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Opening Mechanism of G·T/U Pairs in DNA and RNA Duplexes: A Combined Study of Imino Proton Exchange and Molecular Dynamics Simulation

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Abstract: The opening pathway of wobble pairs dG·T and rG·U has been investigated in four DNA and two RNA duplexes. Using NMR spectroscopy, we measured the imino proton exchange of both G(H1) and T/U(H3), catalyzed by ammonia, tris, and OH^-, and we calculated the free energy surface related to G·T/U opening by molecular dynamics simulations. Taken together the experimental and theoretical results, we suggest that wobble pairs open through a coupled rotation of the bases toward the major groove where exchange of both imino protons takes place with the surrounding water.

Introduction

The exchange of imino protons with water in Watson−Crick base pairs is commonly used to measure the opening and reclosing rates of hydrogen-bonded base pairs in nucleic acid structures. The process is normally interpreted according to a two-state (open−closed) model of the base pair. It is widely accepted that the imino proton cannot exchange from the closed pairs. Exchange with water, catalyzed by proton acceptors, occurs from the open state. Based on imino proton exchange catalyzed by NH_3, the opening and closing rates of Watson−Crick pairs have been measured in a variety of nucleic acid structures including B-DNA,[1] Z-DNA,[2] and RNA[3] duplexes and tRNA.[4] The base pair lifetimes, obtained as the imino proton exchange times at infinite proton acceptor concentration, range from less than 4 ms at 15 °C for the A·U pairs within RNA duplexes to several minutes for some tRNA base pairs. The base pair opening probability is extremely small, 10^{-4} to less than 10^{-7} at 15 °C, and the open pair lifetimes are typically in the range of 10 ns to hundreds of nanoseconds. For this reason, the structure of the open pair and the opening pathway remain inaccessible to experimental investigations. In the absence of added proton acceptor, imino proton exchange is catalyzed by the nitrogen of the complementary base via a concerted proton transfer involving one or several water molecules.[5] This indicates the formation of a water bridge between the imino proton and the cyclic nitrogen in the open base pair. The presence of bridging water molecules in the open state is predicted by molecular dynamics simulations,[6] and such a water molecule is observed in the X-ray structure of a DNA duplex containing a trapped open base pair.[7]

Base pair opening and consequently the rupture of the intervening hydrogen bonds can result from a rotation of the bases toward either the major or the minor groove directions. The imino proton exchange kinetics of drug−DNA complexes whose minor groove is obstructed by the backbone of bis-intercalators such as luzopeptin or echinomycin argues for opening toward the major groove.[8] Minor groove opening is generally considered to be hindered by the steric clash of the exocyclic groups and the proximity of the sugar−phosphate backbone. Nevertheless, recent theoretical investigations of the opening pathway of A·T and G·C pairs indicate that the free energy profiles associated with opening into the major and minor grooves are comparable.[6,9,10]

Wobble rG·U pairs are ubiquitous in RNA structures[11] and the analogous dG·T pairs arise as mismatches in DNA, which need to be corrected by a suitable repair system.[12] In a wobble base pair (Scheme 1), the presence of two imino protons, one on the major groove side, the other on the minor groove side, provides two potential markers to probe the opening directionality. This prompted us to undertake a study that combines imino

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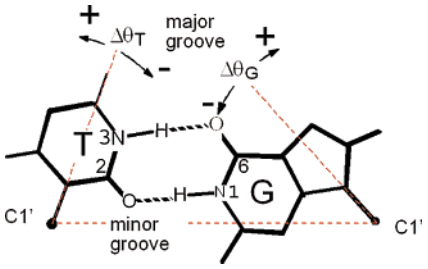
Table 1. Kinetic Data of Imino Proton Exchange of G·T/U Pairs in DNA and RNA Duplexes[a]

		T/U(H3)–(O6)G				G(H1)–(O2)T/U			
name	duplex	τ_0 (ms)	$\alpha\tau_{open}$ (μs)	τ_{int} (s)	E_{ac} (kcal/ mol)	τ_0 (ms)	$\alpha\tau_{open}$ (μs)	τ_{int} (s)	E_{ac} (kcal/ mol)
dTGA1	d(CGCGTGATTACGCG)$_2$	<0.2	<3.8	0.7		0.8	4.9	0.8	
dTGA2	d(CGCGTGACGCGTTAC GCG)$_2$	<0.2	<3.6	0.8	17	1	8.4	1.8	18
dCGG	d(TATACGGATATCTGTA TA)$_2$	<0.2	<3.8	5	24	0.7	5.2	12	25
dGGA	d(CGCGGATTCGCG)$_2$	<0.2	<2	3	19	0.7	4.2	6	20
rCGG	r(UAUACGGAUAUCUGU AUA)$_2$	0.6	6.6	0.6	20	0.6	3.9	0.9	20
rGGA	r(CGCGGAUUCGCG)$_2$	1.2	1.2	5.6	19	1.5	0.8	8.6	19

[a] H-bond lifetimes, τ_0, are derived from imino proton exchange catalyzed by tris or ammonia at 0 °C. The apparent open-state lifetimes, $\alpha\tau_{open}$, are calculated from the apparent dissociation constant, $\alpha K_{diss,NH_3}$, measured at 0 °C using ammonia as catalyst. τ_{int} is the rate of intrinsic catalysis measured at 0 °C and pH 6. τ_{int} values larger than 1 s were determined by extrapolation to 0 °C from exchange times measured between 45° and 10 °C. The activation energy, E_{ac}, is derived from the Arrhenius plot of τ_{int}. The bases of the wobble pairs are in italic in the duplex sequence.

Scheme 1



proton exchange experiments and molecular dynamics simulations of dG·T and rG·U base pair opening in deoxyribonucleotide and ribonucleotide duplexes.

Previous imino proton exchange studies of G·T pairs focused mainly on the effect of the wobble pair on lifetimes and dissociation constants of neighboring pairs. These studies also established that the exchange rate of G·T pairs is very fast in the presence of proton acceptor.[13,14] To avoid spectral overlap of exchange broadened peaks of G and T/U imino protons, we selected sequences where the imino protons are far apart in the NMR spectrum, as predicted by calculated ring currents of their first neighbors.[15] This is the case in particular for the dTGA1 and dCGG duplexes (for sequences, see Table 1). The NMR spectrum of dTGA1 at a strand concentration of about 1 mM shows an equilibrium between the duplex and a hairpin structure containing a GATT loop. For this reason, we synthesized the longer dTGA2 sequence that forms a duplex even in the submillimolar concentration range. The dGGA duplex was synthesized for comparison with the d(CGCGAATTCGCG)$_2$ dodecamer that may be considered as a benchmark for imino proton exchange studies.[16−18] The rGGA and rCGG RNA duplexes were studied for comparison with their DNA counterparts. To evaluate the imino proton accessibility in the open pair, the exchange catalysis was studied using three proton acceptors: the small and neutral NH_3 molecule, the bulky and neutral tris(hydroxymethyl)aminomethane, and the negatively charged hydroxyl ion. Imino proton exchange was also investigated by HPO_4^{2-} catalysis with dTGA1 and dGGA duplexes.

It has only recently become feasible to obtain structural insight into base opening and calculate the free energy associated with the opening process using all-atom molecular dynamics simulations. We developed a special opening restraint and used it in conjunction with umbrella sampling to open base pairs without any prior assumptions as to the conformational consequences of opening. This approach was used previously to study single base opening of A·T and G·C pairs in B-DNA[6] and of A·U pair in A-RNA[19] duplexes and cytosine flipping within the target sequence of the *Hha*I methyltransferase enzyme.[9] In the present study, we determine the free energy and the imino proton accessibility associated with the opening of the G·T/U wobble pairs in selected DNA and RNA duplexes. The combined experimental and theoretical results reveal that the wobble base pairs open into the major groove where exchange of both imino protons takes place with the surrounding water.

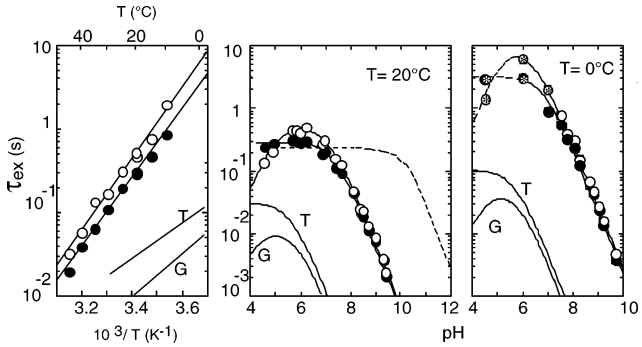


Figure 1. Influence of temperature and pH on the exchange time of G5(H1) (open symbols) and T8(H3) (black symbols) of the wobble pair in dGGA. Left panel: Exchange time vs temperature at pH 6. The exchange times of the monomeric 2′−3′ cyclic deoxyguanosine (G) and deoxythymidine (T) are displayed for comparison. Central panel: Exchange time vs pH at 20 °C. The exchange times of the G·T imino protons are compared to that measured for the A·T Watson−Crick pairs in d(CGCGATCGCG)$_2$ (dashed line). The curves labeled G and T show data for T(H3) and G(H1) in the monomers. Right panel: Exchange times vs pH at 0 °C. The data points labeled by a star are in a range inaccessible to exchange time measurements. These were derived by extrapolation to 0 °C from exchange times measured between 45 and 10 °C. The curves labeled G and T show data for T(H3) and G(H1) in the monomers.

Results

(A) Imino Proton Exchange in Wobble Base Pairs. (1) Exchange in the Absence of Added Catalyst. The effects of temperature and pH on the imino proton exchange times of the dG·T pair in dGGA are shown in Figure 1. The straight line with a slope of −1 above pH 7 is characteristic of OH^- catalysis. If we compare the rates of catalyzed exchange of G5(H1) and T8(H3) in the duplex with those in the G and T monomers at 0 °C, it is clear that the exchange process is slowed by a factor

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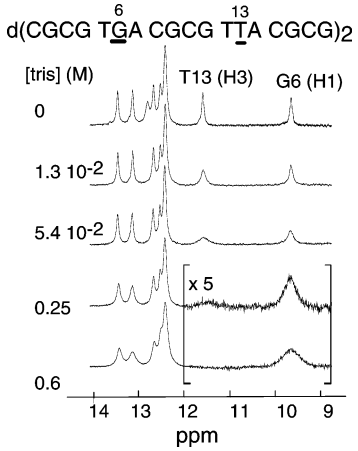

Figure 2. Line broadening induced by exchange at increasing tris concentrations in the imino proton spectrum of dTGA2 at pH 8.1, 0 °C, and 0.1 M NaCl. The broadening reflects the short lifetime of the T(H3)−(O6)G bond.

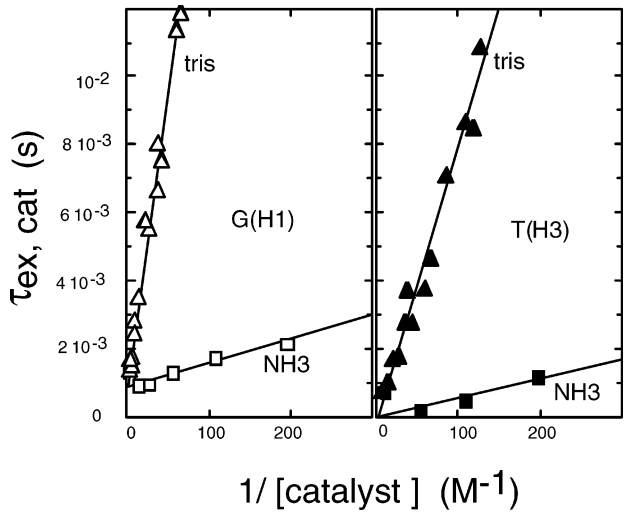

Figure 3. Influence of catalyst concentration on the exchange times of G6(H1) (open symbols) and T13(H3) (black symbols) of the G6·T13 pair in dTGA2 at 0 °C. Catalysis by ammonia was measured at pH 8.8 (squares) and by tris at pH 8.1 (triangles). The exchange times decrease linearly with the inverse catalyst concentrations. Left panel: The exchange time of G(H1) extrapolates at infinite catalyst concentration to a lifetime of 1 ms for the G(H1)−(O2)T bond, both with ammonia and tris as catalyst. Right panel: Extrapolation to infinite catalyst concentrations indicates a lifetime shorter than 0.2 ms for the T(H3)−(O6)G bond.

of 10^{-3} and 2×10^{-3}, respectively. Under pH 7, the exchange time of T8(H3) tends toward a pH-independent plateau of 0.22 and 3 s at 20 and 0 °C, respectively. The exchange time of G5(H1) is maximal around pH 6 and decreases at lower pH, indicating an acid-catalyzed exchange process.

(2) Catalyzed Imino Proton Exchange. The effect of increasing tris concentration on the imino proton spectrum of the dTGA2 duplex is shown in Figure 2. The peak corresponding to T13(H3) is broadened out due to fast exchange at concentrations higher than 0.25 M. In contrast, the exchange time of G6(H1) tends at infinite tris or ammonia concentration toward a limit value of 1 ms, which corresponds to the lifetime of the G6(H1)−(O2)T13 bond (Figure 3). The linear dependence of $\tau_{ex,cat.}$ versus the inverse catalyst concentration (varied from 4 mM to 0.46 M) is consistent with an exchange process occurring from a single open state.

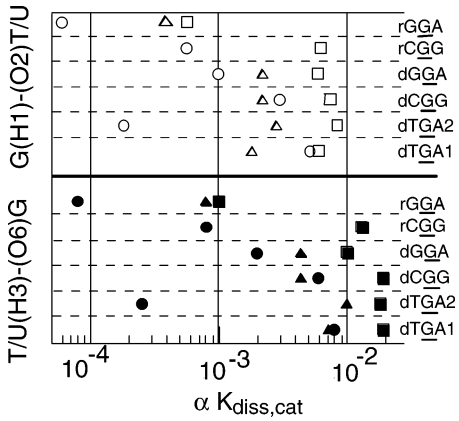

Figure 4. Apparent dissociation constants of the T(H3)−(O6)G and G(H1)−(O2)T bonds derived from imino proton exchange catalyzed by ammonia (squares), tris (triangles), and OH^- (circles) in DNA and RNA duplexes.

The H-bond lifetimes of the G·T/U pairs in the DNA and RNA duplexes studied are displayed in Table 1. The apparent dissociation constants of H-bonds derived from the imino proton exchange catalyzed by tris, ammonia, and OH^- are displayed in Figure 4. Examination of these data shows that the G·T imino proton exchange kinetics in the four DNA duplexes exhibit a number of similar features. The lifetime of the G(H1)−(O2)T bond ranges between 0.7 and 1 ms and that of the T(H3)−(O6)G bond is always shorter than 0.2 ms. The apparent dissociation constants derived from catalysis by ammonia fall in a very narrow range of values: $(6-8.4) \times 10^{-3}$ for the G(H1)−(O2)T and $(1-1.9) \times 10^{-2}$ for the T(H3)−(O6)G bonds. The apparent dissociation constants derived from tris catalysis are 2−5 times smaller than those derived from ammonia catalysis, suggesting a modest reduction of accessibility for the bulkier catalyst. The apparent dissociation constants determined from OH^- catalysis are also smaller, in particular in the dG*G*A and dT*G*A2 duplexes, indicating a reduced accessibility for the negatively charged hydroxyl ion. The observation that the addition of 0.5 M HPO_4^{2-} has no effect on the exchange rate of the imino protons of G·T pairs in the dT*G*A1 and dG*G*A duplexes has most likely the same explanation.

In the two RNA duplexes, the U(H3)−(O6)G and G(H1)−(O2)U bonds have similar lifetimes: 0.6 ± 0.3 ms in rC*G*G and 1.2 ± 0.3 ms in rG*G*A (Table 1 and Figure 5). The dissociation constants of the G(H1)−(O2)U bond are roughly half compared to those measured for the U(H3)−(O6)G bond (Figure 4). It is noteworthy that the H-bond dissociation constant measured for the rG·U pair in rG*G*A is about 10 times smaller than those measured for the other RNA and DNA duplexes.

The lifetimes and dissociation constants of base pairs adjacent to the wobble G·T pairs of dG*G*A were measured at 20 °C. Their lifetimes and open-state lifetimes are compared to those of the corresponding base pairs of the $(dCGCGAATTCGCG)_2$ duplex in Table 2. The lifetimes of the pairs adjacent to the G·T pair are strongly reduced, and their open-state lifetimes are increased by a factor of 13 for A6·T7 and 24 for G4·C9. The G·T mismatch has little effect on the opening kinetics of the second neighbor, C3·G10, whose lifetime is surprisingly longer in the duplex containing the wobble pair.

(B) Molecular Dynamics Simulation of G·T/U Opening. (1) G·T Opening in dGGA. The free energy change associated

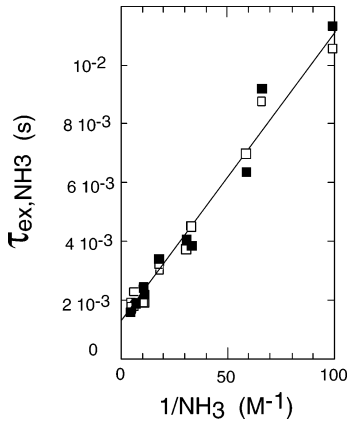

Figure 5. Influence of ammonia concentration on the exchange times of G(H1) (open squares) and U(H3) (black squares) of the G5·U8 pair in rG*G*A measured at pH 8.8, 0 °C, and 0.1 M NaCl. The similarity of the exchange times of G5(H1) and U8(H3) at infinite catalyst concentration suggests a simultaneous opening of G5(H1)−(O2)U8 and U8(H3)−(O6)G5 bonds.

Table 2. Base Pair Lifetimes, Open-State Lifetimes, and Apparent Dissociation Constants of the Watson−Crick Base Pairs Adjacent to the G5·T8 Wobble Pair in dG*G*A Measured at 20 °C[a]

C3·G10	G4·C9	A6·T7		
15	6.2	1.7	X·Y = G·T	base pair lifetime (ms)
6.3	21	20	X·Y = A·T	
18	51	134	X·Y = G·T	open-state lifetime (ns)
3.1	2.1	10	X·Y = A·T	
1.2	8.2	7.9	X·Y = G·T	$\alpha K_{diss,cat.} \times 10^{-6}$
0.5	0.1	0.5	X·Y = A·T	

[a] The lifetimes and the apparent dissociation constants are compared with the values measured at high ammonia concentration in d(CGCGAATTCGCG)$_2$.

with the dG·T opening is shown as a function of base opening angles in Figure 6. Opening of both bases into the major groove provides the lowest free energy pathway. A 10° rotation of T and G disrupts the T(H3)−(O6)G bond at a free energy cost of only about 2.5 kcal/mol and exposes the imino proton T(H3). This partially open state, stabilized by a bifurcated H-bond between T(O2) and (H1/NH2)G, corresponds to a shallow minimum on the free energy surface centered around $\Delta\theta_T$ = 20° and $\Delta\theta_G$ = 10°. Further rotation of the bases into the major groove breaks T(O2)−(H1/NH2)G, and the bases lose all H-bonding interactions at an opening angle of about $\Delta\theta_T$ = 40° and $\Delta\theta_G$ = 20°. As a result, G(H1) becomes exposed in the major groove at a free energy cost of 6 kcal/mol. As seen in earlier works,[6,9] base opening starts with coupled rotation in the quadratic free energy regime, where hydrogen bonds stretch and eventually break. Then unstacking and desolvation effects come into play, and consequently further opening of T is facilitated over that of G.

A simultaneous rotation of 10° of both bases into the minor groove breaks the G(H1)−(O2)T bond at a free energy cost of about 5 kcal/mol. Further opening in this direction requires large free energy investment. The analysis of this opening pathway shows that G continues to open into the minor groove and then turns out of plane, effectively burying its imino proton in the groove, while T remains in a stacked position. The T(H3)−(O6)G bond survives late along this pathway, which hampers the imino proton exchange of thymidine.

A 10° counterrotation of G into the major groove and T into the minor groove causes immediate clashes of the N−H groups which lead to a free energy barrier of 9 kcal/mol. This results

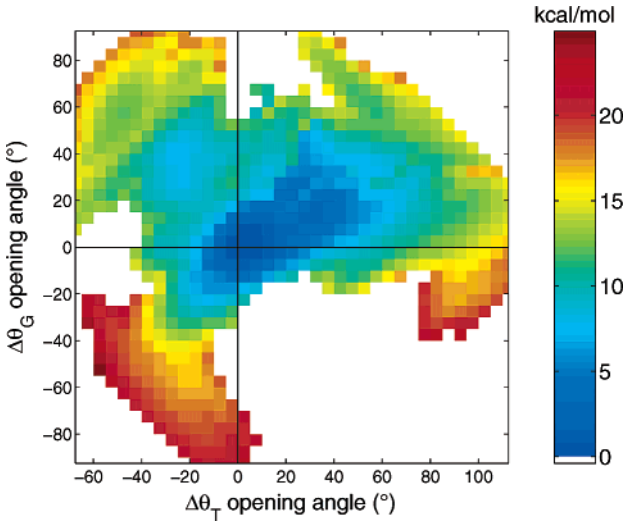

Figure 6. Free energy surface associated with dG·T opening in dG*G*A. The opening angle, $\Delta\theta_T$ and $\Delta\theta_G$, of each base is given relative to their position in the relaxed base pair. Positive and negative opening angles correspond to opening into the major and minor groove, respectively. A coupled opening of the bases into the major and minor grooves corresponds to the top-right and bottom left quarters of the free energy map, respectively. The counterrotation of the bases corresponds to the top-left or bottom-right quarters. The free energy of opening is color-coded as a function of $\Delta\theta_T$ and $\Delta\theta_G$.

in a difference in rise within the base pair and a quick sliding motion in opposite directions. Bases then return to plane and form a new H-bond, T(O4)−(NH2)G, which lead to a local free energy minimum centered around $\Delta\theta_T$ = −20° and $\Delta\theta_G$ = 40°. In this opening mechanism, G(H1) becomes exposed in the major groove and T(H3) in the minor groove. The alternative counterrotation of G into the minor groove and T into the major groove was found to be energetically prohibitive, probably due to the repulsion of the carboxyl groups.

(2) Imino Proton Accessibility to Solvent. Rotation of the bases into the grooves results in a rupture of the intervening hydrogen bonds, but this does not always lead to efficient solvent exposure. Imino protons may be involved in other hydrogen bonding interactions or can be shielded by the neighboring bases. To quantify the degree of solvent exposure, we calculated the percentage surface accessibility of the imino protons along the base pair opening pathways with respect to the values calculated for isolated nucleosides. The analysis of the accessibility of G(H1) and T(H3) shows that the structural ensemble corresponding to a given opening angle is heterogeneous and that the imino proton accessibilities fall in a rather wide range of values. Therefore, we mapped the free energy as a function of the combined opening angles and the percentage accessibility. By integrating over the structural ensemble, we determined the free energy required to achieve a given imino proton accessibility. Results for the coupled opening of bases into the major and minor grooves are shown in Figures 7 and 8. Opening into the major groove provides a 10−40% accessibility to T(H3) for only 3−5 kcal/mol, while a comparable accessibility to G(H1) cost about 7 kcal/mol. Further accessibility requires increasingly more free energy for both T(H3) and G(H1). Opening into the minor groove provides easier accessibility to G(H1) compared to T(H3); however, even the low accessibility values (10−40%) require around 10 kcal/mol. In the alternative pathway, where bases counterrotate, major groove accessibility

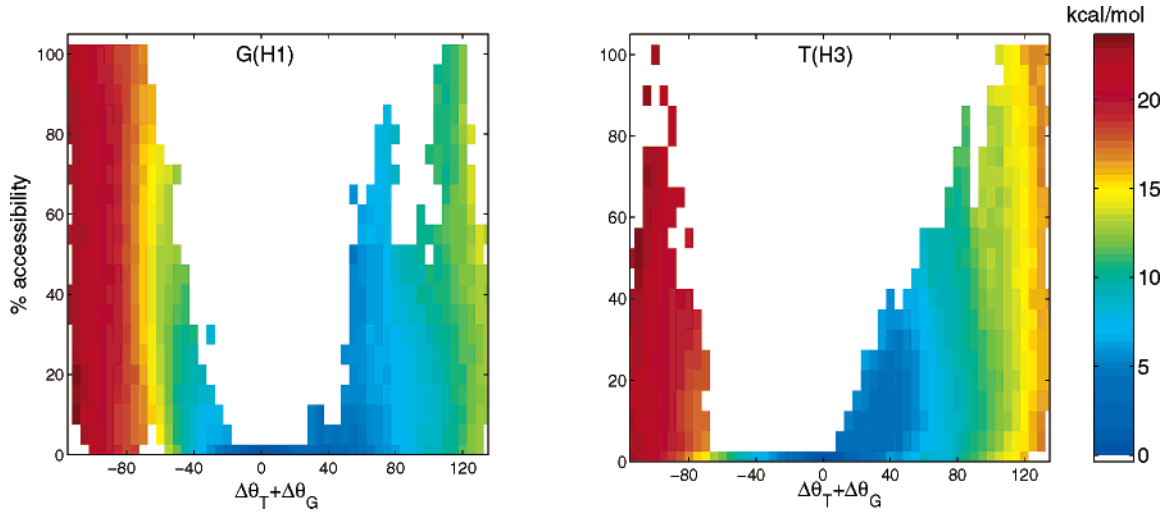

Figure 7. Solvent accessibility of G(H1) (left) and T(H3) (right) in dG*G*A along the opening pathway into the major groove (positive values) and minor groove (negative values). Reaction coordinate is taken as the sum of the individual base opening angles ($\Delta\theta_T + \Delta\theta_G$). The free energy required to reach a given accessibility along the opening pathway is given in color code.

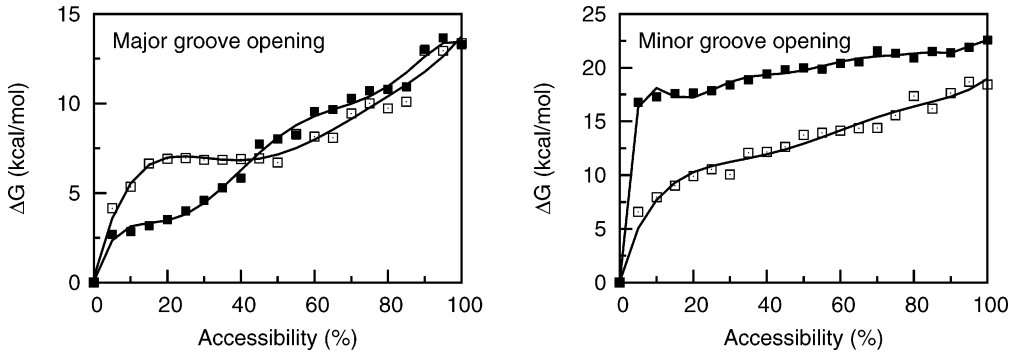

Figure 8. Free energy requirement of fractional imino proton accessibility of T(H3) (black symbols) and G(H1) (open symbols) in dG*G*A: left panel, opening into the major groove; right panel, opening into the minor groove.

for G(H1) costs 9−11 kcal/mol and minor groove accessibility for H3(T) costs 10−13 kcal/mol (data not shown).

(3) Wobble Pair Opening in the dTGA1 and rGGA Duplexes. The opening mechanism of dG·T and rG·U wobble pairs was also investigated in the dT*G*A1 and rG*G*A duplexes. The free energy profiles along the opening trajectories are similar to those described above for dG*G*A, within the ∼2 kcal/mol error of the simulation method. The imino proton accessibility values of the wobble pairs in dT*G*A1 and rG*G*A are also comparable to those described above for the imino protons of the dG·T pair in dG*G*A. Therefore, we focus on the results obtained for dG*G*A in the following discussions.

(4) Stability of G4·C9 in the d(CGCGAATTCGCG)$_2$ and dGGA Duplexes. The calculated free energy required to open the G4·C9 base pair adjacent to dG·T in dG*G*A is about 2 kcal/mol lower than that adjacent to the corresponding Watson−Crick pair in d(CGCGAATTCGCG)$_2$. This is in good agreement with experimental data, since the dissociation constant of G4·C9 is 80 times larger in dG*G*A than that measured in d(CGCGAATTCGCG)$_2$ (Table 2), which corresponds to a free energy difference of about 2.5 kcal/mol. In both duplexes, the lowest free energy pathway corresponds to cytidine opening into the major groove.

Discussion

The imino protons of Watson−Crick base pairs are aligned approximately along the helical axis. Protected by the adjacent base pairs, they are in a hydrophobic environment and cannot exchange from the closed pair. By contrast, the imino protons of G and T/U in a wobble pair are located at the bottom of the minor and major grooves, respectively, and their accessibility to water and proton acceptors must be considered in the closed wobble pair prior to interpreting their exchange rates.

First, it should be remarked that the accessibility calculated for the dG*G*A duplex shows that the imino protons of the closed dG·T pairs are not accessible to water (Figure 7). Furthermore, the observation that the exchange times of G(H1) in dG·T and rG·U pairs and U(H3) in rG·U pairs extrapolate at infinite proton acceptor concentration to nonzero limit values establish that imino proton exchange cannot occur from the closed pair (Figures 3 and 5). Hence, imino proton exchange from G·T/U pairs may be interpreted according to the standard two-state model of the base pair. The exchange times of T(H3) in dG·T pairs at infinite catalyst concentration are, however, indistinguishable from zero (Figure 3), and this could be an argument in favor of an exchange process occurring from the closed pair. Nevertheless, the negligible accessibility calculated for T(H3) in the dG·T pairs and the observation that U(H3) is inaccessible in the closed rG·U pairs in both RNA oligomers studied suggest that the lifetime of the T(H3)−(O6)G bond is shorter than 0.2 ms, the lower limit value accessible to NMR measurements.

(A) Internal Catalysis of Imino Proton Exchange in G·T/U Pairs. At pH higher than 7, imino proton exchange is

controlled by hydroxyl catalysis (Figure 1). The exchange time of the T/U imino proton in the wobble pair reaches a pH-independent plateau around pH 6 in all the duplexes investigated, while that of G reaches a maximal value. The values for exchange time range between 0.6 and 12 s at pH 6 and 0 °C (Table 1) and are roughly proportional to the inverse of H-bond dissociation constants, i.e., the fraction of time during which H-bonds are disrupted.

In the absence of added catalyst (aac), imino proton exchange from Watson−Crick base pairs is controlled by a concerted transfer of protons involving one or several water molecules bridging the imino proton of the open base and the cyclic nitrogen of the complementary base, i.e., C(N3) in the case of a G·C pair and A(N1) for an A·T/U pair, which acts as the intrinsic catalyst.[5] The imino proton exchange rate induced by this process depends on the fraction of time during which the base pair is open, K_{diss}, and on the transfer efficiency, $1/(1 + 10^{\Delta pK})$, where, ΔpK is the pK difference between the imino proton ($pK_{GH1} = 10.2$, $pK_{TH3} = 10.5$ at 0 °C) and the cyclic nitrogen of the complementary base ($pK_{CN3} = 4.3$, $pK_{AN1} = 3.7$).

$$k_{ex,aac} = k_{ex,open} K_{diss}[1/(1 + 10^{\Delta pK})] \quad (1)$$

A compilation of exchange times measured for B-DNA, RNA duplexes, and drug−DNA complexes shows that the concerted transfer rate, $k_{ex,open}$, is in the range of $10^{10}-10^{12}$ s^{-1}.[3,5,8] It may be expected that in wobble pairs G(H1) and T(H3) exchange is catalyzed by the T(O2) and G(O6) oxygens, respectively. The exchange times versus pH of the imino protons of the G·T pair of dG*G*A are compared with that of the central A·T pair in the dCGCGATCGCG duplex[13] in Figure 1. A comparison of the hydroxyl-catalyzed exchange rates of T(H3) in the G·T and A·T pairs shows that the dissociation constant of the T(H3)−(O6)G bond is about 3 orders of magnitude larger than that of the reference A·T pair. Nevertheless, their pH-independent rates, $k_{ex,aac}$, are comparable. According to eq 1, this indicates that the transfer efficiency of T(H3) in the open G·T pair is reduced by 3 orders compared to that in the A·T pair, suggesting that the pK of G(O6) is close to 1, i.e., 3 units lower than pK_{AN1}. Similarly, the exchange rate of G(H1) in the wobble pair is consistent with a concerted exchange process catalyzed by T(O2) with a pK close to zero. The chemical shift versus pH of the H6 proton of the monomeric thymidine (not shown) indicates a protonation of the base around pH 0, which supports the pK value estimated for T(O2).

The enhanced exchange rate of G(H1) at low pH may be assigned, as in the case of G·C pairs, to the shifted pK of G(H1) induced by partial protonation of GN7.[20] The activation energies related to the intrinsic catalysis of the G·T/U imino protons are 17−25 kcal/mol (Table 1). These values include contributions from both the base pair motion and the exchange chemistry and reflect, according to eq 1, the effect of temperature on K_{diss}, $k_{ex,open}$, and the pK of donor and acceptor. It should be noted that this activation energy cannot be compared to the calculated free energy values which are related only to the opening process.

(B) Base Pair Opening Pathways in G·T/U Pairs. The imino proton exchange experiments show that the lifetimes of the two H-bonds in dG·T pairs are distinct. In the four DNA duplexes examined, the lifetime of G(H1)−(O2)T is at least 3 times longer than that of T(H3)−(O6)G and the dissociation constant of G(H1)−(O2)T is about half of that measured for T(H3)−(O6)G. Hence, T(H3) is expected to be exposed more readily in the preferred opening mechanism.

To obtain insight into the nature of the open state, the opening pathways of the wobble pairs into the major and the minor grooves were simulated by biased molecular dynamics. The resulting free energy map displayed in Figure 6 shows that a counterrotation of G into the minor groove and T into the major groove cannot take place due to the repulsive interaction between T(O2) and G(O6). The counterrotation of G into the major groove and T into the minor groove can be achieved through a large free energy barrier of about 9 kcal/mol. The concerted rotation of both bases into the minor groove appears also to be an energetically unfavorable process. In addition, these pathways expose G(H1) prior to T(H3), which is inconsistent with the imino proton exchange experiments.

The lowest free energy pathway corresponds to the opening of both G and T into the major groove. In this opening mechanism the T(H3)−(O6)G bond breaks first which is fully consistent with the shorter lifetime of this H-bond. Hence this mechanism accounts for the experimental data and suggests a two-phase opening process of the dG·T wobble pair. A frequent, small-amplitude opening motion disrupts the T(H3)−(O6)G bond at a cost of 2.5 kcal/mol and a less frequent, large-amplitude motion breaks both T(H3)−(O6)G and G(H1)−(O2)T bonds at a cost of 6 kcal/mol.

The equilibrium dissociation constant, K_{diss}, was calculated for the T(H3) and G(H1) exchange processes along the different pathways. Since it is not known what value of accessibility is needed for an effective exchange, we defined the open state with a series of minimal accessibility values. The results showed that only the K_{diss} of the lowest free energy pathway gave good correspondence with the experimental K_{diss} with use of accessibility values between 10 and 40%. Therefore these results illustrate a proton exchange process in the major groove for both imino protons with relatively low accessibility values. It should be remarked that the experimental exchange times of T(H3) and G(H1) could also be explained by two distinct base opening motions with appropriate probabilities. However, the alternative pathways offer imino proton exposure at a higher free energy cost, and therefore these are less probable.

The molecular dynamics simulations do not show any significant difference between dG·T and rG·U opening. Nevertheless, in the two RNA duplexes studied, the lifetimes of U(H3)−(O6)G and G(H1)−(O2)U are similar and hence suggest a simultaneous breaking of the two H-bonds. A counterrotation of G into the minor groove and T into the major groove could account for such a scenario. This opening pathway is, however, energetically unfavorable (see above). A plausible explanation is that rG·U opening occurs, as in the case of dG·T, by a coupled rotation of the bases into the major groove but the small-amplitude movement that would preferentially break U(H3)−(O6)G does not take place in RNA. A comparison of the H-bond lifetimes in the DNA and RNA sequences suggests that the simultaneous disruption of the H-bonds of rG·U pairs is the result of a base pair opening with an opening amplitude

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comparable to that which breaks the G(H1)−(O2)T bond of the dG·T pairs. It should be noted, however, that the H-bond lifetimes in G·T/U pairs even at 0 °C are very short, close to the limit value accessible to NMR experiments. The difference between the apparent dissociation constants of T/U(H3)−(O6)G and G(H1)−(O2)T bonds is less than an order of magnitude, which corresponds to ∼2 kcal/mol, the limit of the accuracy of our simulation method.

Spontaneous base pair opening has been observed in simulations of DNA containing base analogues.[21] In the study, where an A·T base pair was replaced by an adenine−difluorotoluene pair, a similar mechanism was proposed: a more common partial opening of the bases with only one hydrogen bond lost and a less common full opening with both hydrogen bonds lost. This study describes an asymmetric opening motion where only one of the bases swing out of the double helix. Partial opening of a G·U pair within DNA has been examined in a series of 5 ns unrestrained molecular dynamics simulations in different sequence contexts.[22] This study showed that the U(H3)−(O6)G bond breaks up by a symmetrically coupled rotation of G and U into the major groove. The free energy difference between the closed and the partially open state was calculated to be 1−2 kcal/mol, and the open state lifetime was estimated to be 0.1−1.7 ns, several orders of magnitude shorter than the values derived from proton exchange experiments (Table 1). The G(H1)−(O2)U bond remained, however, intact during these simulations, suggesting a marked difference between the exchange rates of U(H3) and G(H1), which was not observed in our proton exchange experiments. Finally, spontaneous opening of both A·U and G·C base pairs were observed in RNA sequences during 5 ns molecular dynamics simulations.[23] Yet, lifetimes for these base pairs were measured to be ≤0.1 and 30−50 ms, respectively.[3] The discrepancy between the simulated and measured time scales of opening might indicate that not all the opening events result in an effective imino proton exchange, but it can also point to possible bias by the applied force field.[23]

(C) Base Pair Opening of G·T/U and Watson−Crick Pairs. For the G·T/U pairs of r*G*G*A*, r*C*G*G*, d*G*G*A*, and dT*G*A2, the H-bond dissociation constants determined from hydroxyl-catalyzed imino proton exchange are 1−2 orders of magnitude smaller than those derived from exchange catalyzed by NH_3. This feature is unusual. The dissociation constants of the Watson−Crick base pairs of $d(CGCGATCGCG)_2$ derived from the hydroxyl catalysis are only 3−7 times smaller than those determined using NH_3.[24] In the latter duplex, the small systematic reduction of the dissociation constants measured with OH^- and negatively charged catalysts was interpreted as a consequence of the reduced local concentration of the negatively charged species in the vicinity of the DNA. The larger difference observed between the dissociation constants of the wobble pairs in dT*G*A2 and r*G*G*A* duplexes derived from OH^- and NH_3 catalysis may be the indication that the exposed imino proton is closer to the negatively charged phosphate backbone, a region where the effective OH^- concentration is much smaller than that expected from pH measurements. It may be noted that this explanation accounts also for the absence of catalytic effect of HPO_4^{2-} on the imino proton exchange rates in the wobble pairs.

Another unusual feature of the base pair kinetics of wobble pairs is their long open-state lifetime. The values derived from the exchange rate measured with NH_3 catalysis are in the microsecond range, i.e., 10−100 times longer than those of the Watson−Crick base pairs in DNA[13] and RNA[3] duplexes. Molecular dynamics simulation shows that base opening in the major groove allows the formation of a bifurcated H-bond between T(O2) and G(H1/NH2) which could stabilize the open state of the T(H3)−(O6)G bond. Nevertheless, this explanation cannot justify the long open-state lifetime of the G(H1)−(O2)T bond. It may be expected that the reclosing rate of an open base pair is proportional to the free energy difference between the open and closed states in the quadratic region of the free energy.[6] The relative free energy of the open state of a Watson−Crick pair[6] is 7−10 kcal/mol, which is significantly larger than that of a wobble base pair (3−6 kcal/mol). It is therefore possible that this difference results in a longer open-state lifetime of the G·T/U pairs.

Materials and Methods

(A) Oligonucleotide Synthesis and Sample Preparation. The oligonucleotides were synthesized with β-cyanoethyl phosphoramidites on a 2 μM scale for the RNA sequences and on a 10 μM scale for the DNA sequences. These were then purified on a Q sepharose Hiload column using a NaCl gradient. After purification, the oligomers were dialyzed against water and lyophilized. The NMR samples were prepared by dissolving the oligonucleotides in a 90% H_2O, 10% ^2H_2O solution containing 0.1 M NaCl, 1 mM ethylenediamine tetraacetic acid and 0.2 mM 2,2-dimethyl-2-silapentane-5-sulfonate whose methyl peak was set to 0 ppm for chemical shift reference. The duplex concentrations, determined from the UV absorbance using the A^{260} values calculated according to a nearest neighbor model,[25] ranged from 1 to 3 mM. The sample pH was adjusted using 0.1−2 M HCl and NaOH solutions. After addition of NaOH, the RNA samples were immediately vortexed to avoid hydrolysis by a strong local OH^- concentration. The integrity of the RNA samples was controlled by the absence of phosphomonoester and of 2′−3′ cyclic phosphate peaks in their ^{31}P NMR spectra.

For the proton exchange time measurements performed against pH, the sample pH was measured at room temperature after each experiment. For the exchange time measurements performed against tris concentration at 0 °C, the sample pH was determined from the chemical shift, δ_{ppm}, of the tris O-methyl protons according to $pH = 8.4 - \log((3.5 - \delta_{ppm})/(\delta_{ppm} - 3.72))$. For the exchange experiments catalyzed by NH_3, the addition of 1 mM tris to the NMR samples, a concentration with negligible effect on the imino proton exchange rates compared to that of NH_3, similarly provided the pH of the sample. The catalysts were added to the NMR samples from 1 M tris, pH 8.1 and from 6 M ammonia, pH 8.8 stock solutions.

(B) NMR Methods. Unless otherwise stated, all the NMR experiments were performed at 0 °C on a 500 MHz Varian Unity INOVA spectrometer using a penta probe. The imino protons of the wobble pairs were connected in the NOESY spectra collected with mixing times of 70 and 120 ms. G(H1), identified by the strong imino−amino proton cross-peak, was always found upfield shifted compared to that of T/U(H3).

Exchange times longer than 20 ms were measured by magnetization transfer from water. In these experiments, the water magnetization was selectively inversed with a DANTE sequence[26] and the imino proton

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Table 3. Rates of Imino Proton Exchange Catalyzed by Ammonia, Tris, and Hydroxyl Measured at 0 °C Using Thymidine and 2′−3′ Cyclic Guanosine (s^{-1})

	$k_{ex,NH3}$	$k_{ex,tris}$	k_{ex,OH^-}
T	9.1×10^6	1.24×10^6	2.32×10^9
G	1.66×10^7	1.97×10^6	3.3×10^9

magnetization was detected after a variable delay incremented from 1 to 200 ms using an echo sequence as previously reported.[3] Spurious effects due to cross-relaxation were limited by using variable delays shorter than 200 ms. The exchange contribution of the added catalyst was determined from the exchange times measured in the presence (τ_{ex}) and in the absence (τ_{ex0}) of catalyst by

$$\tau_{ex,cat.} = (1/\tau_{ex} - 1/\tau_{ex0})^{-1} \quad (2)$$

Exchange times shorter than 20 ms were determined from the longitudinal relaxation times measured in the presence (T_1) and in the absence of added catalyst (T_{10}). The two imino protons of the wobble pair were inverted with a DANTE sequence and a Jump and Return pulse[27] followed after variable delays incremented from 0.2 ms to $5T_1$. In this case, the exchange contribution of the added catalyst was calculated as

$$\tau_{ex,cat.} = (1/T_1 - 1/T_{10})^{-1} \quad (3)$$

For imino proton exchange experiments catalyzed by high tris concentrations, the tris *O*-methyl NMR peak was selectively saturated by a 0.3 s low-power pulse in order to avoid amplifier saturation.

(C) Imino Proton Exchange Theory. The formalism of catalyzed proton exchange has been extensively described.[18] For an isolated nucleoside, the imino proton exchange rate induced by a proton acceptor is

$$k_{ex,acc} = k_{coll}[acc]/(1 + 10^{\Delta pK}) \quad (4)$$

where k_{coll} is the collision rate, [acc] the proton acceptor concentration, and ΔpK the p*K* difference between the imino proton ($pK_{GH1} = 10.2$, $pK_{TH3} = 10.5$) and the proton acceptor ($pK_{NH3} = 9.3$, $pK_{tris} = 8.4$). Imino proton exchange from G·C and A·T base pairs is a two-step process that requires base pair disruption, followed by a proton transfer to the proton acceptor. The contribution of the proton acceptor to exchange time is given by

$$\tau_{ex,cat.} = \tau_0 + 1/(k_{ex,acc}\alpha K_{diss}) \quad (5)$$

where τ_0 is the base pair lifetime, K_{diss} the base pair dissociation constant, and $k_{ex,acc}$ the proton-transfer rate from the open pair. α is an accessibility factor, which is equal to one for a fully accessible imino proton. According to this model, the plot of $\tau_{ex,cat.}$ versus the inverse of the catalyst concentration is a straight line whose extrapolation to infinite catalyst concentration yields the base pair lifetime, τ_0. The apparent dissociation constant, αK_{diss}, is obtained according to equ 5 as the ratio of the exchange rates measured in the duplex and in the isolated nucleoside (Table 3). The apparent open-pair lifetime, $\alpha\tau_{open}$, is equal to the product $\tau_0\alpha K_{diss}$.

(D) Molecular Dynamics Simulation. Model building, simulations, and analysis were performed with the AMBER 7 suite of programs,[28] using the Parm99 force field.[29] The RNA and DNA duplexes were constructed in canonical A and B conformation, respectively. The duplexes were neutralized by Na^+ counterions, and further Na^+ and Cl^- ions were added in random initial positions (but not closer than 5 Å from the solute or one another) to bring the NaCl concentration to 0.1 M. The system was then solvated with TIP3P water molecules (~5000) within a truncated octahedral box, corresponding to a solvent layer larger than 10 Å. The simulations were carried out using periodic boundary conditions at constant temperature (300 K) and pressure (1 bar), with 5 ps coupling constants. Long-range electrostatic interactions were treated using the particle-mesh Ewald approach with a 8 Å direct space cutoff. Bond lengths involving hydrogen atoms were constrained using SHAKE, and the equations of motion were integrated with a 2 fs time step. The solvent and counterions were relaxed by energy minimization and allowed to equilibrate around the restrained solute during 100 ps at 300 K and constant volume. The simulation was then switched to constant pressure, and the harmonic restraints on the solute atomic positions were gradually reduced over a period of 1 ns. An unrestrained simulation of 4 ns was then performed to create an ensemble of starting structures for the opening simulations.

The opening angle of a base (θ) is calculated as the angle between the C1′−C1′ vector of the base pair and a vector obtained by the projection of the glycosidic bond of the opening base into the plane that is normal to the local helical axis and contains the C1′−C1′ vector.[30] A right-handed base rotation around the axis oriented in the 5′−3′ direction of the strand corresponds to base opening into the major groove (increasing angles) and left-handed rotation to base opening into the minor groove (decreasing angles). In canonical B-DNA, the opening angle has a value of ~55° for the Watson−Crick bases. In the G·T/U wobble base pairs, however, the bases are slightly displaced to maintain H-bonding: T/U into the major groove ($\theta_T = 65°$) and G into the minor groove ($\theta_G = 45°$). Opening angles are reported relative to the values calculated in the relaxed oligomer ($\Delta\theta$) in this work. Imino proton exchange can (a priori) take place as a result of correlated movements of the bases. To sample efficiently the surface defined by the two individual opening angles, reaction coordinates of their linear combinations were used as restraints. Hence, T and G opening was induced either in a coupled rotation of the bases toward the major and minor grooves or by a counterrotation of each base toward a distinct groove (e.g., G toward the major groove and T toward the minor groove).

The opening trajectories were initiated from three independent initial structures to test the stability of the simulations. Base opening was induced by gradually changing the reaction coordinate from its value in the relaxed oligomer (set to 0°) to ±120° by steps of 5°. The reaction coordinates were maintained by a harmonic potential with a 0.05 $kcal.mol^{-1}\cdot deg^{-2}$ force constant. For each set of opening angles, structural ensembles were collected from 100 ps molecular dynamics simulations. The conformation obtained at the end of a sampling window was used as the starting point for the next window. The total simulation time for base pair opening along the different pathways was 30 ns for each oligomer, i.e., 100 ps × 25 window × 4 pathways × 3 independent simulations. During the generation of the structural ensemble, the opening-angle values were recorded to generate biased probability histograms.[31] It was verified that adjacent histograms showed significant overlaps, indicating that all values of the reaction coordinate within the desired range had been properly sampled. These histograms were used to obtain the potential of mean force or free energy associated with base opening by the constant-temperature weighted histogram analysis method (WHAM).[32,33] Convergence of the free energy results was checked by comparing different blocks of data within a trajectory and also between independent trajectories. Both these analyses suggest

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that the results obtained are precise within roughly 2 kcal·mol^{-1}. Atomic surface accessibilities for the imino protons were calculated using a Korobov grid, Pauling atomic radii, and a probe sphere of 1.4 Å radius.[34]

Concluding Remarks

The presence of two imino protons in wobble G·T/U pairs provide a convenient way to study the base opening pathways within DNA and RNA duplexes. NMR spectroscopy and molecular dynamics simulation have been used to obtain kinetic, thermodynamic, and structural insight into the opening mechanism. It has been established that imino proton exchange occurs from the open base pair. The lifetimes of hydrogen bonds between the bases are extremely short: less than 1.5 ms at 0 °C. Molecular dynamics simulations indicate that the lowest free energy pathway corresponds to a coupled rotation of the two bases into the major groove. In DNA, this movement first disrupts the T(H3)−O6(G) bond at a cost of 2.5 kcal/mol. Subsequently, the G(H1)−(O2)T bond breaks at a cost of 7 kcal/mol. In line with simulations, the proton exchange experiments show that the lifetime of the T(H3)−O6(G) bond is always shorter than that of the G(H1)−(O2)T bond. In RNA duplexes, the similarity of the two hydrogen bond lifetimes suggests a more simultaneous disruption of the H-bonds. A comparison of the apparent dissociation constants from imino proton exchange catalyzed by ammonia with those calculated along the lowest free energy pathway shows that an effective proton exchange process can take place at rather low accessibility of the imino proton (10−40% relative to the isolated nucleoside). Modest opening amplitude is also consistent with the reduced effect observed for the negatively charged catalysts (OH^- and HPO_4^{2-}). Similarly, higher steric accessibility is required by the bulkier catalyst, tris, which results in lower apparent dissociation constants.

In the absence of added catalysts, the exchanges of G(H1) and T(H3) are catalyzed by T(O2) with pK = 0 and G(O6) with pK = 1, respectively. The effect of the mismatch on the stability of neighboring base pairs was also investigated. It showed that the lifetimes of adjacent base pairs are strongly reduced, but little influence was observed on the second nearest neighbor. This result indicates that the incorporation of G·T mismatches in DNA causes only local structural perturbations and hence DNA repair systems must involve a complex recognition mechanism.

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