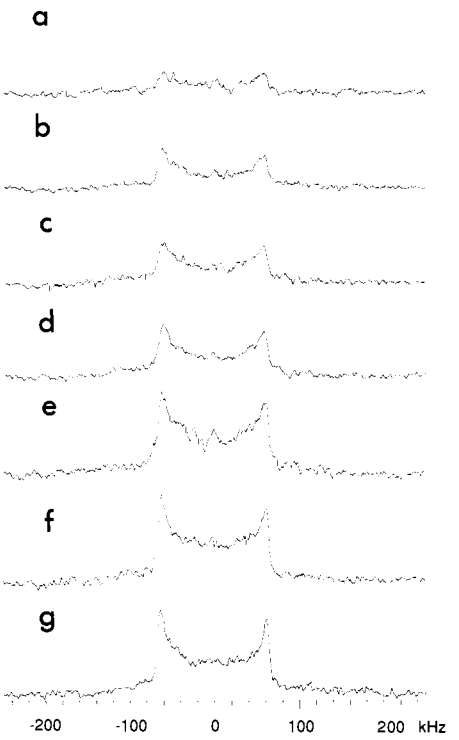

Figure 4. Temperature dependence of ^2H line shape in thymidine-*2′-C,2′-C*-d_2 at (a) 72 °C, (b) 68 °C, (c) 65 °C, (d) 61 °C, (e) 22 °C, (f) −66 °C, and (g) −125 °C. Delay time between the two pulses of the quadrupole echo sequence was 30 μs.

The powder ^2H NMR spectrum of thymidine-*3,3′-O,5′-O*-d_3 at +22 °C is a superposition of three powder patterns (Figure 2b). A room-temperature X-ray structure showed that hydrogen bond lengths, XH···Y, are 2.74 and 2.79, and 2.85 Å for the 3′-, 5′-, and 3-positions, respectively.[16] On the basis of an empirical relationship between X^2H···Y lengths and ^2H quadrupole coupling constants,[17-19] position 3 (N^2H···O) can be clearly distinguished from the others in terms of singularity position in powder spectrum; however, the 3′- and 5′-positions cannot be discriminated because of the similarity in the hydrogen bonding. ^2H T_1 measurements showed that the position 3 deuterium had a T_1 of more than 200 s, while the components corresponding to the 3′- and 5′-positions had $T_1 \approx 30$ s. This relaxation data also support the conclusion that the methyl reorientation is the only motion at the base position of thymidine.

Figure 3 shows the ^2H NMR spectrum of C-8 deuterated deoxyguanosine at +22 °C, whose shape is nearly identical with those of guanosine and adenosine reported earlier.[20] The nonzero

(13) Hiyama, Y.; Roy, S.; Guo, K.; Butler, L. G.; Torchia, D. A. *J. Am. Chem. Soc.* **1987**, *109*, 2525–2526.
(14) Kintanar, A.; Alam, T. M.; Huang, W.-C.; Schindele, D. C.; Wemmer, D. E.; Drobny, G. *J. Am. Chem. Soc.* **1988**, *110*, 6367–6372.
(15) Torchia, D. A.; Szabo, A. *J. Magn. Reson.* **1982**, *49*, 107–121.
(16) Young, D. W.; Tollin, P.; Wilson, H. R. *Acta Crystallogr. Sect. B: Struct. Crystallogr. Cryst. Chem.* **1969**, *B25*, 1423–1432.
(17) Soda, G.; Chiba, T. *J. Chem. Phys.* **1969**, *50*, 439–455.
(18) Hunt, M. J.; Mackay, A. L. *J. Magn. Reson.* **1974**, *15*, 402–414.
(19) Hiyama, Y.; Keiter, E. A.; Brown, T. L. *J. Magn. Reson.* **1986**, *67*, 202–210.
(20) Shindo, H.; Hiyama, Y.; Roy, S.; Cohen, J. S.; Torchia, D. A. *Bull. Chem. Soc. Jpn.* **1987**, *60*, 1631–1640.

Table I. Averaged Spin Lattice Relaxation Times and Quadrupole Splitting of Thymidine-*2'-C,2'-C-d_2*

temp, °C	T_1, ms 76.76 MHz	T_1, ms 38.45 MHz	quadrupole splitting, kHz[a]
+69	80		122
+57	85		122
+22	142	114	122
-12	250		122
-38	500		122
-45		300	122
-66	2500		123
-125	7000		125

[a] Frequency separation between the two singularities.

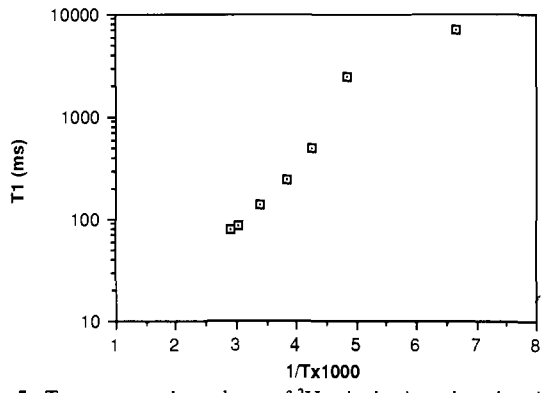

Figure 5. Temperature dependence of ^2H spin-lattice relaxation time in thymidine-*2'-C,2'-C-d_2*.

asymmetry parameter (0.08) is caused by the environment in the imidazole ring rather than motional averaging.[19,21] The ^2H T_1 of deoxyguanosine was 1.5 s at 76.76 MHz and 1.1 s at 38.45 MHz, suggesting the existence of small-amplitude (≤2° root mean square (rms)) fast motion, whose correlation time is about 10^{-9} s. Spin alignment echo heights of deoxyguanosine remained about same as τ_2 increased from 500 μs to 40 ms. These results show that the base position of this nucleoside is also essentially rigid.

Deoxyribose Ring Flexibility. At −125 °C, the ^2H NMR spectrum of thymidine-*2'-C,2'-C-d_2* gave a Pake doublet with a quadrupole splitting of 125 kHz (Figure 4g), a typical value for a rigid C–^2H bond. The ^2H spin-lattice relaxation time of 7 s also supports the idea that the C–H bond is rigid. Because the quadrupole splitting at +65 °C (122 kHz) is nearly identical with the 125-kHz splitting observed at −125 °C, the C–^2H bond undergoes only small-amplitude motion, ≤5° rms, in the −125 to +65 °C temperature range.

The ^2H spin-lattice relaxation times of thymidine-d_2 were measured at the two field strengths listed in Table I. At 22 °C, T_1 was 142 ms at 76.76 MHz and 114 ms at 38.45 MHz. These values are surprisingly small considering the small reduction of the quadrupole splitting observed in the ^2H spectra from −125 to +22 °C (Figure 4). This shows that the small-amplitude motion (rms angle of ≃5°) has a correlation time close to the T_1 minimum: $\tau \approx 10^{-9}$ s. Below −30 °C, the field dependence became more significant, which suggests that correlation time of small-amplitude motion increases and is on the slow side of the T_1 minimum at low temperature. An Arrehnius plot of T_1 against $1/T$ yielded an apparent activation energy of 3.8 kcal/mol (Figure 5). This motion probably corresponds to a conformational change between substates such as between C3'-exo and C2'-endo modulating the C–^2H bond vector by a small amplitude.[22]

Above 68 °C, the total intensity of the ^2H NMR spectra in thymidine-*2'-C,2'-C-d_2* decreased precipitously in the temperature range 70–95 °C as shown in Figure 6. The loss of intensity was caused by a large-amplitude rms 30° motion on the time scale

(21) Tsang, P.; Vold, R. R.; Vold, R. L. *J. Magn. Reson.* **1987**, *171*, 276–282.

(22) Levitt, M.; Warshel, A. *J. Am. Chem. Soc.* **1978**, *100*, 2607–2613.

Table II. Conformational Change Determined by Spin Alignment

temp, °C	population, %[a] C3'-exo	C3'-end	f	τ_c, ms	T_{1Q}, ms
25	95	5	0.90	1	170 (40)
46	84	16	0.73	1	120 (20)
55	50	40	0.4	1	100 (20)
60	≤50	≤50	0.3	1	100 (30)

[a] Relative populations are calculated based upon a two-site jump model. Note that this assumption fails at 55 and 60 °C.

10^{-5}–10^{-7} s, because T_2 became extremely short, less than 40 μs for most orientations, from 68 to 72 °C (Figure 6). Note that no edge of the powder pattern survived, strongly suggesting that the slow motion is multisite jumping. It is interesting that this motion exists only at the deoxyribose ring positions, since the line shape and intensity of the methyl deuterons at 75 °C were essentially identical with those observed at 22 °C (Figure 1a).

In order to investigate whether this large-amplitude slow motion existed at temperatures below +65 °C, we performed a series of spin alignment experiments.[8,9] This measurement is sensitive to motions having correlation times as large as T_{1Q}. Because the T_1 of thymidine-d_2 is more than 100 ms at 25 °C, motions having correlations times in the range 10^{-2}–10^{-4} s can be studied.

In the case of jumps among N inequivalent sites, the correlation function for spin alignment $F(\tau_1, \tau_2)$ has a limiting value, $F(\tau_1 \gg \omega_Q, \tau_2 \gg \tau_c) = \sum P_i^2$ (hereafter f), where τ_c is the correlation time of the motion and p_i is the normalized relative population of ith site.

Alignment echo height, $S(\tau_2)$, can be written as

$$S(\tau_2) \approx (1 - f) \exp(-\tau_2/\tau_c - \tau_2/T_{1Q}) + f \exp(-\tau_2/T_{1Q})$$

At 25 °C, the echo intensity data taken with $\tau_1 = 30$ μs (Figure 7) are accounted for with $f = 0.90$ (±0.02), $\tau_c = 1$ ms (uncertainty in τ_c is 1 order of magnitude), and $T_{1Q} = 170$ (±40) ms (Table II). This means that population of the major conformation is 95%, if one assumes that there are only two conformations. We assign the major conformation as C3'-exo, because this conformation is found in the X-ray structure.[16] At 46 °C, $f = 0.73$ (±0.02), $\tau_c = 1$ ms, and $T_{1Q} = 120$ (±20) ms fit the data well. These parameters indicate that at 46 °C the population of the minor conformation has risen to 16% while population of the C3'-exo conformation has fallen to 84%. At 55 °C the two-site jump model fails to account for the data because the fitted value for $f = 0.4$ (±0.05) whereas the minimum value of f for the two-site jump is 0.5. This result suggests that above 55 °C there are at least three conformations that have substantial populations.

The large uncertainty of correlation times measured by the spin alignment experiment precluded determination of the activation energy of the slow motion in thymidine. However, our spectra suggest that the motion observed by the spin alignment experiment becomes fast enough ($\tau_c \leq 10^{-4}$ s) to show the T_2 distortion above 65 °C. We estimate an activation energy for the slow motion between 5 and 10 kcal/mol, because the time scale of this motion is substantially slower than that of the small-amplitude nanosecond fluctuation, which has an apparent activation energy of 3.8 kcal/mol.

The ^2H NMR spectrum of thymidine-*3,3'-O,5'-O-d_3* at 75 °C (Figure 2a) differed from the spectrum at 22 °C in two respects: (1) the intensity decreased by a factor of 2.5 upon going from 22 to 75 °C, and (2) the overall breadth of powder spectrum increased by 20 kHz from 22 to 75 °C. Because the methyl deuteron data showed that the base was rigid up to at least 95 °C, the total loss of signal can be attributed to short T_2 at 3'- and 5'-positions caused by a large-amplitude slow motion. Whether or not this motion is the one that was observed at the C2'-position is not determined because there are other possible rotations such as either along the C3'–O3' axis or along the C4'–C5' axis. At 22 °C the 3-position (N–^2H) makes a hydrogen bond to the 3'-oxygen of neighboring molecule in the crystal. However at the high temperature, the 3'-oxygen may not be available for hydrogen bond formation because of the large-amplitude motion of the deoxyribose ring. Non-hydrogen-bonded N–^2H deutrons have a

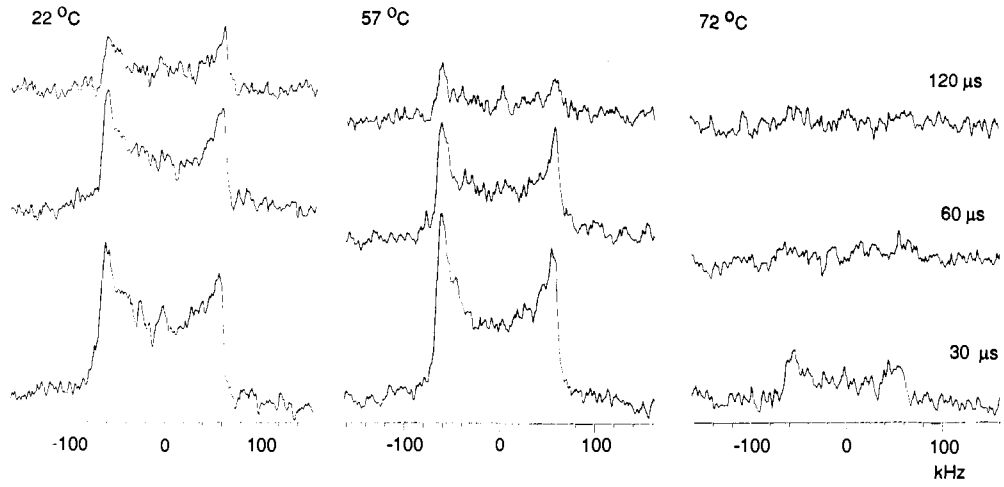

Figure 6. Temperature dependence of 76.76-MHz ^{2}H NMR spectra in thymidine-2′-*C*,2′-*C*-d_2 with echo delay time of 120, 60, and 30 μs. Conditions: 800 scans; 2000-Hz Lorentzian broadening added.

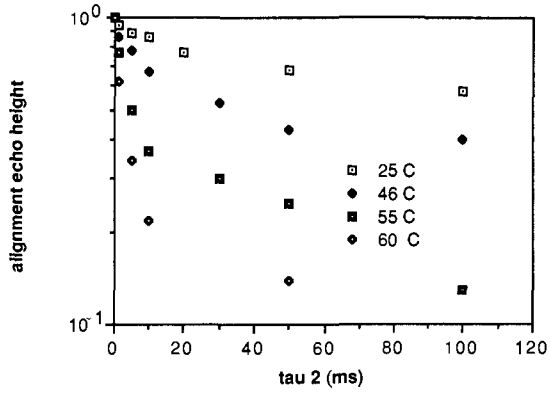

Figure 7. Spin alignment echo heights of thymidine-2′-*C*,2′-*C*-d_2 (a) at +25 °C, (b) at +46 °C, (c) at +55 °C, and (d) at +60 °C. Echo heights were normalized to three-eighths of the quadrupole echo at each temperature. τ_1 = 30 μs.

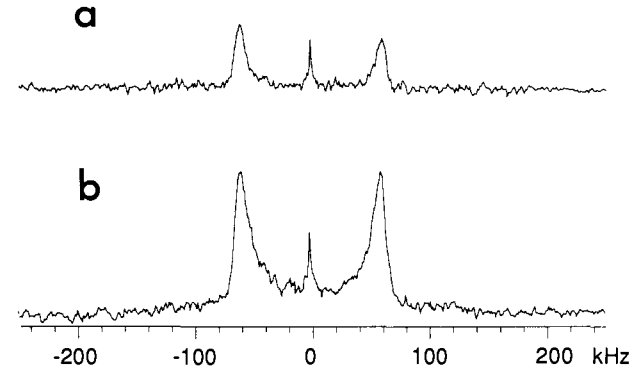

Figure 8. ^{2}H NMR spectra of thymidine-2′-*C*,2′-*C*-d_2 contaminated with Tris, at 22 °C (a) with echo delay of 60 μs and (b) with echo delay of 30 μs.

larger quadrupole coupling constant (over 200 kHz) than their hydrogen-bonded counterparts.[18,19] Therefore, the absences of hydrogen bonding at high temperature presumably cause the larger breadth of the powder spectrum observed at 75 °C as compared with 22 °C.

In contrast with the spectrum of salt-free thymidine, the spectrum of crystalline thymidine-2′-*C*,2′-*C*-d_2 containing Tris (nonpurified sample) showed significant T_2 distortion at 22 °C (Figure 8). This distortion indicates that a large-amplitude (≥30° rms), slow (correlation time $\simeq 10^{-6}$ s) motion exists in the thymidine/Tris sample even at 22 °C. Since Tris does not bind covalently to thymidine, it is likely that differences in crystalline packing cause the difference in motion observed in the thymidine and thymidine/Tris samples. The T_1 of the thymidine/Tris sample at 22 °C, 76.76 and 38.45 MHz, was about 120 ms, which suggests that the sugar ring also undergoes small fast (nanosecond) motion in addition to the slow motion.

Discussion

The major advantage of solid-state NMR is that one can study internal molecular motions that are usually masked in solution by overall isotropic molecular tumbling. In the nucleoside crystals, overall molecular motion is absent as base positions are essentially rigid, although the ^{2}H NMR study by Kintanar et al.[14] concluded that there is a fast small-amplitude libration of the base because of a relatively short relaxation time at the C6 deuteron. In contrast, there are two types of motions at the C–^{2}H position of the deoxyribose rings: (1) small amplitude (≤5° rms) on the nanosecond time scale and (2) large amplitude (30° rms) on the milisecond to microsecond time scale. The former motion can be attributed to small a conformational change such as between either C3′-exo and C2′-endo or C2′-exo and C3′-endo. This nanosecond motion does not exist at the 3′- or 5′-hydroxyl positions since these sites have large T_1 values at 22 °C.

The time scale of the large-amplitude motion ranged from 1 ms in pure thymidine to 1 μs in thymidine/Tris and in deoxyguanosine. Two possible sources that can change the time scale (or activation energy) of this dynamic process are (a) crystalline packing and (b) hydrogen bonding. In phenylalanine crystals, the activation energy of 180° phenyl ring flip varies from 5 to 11 kcal/mol.[12] The L-methionine crystal unit cell contains two molecules that differ greatly in their internal flexibility.[23] In a cyclic peptide, one of the proline rings is flexible while the other is not.[24] These results clearly showed that crystalline packing can affect the amplitude and activation energy of large-amplitude internal molecular motions. Other studies show that the saturated five-membered rings have the capability of changing conformation rapidly (nanosecond or faster) at room temperature. For instance, the proline ring in the crystals shows large amplitude (30° rms) and very rapid (picosecond) motion at 22 °C.[25] Cyclopentane undergoes rapid internal motion below −150 °C.[26] The presence of hydroxyl groups enables the five-membered ring to form hydrogen bonds that can add at least 2 kcal/mol to the activation energy associated with a conformational change. Several theoretical calculations on ribose ring dynamics have predicted a small activation energy ca. 2 kcal/mol for the C2′-endo ↔ C3′-endo

(23) Sparks, S. W.; Young, P. E.; Budhu, N.; Torchia, D. A. *J. Am. Chem. Soc.* **1988**, *110*, 3359–3367.
(24) Sarkar, S. K.; Torchia, D. A.; Kopple, K. D.; VanderHart, D. L. *J. Am. Chem. Soc.* **1984**, *106*, 3328–3331.
(25) Sarkar, S. K.; Young, P. E.; Torchia, D. A. *J. Am. Chem. Soc.* **1986**, *108*, 6459–6464.
(26) Mack, J. W.; Torchia, D. A. Manuscript in preparation.

interconversion and nearly no activation energy for small conformational change (e.g., C3'-exo <<*mr C2'-endo).[27,28] These calculations did not take account of crystalline packing or hydrogen bonding.

(27) Olson, W. K.; Sussman, J. L. *J. Am. Chem. Soc.* **1982**, *104*, 270-278.
(28) Olson, W. K. *J. Am. Chem. Soc.* **1982**, *104*, 278-286.

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Registry No. D_2, 7782-39-0; thymine-*methyl*-d_3, 68941-98-0; 2-deoxyribose 1-phosphate, 17039-17-7; thymidine-2',2'-d_2, 100447-82-3; thymidine, 50-89-5.

Hyperfine Interactions of ^{57}Fe in Human Transferrin: An ENDOR Spectroscopic Study

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Abstract: Electron nuclear double resonance (ENDOR) spectra have been obtained for high-spin $^{57}Fe(III)$ complexes of human serum transferrin and ethylenebis[(*o*-hydroxyphenyl)glycine] (EHPG), a compound used to model the specific sites of transferrin. The pseudonuclear Zeeman effect (PNZE), representing interaction of electronic Zeeman and hyperfine terms of the spin Hamiltonian, is primarily responsible for splitting of ENDOR lines, the nuclear Zeeman term making a negligible contribution to line separation. The sensitivity of the PNZE to zero-field splittings makes it possible to extract fine structure as well as hyperfine parameters of the spin Hamiltonian from analysis of the ^{57}Fe ENDOR spectra. Differences between the two sites of transferrin are reflected in *D*, the axial parameter of the fine structure operator.

The study of hyperfine interactions by EPR spectroscopy has provided valuable information regarding the ligand structure of metal-binding sites in metalloproteins and the electronic configuration of the bound metal ions. Transferrin, a protein functioning in the transport and delivery of iron to cells, has been the subject of numerous spectroscopic studies probing the ligand structure of its two specific metal-binding sites. EPR, pulsed EPR, and electron nuclear double resonance (ENDOR) spectroscopies have been particularly productive in the study of the Cu(II) and VO^{2+} complexes of transferrin, revealing differences between the two sites,[2,3] the presence of a single histidyl nitrogen ligand,[4,5] the coordination to the metal ion of the synergistic anion required for metal binding,[5] and the accessibility of bound metal ion to solvent.[6] Despite these successes, details of hyperfine structure in the physiologically important Fe(III) complexes of transferrin have been elusive as they are obscured by relatively large line widths in the EPR spectra (>15 G).

We now report observation of hyperfine structure in ^{57}Fe-(III)-transferrin complex by means of electron nuclear double resonance spectroscopy. A computer fit of experimental data to an appropriate spin Hamiltonian has permitted determination of the hyperfine coupling and zero-field parameters in the protein's physiological iron-bearing state. Site-specific information was obtained by selectively loading one metal-binding site at a time with ^{57}Fe. Similar studies were carried out on ethylenebis[(*o*-hydroxyphenyl)glycine] (EHPG), a compound that has been used to model the Fe(III)-binding sites of transferrin.[7-10]

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(2) Zweier, J. L.; Aisen, P. *J. Biol. Chem.* **1977**, *252*, 6090.
(3) Chasteen, N. D.; White, L. K.; Campbell, R. F. *Biochemistry* **1977**, *16*, 363.
(4) Roberts, J. E.; Brown, T. G.; Hoffman, B. F.; Aisen, P. *Biochem. Biophys. Acta* **1983**, *747*, 49.
(5) Zweier, J. L.; Aisen, P.; Peisach, J.; Mims, W. B. *J. Biol. Chem.* **1979**, *254*, 3512.
(6) Zweier, J. L. *J. Biol. Chem.* **1980**, *255*, 2782.
(7) Gaber, B. P.; Miskowski, V.; Spiro, T. G. *J. Am. Chem. Soc.* **1974**, *96*, 6868.
(8) Pecoraro, V. L.; Harris, W. R.; Carrano, C. J.; Raymond, K. N. *Biochemistry* **1981**, *20*, 7033.
(9) Spartalian, K.; Carrano, C. J. *J. Chem. Phys.* **1983**, *78*, 4811.

Materials and Methods

Sample Preparation. Human serum transferrin was purchased from Calbiochem-Behring. Since this commercial preparation displayed a homogeneous band on SDS-PAGE and bound two Fe atoms/molecule, it was used as received. Protein was freed from iron and chelating agents by previously reported methods.[11] The resulting apoprotein was used to prepare the diferric and monoferric transferrins preferentially loaded at either the N- or C-terminal sites with ^{56}Fe (as $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ in 1 mM HCl) or ^{57}Fe (as $^{57}Fe_2O_3$ in 6 N HCl).[12,13] After iron loading, all samples were dialyzed against two changes of Hepes buffer (0.05 M Hepes and 0.1 M KCl (pH 7.4)) and concentrated in a collodion membrane ultrafiltration apparatus (Schleicher and Schuell). Samples were transferred to Wilmad precision EPR tubes and stored at liquid nitrogen temperature. Purity of the monoferric preparations was verified by urea gel electrophoresis[11] (Figure 1).

The pure meso isomer of EHPG was the generous gift of Dr. Carl Carrano. Samples for EPR spectroscopy were prepared by adding the ligand to a solution of $FeCl_3$ obtained from 99.9% pure iron wire or 95.6% ^{57}Fe metal. Complete dissolution of the EHPG was attained by adjustment to pH 0.8 with HCl and addition of a slight molar excess of Fe(III). Samples were then brought to 1 mM EHPG, 2 mM $NaClO_4$, 50 mM Hepes, and 100 mM KCl (pH 7.4). The complex gave a single band by thin-layer chromatography, in contrast to the two bands obtained from commercial EHPG (Sigma).

Reagents. Isotopically enriched $^{57}Fe_2O_3$ (90.68%) was supplied by Oak Ridge National Laboratory; ^{57}Fe metal was from Isotec. Other reagents were of the highest grade obtainable. To minimize contamination by extraneous metal ions, all buffers were treated with Chelex 100. Glassware and electrophoresis apparatus were rendered iron free by soaking in 4 N HNO_3 followed by extensive washing with doubly distilled deionized water.

Spectroscopy. EPR spectra were obtained at 77 K with either an IBM-Bruker ESR 200D-SRC or a Varian E-9 spectrometer with TE_{102} cavities. Wilmad precision EPR tubes were used for all samples.

ENDOR experiments were performed at 2.3 K with a Bruker EN810 ENDOR accessory with a 200-W ENI amplifier and a TM_{110} ENDOR cavity mounted in an Oxford ESR 10 cryostat. Data were obtained with

(10) Patch, M. G.; Simolo, K. P.; Carrano, C. J. *Inorg. Chem.* **1983**, *22*, 2630.
(11) Aisen, P.; Leibman, A.; Zweier, J. *J. Biol. Chem.* **1978**, *253*, 1930.
(12) Zak, O.; Aisen, P. *Biochemistry* **1988**, *27*, 1075.
(13) Thompson, C. P.; Grady, J. K.; Chasteen, N. D. *J. Biol. Chem.* **1986**, *261*, 13128.

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