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Determination of internal dynamics of deoxyriboses in the DNA hexamer d(CGTACG)$_2$ by ^{1}H NMR*

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Abstract. The conformations and internal dynamics of the deoxyriboses of d(CGTACG)$_2$ have been determined by NMR measurements at 15 °C. The conformations of the sugars were determined using coupling constants and time-dependent NOE measurements. The J-splitting patterns of the H1′, H2′ and H2″ resonances show that the sugars exist as mixtures of conformations near C2′ endo (south) and C3′ endo (north). The population of the south conformation was larger for the purines than for the pyrimidines. The overall tumbling time of the molecule in ^2H_2O was determined from measurements of the cross relaxation rate constant for the H6-H5 vectors of the two cytosine residues. Order parameters were determined for the H1′-H2″, H2′-H2″ and H2′-H3′ vectors from measurements of cross relaxation rate constants, making use of multi-spin analysis of the NOE build up rates. These order parameters are weakly dependent of the base sequence, and except for the terminal Cyt 1 residue, the H2′-H2″ and H2′-H3′ vectors are near unity, indicating the absence of rapid pseudorotation on the nanosecond time scale. However, the order parameter for the H1′-H2″ vector is significantly smaller than expected for rapid pseudorotation indicating the presence of other motions of the sugars. This motion must be about an effective axis parallel to the H2′-H2″ vector, and to occur with an angular fluctuation of about 30°.

The results show that to obtain highly refined structures for nucleic acids by NMR the effects of spin diffusion and motional averaging cannot be ignored.

Key words: Cross relaxation, DNA dynamics

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Introduction

The sequence dependence of the structure and internal dynamics of oligodeoxynucleotides is of considerable importance in understanding the mechanism of recognition of specific DNA sequences by cognate proteins such as repressors, restriction endonucleases and RNA polymerase. The sequence-dependent properties of DNA may also be important in regulating the expression of genes. Further, an understanding of the internal dynamics of DNA fragments has practical significance for the determination of structures using NMR methods. The determination of the solution structure of DNA fragments in most laboratories relies on the estimation of distances between pairs of protons using the nuclear Overhauser effect (NOE) (Nilges et al. 1987; Patel et al. 1987). Unfortunately, the NOE, or more precisely the cross relaxation rate constant, depends not only on the interproton distance, but also on the correlation time, which is usually taken to be equal to the overall tumbling time for a rigid molecule. If internal motions are of significant amplitude, estimated distances are at best a non-linear average over all conformations sampled, leading to derived structures that may be incorrect in detail (Jardetzky 1980; Jardetzky and Roberts 1981; Lefèvre et al. 1987). Further, if the internal motions are fast compared with the overall tumbling, the spectral density functions depend both on the distance fluctuations and the frequency of the motion. Although the overall conformational properties of DNA fragments can be determined from NOE data even when internal motions are ignored, a detailed picture of the sequence-dependent variation of the local structure requires high precision distance information, and any internal motions that occur must be taken into account. It is therefore important to map out mobility in different parts of the molecule to decide on the magnitude of errors that can arise from ignoring motion. Conformational averag-

ing can have large effects on observed NOE intensities (Jardetzky and Roberts 1981; Lane 1988).

The presence of conformational equilibria in macromolecules is an additional complication that must be considered. The existence of multiple potential minima has been detected in peptides (Gariépy et al. 1986; Kessler et al. 1988) in nucleotides (Birdsall et al. 1975) and in nucleic acids (Rinkel et al. 1987; Bax and Lerner 1988; Lefèvre et al. 1988; Zhou et al. 1988). Altona and coworkers have shown that the conformation of deoxyriboses in nucleic acids can be accurately described by pseudorotation (Altona and Sundaralingam 1972; Rinkel et al. 1987; Rinkel and Altona 1987). They have presented convincing experimental evidence that the sugars exist as a mixture of C2′ endo and C3′ endo conformations. This conformational equilibrium can be determined from spin-spin coupling constants (Rinkel and Altona 1987). However, according to Olson (Olson and Sussman 1982; Olson 1982) the energy difference between C2′ endo and C3′ endo is only 1 to 2 kcal/mol, and the energy minima are rather shallow. Presumably a range of conformations is significantly populated (Olson and Sussman 1982; Olson 1982), so that a precise determination of the phase angle has no clear meaning. However, a consistent representation of the pucker equilibrium can be obtained by combining information from coupling constants and NOEs.

There has been relatively little experimental work reported on the internal dynamics of short DNA sequences in solution, though theoretical considerations and analysis of Debye-Waller factors from single crystal X-ray analyses indicate that the greatest internal mobility is shown by the phosphates, followed by the deoxyriboses, and the least motion by the bases (McCammon and Harvey 1987; Nelson et al. 1986).

The sensitivity of the proton can be used to determine some aspects of internal motion, provided that the NOE for proton-proton vectors of fixed length can be measured (Olejniczak et al. 1984; Lane et al. 1986; Lane 1989). Fortunately, there are several vectors in DNA whose lengths are essentially unchanged by internal motion (e.g. Cyt H6-H5, d-ribose H2′-H2″, H2′-H3′ and H1′-H2″, Wüthrich 1986; Lefèvre et al. 1987; van de Ven and Hilbers 1988). In the absence of internal motions, the correlation time for different proton-proton vectors will be identical. The correlation time for Cyt H6-H5 vectors seems to be close to the overall tumbling time of short oligonucleotides (Lane et al. 1986), and can therefore be used as a reference vector for determining order parameters for other vectors (Lipari and Szabo 1982).

A report of the mobility of the deoxyriboses in the hexamer $d(CGTACG)_2$ has appeared (Groneborn et al. 1984), in which the cross relaxation rate constants for the H2′-H2″ vectors were compared with those for the Cyt H6-H5 vectors. They showed that the estimated cross relaxation rate constant for the H2′-H2″ vector required an order parameter of about 0.3. However, Nerdal et al. (1988) have recently challenged this conclusion, based on cross peak intensities observed in NOESY spectra recorded with very short mixing times.

In this paper we report measurements of coupling constants and time dependent NOEs in $d(CGTACG)_2$ for a large number of protons, using at least five irradiation times to sample the NOE time courses. To overcome the effects of spin diffusion, which are particularly severe in the sugars (Chazin et al. 1986; Lefèvre et al. 1987), we have analysed the NOE time courses using complete spin systems. Correlation times of several proton-proton vectors in the self complementary hexamer $d(CGTACG)_2$ have been determined, extending the findings and methods of Clore and Gronenborn (1984). We compare the derived order parameters with calculations for a variety of models.

Materials and methods

Materials

The self complementary hexamer $d(CGTACG)_2$ was synthesised using phosphoramidite chemistry on a Beckman synthesiser and purified by FPLC using a gradient of ammonium bicarbonate. Fractions were lyophilised to remove the ammonium bicarbonate, and then dissolved in buffer containing 20 m*M* sodium phosphate, 160 m*M* KCl, 0.2 m*M* EDTA, pH 7.0. The sample was annealed by heating to 80 °C for 10 min, and allowed to cool slowly. The annealed oligonucleotide was then lyophilised twice from 99.8% D_2O, and finally dissolved in 0.5 ml 99.98% D_2O (Aldrich).

Methods

NMR Spectra. ^{1}H NMR spectra were recorded at 500 MHz on a Bruker AM 500 instrument and at 400 MHz on a Bruker WB 400. Typically spectra were obtained using a spectral width of 5,000 Hz over 16,384 complex points giving a digital resolution of 0.61 Hz per point. NOESY spectra were recorded using the time proportional phase increment method (Marion and Wüthrich 1983) to give absorption mode spectra. 512 free induction decays consisting of 2,048 points were recorded over a spectral width of 4,000 Hz. The final matrix was filled in F1 to 2,048 points with zeroes prior to Fourier transformation, using a Gaussian window function in both dimensions. A double-quantum-filtered COSY spectrum was recorded using 4,096 points in F2 and 1,024 points in F1, zero filled to

2,048 points, with a digital resolution of 1.7 Hz per point. A high digital resolution HOHAHA spectrum was acquired using 4,096 points, zero filled to 8,192 points in F2 and 1,024 points in F1 over a spectral width of 3,400 Hz. The spin lock field was generated using the decoupler and an MLEV 17 scheme (Bax and Davis, 1985) with B1 = 8 kHz. Driven truncated NOE data were collected using the method of Wagner and Wüthrich (1979), with 16 acquisitions interleaved on and off resonance. Cross relaxation rate constants, σ, for the Cyt H6-H5 vectors were determined from the time dependence of the NOE according to:

$$\mathrm{NOE}(t) = (\sigma/R_1)\,[1-\exp(-R_1\,t)]\,, \tag{1}$$

where R_1 is the effective spin-lattice relaxation rate constant. For other vectors, the cross relaxation rate constants were determined by integration of the Bloch-Solomon equations for appropriate spin systems as previously described (Lane 1988; Lefèvre et al. 1987). Correlation times were estimated from the cross relaxation rate constants using the relationship:

$$\sigma = \alpha[6J(2\omega)-J(0)]/r^6\,, \tag{2A}$$

where α is a nuclear constant whose value if 56.92 Å^6 ns^{-2}, r is the interproton separation, and $J(\omega)$ is the spectral density function. For a rigid sphere, the spectral density function is given by:

$$J(\omega) = \tau/(1+\omega^2\tau^2)\,, \tag{2B}$$

where ω is the Larmor frequency and τ is the correlation time. The hexamer is essentially a squat cylinder of dimensions 20 Å by 20 Å, and is therefore expected to tumble isotropically in solution. In the presence of internal motions, however, the spectral density function is not necessarily a single Lorentzian [cf Eq. (2A)]. Provided that the internal motion is fast compared with overall tumbling, the spectral density function can be described as a sum of two Lorentzians (Lipari and Szabo 1982):

$$J(\omega) = S^2\tau_R/(1+\omega^2\tau_R^2)+(1-S^2)\tau_e/(1+\omega^2\tau_e^2)\,, \tag{3}$$

where S^2 is an order parameter that characterises the amplitude of the motion. In a rigid body, S^2 is unity, whereas for complete orientational freedom, the order parameter is zero. τ_R is the correlation time for overall tumbling, and τ_e is a composite correlation time defined as:

$$\tau_e = \tau_i\tau_R/(\tau_i+\tau_R)\,, \tag{4}$$

where τ_i is the correlation time for internal motion.

In the limit of very fast internal motion, $\tau_i \ll \tau_R$, the effective correlation time τ_e becomes equal to τ_i. Under these conditions, using the definition in Eq. (3), Eq. (2A) becomes:

$$\sigma = (\alpha/r^6)[6S^2\tau_R/(1+4\omega^2\tau_R^2)+5(1-S^2)\tau_i-S^2\tau_R\,. \tag{5}$$

It has been shown that the dependence of the cross relaxation rate constant of H1′-H2″ vectors in DNA is a linear function of viscosity/temperature, which is evidence for rapid internal motion (Lefèvre et al. 1987). Further, if the amplitude of the motion is not too large, the term in $(1-S^2)\tau_i$ is negligible compared with the other terms (τ_i small). Equation (2) reduces to the expression for a rigid body scaled by the order parameter, S^2. In this instance, only the order parameter can be determined.

The determination of the order parameter reduces to measuring the overall correlation time τ_R, and cross relaxation rate constants for a variety of proton-proton vectors. From Eqs. (2) and (5), the ratio of the cross relaxation rate constants for two vectors in the limit of very fast internal motion is:

$$\sigma_1/\sigma_2 = S_1^2 r_2^6/S_2^2 r_1^6 \tag{6}$$

Provided that the distances are known, the ratio of the order parameters can be estimated. The order parameter for the H6-H5 vector of cytosine residues (r = 2.45 Å) is approximately equal to unity (Lane et al. 1986). Hence, the order parameter for sugar vectors can be determined. The most readily measured cross relaxation rate constants for sugar protons for which the distance is independent of conformation are the H2′-H2″ (r = 1.79 Å), H1′-H2″ ($r = 2.3 \pm 0.03$ Å; this distance is very slightly dependent on the sugar pucker, varying from 2.32 Å in C2′ endo to 2.3 Å in C3′ endo. This yields at most a 5% error in the r^6 term), and H2′-H3′ ($r = 2.32 \pm 0.02$ Å). These vectors also point in different directions, so are sensitive to different internal motions.

In the Lipari-Szabo formalism, S^2 is model-independent, and gives only an indication of the degree of spatial order on the sub-nanosecond time scale. However, order parameters can be calculated for specific motional models, using the method of Tropp (1980). The equivalence of the formalisms of Tropp (1980) and Lipari and Szabo (1982) is demonstrated in the Appendix. As the dipolar interaction is averaged by motion, it is necessary to calculate the effective Hamiltonian under the influence of motion. This amounts to calculating the square of the average dipole strength:

$$S^2 = \sum_{m=-2}^{2}\left|\sum_{i=1}^{N} p_i\,Y_{2m}(\theta_i,\phi_i)r_i^{-3}\right|^2\,, \tag{7}$$

where $Y_{2m}(\theta_i,\phi_i)$ are the spherical harmonics, r_i is the internuclear distance, p_i is the probability of state i, and N is the number of conformations to be averaged.

If coordinates of the pairs of protons are known for the conformations that are being averaged, the order parameter for that motion can be calculated using Eq. (7), and compared with the measured values.

Sugar pucker equilibria from coupling constants. Coupling constants were derived where possible from high digital resolution spectra. Sums of coupling constants were also obtained from cross sections of high digital resolution HOHAHA and double-quantum-filtered COSY spectra. Coupling constants are determined to within ± 0.5 Hz. The sugar conformations were analysed using the pseudorotation formalism (Altona and Sundaralingam 1972; Rinkel et al. 1987), and the Karplus equation. The Karplus equation relates the coupling constant to the dihedral angle. For convenience, we have reparametrised the Karplus equation in terms of the pseudorotation phase angle, P as follows:

$$J_i = A\cos(\phi_i+\delta_2) + B\cos^2(\phi_i+\delta_2) \quad (8)$$

$$\phi_i = \phi_m \cos(P+\delta_i) \quad (9)$$

where A, B, δ_2 are constants, ϕ_i is the torsion angle in the ring, and ϕ_m is the maximum pucker amplitude, which is usually near 37° ($\pm 3°$) (Rinkel and Altona 1987). Coupling constants were fitted to sets of equations as:

$$J_i = f_S \cdot J_i(P_S) + (1-f_S)\, J_i(P_N) \quad (10)$$

where P_s is the phase angle of the major conformation (south, P near 162°, C2′ endo), P_N is the phase angle of the minor conformation (north, P near 9°, C3′ endo), and f_S is the fractional population of the south conformation. Because at most three coupling constant per sugar were obtained, the value of P_N was kept fixed at 9°, though calculation showed that varying P_N from 0 to 30° had only a minor influence on the determination of the other parameters. Varying ϕ_m from 33° to 39° produces changes in J_i for a given P only within the error bounds, which are about ± 0.5 Hz including the experimental error and the errors in the parameterisation of the Karplus equation owing to uncertainty of the value of P and ϕ_m to be used in the calibration curve. We have therefore maintained ϕ_m at 37°, leaving only two parameters to be determined, namely P_s and f_S.

Results

Assignment of the ^1H spectrum of d(CGTACG)$_2$

The proton spectrum was assigned by using NOESY, 2-quantum filtered COSY and HOHAHA (Hare et al. 1983; Clore and Gronenborn 1983; Scheek et al. 1984; Weiss et al. 1984; Bax and Davis 1985; Wüthrich 1986; Flynn et al. 1988). Figure 1 A shows part of a NOESY spectrum and the magnetisation transfer pathways. Part of a HOHAHA spectrum is shown in Fig. 1 B, giving the spin-spin connectivities in the deoxyriboses. The assignments of the non-exchangeable protons are given in Table 1. The assignments agree with those obtained by Gronenborn et al. (1984), except for minor differences in chemical shifts attributable to the different temperature (15 °C versus 5 °C).

The imino protons were assigned using NOE difference spectra recorded in ^1H_2O as described in the Materials and methods. A typical one dimensional spectrum is shown in Fig. 1 C. The broad peak at 13.06 ppm disappears at higher temperatures, and can therefore be assigned to CG1, 6. On irradiation of the resonance at 12.78 ppm, which is expected to be an imino proton of a CG base pair, an NOE is observed to the resonance at 13.56 ppm. Further, irradiation of the peak at 13.56 ppm yields an NOE both of the peak at 12.78 ppm, and of a singlet at 7.5 ppm, which is the H2 of A4. Hence the lowest field peak is the imino proton of TA3, AT4, and the highest field peak belongs to GC2/CG6.

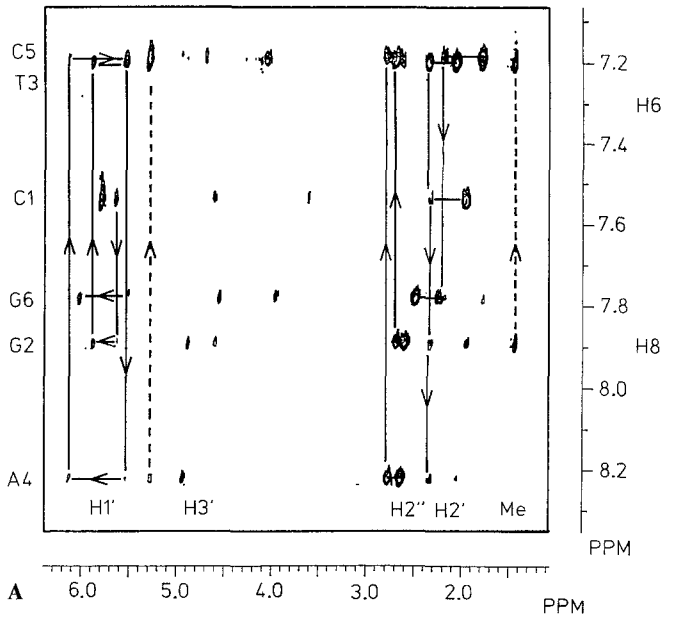
Analysis of coupling constants: sugar puckering

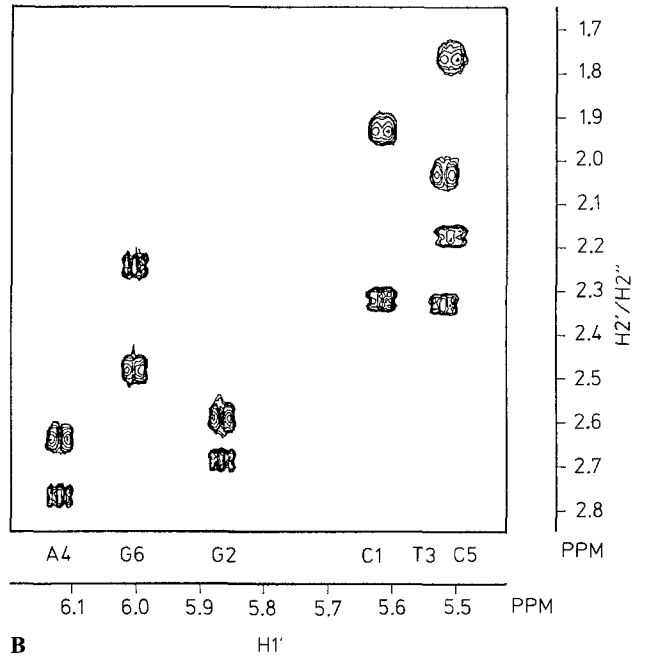
Figure 2 A shows the H1′ region of the hexamer. Of the four resolved H1′ resonances, there are clear differences in the coupling patterns to H2′ and H2″. Thus, C1 H1′ and G6 H1′ are triplets, whereas A4 H1′ and G2 H1′ are double doublets. Both patterns are characteristic and indicate that a south conformation is the dominant conformer (cf Fig. 2 A). Triplets are ex$\}$ected when $^3J_{1'2'} = {}^3J_{1'2''}$, which occurs when P$\approx$90° and P$\approx$215°, or when the fraction south is about 0.6 ($P_s \approx 162°$, $P_N \approx 9°$). Double doublets are characteristic for P in the range 120° to 170° when the fraction south is larger than about 0.8. Hence, C1 and G6 have rather different sugar conformations from A4 and G2. Additional information on the sugar conformation can be obtained from the coupling constants involving H3′. Because the resolution of the one dimensional spectrum for the nucleotides T3 and C5 was insufficient to determine their coupling constants, we have used

Table 1. Assignments of the protons in d(CGTACG)$_2$ at 15 °C. Protons were assigned as described in the text. Chemical shifts are referenced to internal DSS

Base	H8/6	H5/ H2/Me	H1′	H′$_2$	H″$_2$	H′$_3$	H′$_4$	H′$_5$/H″$_5$	NH[a]
C1	7.52	5.75	5.60	1.91	2.30	4.57	3.96	3.60	–
G2	7.88	–	5.85	2.58	2.66	4.86	4.24	4.00/3.90	12.78
T3	7.20	1.41	5.50	2.04	2.31	4.73	4.10	4.02	13.56
A4	8.22	7.48	6.10	2.62	2.75	4.92	4.32	4.05/3.96	–
C5	7.19	5.26	5.48	1.75	2.14	4.68	4.14	4.06/4.04	–
G6	7.77	–	5.99	2.49	2.23	4.53	4.05	3.96	13.06

[a] Chemical shift at 278 K





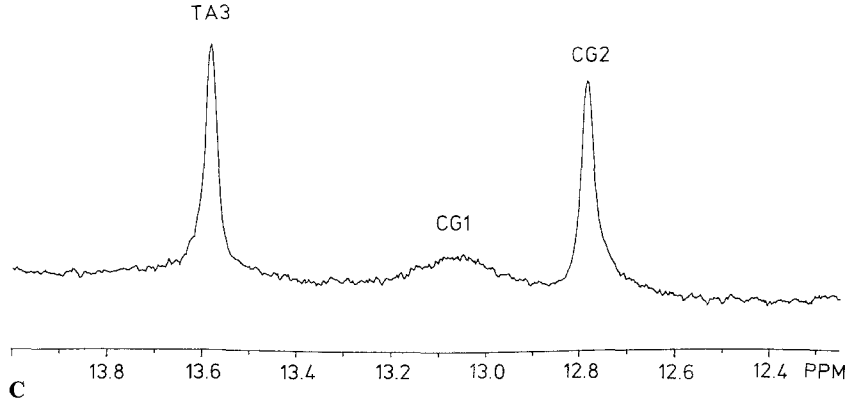


Fig. 1A–C. NMR spectra of $d(CGTACG)_2$ at 288 K. The sample was prepared as described in the Materials and methods. **A** Phase sensitive NOESY spectrum. 2,048 points were acquired in F2, and 512 in F1 zero-filled to 2,048 points over a spectral width of 3,400 Hz (digital resolution = 3.3 Hz per point). Free induction decays were multiplied by a 60° shifted sine-squared function in both dimensions prior to Fourier transformation. The mixing time was 300 ms. Continuous lines connect sequential NOEs, and *dashed lines* interbase NOEs. **B** HOHAHA spectrum with a mixing time of 25 ms. 4,096 points zero-filled to 8,192 points in F2 and 1,024 points in F1 were acquired over a spectral width of 3,400 Hz (digital resolution = 0.83 Hz per point in F2). Free induction decays were multiplied by a 45° shifted sine-squared function in F2, and by a 60° shifted sine-squared function in F1 prior to Fourier transformation. **C** Imino protons of CGTACG. The solvent resonance was suppressed using the 1,331 sequence (Hore 1983). 16,384 points zero-filled to 32,768 points were acquired over a spectral width of 11,000 Hz. 2 Hz line broadening was added to the spectra. The temperature was 276 K

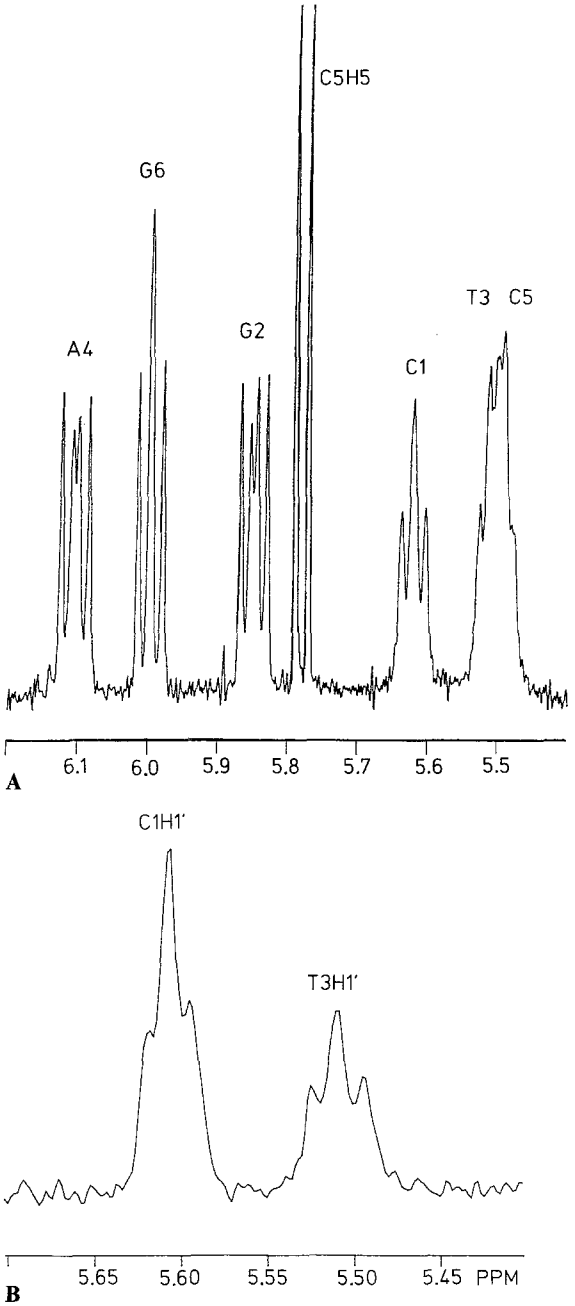


Fig. 2 A and B. Spin-spin splitting in the deoxyriboses. **A** Spectrum of the H1′ region; 32,368 points were acquired over a spectral width of 4,000 Hz, for a digital resolution of 0.25 Hz per point. The resolution was enhanced using a Lorentz to Gauss transformation (LB = −2.5, GB = 0.25). **B** Cross sections from the HOHAHA spectrum (Fig. 1 B). The cross sections are parallel to F2 (digital resolution = 0.83 Hz per point), showing the fine structure of the C1 H1′-C1 H2′ and T3 H1′-T3 H2′ cross peaks

HOHAHA to obtain sums of coupling constants. Figure 2 B shows cross sections from a HOHAHA spectrum (digital resolution = 0.9 Hz per point). Note that because the components of the cross peaks are in phase, the signal-to-noise ratio is much higher than in a phase-sensitive COSY spectrum, where the cross

Table 2. Spin-spin coupling in the deoxyriboses of d(CGTACG)$_2$ $^3J_{1'2'}$, $^3J_{1'2''}$ were determined from 1 dimensional spectra. Sums of coupling constants $\Sigma_{1'} = {}^3J_{1'2'} + {}^3J_{1'2''}$, $\Sigma_{2'} = {}^3J_{2'1'} + {}^2J_{2'2''} + {}^3J_{2'3'}$ were obtained from cross sections of high digital resolution HOHAHA spectra. P_S is the pseudorotation phase angle of the major (south) conformation and f_S is the fraction of the major conformation present. P_s and f_S were determined as described in the text with $\phi_m = 36°$, and $P_N = 9°$. The error on f_S is about ±0.05, and on P_S about ±10°

Base	$J_{1'2'}$	$J_{1'2''}$	1D		2D		P_s	f_S
			$\Sigma_{1'}$	$\Sigma_{2'}$	$\Sigma_{1'}$	$\Sigma_{2'}$		
	Hz							
C1	7.5	6.3	13.8	28.0	13.6	27.0	162	0.67[b]
G2	8.7	6.2	14.9	nd[a]	14.8	27.5	173	0.85
T3	nd	nd	nd	28.0	13.5	30.0	120	0.65[b]
A4	9.2	6.4	15.6	nd	15.1	27.0	187	0.93
C5	nd	nd	nd	29.0	14.7	28.0	167	0.83
G6	8.4	7.1	15.5	28.5	14.8	29.0	162	0.90

[a] nd: not determined
[b] Value from NOEs was 0.73

peak components are in antiphase, and tend to cancel. The coupling constant data are summarised in Table 2. The sums of coupling constants derived from one dimensional spectra agree well with those obtained from the HOHAHA spectrum. It is therefore possible to use the sums of coupling constants obtained from the HOHAHA spectrum for those resonances that are not resolved in one dimensional spectra (e.g. T3 H1′, C5 H1′).

The coupling constants were analysed according to Eqs. (8–10), from which P_s and f_S were obtained for each sugar (Table 1). As Table 2 shows, G2, A4 and G6 attain high conformational purity, whereas C1, T3 and C5 have significant amounts of the C3′ endo conformation. We have also used the graphical method of Rinkel and Altona (1987), which gave essentially identical results. The greater conformational purity of purines over pyrimidines seems to be a general property (Rinkel et al. 1987; Lefèvre et al. 1987; Bax and Lerner 1988). Given error bands of about ±0.5 Hz, and the relative flatness of the curves in the range $100 < P < 180°$ (Rinkel and Altona 1987), precise values for P are difficult to determine. Indeed, the potential minima for pseudorotation are broad (Olson 1982; Olson and Sussman 1982), suggesting that a unique value for the pseudorotation phase angle may not exist. On the other hand, the coupling constants are quite sensitive to the fraction of the south conformation, so that this parameter is relatively well determined. The probable error on the determination of f_S is about ±0.05. The fractions south were also confirmed by NOESY measurements using short mixing times (not shown), in particular using the NOE

H3′-H8/6. This NOE is very sensitive to the fraction C3′ endo, as the distance varies between about 2.7 Å in C3′ endo to greater than 4.3 Å in C2′ endo for a glycosidic torsion angle near −100°. These data indicated values of f_S of about 0.7 for C1 and T3, and larger than 0.85 for the other four nucleotides. Similar conclusions were obtained for the double-quantum filtered COSY experiment (Widmer and Wüthrich 1987) (data not shown).

Determination of the overall tumbling time

The hexamer has a molecular weight of 3,600, a length of about 20 Å, and a diameter of about 20 Å. It is therefore reasonable to treat this molecule as an isotropic rotor. There are two fixed length proton-proton vectors in the molecule that can be used to determine the overall correlation time, namely C1 and C5 H6-H5. Therefore we measured the time dependence of the NOE intensity on irradiation of the H6 protons, at two temperatures (15 °C and 25 °C). Figure 3A shows a typical NOE difference spectrum, obtained by saturating the H6 resonance of C5 for 0.2 s. The dependence of the NOE intensity on the irradiation time is shown in Fig. 3B. The cross relaxation rate constants were extracted by fitting the data to Eq. (1). The value of the correlation time was then estimated from the cross relaxation rate constant according to the Eq. (2), using $r = 2.45$ Å. We have also measured the cross relaxation rate constants for the T H6-Me vector. The correlation time was estimated using a value of $r = 2.7$ Å (Lane et al. 1986).

The value of the correlation time was 2.4 ± 0.2 ns for each vector at 15 °C, and 1.8 ± 0.2 ns at 298 K, consistent with the proposal that this molecule behaves as an isotropic rotor. These values are also close to what is calculated for a rigid, hydrated sphere using the Stokes-Einstein equation (Lane et al. 1986), and the viscosity of D_2O (Wilbur et al. 1976).

Internal motions in the deoxyriboses

An apparent correlation time, τ_{app}, can be calculated from Eq. (2) assuming a rigid body. It has previously been shown that τ_{app} of the H1′-H2″ vectors are smaller that of the Cyt H6-H5 vector in DNA fragments, which was attributed to rapid pseudorotation (Lane et al. 1986; Lefèvre et al. 1987). Figure 4 shows how the conformation of the ring changes in the transition from C2′ endo to C3′ endo. To test this hypothesis further, we have measured the cross relaxation rate constant for each of the six H1′-H2″ vectors in $d(CGTACG)_2$ at 15 °C in the same manner as for the H6-H5 vectors. Figure 5 shows NOE build up curves for H2′ and H2″ obtained from irradiation of the H1′ protons of A4 and C1. Further, we have determined cross relaxation rate constants from NOE build up

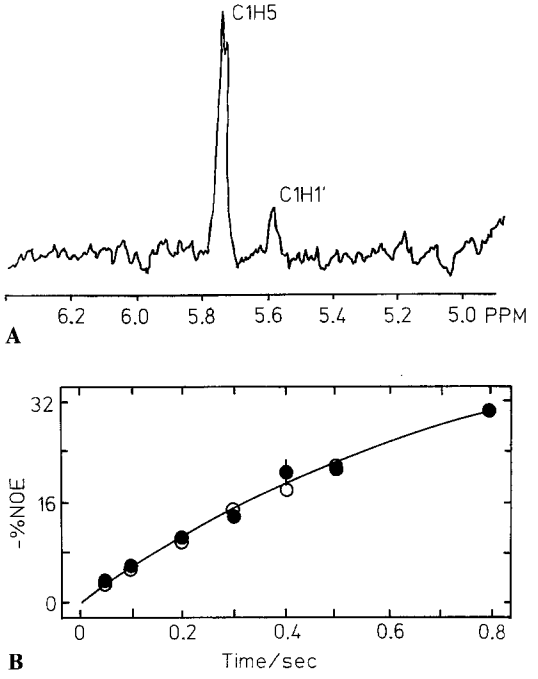


Fig. 3A and B. Correlation time for the Cyt H6-H5 vectors. The H6 resonances were saturated as described in the Materials and methods. **A** NOE difference spectrum obtained after irradiating Cyt 1 H6 for 200 ms. Line broadening = 3 Hz. **B** NOE time courses for Cyt 1 and Cyt 5 H6-H5 vectors. (o) Cyt 1, (•) Cyt 5. The line is a regression fit to Eq. (1)

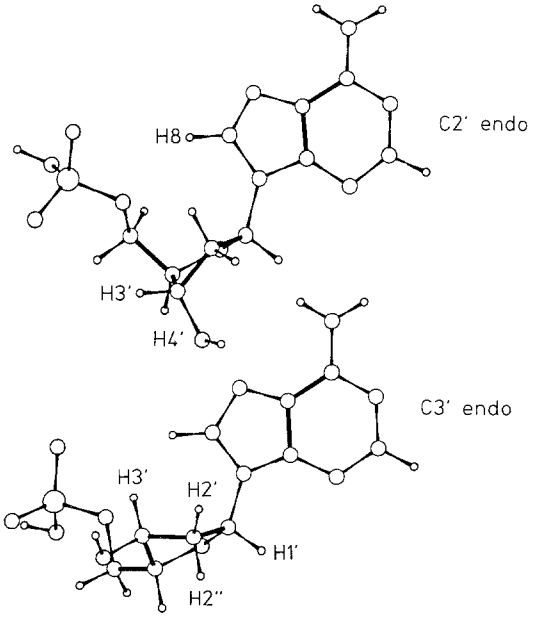


Fig. 4. Conformation of C2′ endo and C3′ endo nucleotides. The dAMP nucleotides were generated using QUANTA (Polygen). The bases are shown in the same orientation, and the glycosidic torsion angle is −110°. Upper C2′ endo, lower C3′ endo

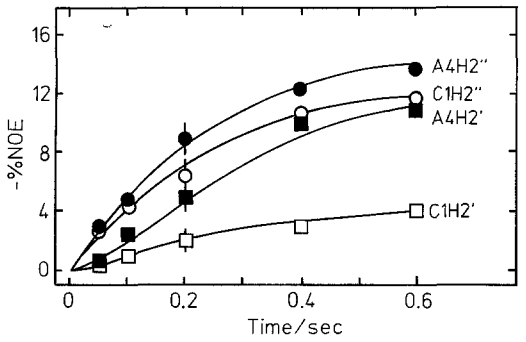


Fig. 5. Correlation times for the H1'H2'' and H2'H2'' vectors. NOE time courses for Cyt 1 and Ade 4 on irradiation of H1'. (■) H2', (●) H2''. *Filled symbols:* Ade 4; *open symbols:* Cyt 1. The lines are regression fits using the integration of the Bloch-Solomon equations for a spin system consisting of H8, H1', H2', H2'', H3' and H4'

curves for some of the H2'-H2'' and H2'-H3' vectors. Because the protons within deoxyribose are strongly dipolar coupled, it is necessary to take into account the effects of spin diffusion. For example, irradiating the H2'' proton leads to a strong direct NOE to the H2' proton, and a smaller direct NOE to the H1'. The high rate of magnetisation transfer from H2'' to H2' means that there is a significant contribution to the H1' NOE from the H2', even though this distance is about 3 Å. The observed NOE H2'' to H1' is therefore larger than the true NOE, which in turn would yield an overestimate of the cross relaxation rate constant if analysed as a two-spin system. The true cross relaxation rate constant was therefore obtained by calculating the time course of the magnetisation transfer (Lefèvre et al. 1987; Lane 1988) including all the protons in the nucleotide, and comparing the result with the observed NOE time course. The cross relaxation rate constant was refined using a least squares regression Marquardt algorithm (Press et al. 1986), in which the appropriate cross relaxation rate constants and spin-lattice relaxation rate constants were parameters to be determined. Initial values for cross relaxation rate constants were obtained from coordinates for a nucleotide and Eq. (2). Relaxation rate constants that are not in the relaxation pathway for the spins irradiated and observed were held constant. The starting conformation had negligible influence on the values of the fitted cross-relaxation rate constants. The multispin analysis also provides estimates of the cross relaxation rate constants between protons that are not directly observed. For example, irradiating H1' and observing H2' and H2'' yields the σ values not only for the H1'-H2' and H1'-H2'' vectors, but also for the H2'-H2'' vector, which can be compared with the value obtained by irradiating H2' or H2''. The derived cross relaxation rate constants are summarised in Table 3.

Table 3. Correlation times and order parameters for ^{1}H-^{1}H vectors in $(CGTACG)_2$. Cross relaxation rate constants, σ were determined from NOE build up curves at 288 K as described in the text. τ_{app} is the apparent correlation time calculated from σ using Eq. (2). The order parameters, S^2 were calculated as the ratio of the cross relaxation rate constant of vector i to that expected for the same vector whose correlation time is 2.4 ns. Distances were 2.45 Å for C H5-H6, 2.30±0.03 Å for H1'-H2'', 2.32±0.03 for H3'-H2' and 1.79±0.01 Å for H2'-H2''. Except where noted, errors on σ values are <10%

Base	Vector	$-\sigma$ [s^{-1}]	τ_{app} [ns]	S^2
C1	H6-H5	0.61	2.38	0.97
C5	H6-H5	0.61	2.38	0.97
T3	H6-Me	0.35	2.44	1.00
C1	H1'-H2''	0.60	1.65	0.67
	H2''-H1'	0.45	1.3	0.50
	H2''-H2'	2.6	1.6	0.64
	H2''-H2'[a]	3.0	1.8	0.74
G2	H1'-H2''	0.6	1.65	0.67
	H2''-H2'[a]	3.6	2.15	0.89
	H3'-H2'	0.9	2.65	1.0
T3	H1'-H2''	0.66	1.8	0.73
	H2''-H1'	0.74	2.0	0.82
	H2''-H2'	3.5	2.1	0.86
	H2''-H2'[a]	3.9	2.25	0.96
A4	H1'-H2''	0.69	1.75	0.76
	H2''-H2'[a]	3.5	2.1	0.86
	H3'-H2'	0.8	2.25	0.94
C5	H1'-H2''	0.65	1.75	0.72
	H2''-H1'	0.66	1.8	0.73
	H2''-H2'	2.63	1.61	0.65
	H2''-H2[a]	3.0	1.82	0.74
G6	H1'-H2''	0.7	1.9	0.78
	H2''-H1'	0.6	1.65	0.67
	H2''-H2'	3.7	2.2	0.92
	H2''-H2'[a]	3.3	2.0	0.81

[a] Indirectly calculated from multi spin fit for which the error is approximately ±15%

Pseudorotation within the sugar rings is the only motion that could lead to a change in the length of the H-H vectors. However, this motion has only small effects on the H1'-H2'' and H2'-H3' distances (<0.05 Å), and an insignificant effect on the H2'-H2'' distance, leading to errors in the determination of the cross relaxation rate constants of less than 10%, which is comparable to the statistical error of the measurements. Hence, from Eq. (2) and (3), the apparent correlation time for any given vector can be obtained, provided that the distance r is known. The apparent correlation correlation times for the different vectors are also given in Table 3. Interestingly, the apparent correlation times for most of the H2'-H2'' and H2'-H3' vectors are close to those observed for the Cyt H6-H5 vectors. This is in contrast to the results of Gronenborn et al. (1984), who obtained apparent correlation times for the H2'-H2'' vectors three to four times smaller than for the Cyt H6-H5 vectors. The most

likely explanation for this discrepancy is that because the observed protons relax very rapidly, the initial slope of the NOE time course is seriously underestimated, a problem that can be avoided by fitting well-sampled time courses to the appropriate equations. We have calculated the order parameters for the various vectors using Eq. (6) (see Table 3). We find that the order parameters for the H1′-H2″ vectors are significantly smaller than unity, even when analysed using multispin calculations. Further, there is excellent agreement between estimates of σ calculated by irradiating H2″ while observing H1′ and irradiating H1′ while observing H2″. We conclude that there is some rapid internal motion (i.e. faster than about 1 ns) affecting the relaxation properties of these protons.

We have also estimated relative order parameters for these vectors from NOESY spectra, which were run using relaxation delays of 3 T_1. The cross peak volumes of the sugar vectors were compared with the cross peak volume of the two Cyt H6-H5 vectors (data not shown). Although these data were of lower precision than the one dimensional NOE experiments, the same trends were observed, with S^2 (H1′-H2″) $< S^2$ (H2′-H2″), and the order parameters for C1 significantly smaller than for the other five nucleotides. The derived order parameters (Table 3) are very similar to those obtained using a different method of computation (P. Koehl and J.-F. Lefèvre, personal communication) on the same data. Hence, the results represent a property of the data, and not of the method of data reduction. Order parameters for the H1′-H2″ and H2′-H3′ vectors were also determined at 298 K. Values similar to those at 288 K were obtained, with $S^2_{H2'\text{-}H3'} > 0.95$ and $S^2_{H1'\text{-}H2''} \approx 0.6$ for all vectors except Cyt 1, which was about 0.4.

An obvious motion that might account for the observed correlation times is the pseudorotation that is known to occur in this molecule (see above). If the rate of transition between the south and north states is fast compared with molecular tumbling, then the order parameter can be calculated from Eq. (7) (Tropp 1980). Figure 6 shows the dependence of the calculated order parameter on the mole fraction of the C2′ endo state (f_S). Because the order parameter is a population average, the greatest effect of the motion occurs when the two states are equally populated: the minimum value of S^2 for the H2′-H2″ vector is about 0.55 and 0.4 for the H2′-H3′ vectors. The order parameter for the H1′-H2″ vector is less sensitive to pseudorotation (minimum $S^2 = 0.75$). Varying the maximum pseudorotation amplitude (ϕ_m) between 33° and 40° changed the calculated values of S^2 by not more than 0.04. The calculated order parameters for this motion are ranked S^2 H1′-H2″ > H2′-H2″ > H2′-H3′, whereas the reverse ranking is observed experimentally (cf Table 3). Further, there is no correlation between the

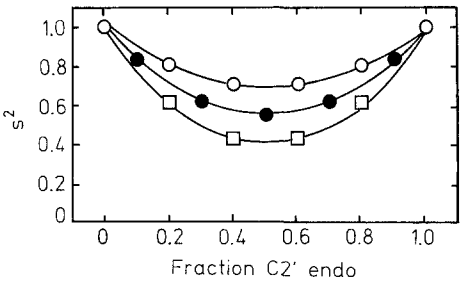


Fig. 6. Dependence of the order parameter on fraction C2′ endo. The order parameter, S^2 was calculated using the method of Tropp (1980) assuming rapid interconversion of C2′ endo and C3′ endo. (●) H2′H2″ vector, (□) H2′H3′ vector, (○) H1′H2″ vector

observed order parameters and the fraction south, as would be expected if pseudorotation were the dominant motion (Fig. 6). For example, the fractions south of A4 and G6 are >0.9, which would imply order parameters of 0.85 (H1′-H2″), 0.75 (H2′-H2″) and 0.65 (H2′-H3″), compared with observed order parameters of 0.72, 0.86 and 0.94 for A4. Hence there must be a motion other than pseudorotation that affects the correlation time of these vectors. In particular, the relatively low value of the order parameter for the H1′-H2″ vectors even when the order parameter for the H2′-H2″ vectors is high indicates that the motion affects the H1′-H2″ vector much more than it does the H2′-H2″ vector. This may be because these vectors are nearly orthogonal. Rotation about an axis parallel to H2′-H2″ would have no influence on the H2′-H2″ vector, but would have the maximum possible effect on the H1′-H2″ vector.

We have also calculated the effect of varying the internal correlation time from 0.01 to 2.0 ns on the calculated order parameters. The main conclusion is that for the order parameters observed (i.e. >0.6), the cross relaxation rate constants are rather insensitive to the frequency of the internal motion (results not shown). This is in agreement with the findings of Lipari and Szabo (1982).

Discussion

The hexamer $d(CGTACG)_2$ presents an ideal case for a detailed analysis of the conformation and dynamics of a nucleic acid by NMR because of its well resolved and simple spectrum. It therefore provides a useful test case for determining the quality of a structural analysis using NMR data alone. The following points can be made.

First, the deoxyriboses in duplex DNA exist as a mixture of north and south conformations, in agreement with the findings of Rinkel et al. (1987) and Bax and Lerner (1988) for other sequences. The data suggest that sugars attached to pyrimidines tend to adopt

a wider range of conformations than sugars attached to purines, both in terms of the average phase angle of the south conformation, and the fraction of the north conformer present.

Second, pseudorotation has only small effects on the correlation times for intra-sugar vectors, though there is at least one other motion that affects the correlation time for the H1′-H2″ vector. Nevertheless, the smallest order parameter measured is about 0.6 (Table 3), which indicates that the assumption of a single correlation time equal to the overall tumbling time may be reasonable in practice. This assumption is implicit in deriving distances for distance geometry and restrained molecular dynamics calculations. These results also agree with Nerdal et al. (1988), who found that internal motions of H2′-H2″ vectors were less significant than those reported by Gronenborn et al. (1984). Interestingly, the order parameter for the H1′-H2″ vector is essentially independent of the sequence, or of the length of the DNA duplex; similar values were also found for a 20 base pair duplex (Lefèvre et al. 1987). The motion must be an intrinsic property of nucleotides.

It is not possible to determine the nature of the motions that give rise to order parameters smaller than unity; only specific motional models can be ruled out. For example, as shown above, pseudorotation is not the dominant motion affecting the H1′-H2″ vectors. However, some information about the angular fluctuation and the axis of the fluctuation can be gleaned from the following argument. According to Lipari and Szabo (1982), the order parameter can be related to the angle, ϕ, of a cone in which the vector moves, as:

$$S^2 = 0.25 \cos(\phi)\,(1+\cos^2(\phi))^2\,. \tag{11}$$

For an order parameter $S^2 = 0.6$, $\phi = 33°$. In the purines, the fraction south conformation is near unity (cf Table 2), so that pseudorotation cannot significantly affect the order parameter for any vector. We have not found any motion about a single axis that accounts for the observed order parameters. It is possible that the observed order parameters reflect coupled motions of small amplitude about several axes.

The order parameters for the H1′-H2″ and H2′-H2″ vectors are smallest for C1, which lacks a 5′ base with which to stack. The first base pair is most subject to end effects, as is shown by the ready exchange of the CG1 imino proton (Fig. 1 C). It is therefore likely that C1 has greater motional freedom than internal bases.

Although internal motions of the sugars have only small effects on the intrasugar NOEs, this cannot be true for sugar to base NOEs. The distances between base protons and sugar protons are very different for south and north conformations (Lefèvre et al. 1987; Wüthrich 1986, and see Fig. 4). Hence, any determination of the glycosidic torsion angle and helical parameters from NOE measurements must take the sugar pucker equilibrium specifically into account. This is considerably simpler for model building methods that refine structure against NOEs (or equivalently against σ values) (Lefèvre et al. 1987; Borgias and James 1988), than for methods based on distances, which are assumed to be unique.

Appendix

Equivalence of the Tropp and Lipari-Szabo models for fixed length vectors

For a fixed length vector, r, the model of Tropp (1980) gives a recipe for averaging the spectral density functions $J(\omega)$ due to rapid jumps between N states, with isotropic tumbling. According to Tropp, the spectral density function is given by:

$$J(\omega) = \left\{\sum_{m=-2}^{2} f(m)\right\}\tau/(1+\omega^2\tau^2) + \left\{\sum_{m=-2}^{2} g(m)\right\}\tau_e/(1+\omega^2\tau_e^2)\,, \tag{A1}$$

where

$$f(m) = (1/N)\left|\sum_{i=1}^{N} Y_{2m}(\theta_i, \phi_i)/r_i^3\right|^2 \tag{A2a}$$

and

$$g(m) = (1/N)\sum_{i=1}^{N}\left|Y_{2m}(\theta_i, \phi_i)/r_i^3\right|^2 - f(m) \tag{A2b}$$

$$\tau_e = \tau\,\tau_i/(\tau+\tau_i)\,, \tag{A3}$$

where τ is the overall tumbling time, and τ_i is the correlation time for the internal motion.

Y_{2m} are the spherical harmonics, defined as:

$$Y_{20} = (3\cos^2\theta - 1)/2 \tag{A4}$$
$$Y_{2\pm1} = (3/2)^{1/2}\cos\theta\sin\theta\exp(\pm i\phi)$$
$$Y_{2\pm2} = (3/8)^{1/2}\sin^2\theta\exp(\pm 2i\phi)\,.$$

In Eq. (A4) the spherical harmonics have been normalised by the factor $(5/4\pi)^{1/2}$, thereby differing from the expressions given by Weissbluth (1978).

In the following, the polar and azimuthal angles, θ, ϕ will be omitted.

If r_i is unchanged by reorientation, the frequency terms can be factored out of Eq. (A1) as:

$$J(\omega) = J^0(\omega\tau)\sum_{m=-2}^{2}(1/N)\left|\sum_{i=1}^{N} Y_{2m}\right|^2 + J^0(\omega\tau_e)\left\{\sum_{m=-2}^{2}(1/N)\sum_{i=1}^{N}|Y_{2m}|^2 - \sum_{m=-2}^{2}(1/N)\left|\sum_{i=1}^{N} Y_{2m}\right|^2\right\}, \tag{A5}$$

where

$$J^0(\omega t) = \tau/(1+\omega^2\tau^2)\,.$$

Using the definitions in Eq. (A 4), it can be shown that the term

$$\sum_{m=-2}^{2}(1/N)\sum_{i=1}^{N}|Y_{2m}|^2$$

in Eq. (A 5) is equal to unity whereas the terms $\sum_{m=-2}^{2}(1/N)\left|\sum_{i=1}^{N}Y_{2m}\right|^2$ can be identified with the order parameter, S^2, that described the amplitudes of the angular fluctuation due to transition between states *i*.

Hence, Eq. (A 5) reduces to:

$$J(\omega) = S^2 J^0(\omega\tau) + (1-S^2)J^0(\omega\tau_e) \qquad (A\,6)$$

which is identical to Eq. (5 A) with the equation given by Lipari and Szabo (1982). Hence, while the formalism of Lipari and Szabo (1982) is particularly convenient for analysing experimental data, requiring at most three independent parameters, it is possible to use the formalism of Tropp (1980) to calculate the value of S^2 for specific motional models.

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