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^{13}C NMR Studies of the Molecular Dynamics of Selectively ^{13}C-Enriched Ribonuclease Complexes†

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ABSTRACT: ^{13}C spin–lattice (T_1) relaxation times determined at four frequencies (25, 68, 100, and 125 MHz) have been used to probe the molecular dynamics of ribonuclease S′ complexes prepared from synthetic amino-terminal peptides containing ^{13}C enrichment (ca. 90%) at selected sites [Niu, C., Matsuura, S., Shindo, H., & Cohen, J. S. (1979) *J. Biol. Chem. 254*, 3788]. It was found that the motion of the C^{α}–H bond of Ala-5 could not be determined by isotropic reorientation alone. The time scale and spatial restriction on the internal motion of this residue were determined by the model-free approach of Lipari and Sazbo [Lipari, G., & Szabo, A. (1982) *J. Am. Chem. Soc. 104*, 4546–4559]. It was found that the C^{α}–H bond, in addition to an overall correlation time of 20 ns, underwent internal motion with a correlation time of 0.5 ns and a generalized order parameter $\mathcal{S}$ corresponding to a cone semiangle of 23 °C. The C^{β}–H bond had a correlation time of 37 ps, reflecting the fast rotation of the methyl group, and had an $\mathcal{S}$ value close to that expected if the C^{α}–C^{β} and C^{α}–H bonds have the same degree of spatial restriction.

NMR spectroscopy is now widely used in studies of the conformation and mobility of proteins in solution (Cohen et al., 1983). But there have in general been many fewer applications of ^{13}C NMR than of ^{1}H NMR to proteins because of the lower sensitivity of ^{13}C NMR (1.6%) and its lower natural abundance (1.1%) compared to ^{1}H. However, it is particularly useful to observe ^{13}C relaxation in order to determine mobility in proteins, because of the averaging of proton relaxation rates due to spin diffusion in proteins (Kalk & Berendsen, 1976).

Several years ago, Niu et al. (1979) synthesized selectively ^{13}C-labeled amino-terminal peptides of ribonuclease (RNase) in order to study their fully enzymatically active RNase S′ complexes. The greater sensitivity resulting from use of the high ^{13}C enrichment (ca. 90%), and the absence of ambiguity in the assignments of the signals observed well above the background of natural abundance resonances, allowed single carbon atom sites in this enzyme to be studied in detail. Several examples of selectively ^{13}C-labeled proteins have been published (Eakin et al., 1975; Jones et al., 1976; Blakley et al., 1978; Deber et al., 1978; Schejter et al., 1978; Cohen et al., 1979; Harina et al., 1980; Matta et al., 1980; Wooten et al., 1981). These have frequently been prepared biosynthetically, resulting in the presence of multiple peaks in the spectrum and the need for subsequent assignment.

We now wish to report ^{13}C relaxation time measurements on the ^{13}C-labeled RNase complexes and their analysis in terms of protein mobility. We have carried out these relaxation time determinations at several observing frequencies in order to satisfy the needs for rigorous analysis, particularly to distinguish between different mechanisms of relaxation that may be operative (Doddrell et al., 1972; Lyerla & Levy, 1974; Norton et al., 1977). A few examples of ^{13}C relaxation time measurements of individually resolved resonances in proteins at several frequencies have been reported previously (Jones et al., 1976; Richarz et al., 1980; Ribeiro et al., 1980; Norton et al., 1977). Earlier work tended to be at a single lower field strength [for review, see Egan et al. (1977) and Howarth & Lilley (1978)].

Recently, a new model-free approach to the analysis of NMR relaxation data of macromolecules in solution has been developed (Lipari & Szabo, 1982). We have applied this theory to the ^{13}C relaxation data for the Ala-5 residue of RNase S′ with data obtained at four frequencies. Our conclusions are that, in addition to the overall motion, a component of local mobility is needed to satisfactorily explain the relaxation time data. Using the Lipari–Szabo theory, we are able to estimate the time scale and the amplitude of this local motion.

Experimental Procedures

Materials. The three ^{13}C-enriched (1–15) RNase amino-terminal peptides were synthesized and purified as previously

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reported (Niu et al., 1979). Bovine pancreatic RNase S protein (type XII-S, grade XII-PR) and the sodium salt of cytidine cyclic 2′,3′-phosphate were obtained from Sigma Chemical Co. RNase A (phosphate free) used to calibrate the assays was purchased from Worthington Biochemical Corp.

Instrumentation. The ^{13}C NMR spectra were recorded with five pulse Fourier transform NMR spectrometers using broad-band proton decoupling in each case. They were as follows: JEOL FX-100 (25.1 MHz, two instruments); NIH-modified spectrometer with a Bruker Magnet and Nicolet 1180 computer (67.9 MHz); Bruker WH-400 (100.6 MHz), at the National burea of Standards; Nicolet NT500 (125.7 MHz).

Temperature was carefully controlled, typically at 27 ± 1 °C, and was kept below 40 °C at all times to avoid protein denaturation (Bock et al., 1980). Generally, temperature equilibration was accomplished with cooled nitrogen gas and a feedback heating system. To minimize radiative heating due to the high ionic nature of the protein–salt solutions, bilevel decoupling was used in those cases where the cooling gas was not sufficiently cold to equilibrate temperature at the value desired. Temperatures were set with a protein-free control sample. checked after equilibration of the actual sample, and the final quoted value was that determined immediately after the accumulation was completed. Temperature was measured with a Doric digital thermometer and a copper–constantan thermocouple in a glass sleeve immersed into the solution immediately following sample ejection.

A Gilford 240 UV spectrophotometer was used for RNase assays and protein concentration measurements. Values of pH were made at room temperature with a Radiometer Model 26 pH meter and an Ingold long thin (3 mm) combination electrode. Least-squares fitting of the relaxation data was carried out with the Digital Equipment Corp. PDP10 of the Computer Division at NIH using the MLAB interactive mathematical modeling program.

Methods. The three semisynthetic RNase S′ complexes were prepared as described previously (Niu et al., 1979). A solution of the RNase (1–15) peptide was added to the S protein (ca. 100 mg) in 0.15 M phosphate buffer (5 mL, pH 6.9). The solution was stirred at 4–6 °C for several hours, and excess peptide and buffer were removed by dialysis with an Amicon ultrafiltration system. In several cases, phosphorus analysis established the efficacy of this process for the removal of phosphate, NMR confirmed the removal of excess peptide, and assay idicated the formation of the product. The solutions were concentrated to ca. 2 mL, centrifuged, and kept chilled in an ice bath before NMR studies.

RNase assays were done spectrophotometrically by using the method of Crook et al. (1960) with cytidine cyclic 2′,3′-phosphate as substrate in tris(hydroxymethyl)aminomethane (Tris) buffer (0.1 M, 0.1 M NaCl, pH 7.3) at 290 nm and 25 °C. Concentrations of RNase A and S were measured with an extinction coefficient of 0.695 for 1 mg/mL at 280 nm. The specific activity was measured both preceding and following individual NMR experiments; values in the range 70–100% were routinely obtained and were considered acceptable.

The ^{13}C NMR spectra were obtained by using ca. 50 mg of RNase S′ complex in ca. 1 mL 0.1 M NaCl (pH 6.5) in Wilmad 10-mm precision-bore NMR tubes fitted with a water-filled glass antivortex insert. Approximately 15% D_2O was used in the final solution as an electronic lock signal. Chemical shifts are quoted positive downfield and were measured relative to [^{13}C]acetonitrile, added as 3 μL of a 10% solution in D_2O, and may be referenced from tetramethylsilane, which absorbs 1.24 ppm upfield from acetonitrile. The 90° pulse widths were determined prior to T_1 measurements for each NMR spectrometer.

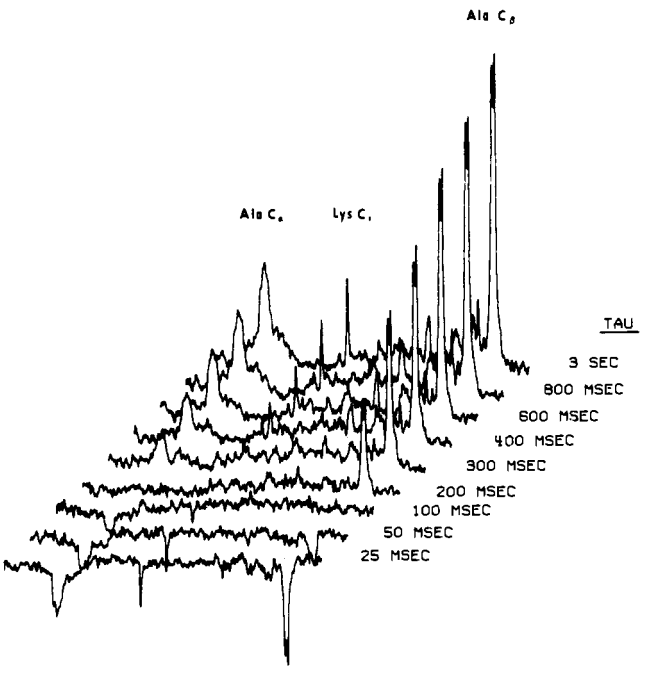

FIGURE 1: Stacked plot of partially relaxed ^{13}C NMR spectra at 68 MHz of [[U-^{13}C]Ala^5]RNase S′ (55 mg/mL in 0.1 M NaCl, 27 °C) for the determination of T_1 by the fast inversion recovery Fourier-transform method (500 scans, delay between scans 3.0 s, acquisition time 541 ms).

Theory

Spin–Lattice Relaxation Time Measurements. The standard method for T_1 relaxation time measurements is the inversion recovery method (Vold et al., 1968). This consists of the pulse sequence $(180°-\tau-90°-d)_n$, and an example of its application to an RNase S′ complex is shown in Figure 1. The equation used to ideally describe the intensity data may be written

$$I_\tau = I_\infty(1 - 2e^{-\tau/T_1}) \quad (1)$$

where τ is the adjustable delay between pulses, d is a delay time, and T_1 is the only fitted parameter. For this method to give accurate values, it is necessary to set $d > 5T_1$ and to measure the intensity I_∞ for τ very long. It is preferable to fit the linear form

$$\ln (I_\infty - I_\tau) = B - \tau/T_1 \quad (2)$$

with two parameters, T_1 and B, where B is an adjustable intercept rather than strictly being $\ln 2I_\infty$, since the first pulse (nominally 180°) may not be exactly twice the second pulse (90°). When the T_1 value is long, this inversion recovery procedure can take an appreciable time; characteristically in the cases reported here, with T_1 of several seconds, with several thousand acquisitions to obtain sufficient signal to noise, and with at least six τ values, the overall time required is of the order of 2 days. When the T_1 value is very long (or suspected to be so), this time is excessive. However, an alternative approach is to use the so-called fast inversion recovery method (Canet et al., 1975; Levy & Peat, 1975), in which d is set equal to ca. $3T_1$ and no I_∞ measurement is made. The data generated by this procedure may be fit with a three-parameter version:

$$\ln (I_i - I_\tau) = B - \tau/T_1 \quad (3)$$

in which I_i is now an adjustable parameter. This saves time in not requiring a very long τ value spectrum to be accumulated. In general, we have used this procedure in measuring and fitting the data reported here. For measuring NOE values, the delays recommended are longer than for T_1 determinations; for example, $d = 9T_1$ (Harris & Newman, 1976). We were able to accomplish this at the two lower frequencies (68 and 100 MHz), but at 125 MHz, where the T_1 value was longer, due to time constraints, we were only able to use ca. $3.5T_1$. There are several statistical analyses in the literature regarding the choice of optimal parameters for relaxation time measurements (Sass & Ziessow, 1977; Pitner & Whidby, 1979; Becker et al., 1980), and in this work we took appropriate precautions in choosing experimental parameters. The fitted T_1 values are shown in Table I, without the calculated errors, which were usually less than the expected error is determination of ca. 10%. The average values of the 10 natural abundance Lys C^ϵ T_1's are also given in the table for comparison, although these exhibit rapid side chain motion and were not analyzed. It should be noted that ^{13}C–^{13}C dipolar relaxation in the enriched Ala-5 complex is not expected to play a significant role because of the smaller value of γ_C than γ_H.

Analysis of Relaxation Parameters. In this section we summarize the model-free approach of Lipari & Szabo (1982a, b). This approach is based on the realization that the information of *fast internal* motions contained in relaxation parameters is limited because of the nature of the NMR experiment. When the internal motions (but not the overall motion) are in the extreme narrowing limit, the unique information on the internal motion of a particular protonated carbon can be completely specified by a generalized order parameter ($\mathcal{S}$) and an effective correlation time (τ_e), which are well-defined measures of the spatial restriction and the time scale of the motion, respectively. The simple procedure (see below) for extracting these parameters from relaxation data is rigorous in this limit; when the internal motions are slower, the procedure is a useful approximation. Once these parameters have been obtained, they can be modeled (in a variety of ways) if a physical picture of the motion is desired. Thus, the model-free approach actually simplifies the process of modeling data.

When the internal motions are very fast and the overall motion is known, one needs in principle only two measurements to determine $\mathcal{S}^2$ and τ_e for a particular ^{13}C–H vector. For example, if T_1 and $\tilde{T}_1$ are spin–lattice relaxation times at two magnetic fields for a given nucleus (e.g., they are NT_1's for $^{13}C^\beta$–H of Ala) and $(T_1)_0$ and $(\tilde{T}_1)_0$ are the corresponding numbers for the overall motion (e.g., for C^α–H of Ala or the α-carbon envelope), then

$$\mathcal{S}^2 = \frac{T_1^{-1} - \tilde{T}_1^{-1}}{(T_1^{-1})_0 - (\tilde{T}_1^{-1})_0} \qquad (4)$$

where $\mathcal{S}$ is the generalized order parameter of the C^β–H vector in a frame that is attached to the carbon(s) that determine(s) the overall motion. There exist a number of examples [see Tables II and XI of Lipari & Szabo (1982b)] that show that multifield data are actually redundant (i.e., one can obtain essentially the same $\mathcal{S}$ and τ_e if one uses only a part of the data set). However, it is important to obtain multifield data for a new system for two reasons. First, even if the internal motions are in the extreme narrowing limit, eq 4 is extremely sensitive to experimental errors. For proteins, T_1's are accurate to ±10–20%, and errors in NOE's can be even larger. Least-squares fitting an overdetermined data set minimizes

Table I: ^{13}C NMR Parameters for ^{13}C-Enriched RNase S' Complexes[a]

complex	residue	^{13}C	δ (ppm)	observing frequency: 25 MHz T_1 (s)	25 MHz temp (°C)	68 MHz T_1 (s)	68 MHz temp (°C)	68 MHz NOE	68 MHz temp (°C)	100 MHz T_1 (s)	100 MHz temp (°C)	100 MHz NOE	100 MHz temp (°C)	125 MHz T_1 (s)	125 MHz temp (°C)	125 MHz NOE	125 MHz temp (°C)
[[U-^{13}C]Ala^5]RNase S'	Ala-5	C^0	176.7	0.56	27	1.35[c]	20	1.1[c]	20	2.9	27	1.1	27				
		C^α	51.7	0.074	27	0.38	25	1.5[c]	20	0.49	27	1.3	27	0.66	27	1.2	27
		C^β	15.3	0.14	27	0.30	25	2.5[c]	20	0.38	27	2.0	27	0.43	27	1.9	27
	Lys[b]	C^ϵ	38.5	0.23	27	0.43	25			0.58	27	2.3	27	0.63	27	2.1	27
[[ϵ-^{13}C]His^{12},	His-12	C^ϵ	135.8	0.087	27	0.24	26	1.1	22	0.47	27	1.6	27				
[γ-^{13}C]Asp^{14}]RNase S'	Asp-14	C^γ	175.6	0.50	27	0.62	26	1.3	22	1.9	27	1.2	27				
[[ϵ-^{13}C]Met^{13}]RNase S'	Met-13	C^ϵ	11.4			1.15[c]	20	1.2[c]	20	2.0	27	1.8	27				

[a] Chemical shifts (δ) are downfield from $^{13}CH_3CN$ as internal standard. All T_1's were evaluated by three-parameter exponential fits, and the calculated standard errors were <±10%. [b] Natural abundance ^{13}C signal of lysine residues at positions 1, 7, 31, 37, 41, 61, 66, 91, 98, and 104. [c] From Niu et al. (1979).

Table II: Calculated Relaxation Parameters for Ala-5 of [[U-^{13}C]Ala^5]RNase S′ Assuming the C$^\alpha$–H Vector Reorients Isotropically[a]

	C$^\alpha$		C$^\beta$	
ω (MHz)	T_1 (s)	NOE	T_1 (s)	NOE
25	0.045 (0.074)	1.22	0.14 (0.14)	1.75
68	0.24 (0.38)	1.16 (1.5)	0.32 (0.30)	2.44 (2.5)
100	0.52 (0.49)	1.16 (1.3)	0.38 (0.38)	2.67 (2.0)
125	0.80 (0.66)	1.16 (1.2)	0.41 (0.43)	2.76 (1.9)

[a] The experimental results are in parentheses. Best fit parameter values: C$^\alpha$, τ_M = 9.09 ns; C$^\beta$, τ_e = 37 ps, $\mathcal{S}^2$ = 0.076.

Table III: Calculated Relaxation Parameters for Ala-5 of [[U-^{13}C]Ala^5]RNase S′ Assuming the C$^\alpha$–H Vector Undergoes Internal Motion[a]

	C$^\alpha$		C$^\beta$	
ω (MHz)	T_1 (s)	NOE	T_1 (s)	NOE
25	0.080 (0.074)	1.45	0.14 (0.14)	1.87
68	0.33 (0.38)	1.71 (1.5)	0.32 (0.30)	2.48 (2.5)
100	0.54 (0.49)	1.64 (1.3)	0.37 (0.38)	2.59 (2.0)
125	0.70 (0.66)	1.56 (1.2)	0.40 (0.43)	2.64 (1.9)

[a] The experimental results are in parentheses. Best fit parameter values: C$^\alpha$, τ_M = 20.1 ns, τ_e^α = 0.52 ns, $\mathcal{S}_{\alpha H}^2$ = 0.786; C$^\beta$, τ_e = 35 ps, $\mathcal{S}^2$ = 0.137.

the influence of such errors. Second, multifield data can be used to establish the existence of slower internal motions (which are of course still faster than the overall motion).

We now briefly summarize some of the relevant expressions that are used to analyze relaxation data in the model-free approach. The spectral density, which determines the relaxation parameters of nucleus 1, is

$$J(\omega) = 2\int_0^\infty (\cos \omega)tC_i(t)\, dt \quad (5)$$

where the correlation function is given by

$$C_i(t) = C_0(t)[\mathcal{S}_i^2 + (1 - \mathcal{S}_i^2)e^{-t/\tau_e^i}] \quad (6)$$

where $C_0(t)$ is the correlation function describing the overall motion of the molecule. For example, if the overall motion is isotropic

$$C_0(t) = (1/5)e^{-t/\tau_M} \quad (7)$$

where τ_M is the rotational correlation time.

Results and Discussion

The measurement of NMR relaxation rates has become a principal source of information regarding the local mobility of groups in macromolecules (London, 1980; Shindo, 1983). However, the interpretation of the relaxation rates depends upon the mathematical model applied. In the present work, we have used the "model-free" approach (Lipari & Szabo, 1982a), which makes few assumptions about the nature of the molecular processes operating. We have utilized the selectively ^{13}C-enriched RNase S′ system (Niu et al., 1979), particularly focusing on the uniformly labeled Ala-5 complex. Analysis of the relaxation data for non-hydrogen-containing enriched carbon atoms (such as carbonyl or C$^\epsilon$ of His) was not attempted due to the complication of relaxation mechanisms other than dipole–dipole (Norton et al., 1977), although in general it was found that these values increased with observing field strength (Table I). However, the Ala-5 C$^\alpha$H and C$^\beta$H$_3$ are expected to fully conform to this mechanism and provide an interesting case of backbone and side-chain carbon atoms, which enable overall and local conformational flexibility to be delineated.

We now turn to the analysis of the experimental results, shown in Table I. First, we assume that the C$^\alpha$–H vector reorients isotropically and use eq 7 to fit simultaneously all the C$^\alpha$ data, treating τ_M as an adjustable parameter. We then fit all the C$^\beta$ relaxation data by using eq 6 with $C_0(t)$ as determined above and by varying $\mathcal{S}^2$ and τ_e. The results are shown in Table II. The fit to the C$^\alpha$ data is poor, indicating that the motion of the C$^\alpha$–H vector cannot be described by a single correlation time. The fit to the C$^\beta$ data is quite good; the predicted NOE's are however too large for the two highest fields. The order parameter of the C$^\beta$–H vector relative to a frame that reorients isotropically with a correlation time of 9.09 ns is 0.076. Assuming that the motions of and about the C$^\alpha$–C^b bond are independent, the generalized order parameter for a methyl group can be decomposed as

$$\mathcal{S}^2 = \mathcal{S}_{2\beta}^2[(3/2)\cos^2\beta - 1/2]^2 \quad (8)$$

where $\mathcal{S}_{\alpha\beta}$ is the generalized order parameter for the C$^\alpha$–C$^\beta$ bond and β is the angle between the C$^\beta$–H and C$^\alpha$–C$^\beta$ bonds. Assuming ideal tetrahedral geometry

$$\mathcal{S}^2 = 0.111\mathcal{S}_{\alpha\beta}^2 \quad (9)$$

Thus, $\mathcal{S}^2$ = 0.076 indicates that relaxation of the methyl group cannot be described by methyl rotation alone but that C$^\alpha$–C$^\beta$ bond must undergo internal motion.

The fact that the C$^\alpha$ data cannot be reproduced by using a single correlation time indicates that the overall motion is anisotropic and/or that local motion exists. Indeed, ribonuclease is not spherical; its shape is closer to a cylinder with an axial ratio close to 1.8. For a cylinder with an axial ratio of 2, the two rotational diffusion coefficients differ by a factor of 2, and consequently, the three correlation times that appear in the spectral density are in the ratio 6:7:10. This spread is much too small to account for the C$^\alpha$ data, and thus, local motions of the C$^\alpha$–H vector must exist. This is consistent with the conclusion reached above concerning the motion of the C$^\alpha$–C$^\beta$ bond. Assuming that the overall motion is adequately represented by a single correlation time (τ_M) and that the internal motion of the C$^\alpha$–H vector is described by $\mathcal{S}_{\alpha H}^2$ and τ_e^α, we can use the following correlation function to fit the C$^\alpha$ data:

$$C_0(t) = (1/5)e^{-t/\tau_M}[\mathcal{S}_{\alpha H}^2 + (1 - \mathcal{S}_{\alpha H}^2)e^{-t/\tau_e^\alpha}] \quad (10)$$

treating τ_M, $\mathcal{S}_{\alpha H}^2$, and τ_e^α as adjustable parameters. Although formally eq 10 is the same as the expression used in the model-free approach to treat highly anisotropic overall motion [see eq 7 of Lipari & Szabo (1982b)], in the present case the parameters have a well-defined meaning. After fitting the C$^\alpha$ data, we then fit the C$^\beta$ data by using eq 6 with $C_0(t)$ given by eq 10 (and not by eq 7 as previously). The results are shown in Table III. The fit to the C$^\alpha$ data is much better than those in Table II. The order parameter for the C$^\alpha$–H bond (i.e., 0.786) when modeled within the framework of motion-in-a-cone model corresponds to a semiangle of 22.7°, indicating internal motions of significant amplitude. The effective correlation time for this motion (τ_e = 0.52 ns) is considerably slower than the correlation time for motion of the C$^\beta$–H vector (τ_e^α = 37ps) as would be expected since the latter time reflects fast rotations of the methyl group about the C$^\alpha$–C$^\beta$ axis. The quality of the fit to the C$^\beta$ data is similar to that in Table III. However, the resulting order parameter ($\mathcal{S}^2$ = 0.137) now is a measure of the restriction of the motion relative to a frame that is attached to the C$^\alpha$–H bond, which is not longer moving isotropically. Assuming that the C$^\alpha$–H and the C$^\alpha$–C$^\beta$ bonds have the same order parameter, then it follows from eq 9 that

the above order parameter of the C^{β}–H bond reflects only the rotation of the methyl group and should have a value near 0.111. On the basis of these considerations, the extracted value of 0.137 is reasonable, and its closeness to 0.111 supports the validity of the analysis.

It should be emphasized that the fits shown in Table III, while quite good for the T_1's, overestimate the NOE's at the two highest fields. This may be an indication that the NMR experiment is detecting rather slow internal motions. In other words, one may be in a regime where the model-free approach begins to break down [see the discussion of the range of validity of this approach in Lipari & Szabo (1982a)]. However, since the errors in the experimental NOE's may be quite large (Canet et al., 1976; Harris & Newman, 1976) and since the quality of the fits and the values of the resulting parameters [i.e., the predicted $\mathcal{S}^2$ for the methyl group (0.137) is close to the ideal value of 0.11)] are reasonable, it does not appear that one can quantify the nature of the internal motions further by using the available data.

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