opposite A at a significantly lower rate than AP opposite C (Mhaskar & Goodman, 1984). We have shown in this paper that the geometry of the AP·A mismatch is wobble with both bases oriented anti. Perhaps the apparent preference of DNA polymerase to form the less stable AP·C mispair is an example of how active site geometric constraints allow the polymerase to discriminate against even relatively stable base mispairs that form other than Watson–Crick geometry.

Registry No. AP, 452-06-2; d[CGA(AP)GGC]·d(GCCACCG), 105356-93-2; adenine, 73-24-5; guanine, 73-40-5; cytosine, 71-30-7.

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NMR Studies of the Stable Mismatch Purine–Thymine in the Self-Complementary d(CGPuAATTTCG) Duplex in Solution†

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ABSTRACT: One- and two-dimensional nuclear Overhauser effect experiments demonstrate that a single hydrogen bond between a T imino proton and purine N3 is sufficient to hold the base pair dPu·dT in d(CGPuAATTTCG) by a Watson–Crick fashion rather than a Hoogsteen type. In addition, the dPu·dT base pair is well stacked with neighboring base pairs. The spin–lattice relaxation measurements at 30 and 35 °C of two decamers, d(CGPuAATTTCG) and d(CGAAATTTCG), reveal that the elimination of two single hydrogen bonds of dA·dT base pairs (due to the substitution of adenine for purine) in the sequence results in an increase in the overall imino proton exchange rate from 7 to 36 s^{-1} at the site of mismatch.

Recently we have demonstrated by dissociation kinetic experiments that the incorporation of a single mismatched base pair destabilizes DNA duplexes to some extent, depending on the nature of the mismatched base pair incorporated (Ikuta et al., 1987). In that system, stable mismatched G·A and G·T slightly destabilize a duplex while unstable mismatches of A·A, T·T, C·T, and C·A significantly destabilize it relative to a perfectly matched duplex. Similar high-stability duplexes involving guanosine residues have been reported for homopolymer and oligonucleotide duplexes containing G·A and G·G interactions (Dodgson & Wells, 1977; Gilliam et al., 1975). Aboul-ela et al. (1985) reported on studies of mismatched bases and concluded that G·G as well as G·T (Patel et al.,

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1982) and G·A (Patel et al., 1984; Kan et al., 1983) form hydrogen bonds. These and other (Kennard, 1986) results provide direct information on the stability of the mismatched duplexes and a rule to predict the formation of stable mismatches. The rule (Ikuta et al., 1987) that a single imino proton is necessary to form a stable mismatch accounts for the stability of mismatched duplexes, though the stacking of base pairs also significantly contributes to it (Cantor & Shimmel, 1980; Bloomfield et al., 1974). However, the mode of the base pairing and conformation of the mismatched bases in solution can be determined only by NMR spectroscopy. Besides the stability of the duplex, dA·dT can readily adopt a Hoogsteen-type base pair when the DNA octamer d(GCGTACGC) interacts with the bis-intercalator antibiotic triostin A (Quigley et al., 1986). We have used proton NMR extensively to investigate the conformation and dynamics of mismatched base pairs between deaminoadenine (purine) and thymine. In this paper, we report that purine pairs with thymine in a Watson–Crick manner and the dPu·dT[1] base pair is well stacked in the DNA duplex. However, the stability of the duplex decreases due to lack of an $-NH_2\cdots O=C<$ interaction.

Materials and Methods

Materials. 2′-Deoxynebularine was prepared following published procedures (Nair & Chamberlain, 1984). The two DNA molecules d(CGAAATTTCG) and d(CGPuAATTTCG) were synthesized by the phosphotriester method (Tan et al., 1983; Eritja et al., 1986). Positive counterions of the DNA were changed to sodium by a procedure described before (Ikuta et al., 1986). The DNA oligomers, 260 OD units at 260 nm, were dissolved in 0.3 mL of D_2O or 80% H_2O/20% D_2O, containing 10 mM sodium phosphate and 0.1 M NaCl at pH 6.4. All NMR experiments were carried out with samples in 5-mm NMR tubes.

For spectra in D_2O, the samples were exchanged twice with 99.996% D_2O (Cambridge Isotopes) and lyophilization. The samples were then dissolved in 0.3 mL of the same D_2O and transferred to 5-mm NMR tubes.

NMR Measurements. NMR experiments were performed on Nicolet NT-300 instrument with a 293-C pulse programmer. Nuclear Overhauser effects were recorded by one-dimensional difference spectroscopy (Hare & Reid, 1982; Gronenborn et al., 1984), with presaturation pulses of defined length described in the figure captions and relaxation delay of 4 s. Low-field spectra were obtained in 80% H_2O/20% D_2O with a 1–1 hard pulse sequence (Kime & Moore, 1983). Pure absorption-phase NOESY spectra (States et al., 1982) were obtained with 1024 points in t_2 and 256 points in t_1. A total of 64 scans were accumulated with 3-s delays between acquisitions. Chemical shifts (ppm) were determined relative to the chemical shift of water (or the residual HOD peak).

Results and Discussion

Figure 1 shows the sequences of the two self-complementary 10 base pair duplexes along with the numbering system used.

NOE between Exchangeable Protons and Aromatic Protons. NMR spectra of the low-field and aromatic region of d(CGPuAATTTCG) at 18 °C is presented in Figure 2A. In the 12–14 ppm low-field region, four different signals are seen; among them, two signals at 13.70 and 13.81 ppm are easily assigned to the thymine imino proton (T imino) of the dA-dT base pairs at positions 4 and 5. The remaining two signals

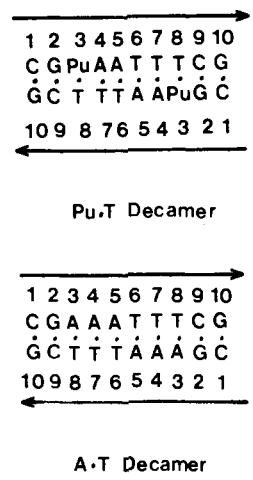

FIGURE 1: Sequences of two decamers are shown with the numbering system used. Pu denotes purine.

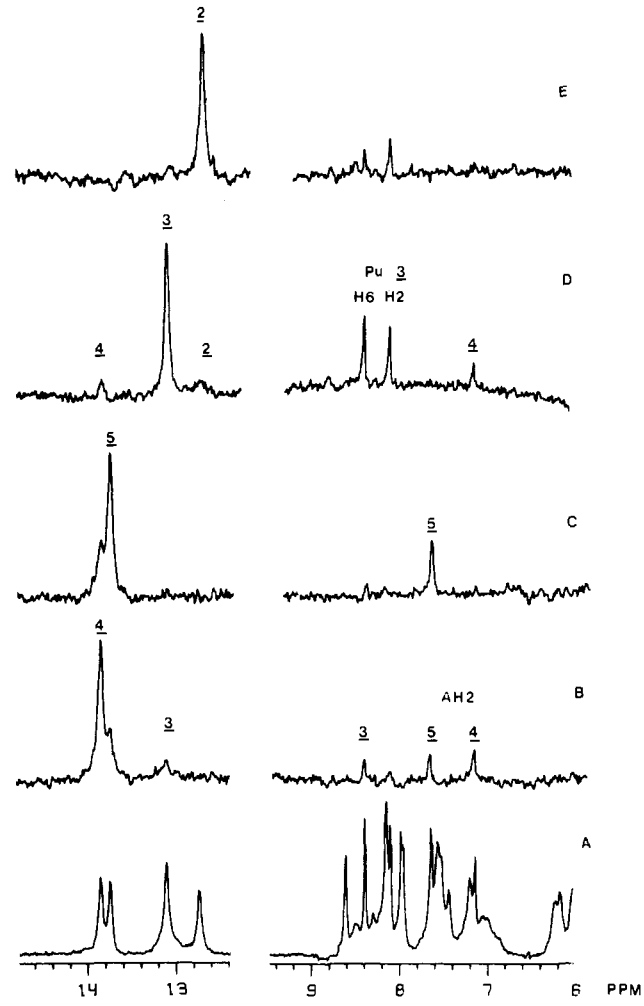

FIGURE 2: NOE measurements on the Pu·T decamer at 13 °C. (A) Saturating pulse applied to a resonance-free region (off-resonance); (B) difference spectra following a 1-s selective presaturation of the 13.81 ppm T imino at position 4; (C) 13.70 ppm T imino at position 5; (D) 13.70 ppm T imino at position 3; (E) 12.70 ppm G imino at position 2. Each resonance is numbered to the corresponding base pair on the top of the signal.

at 13.07 and 12.70 pm are due to the guanine imino proton (G imino) of the dG·dC base pairs at positions 1 and 2 and the T imino of the purine–thymine (dPu·dT) base pair at position 3. To unambiguously assign the resonances to particular base pairs, we have carried out a series of one-dimensional NOE experiments as shown in Figure 2 (Roy & Redfield, 1981; Hare & Reid, 1982). Saturation of T imino

[1] Abbreviations: dPu, 9-(β-D-2-deoxyribofuranosyl)purine or 2′-deoxynebularine; NOE, nuclear Overhauser effect.

Table I: Chemical Shifts of Imino Proton Resonances in the Purine Decamer Duplex at 13 °C

base pair	chemical shifts (ppm)
dG·dC (1, 10)	13.07
dG·dC (2, 9)	12.70
dPu·dT (3, 8)	13.07
dA·dT (4, 7)	13.81
dA·dT (5, 6)	13.70

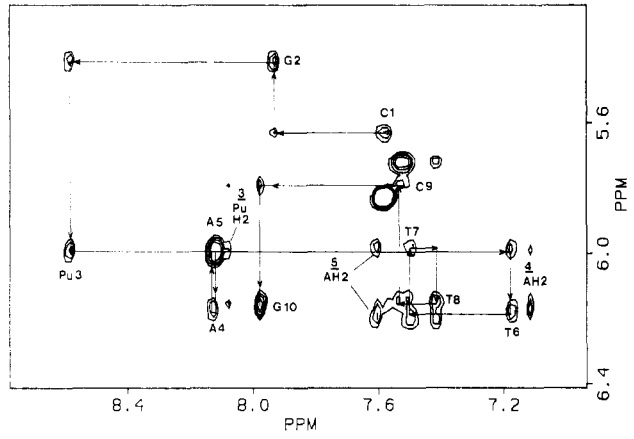

FIGURE 3: Selected portion of the NOESY spectrum of the Pu·T decamer obtained with a mixing time of 500 ms at 13 °C. The cross-peaks between the aromatic region and the anomeric H1–CH_5 region were traced sequentially from C-1 to G-12 except the interaction between TH1′ at position 7 and TH6 at position 8. The cross-peaks between AH2, PuH2, H1′ are also noted.

at 13.70 ppm results in a large intra base pair NOE at the 7.62 ppm AH2 resonance (Figure 2C), but no NOE is observed at 13.07 and 12.70 ppm where the G imino and T imino of base pairs 1, 2, and 3 resonate. This permits assignment of the 13.70 ppm signal to the T imino at position 5 and the 7.62 ppm signal to AH2 in the same residue. Consequently, the other imino resonance at 13.81 ppm is assigned to the T imino at position 4. Saturation of the T imino at position 4 results in the small inter base pair NOE on the T imino at position 3 and two large NOEs arising from AH2 at positions 4 and 5 (Figure 2B). The imino–imino inter base pair NOE reveals that the dPu·dT mismatch at position 3 is held together through a single hydrogen bond and is well stacked with the dA·dT base pair at position 4. Conversely, saturation of the 13.07 ppm T imino of the dPu·dT base pair at position 3 results in the inter base pair NOE at positions 2 and 4. Strong intra base pair NOEs at 8.39 and 8.10 ppm are quite narrow due to longer spin–spin relaxation time, T_2 (Figure 2D). This is characteristic of the aromatic resonance of purine bases (Figure 2D). The stacking of the dG·dC base pair at position 2 with the dPu·dT base pair at position 3 was also observed (Figure 2E). The assignment of imino resonances along with their chemical shifts is listed in Table I. The assignment of the signals at 8.39 and 8.10 ppm was essential to determine the mode of base pairing, Watson–Crick vs. Hoogsteen, and was made by the pure absorption phase NOESY experiments described below.

NOE between Aromatic Protons and Their Anomeric H1′ Protons. We recorded NOESY spectra of d-(CGPuAATTTCG) at 13 °C with a mixing time of 500 ms to determine the mode of the dPu·dT base pair. Figure 3 shows a portion of the NOESY spectra giving the connectivity between the aromatic protons and anomeric H1′ sugar protons. The assignments were made by a standard sequential procedure (Hare et al., 1983; Feigon et al., 1982) that is based on the fact that (i) the purine H8 or pyrimidine H6 is close to

Table II: Proton Chemical Shifts (ppm) of Base and T-CH_3 Resonances of d(CGPuAATTTCG) in 0.1 M NaCl–10 mM Sodium Phosphate Buffer at 13 °C, pH 6.4

	H8	H6	H5/CH_3	H2
C-1		7.58	5.87	
G-2	7.95			
Pu-3	8.60	8.39		8.10
A-4	8.14			7.14
A-5	8.14			7.62
T-6		7.20	1.36	
T-7		7.53	1.67	
T-8		7.43	1.77	
C-9		7.55	5.76	
G-10	7.99			

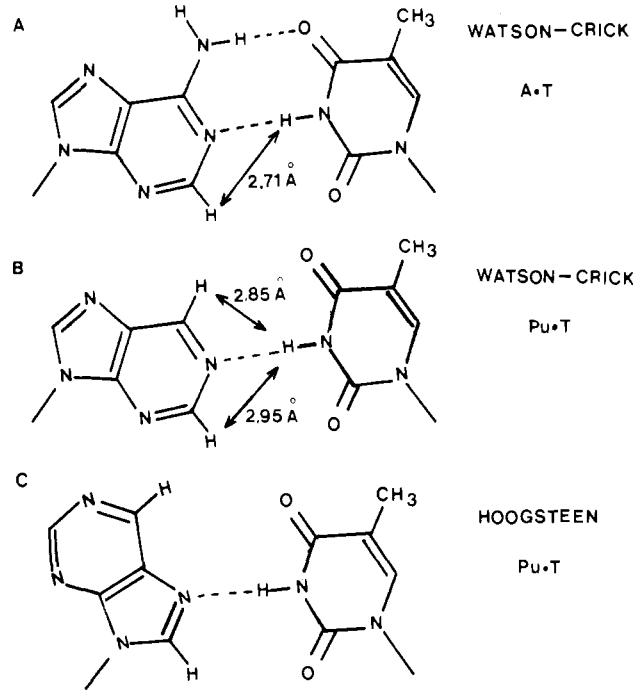

FIGURE 4: Watson–Crick model of dA·dT base pairing (A) and dPu·dT base pairing (B) and Hoogsteen model of dPu·dT base pairing (C).

the H1′ of its own sugar and to the H1′ of the neighboring sugar at the 5′ side and (ii) each base must have either an H6 or H8 proton. Indeed, all the cross-peaks between aromatic resonances and the anomeric sugar H1′ protons were observed except the interaction between TH1′ at position 7 and at position 8 TH6 (Figure 3). At this point we are not sure if the lack of cross-peaks is due to motion or extended distances. The assignment of aromatic resonances along with their chemical shifts is listed in Table II. Besides the connectivity between the aromatic and H1′ resonance, the cross-peaks arising from the following interactions are observed: (i) within a strand, 3-PuH2 → 3-H1′, 4-AH2′ → 4-H1′ and 5-AH2 → 5-H1′; (ii) between strands, 5-AH_2 → 6-H1′. In contrast to the PuH2 resonance, the other aromatic proton PuH6 has no cross-peak, indicating this proton is completely isolated from other protons.

Geometry of the dPu·dT Base Pair. NOE difference spectra (Figure 2D) demonstrates that purine pairs with thymine; however, the dPu·dT base pair can be held in two different manners: Watson–Crick (Figure 4B) and Hoogsteen (Figure 4C). If it were to adopt a Hoogsteen type of base pairing, NOEs between T imino and PuH6 and PuH8 would be expected. In contrast to Hoogsteen dPu·dT base pairing, Watson–Crick base pairing would have given cross-peaks between T imino and PuH6 and PuH2, instead of PuH8. Examination of NOE difference spectra (Figure 2) clearly

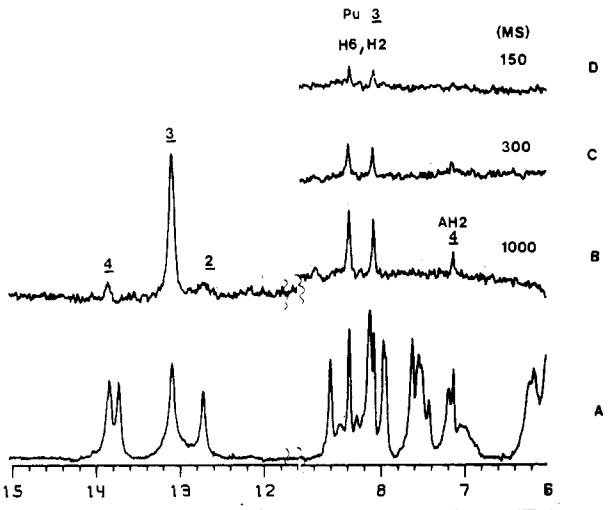

FIGURE 5: Pre-steady-state NOE measurement on the Pu·dT decamer in 80% H_2O/20% D_2O at 13 °C. (A) The 300-MHz ^1H NMR spectrum between 6 and 15 ppm. Difference spectra (off-resonance minus on-resonance presaturation) of T imino at position 3 for 1000 (B), 300 (C), and 150 ms (D).

demonstrates that the T imino of the dPu·dT base pair strongly interacts with PuH2 and PuH6. Therefore, we conclude that the dPu·dT base pair is held with Watson–Crick fashion (Figure 4B).

The next question is what is the interproton distance between the T imino and PuH2 and the T imino and PuH6 compared with those of dA·dT base pairs (Figure 4A). We have measured the cross-relaxation rates of these protons by a pre-steady-state NOE technique (Clore & Gronenborn, 1984). The cross-relaxation rate σ_{ij} between i and j can be determined from a measurement of the initial slope of NOE experiment vs. irradiation time (Wagner & Wuthrich, 1979; Dobson et al., 1982) and is related to the rotational correlation time τ_c as

$$\sigma_{ij} = \frac{\gamma^4 h^2}{10 r_{ij}^6}\left(\tau_c - \frac{6\tau_c}{1 + 4\omega^2\tau_c^2}\right)$$

where ω is the spectrometer frequency. Therefore, if the rotational correlation time τ_c is known, the interproton distance r_{ij} is easily extracted. A series of NOE difference spectra of the Pu·T decamer (Figure 5) exhibits the change in intensity of the purine H2 and H6 resonances at position 3 following the saturation of the T imino resonance for different lengths of time. The cross-relaxation rates of these two protons were obtained from the initial slope of the NOE intensity vs. irradiation time curve. We calibrated this method by using a similar duplex A·T decamer which contains a dA·dT base pair in place of a dPu·dT base pair at the same position 3 in the sequence (Figure 1). The interproton distance of 2.71 Å between T imino and AH2 is used to extract an effective rotational correlation time, τ_c, of 7 ns, which is also used for the Pu·T decamer. We obtained cross-relaxation rates of 0.75 s^{-1} for the T imino → PuH6 interaction and 0.60 s^{-1} for the T imino → PuH2 interaction. Calculated interproton distances are 2.85 Å for T imino → PuH6 and 2.95 Å for T imino → PuH2. These results are summarized in Table III.

We conclude that (i) the single hydrogen bond between T imino and purine N_3 is sufficient to hold the dPu·dT base pair in Watson–Crick geometry and (ii) the interproton distance between T imino and PuH2 in the dPu·dT base pair is longer than the corresponding distance in the dA·dT base pair that contains an additional hydrogen bond between an amino group and a carbonyl group.

Table III: Cross-Relaxation Rates σ_{ij} and Interproton Distance r_{ij} between the Imino Proton of Thymine/Purine and H2/H6 in the Decamer d(CGXAATTTCG) Sequence Obtained by Pre-Steady-State NOE Measurement at 13 °C, Where X = A or Pu

	imino → AH2	imino → PuH2	imino → PuH6
σ (s^{-1})	1.0	0.60	0.75
r (Å)	2.71	2.95	2.85
τ_C (ns)	7.0	7.0	7.0

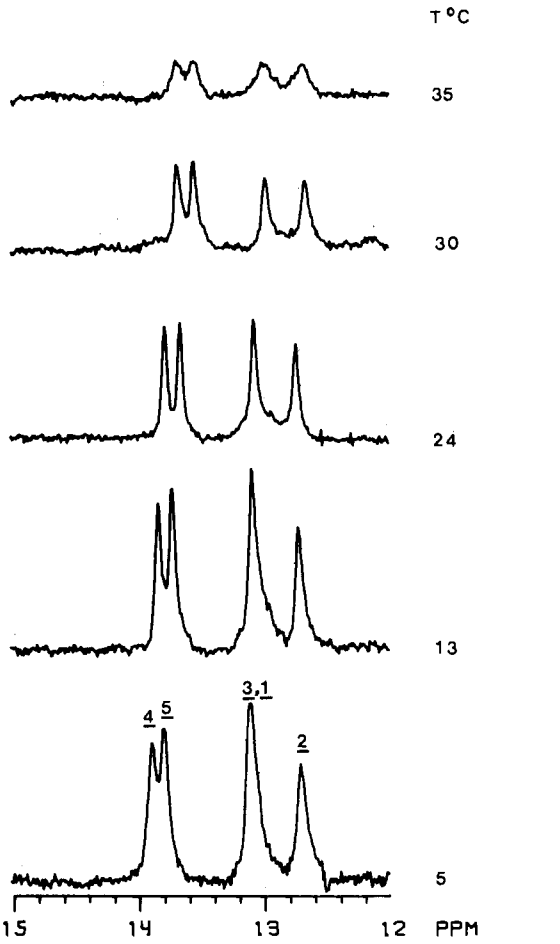

FIGURE 6: Temperature dependence of the 300-MHz low-field spectra (imino resonances) of the dPu·dT decamer.

Temperature-Dependent Imino Resonances and Overall Imino Proton Exchange. Figure 6 shows the low-field imino proton spectra of the Pu·T decamer at various temperatures ranging from 5 to 35 °C at pH 6.4. At 5 °C the peak area of the signal at 13.08 ppm increases nearly 1.5 times relative to that of other resonances resulting in the assignment of G imino at position 1. On raising the temperature to 24 °C, rapid base pair opening at the end of the helix causes the G imino resonance at position 1 to disappear (Patel & Hilbers, 1975; Kan et al., 1975).

However, the imino proton resonances of the base pairs inside the duplex are still observable up to 35 °C, though their line width rapidly increases. The comparison of the line widths between the Pu·T decamer and the A·T decamer (data not shown) revealed that the substitution of the dA·dT base pair with dPu·dT at the same position 3 results in an increase in the overall imino proton exchange rate. For example, the line widths of the T imino at position 3 of the Pu·T decamer and the A·T decamer were 52 and 13 Hz at 35 °C, corresponding to the overall imino proton exchange rate of ~132 and ~9 s^{-1}, assuming that the natural line width of the T imino was ~10 Hz. In order to compare the overall imino proton exchange rate more quantitatively, the spin–lattice relaxation

Table IV: Comparison of Observed Imino Proton Spin–Lattice Relaxation Rates R_1 (s^{-1}) in d(CGAAATTTCG) and d(CGPuAATTTCG) by the Inversion Recovery Method

	imino proton			
	2	3	4	5
d(CGAAATTTCG) at 13 °C	9.0	6.5	6.5	7.5
d(CGAAATTTCG) at 35 °C	20	11	4.0	5.0
d(CGPuAATTTCG) at 30 °C	42	40	19	17

rates, R_1, of two decamers were measured by inversion-recovery techniques under identical conditions.

In general, spin–lattice relaxation rates, R_1, of imino protons are due mainly to their dipolar interaction below room temperature (Early et al., 1981a,b); however, solvent exchange following base pair opening predominantly contributes to it above room temperature (Johnston & Redfield, 1977). This is expressed by

$$R_1(\text{observed}) = R_1(\text{dipolar}) + R_1(\text{exchange})$$

As a result, the difference in relaxation rates at two temperatures gives the overall exchange rate by solvent exchanging (Johnston & Redfield, 1981). The spin–lattice relaxation rates, R_1, of imino proton resonances in the A·T decamer at 13 °C appear to be around 7–9 s^{-1} (Table IV). On raising the temperature to 35 °C, R_1 of base pairs 2 and 3 increases to 20 and 11 s^{-1}, nearly a factor of 2, but those of base pairs 4 and 5, which were located in the center of the decamer, remained unchanged. At this temperature, 35 °C, the observed R_1 of 4–5 s^{-1} for positions 4 and 5 in the A·T decamer can be due only to the dipolar contribution. In contrast, R_1 of imino protons in the Pu·T decamer were not accurately measurable due to broad line widths caused by rapid solvent exchange. Instead, they were measured at 30 °C to be ~40 s^{-1}, which is much larger R_1 than those of the A·T decamer (Table IV). Substitution of the dA·dT base pair with a dPu·dT base pair in the decamer, in other words the elimination of two single hydrogen bonds, —NH_2···O=C<, from positions 3 and 8 in the sequence, results in an increase in the overall imino proton exchange rate at 30 °C from 7 to 36 s^{-1} at the mismatched site.

ACKNOWLEDGMENTS

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Registry No. d(CGPuAATTTCG), 109686-33-1; d(CGAAATTTCG), 109686-34-2; purine, 120-73-0; thymine, 65-71-4; guanine, 73-40-5; cytosine, 71-30-7; adenine, 73-24-5.

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