Deuterium isotope effects and fractionation factors of hydrogen-bonded A:T base pairs of DNA

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Abstract

Deuterium isotope effects and fractionation factors of N1$\cdots$H3–N3 hydrogen bonded Watson–Crick A:T base pairs of two DNA dodecamers are presented here. Specifically, two-bond deuterium isotope effects on the chemical shifts of ^{13}C2 and ^{13}C4, $^2\Delta^{13}$C2 and $^2\Delta^{13}$C4, and equilibrium deuterium/protium fractionation factors of H3, Φ, were measured and seen to correlate with the chemical shift of the corresponding imino proton, δ_{H3}. Downfield-shifted imino protons associated with larger values of $^2\Delta^{13}$C2 and $^2\Delta^{13}$C4 and smaller Φ values, which together suggested that the effective H3–N3 vibrational potentials were more anharmonic in the stronger hydrogen bonds of these DNA molecules. We anticipate that $^2\Delta^{13}$C2, $^2\Delta^{13}$C4 and Φ values can be useful gauges of hydrogen bond strength of A:T base pairs.

Introduction

Hydrogen bonds are central to the structure and function of nucleic acids (Saenger, 1984; Jeffrey and Saenger, 1991). Recently, trans-hydrogen bond $^{h2}J_{NN}$ and $^{h1}J_{NH}$ scalar couplings were measured in uniformly ^{15}N-labeled DNA and RNA molecules (Dingley and Grzesiek, 1998; Pervushin et al., 1998; Wilkens et al., 2002) and shown to report on hydrogen bond distances as evidenced by their correlation to the isotropic chemical shifts of the imino protons (Dingley et al., 1999) and through calculations (Barfield et al., 2001; Wilkens et al., 2002).

Isotope effects on chemical shifts are also measures of hydrogen bond strength. A downfield shift of ∼0.34 ppm of an adenosine ^{15}N1 resonance of a DNA duplex was observed upon substitution of ^{2}H3 for ^{1}H3 of the base-paired deoxythymidine residue, which was a demonstration of a trans-hydrogen bond isotope effect (Wang et al., 1991). Two-bond deuterium isotope effects on ^{13}C chemical shifts, defined

as $^2\Delta^{13}$C $= \delta^{13}$C(^{1}H) $- \delta^{13}$C(^{2}H), gauge hydrogen bond strength as shown from linear correlations with the isotropic chemical shift of the hydrogen-bonded proton in a variety of small compounds (Reuben, 1986b; Dziembowska et al., 1997) and by calculations (Reuben, 1986b; Abildgaard et al., 1998). Previously, $^2\Delta^{13}$C2 and $^2\Delta^{13}$C4 of deoxythymine and uridine have been observed to increase upon formation of hydrogen-bonded homo- and heterodimers (Gmeiner and Poulter, 1988; Reuben, 1987). As a result of these studies we were interested in the potential usefulness of $^2\Delta^{13}$C values as markers of hydrogen bond strength in DNA.

Equilibrium deuterium/protium fractionation factors (Kreevoy, 1976; Cleland, 1980; Schowen and Schowen, 1982; Harris and Mildvan, 1999), Φ, are another informative probe of hydrogen bonds and have been measured in isotopically enriched proteins (Loh and Markley, 1994; Bowers and Klevit, 1996; LiWang and Bax, 1996; Khare et al., 1999). The fractionation factor of a solvent-exchangeable site measures the preference for deuterium over hydrogen, relative to solvent. Protein backbone amides of α helices have

lower Φ values than β strands on average, and recent evidence suggests that α-helical hydrogen bonds provide at least 0.7 kcal/mol more stability than those of β strands (Shi et al., 2002).

Previously, a Φ value of 0.89 was measured at the imino site of thymidine in a 2:1 mixture of adenosine and deoxythymidine in Me_2SO-d_6 and using CH_3OD as the deuterium source (Reuben, 1986a). Thus, we were interested whether Φ values of imino sites of DNA in an aqueous solution would be useful as gauges of hydrogen bond strength. Here we report $^2\Delta^{13}C2$, $^2\Delta^{13}C4$ and Φ values of the imino site of deoxythymidine 5'-monophosphate (dTMP) and of deoxythymidine residues of [d(CGCGAATTCGCG)]$_2$ and [d(CGCGTATACGCG)]$_2$, which will be referred to as **1** and **2** hereafter.

Materials and methods

Non-isotope-enriched samples of **1** and **2** were dissolved in an aqueous buffer containing 125 mM NaCl, 50 mM NaH_2PO_4, 0.75 mM EDTA, 0.2 mM DSS, 0.02% NaN_3, pH 7. Final DNA duplex concentrations were approximately 3.5 and 6 mM for **1** and **2.** All NMR spectra were collected at 14.1 T and 25 °C. Non-exchangeable proton chemical shift assignments of **1** (Hare et al., 1983) and **2** (Cheng et al., 1992) were obtained from previous studies and used to assign our NOESY spectra of **1** and **2** in D_2O. Assignments of imino and amino chemical shifts were performed using WATERGATE NOESY spectra (Piotto et al., 1992) of samples in 95% H_2O, 5% D_2O. Assignments of ^{13}C aromatic chemical shifts of deoxythymidine residues were determined from a combination of one-bond and long-range ^1H, ^{13}C heteronuclear single-quantum correlation (HSQC) spectra (Bax et al., 1996). All chemical shifts were referenced to internal DSS (Markley et al., 1998).

Under the experimental conditions used here the imino protons of the deoxythymidine residues of **1** and **2** were in slow exchange with the solvent. Thus, values of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$ could be measured using long-range ^1H, ^{13}C HSQC spectra that correlate C7($^1H7$)$_3$ and $^{13}C4$, and $^1H6$ and $^{13}C2$ spins of **1** and **2** equilibrated in an equimolar mixture of D_2O and H_2O. The isotopic ratio was chosen to (1) maximize the sensitivity of both isotopomers and (2) minimize the effect of possible errors in molar ratios of H_2O/D_2O. Equimolar solvent mixture required that the ratio of volumes of D_2O/H_2O = 1.004 at 25 °C (Weast, 1988),

Table 1. Equilibrium deuterium/protium measurements of **1**, **2** and dTMP[a]

	$^2\Delta^{13}C2$	$^2\Delta^{13}C4$	Φ[b]	δ^c_{H3}
1, T7	128 ± 1	168 ± 1	0.88 ± 0.03	13.63
1, T8	129 ± 2	168 ± 2	0.83 ± 0.02	13.76
2, T5	123 ± 3	164 ± 1	0.93 ±0.01	13.34
2, T7	119 ± 3	162 ± 2	0.94 ± 0.04	13.19
dTMP	60 ± 1	76 ± 1	1.10 ± 0.02	11.0–10.5[d]

[a]Units of $^n\Delta A = \delta_A(^1H3) - \delta_A(^2H3)$ are in ppb and units of δ_{H3} are in ppm relative to internal DSS. Shown for **1** and **2** are average values from five separate data sets and the uncertainties of the average values.
[b]Equilibrium deuterium/protium fractionation factors of the imino sites of thymine.
[c]Isotropic chemical shifts of the imino protons of thymine relative to DSS in ppm.
[d]As the resonance of the imino proton of dTMP in H_2O was not observable due to fast exchange with the solvent, we assumed that it would be similar to experimentally measured values of $^1H3$ of apparently non-hydrogen bonded uridine and deoxythymidine residues of RNA and DNA molecules in aqueous solutions (Escaja et al., 2000; Dejong et al., 2002).

and our uncertainty in the ratio was ±0.004. The same spectra were used to obtain Φ values from the peak heights of the correlations between C7($^1H7$)$_3$ and $^{13}C4$, after correction for differential relaxation in a manner analogous to that described earlier (LiWang and Bax, 1996). Spectra were collected as data matrices of 512* × 80* along t_2 (71.1 ms) and t_1 (58.9 ms), respectively (n^* represents n complex data points). Final digital resolutions were 6.2 and 5.3 Hz along F_2 and F_1, respectively. Each spectrum, which required 75 h for data collection, used an interscan delay of 2.5 s and a period of 60 ms for each INEPT and reverse INEPT period. Peak positions were determined using contour averaging as described earlier (Wang and Bax, 1996) and peak heights were measured using polynomial interpolation with the program PIPP (Garrett et al., 1991). A total of five such long-range ^1H, ^{13}C HSQC spectra were collected for **1** and **2**. Between recording three of the spectra of sample **1**, the sample was lyophilized and resuspended in 50% H_2O, 50% D_2O in order to estimate the precision of sample preparation: uncertainties in Φ for **1** were the same as those for **2**, which was not lyophilized between data sets (Table 1).

Unlike for **1** and **2**, the exchange rate of the imino hydrogen with solvent was fast for dTMP and necessitated the use of different experiments. Thus, values of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$ were determined from two long-range ^1H, ^{13}C HSQC spectra, one of which was
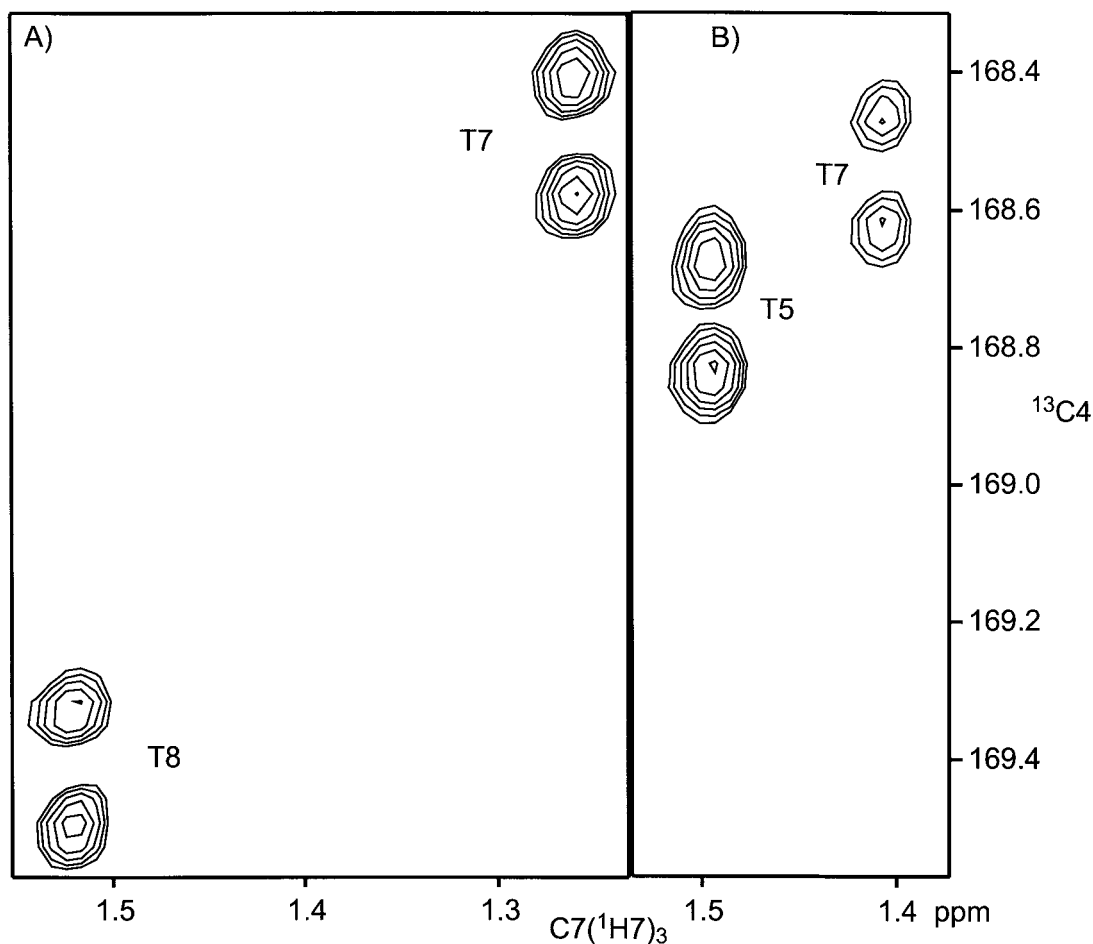
Figure 1. Small regions of long-range HSQC spectra of (A) **1** and (B) **2** in equimolar mixtures of H_2O and D_2O, which show the correlations between C7(^{1}H7)$_3$ and ^{13}C4 of the deoxythymidine residues. These spectra were taken from one of the five data sets collected, which were used to obtain $^2\Delta^{13}$C2, $^2\Delta^{13}$C4 and Φ values.

collected in H_2O and the other in D_2O. The chemical shifts of dTMP in 100% H_2O were measured using a concentric NMR tube in which the solution of dTMP was placed in the outer chamber and field-frequency locking was achieved by filling the inner chamber with D_2O. Due to fast exchange, determination of the Φ value of the imino site of dTMP was carried out using several different D_2O/H_2O solvent ratios as described by Jarrett and Saunders (1985). Data were acquired as two-dimensional matrices of 1792* × 150* points along t_2 (299 ms) and t_1 (1.837 s), respectively. Sample concentrations were 100 mM of unlabeled dTMP. No changes were observed in the non-exchangeable ^{1}H and ^{13}C chemical shifts at concentrations of 10 and 100 mM dTMP in D_2O, which indicated that intermolecular interactions, if any, were identical at both concentrations. Buffer conditions were 125 mM NaCl,

50 mM NaH_2PO_4, 0.75 mM EDTA, 0.2 mM DSS, 0.02% NaN_3, pH 7.

Results

Shown in Figure 1 are small regions of long-range ^{1}H, ^{13}C HSQC spectra showing correlations between C7(^{1}H7)$_3$ and ^{13}C4 of deoxythymidine residues of **1** and **2** in a solvent mixture of 50% H_2O, 50% D_2O. The correlations were split along the ^{13}C4 dimension with a median value of 165 ± 3 ppb as a result of the two-bond isotope shift, $^2\Delta^{13}$C4. The upfield and downfield 'doublet' components correspond to deuterated and protonated states of the imino site of thymine, respectively. The upfield component was assigned to the deuterated isotopomer from spectra collected in
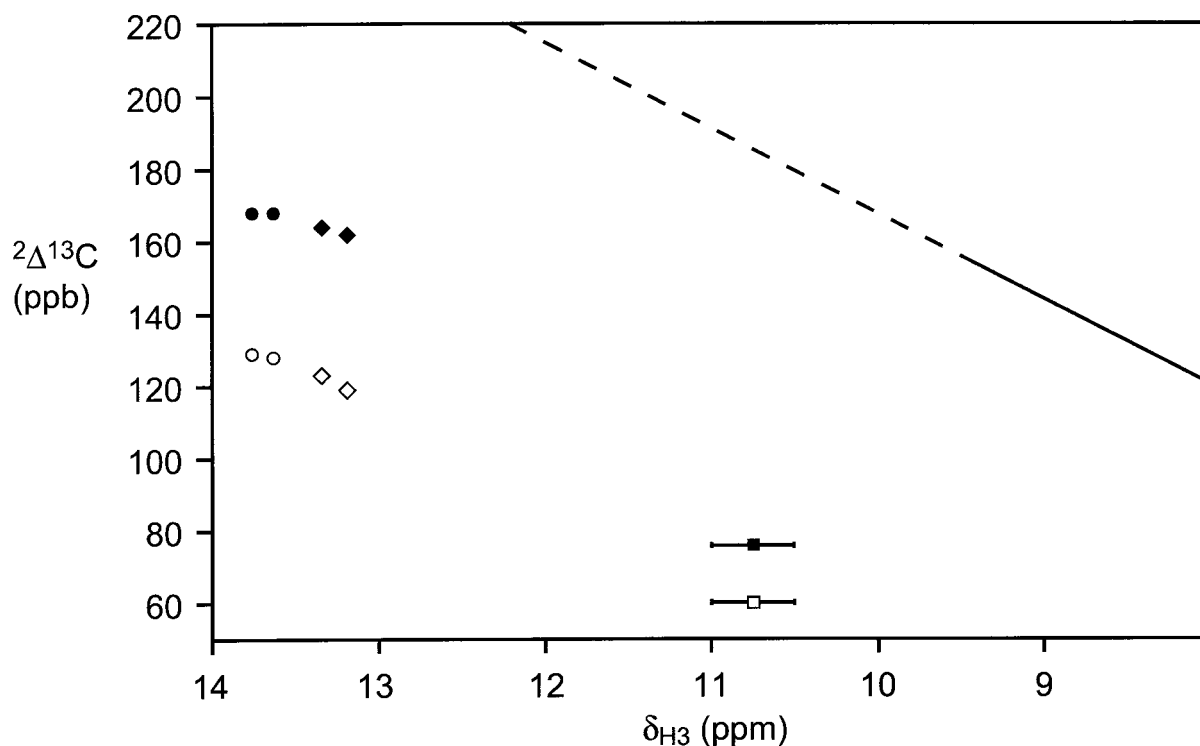
Figure 2. A plot of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$ of thymine versus the corresponding value of δ_{H3}. Open and solid symbols are used for values of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$, respectively. Circles, diamonds, and square are used for **1**, **2** and dTMP, respectively. The uncertainties in the values of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$, which did not exceed ± 3 ppb, do not extend beyond the size of the symbols. The uncertainties in the chemical shifts of $^1H3$ of **1** and **2** were smaller than the width of the symbols. The value of δ_{H3} of dTMP was assumed to be similar to those of apparently non-hydrogen bonded imino protons of uridine and deoxythymidine residues of RNA and DNA molecules (Escaja et al., 2000; Dejong et al., 2002). The solid line is from a linear fit of $^2\Delta^{13}C$ versus δ_{NH2} of intramolecular hydrogen bonded amino protons of a variety of aniline derivatives and is given by $^2\Delta = -68.4 + 23.6\delta_{NH2}$ (Reuben, 1987). The dashed line is an extrapolation of the fit of the aniline data beyond the original experimental data points (not shown).

D_2O. Deuterium two-bond isotope shifts at $^{13}C2$ were also observed through weaker correlations to $^1H6$ in the long-range spectra and had a median value of 124 ± 5 ppb in **1** and **2** (Table 1). Values of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$ of dTMP were significantly lower than those of **1** and **2** (Table 1) and the same as those measured in monomeric $2'$, $3'$, $5'$-O-tribenzoyluridine in chloroform within experimental uncertainty (Gmeiner and Poulter, 1988). Shown in Figure 2 is a plot of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$ of dTMP and of the deoxythymidine residues of **1** and **2** against the corresponding values of δ_{H3}. The chemical shifts of $^1H3$ of **1** and **2** were measured from NOESY spectra (data not shown). As the resonance of the imino proton of dTMP in H_2O was not observable due to fast exchange with the solvent, we assumed that it would be similar to experimentally measured values of $^1H3$ of apparently non-hydrogen bonded uridine and deoxythymidine residues of RNA and DNA molecules in aqueous solutions (Escaja

et al., 2000; Dejong et al., 2002). As seen in Figure 2, downfield-shifted δ_{H3} values associated with larger values of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$. The line shown was taken from a linear fit of $^2\Delta^{13}C$ versus δ_{NH2} of intramolecular hydrogen-bonded amino protons of a variety of aniline derivatives (Reuben, 1987). The discrepancy between the aniline and DNA data was due to the fact that the corresponding hydrogen bonding groups were different. The DNA hydrogen bonds were of the N$\cdots$H–N type whereas those of the aniline derivatives were intramolecular and of the O$\cdots$H–N type with amino instead of imino protons. Thus, the discrepancy probably contained a significant horizontal component.

Figure 3 shows Φ values of the imino site of dTMP and of the deoxythymidine residues of **1** and **2** plotted against the corresponding chemical shift of $^1H3$. A trend was observed where downfield-shifted δ_{H3} values associated with smaller values of Φ. As the ex-
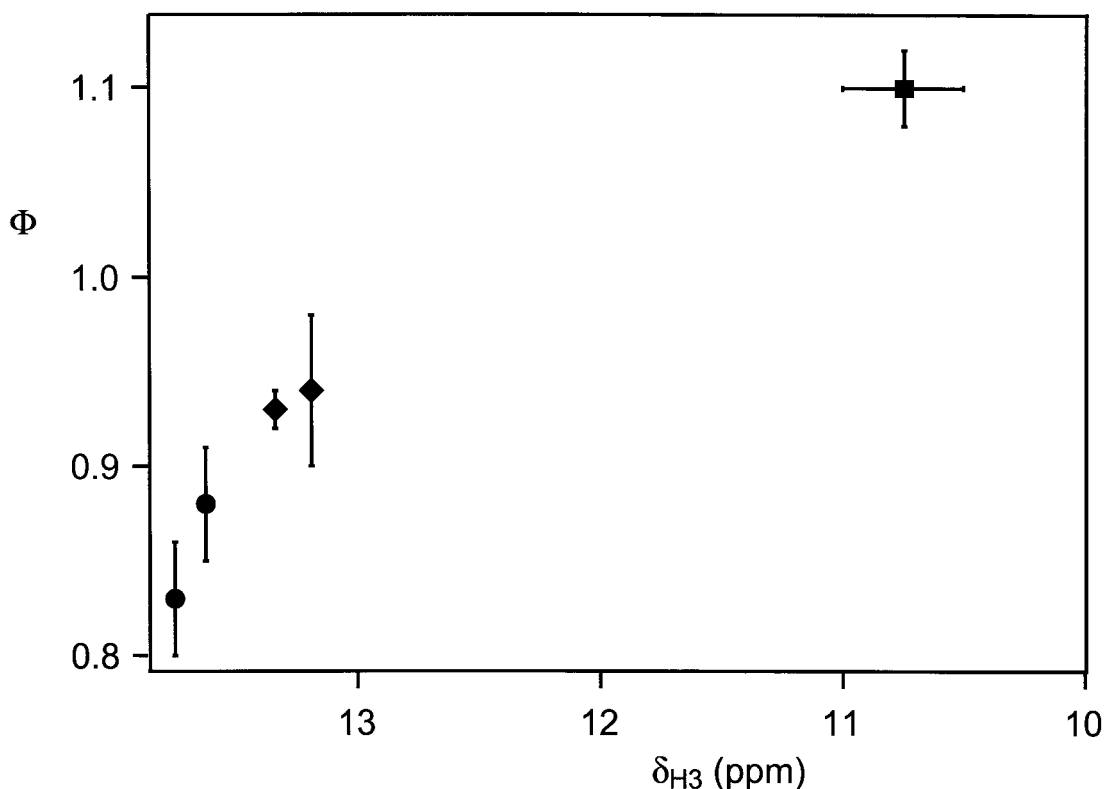
Figure 3. A plot of Φ values of the imino site of dTMP and of the deoxythymidine residues of **1** and **2** against the corresponding δ_{H3} values. Circles, diamonds, and square are used for **1**, **2** and dTMP, respectively. The uncertainties in the chemical shifts of ^{1}H3 of **1** and **2** were smaller than the width of the symbols. The value of δ_{H3} of dTMP was assumed to be similar to those of apparently non-hydrogen bonded imino protons of uridine and deoxythymidine residues of RNA and DNA molecules (Escaja et al., 2000; Dejong et al., 2002).

change rate of the imino proton of dTMP with solvent was fast, the Φ value of the imino site was determined using seven different solvent mixtures of dTMP: 0%, 5%, 20%, 40%, 60%, 80%, and 100% D_2O. Figure 4 shows a plot of ν_{av}/ν_{D_2O} versus D_2O mole fraction of dTMP, where $\nu_{av} = \delta_{H_2O} - \delta_{av}$, δ_{av} and δ_{H_2O} are the chemical shifts of $^{13}C4$ observed in a given mole fraction of D_2O and 100% H_2O, respectively, $\nu_{D_2O} = \delta_{H_2O} - \delta_{D_2O}$, and δ_{D_2O} is the chemical shift of $^{13}C4$ in 100% D_2O. The Φ value, 1.10 ± 0.02, was obtained from a non-linear least squares fit to the data and regression analysis using the following equation (Jarret and Saunders, 1985):

$$\frac{\nu_{av}}{\nu_{D_2O}} = \frac{\Phi \times (\%\text{water} - D)}{(\%\text{water} - H) + \{\Phi \times (\%\text{water} - D)\}} \quad (1)$$

The experimental approach used to determine Φ values in **1** and **2** was analogous to that described previously for the slowly exchanging amides of proteins (LiWang and Bax, 1996). As the samples were equilibrated in a solvent mixture of 50% H_2O, 50% D_2O,

the fractionation factor equals the population ratio of deuterated over protonated states. Although Φ values can be obtained from peak volumes in long-range ^{1}H, ^{13}C HSQC spectra (Figure 1), measurement of peak heights was found to be more reproducible than volume determination here and earlier (LiWang and Bax, 1996). Thus, integrated intensities were derived from peak heights, which must be corrected for the small increase in transverse relaxation rates of $C7(^1H7)_3$ and $^{13}C4$ of thymine when the N3 of thymine is protonated rather than deuterated. Calculated in this manner,

$$\Phi = A_1 \times A_2 \times I_D/I_H, \quad (2)$$

where I_D and I_H are the peak heights of the correlations of $C7(^1H7)_3$, $^{13}C4\{^2H3\}$ and $C7(^1H7)_3$, $^{13}C4\{^1H3\}$, respectively, and A_1 and A_2 are the transverse relaxation correction factors along the F_1 and F_2 dimensions. The effective correlation time was estimated to be 4.3 ns using hydrodynamic theory (Tirado and de la Torre, 1980; Eimer et al., 1990) for a 12 base-pair DNA duplex in a solution of 50% H_2O, 50% D_2O
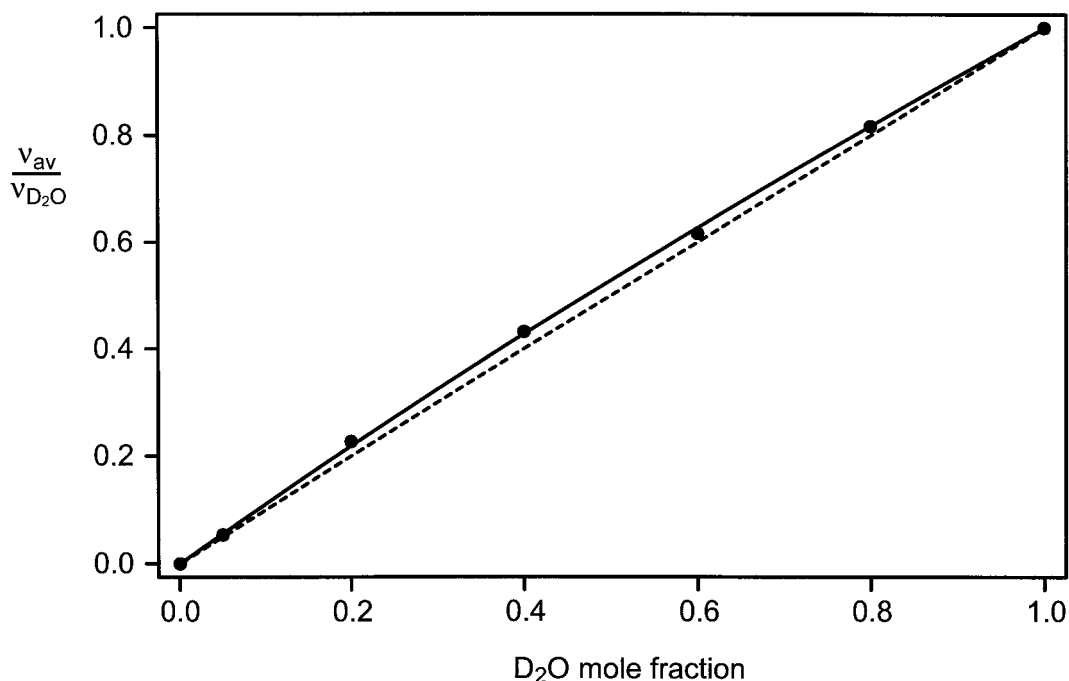
Figure 4. Plot of ν_{av}/ν_{D_2O} versus D_2O mole fraction for dTMP. The Φ value, 1.10 ± 0.02, was obtained from a fit to equation 1, shown as a solid line, while the diagonal is shown as a dashed line.

at 25 °C (Weast, 1988). An NMR relaxation study of ^{13}C-labeled purines of DNA decamers yielded average values of $S^2 = 0.8 \pm 0.1$ and $\tau_e = 20 \pm 20$ ps for internal motions of the duplexes (Kojima et al., 1998) and these average values were used here. As such, the transverse relaxation rate of $^{13}C4\{^1H3\}$ was calculated to be faster than that of $^{13}C4\{^2H3\}$ by 0.4 s^{-1} when considering only the difference in dipole-dipole interactions induced upon protonation/deuteration. We were uncertain of the isotope effect on the individual components of the $^{13}C4$ shielding tensor and assumed that the CSA of $^{13}C4$ (Gregory et al., 1997) was identical for both isotopomers. The effect of the differential relaxation rates on peak heights was determined by applying 0.4 s^{-1} of additional line broadening along the F_1 dimension, which yielded a value of 0.97 for A_1. The different transverse relaxation rates of $C7(^1H7)_3\{^1H3\}$ and $C7(^1H7)_3\{^2H3\}$ (Dellwo and Wand, 1993) during fixed periods and t_2 resulted in a value of 0.997 for A_2. Inversion-recovery measurements showed $C7(^1H7)_3$ of **1** to have an average non-selective T_1 relaxation time of ~ 2 s in 50% H_2O, 50% D_2O. The distance range between H3 and $C7(H7)_3$ is >4.5 Å and there should be no measurable dependence of the relative signal intensities of $C7(^1H7)_3\{^1H3\}$ and $C7(^1H7)_3\{^2H3\}$ on the interscan delay (2.5 s),

as estimated from calculated longitudinal relaxation rates (Dellwo and Wand, 1993). Likewise, exchange of imino protons with excited water protons should not affect measured fractionation factors using the experimental approach described here. It was also assumed that isotopic fractionation at any given site was independent of neighboring sites. It is worth noting that the observed trend between Φ values and δ_{H3} would persist even in the presence of systematic errors in our relaxation analysis, as such errors would influence Φ values of **1** and **2** equally.

Discussion

Hydrogen bonding to an electronegative atom shifts the hydrogen-bonded proton to higher frequencies (Becker, 1996) and the chemical shifts of exchangeable protons in proteins (Wagner et al., 1983) and nucleic acids (Wüthrich, 1986) are sensitive to hydrogen bonding. However, chemical shifts are also modulated by other geometric and ring current effects (Giessner-Prettre and Pullman, 1987; Case, 1995), and it is difficult to use the isotropic chemical shift as a quantitative measure of the hydrogen bond strength of proteins and nucleic acids. Thus, there has been

much interest in using other probes of hydrogen bond strength that are less susceptible to other effects. However, all of the new methods require isotopic enrichment of the sample, which is more technically demanding to accomplish for nucleic acids than for proteins (Batey et al., 1995; Smith et al., 1997). One attractiveness of $^2\Delta^{13}C2$, $^2\Delta^{13}C4$ and Φ values as potential gauges of hydrogen bond strength of A:T base pairs is that even without isotopic enrichment they can be measured with significant precision and accuracy.

The Φ data reported here reveals differences in the effective vibrational potentials of the H3–N3 bonds of **1, 2** and dTMP. The trend of decreasing Φ values with increasing δ_{H3} (Figure 3) suggested that the N1$\cdots$H3–N3 hydrogen bond of a Watson-Crick A:T base pair widens the effective vibrational potential of the H3–N3 bond. The correlation between $^2\Delta^{13}C$ and δ_{H3} observed here (Figure 2) is corroborated by studies showing that the contribution of molecular zero-point vibrational motions to electronic shielding of nuclei depends on the anharmonicity of the potential surface (Ruud et al., 2001). Increasing values of $^2\Delta^{13}C2$ and $^2\Delta^{13}C4$ with increasing δ_{H3} suggested that stronger N1$\cdots$H3–N3 hydrogen bonds of A:T base pairs also possess more anharmonic H3–N3 vibrational potentials.

The sequences of **1** and **2** were chosen largely because their deoxythymidine imino proton chemical shifts were well separated, which indicated differences in their hydrogen bond strengths. Also, studies showed that the A:T base pairs of **1** have longer base pair lifetimes (Patel et al., 1983; Leijon and Gräslund, 1992; Nuutero et al., 1994) and should possess a higher propeller twist relative to **2** (Yoon et al., 1988; Yanagi et al., 1991; Shui et al., 1998). However, the distances between N1 and N3 of the A:T base pairs of **1** in the 1.4 Å crystal structure were the same within uncertainty (Shui et al., 1998). In addition, the structure of **2** was determined using NMR where 'standard' hydrogen bond restraints were employed (Cheng et al., 1992). Thus, no comparisons between our measurements and hydrogen bond geometry could be made.

Although δ_{H3}, $^{h2}J_{NN}$ and $^{h1}J_{NH}$ are already sensitive measures of hydrogen bond strength, the availability of experimental $^2\Delta^{13}C2$, $^2\Delta^{13}C4$ and Φ values may be useful to validate computationally-developed hydrogen bond potentials. We anticipate $^2\Delta^{13}C2$, $^2\Delta^{13}C4$ and Φ values to display significant correlations with values of $^{h2}J_{NN}$ and $^{h1}J_{NH}$. It should be noted that measurement of Φ requires significantly more signal averaging than for $^2\Delta^{13}C4$. The $^2\Delta^{13}C4$ and Φ measurements took advantage of the sensitivity of the methyl group of thymine. Thus, analogous measurements of cytosine and uridine residues in DNA and RNA at natural abundance ^{13}C will be hampered by lower signal-to-noise.

Conclusions

The data presented here indicate that $^2\Delta^{13}C2$, $^2\Delta^{13}C4$ and Φ values can be useful as gauges of hydrogen bond strength of N1$\cdots$H3–N3 hydrogen bonds of Watson-Crick A:T base pairs, as evidenced by their correlations with δ_{H3} values of the associated imino protons. Measurements of $^2\Delta^{13}C$ and Φ report on the anharmonicity and width of the effective H3–N3 vibrational potential, respectively. We anticipate that a quantitative relationship between these parameters and hydrogen bond strength can be established through a combination of empirical approaches and a simultaneous treatment of $^2\Delta^{13}C$ and Φ using *ab initio* calculations.

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