Probing conformational transitions towards mutagenic Watson–Crick-like G·T mismatches using off-resonance sugar carbon $R_{1\rho}$ relaxation dispersion

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Received: 14 March 2020 / Accepted: 13 July 2020 / Published online: 12 August 2020 © The Author(s) 2020

Abstract
NMR off-resonance $R_{1\rho}$ relaxation dispersion measurements on base carbon and nitrogen nuclei have revealed that wobble G·T/U mismatches in DNA and RNA duplexes exist in dynamic equilibrium with short-lived, low-abundance, and mutagenic Watson–Crick-like conformations. As Watson–Crick-like G·T mismatches have base pairing geometries similar to Watson–Crick base pairs, we hypothesized that they would mimic Watson–Crick base pairs with respect to the sugar-backbone conformation as well. Using off-resonance $R_{1\rho}$ measurements targeting the sugar C3′ and C4′ nuclei, a structure survey, and molecular dynamics simulations, we show that wobble G·T mismatches adopt sugar-backbone conformations that deviate from the canonical Watson–Crick conformation and that transitions toward tautomeric and anionic Watson–Crick-like G·T mismatches restore the canonical Watson–Crick sugar-backbone. These measurements also reveal kinetic isotope effects for tautomerization in D₂O versus H₂O, which provide experimental evidence in support of a transition state involving proton transfer. The results provide additional evidence in support of mutagenic Watson–Crick-like G·T mismatches, help rule out alternative inverted wobble conformations in the case of anionic G·T−, and also establish sugar carbons as new non-exchangeable probes of this exchange process.

Keywords Tautomers · Anions · Replication error · Nucleic acid dynamics · Chemical exchange · Sugar pucker

Introduction
In their paper describing the structure of the DNA double helix, James Watson and Francis Crick proposed that spontaneous mutations could arise when nucleotide bases adopt rare and energetically less favorable tautomeric forms as this could allow wobble mismatches such as A·C and G·T to adopt a geometry similar to that of canonical G·C and A·T Watson–Crick (WC) base pairs (bps) (Watson and Crick 1953). Two decades later, Topal and Fresco extended this idea, and proposed that the probability with which rare tautomers form could determine the probability of misincorporating mismatches during replication and translation (Topal and Fresco 1976; Topal and Fresco 1976. During the same period, it was shown that WC-like G·T mismatches could also form via ionization of the thymine base Lawley and Brookes 1962; Sowers et al. 1987; Lawley and Brookes 1961. This anionic WC-like species could explain the increase in G·T misincorporation probability with increasing pH. Yu et al. (1993) Together, the tautomeric and anionic WC-like G·T mismatches could rationalize why chemical modifications that stabilize or mimic either tautomeric (Harris et al. 2003; Budowsky 1976; Woodside and Guengerich 2002; Cantara et al. 2013; Vendeix et al. 2012; Kurata et al. 2008; Ikeuchi et al. 2010) or ionic (Yu et al. 1993; Strebitzer et al. 2018; Weixlbaumer et al. 2007) forms of the bases increase the probability of errors during replication and translation.
Studies over the past decade have provided additional structural and kinetic evidence in support of WC-like mismatches having important roles in the generation of errors during replication and translation. WC-like mismatches have been observed in crystal structures of DNA polymerases (Wang et al. 2011; Bebenek et al. 2011; Koag et al. 2014; Koag and Lee 2018; Sharma et al. 2013) and the ribosome (Rozov et al. 2018; Rozov et al. 2016; Rozov et al. 2015; Demeshkina et al. 2012) in catalytically active conformations. Spontaneous transitions between wobble G·T/U mismatches and short-lived, low-abundance WC-like tautomeric and anionic G·T/U mismatches have also been observed in solution using off-resonance $R_{1\rho}$ NMR relaxation dispersion (RD) experiments (Rangadurai et al. 2019; Palmer and Massi 2006) in both DNA and RNA duplexes (Fig. 1a). Szymanski et al. (2017), Kimsey et al. (2018) and Kimsey et al. (2015) WC-like G·T mismatches were shown to form with probabilities that could quantitatively explain the ~1000 fold reduction in misincorporation rate for G·T mismatches compared to G-C bps (Kimsey et al. 2018). Beyond their roles in the generation of errors during replication and translation, WC-like mismatches have also been proposed to play roles in the generation of errors during transcription (Phillips and Brown 1967; Singer and Spengler 1981; Li et al. 2014) and in DNA damage repair (Freudenthal et al. 2015).

Dynamic transitions between wobble and WC-like G·T/U mismatches (Fig. 1a) (Szymanski et al. 2017; Kimsey et al. 2018; Kimsey et al. 2015) have so far been studied by measuring off-resonance $R_{1\rho}$ NMR RD data for nitrogen and carbon spins in the nucleotide bases. These include the imino nitrogens (G-N1 and T/U-N3), which experience large downfield shifts (~50 ppm) upon deprotonation, as well as the aromatic carbons (G-C8 and T-C6) and amino nitrogen (G-N2) atoms, which experience smaller downfield shifts (~2–4 ppm). These RD measurements (Kimsey et al. 2018; Kimsey et al. 2015) combined with single atom substitution experiments have identified three distinct WC-like G·T/U mismatches stabilized by WC-like hydrogen-bonds.

Fig. 1 a Exchange between wobble and, tautomeric and anionic WC-like G·T mismatches. Green circles denote RD probes used to characterize the exchange in previous studies (Szymanski et al. 2017; Kimsey et al. 2015). b A-T and G-C Watson–Crick bps in DNA. Orange circles denote RD probes used to characterize formation of WC-like mismatches in this study. c DNA constructs used in this study.
exchange process, determining the sugar-backbone conformation, and establishing sugar RD as a new probe of this exchange chemistry during replication.

Beyond confirming the identity of the phosphate oxygen atom, which is integral for polymerization chemistry during replication.

In principle, one can probe such transient conformational changes in the sugar-backbone using RD measurements targeting the sugar carbons C3′ and C4′ (Shi et al. 2018; Clay et al. 2017). Beyond confirming the identity of the transient low-abundance species measured by NMR RD does indeed represent a WC-like G·T mismatch, one would expect that such a transition might restore a canonical WC-like sugar-backbone conformation (Fig. 1b). In principle, one can probe such transient conformational changes in the sugar-backbone using RD measurements targeting the sugar carbons C3′ and C4′ (Shi et al. 2018; Clay et al. 2017). Beyond confirming the identity of the transient low-abundance species measured previously by RD and establishing sugar RD as a new probe of this exchange process, determining the sugar-backbone conformation of Watson–Crick like G·T mismatches is important for assessing their mutagenicity as it defines the placement of the phosphate oxygen atom, which is integral for polymerization chemistry during replication.

Here, we used off-resonance sugar carbon $R_{1\rho}$ measurements, a structure survey, and computational Molecular Dynamics (MD) simulations to characterize changes in the sugar-backbone accompanying the formation of tautomeric and anionic WC-like G·T mismatches. The results show that the G·T wobble mismatch adopts a non WC-like sugar-backbone conformation and that transitions toward either tautomeric or anionic WC-like G·T mismatches restore the canonical WC-like sugar-backbone conformation. These measurements extend the definition of a WC-like bp to encompass the sugar and backbone, help rule out alternative inverted wobble conformations in the case of anionic G·T− mismatches, and also offer new non-exchangeable probes of this exchange process that can enable measurements over a broad range of structural environments.

**Materials and methods**

**Crystal structure survey of G·T mismatches**

All crystal structures containing DNA with resolution better than 3 Å as of April 27th 2017 were downloaded from the Protein Data Bank (PDB) (Berman et al. 2000). Structures were analyzed using an in-house Python script to identify wobble G·T mismatches that are flanked by two WC bps on either side to mimic a duplex like environment. A total of 15 G·T mismatches belonging to 14 distinct structures were identified out of a total of 5906 deposited structures in the PDB containing nucleic acids (Supplementary Table S1). The torsion angles and C1′–C1′ distance of the mismatched bps were computed and compared with a set of unmodified G·C and A·T Watson–Crick bps from free (not bound to proteins/ligands) DNA structures placed in a similar structural context with B form helical geometry as determined using DSSR (Lu et al. 2015). The BI/BII phosphate conformation was determined by first converting the ε and ζ torsion angles to the [0°, 360°] range, taking the difference ε−ζ, and then converting the resultant angle into the [0°, 360°] range (see Figs. 2a and 3). A given phosphate was classified as BI if this difference was in the range [20°, 200°], otherwise it was classified as BI.

To define the movements of the G and T bases required to form a wobble G·T mismatch when starting from a WC bp (Fig. 2b), the coordinates for the G·T mismatches along with one WC bp on either side of the mismatch were extracted from the above crystal structures. An idealized triplet of bps with a G·C bp in the middle was constructed using 3DNA (Lu and Olson 2003). Triplets of G·T mismatches were superimposed on the idealized triplet of bps using the sugar atoms of the bps neighboring the mismatch and the idealized G·C bp. An overlay of the G·T mismatches with the idealized G·C bp after superposition is shown in Fig. 2b.

**MD simulations**

All MD simulations were performed using the ff99 AMBER force field (Cheatham et al. 1999) with bsc0 corrections for DNA (Perez et al. 2007), using periodic boundary conditions as implemented in the AMBER MD simulation package (Salomon-Ferrer et al. 2013). DNA duplexes with G·T or G-C/A-T WC bps used in the MD simulations (Supplementary Figure S2) were derived from the hairpins (GT-CGC/GT-GGC/AT-CAC/AT-GAC/GC-CGC/GC-GGC) used for the NMR measurements (Supplementary Figure S4) by elongating by two G-C/A-T bps on either end. Starting structures of the WC base paired helices for the simulations were generated by creating idealized B-DNA helices using 3DNA (Lu et al. 2015). Corresponding starting structures containing G·T mismatches were obtained by mutating the WC base paired structures. Starting structures were solvated using a truncated octahedral box of SPC/E (Berendsen et al. 1987) water molecules with box size chosen such that the boundary was at least 10 Å away from the DNA atoms. Na+ ions treated using the Joung–Cheatham ion parameters (Joung and Cheatham 2008) were then added to neutralize the charge of the system. Minimization, equilibration and production runs (500 ns) were then performed as described previously (Rangadurai et al. 2018). A set of evenly (5 ps)
spaced snapshots was used for subsequent analysis using the CPPTRAJ (Roe and Cheatham 2013) suite of programs.

**Sample preparation**

**Unlabeled DNA oligonucleotides**

All unlabeled and unmodified DNA oligonucleotides (GT-CGC, GT-GGC, AT-CAC, AT-GAC, GC-CGC and GC-GGC, see Fig. 1c. Supplementary Figure S4) were purchased from Integrated DNA Technologies with standard desalting purification. Unlabeled DNA oligonucleotides containing N1-methyl deoxy Guanosine (m1G, GT-CGC m1G6) or deoxyisoGuanosine (isoG, GT-CGC isoG and GT-GGC isoG) (Supplementary Figure S4) were synthesized in-house using a MerMade 6 oligo synthesizer. In particular, standard (bz-A, ac-C, n-ibu-G and T) and modified DNA phosphoramidites (n,n-dmf-m1G, Chemgenes and dmf-isoG, Glen Research) were used with a coupling time of 1 min, with the final 5'-DMT group being retained during synthesis. The oligonucleotides were then cleaved from the supports (1 μmol, 1000 Å) using ~ 1 mL of AMA (1:1 ratio of ammonium hydroxide and methylamine) for 30 min and deprotected at room temperature for 2 h. The oligonucleotides were then purified using Glen-Pak DNA cartridges followed by ethanol precipitation.

**Site-labeled DNA oligonucleotides**

Site labeled DNA oligonucleotides (slGT-CGC and slGT-GGC) uniformly 13C, 15N labeled at positions T5 and G15 (Supplementary Figure S4), were purchased from the Yale Keck Oligonucleotide Synthesis Facility with cartridge purification.

**Fully labeled DNA oligonucleotides**

Uniformly 13C/15N isotopically labeled flGT-CGC (Supplementary Figure S4) was biochemically synthesized using the Zimmer and Crothers method (Zimmer and Crothers 1995). A chemically synthesized DNA hairpin template containing a 3′ ribonucleotide (IDT) was used in addition to Klenow fragment DNA polymerase (New England Biolabs) and 13C/15N isotopically labeled deoxy nucleoside triphosphates (Silantes). The reaction mixture was centrifuged to remove pyrophosphate and then incubated with NaOH (final concentration 0.3 M) for 3 h at 55 °C for cleavage of the target oligonucleotide from the template. The mixture was then concentrated to 1.5 mL using a 3 kDa molecular weight cutoff centrifugal concentrator (Millipore Sigma). This was followed by addition of 1.5 mL of a formamide based denaturing loading dye. The mixture was then heated at 95 °C for 5 min for denaturation, and then loaded onto a denaturing gel.
(20% polyacrylamide/8 M urea) for separation of the target oligonucleotide from other nucleic acid species. Gel bands corresponding to the target single strands were identified by UV-shadowing and subjected to electroelution (Whatman, GE Healthcare) followed by ethanol precipitation.

Sample annealing and buffer exchange

Oligonucleotides (following ethanol precipitation or as purchased) were re-suspended in water. The samples were annealed by heating at T = 95 °C for ~5 min followed by cooling on ice for ~1 h. Following annealing, the samples were exchanged three times into the desired buffer using centrifugal concentrators (4 mL, Millipore Sigma) with a 3 kDa molecular weight cutoff. 10% by volume of D$_2$O was added to the samples prior to the NMR experiments for establishing C1’-H1’ and C8-H8 assignments (see below). Samples were then lyophilized once and re-suspended in D$_2$O for establishing C3’-H3’ and C4’-H4’ assignments (see below) and performing RD measurements unless mentioned otherwise.

NMR buffer

Sodium phosphate buffer for NMR measurements was prepared by the addition of equimolar solutions of sodium phosphate monobasic and dibasic salts, sodium chloride, and EDTA to give final concentrations (unless mentioned otherwise) of 15 mM (phosphate), 25 mM and 0.1 mM respectively. The pH was then adjusted by the addition of phosphoric acid or sodium hydroxide, after which the buffers were brought up to the desired volume and filtered and stored for further usage.

NMR spectroscopy

All NMR experiments were performed on a 700 MHz Bruker Avance 3 spectrometer equipped with a triple-resonance HCN cryogenic probe. All experiments were performed at pH 6.9 and at T = 25 °C in NMR buffer unless stated otherwise. The NMR data was processed and analyzed using NMRpipe (Delaglio et al. 1995) and Goddard and Kneller (2008).
Resonance assignments

The sugar C1′-H1′ and base C6-H6/C2-H2/C8-H8 resonances were assigned using 2D [1H, 1H] Nuclear Overhauser Effect Spectroscopy (NOESY) and 2D [13C, 1H] Heteronuclear Single Quantum Coherence (HSQC) spectra of samples in 90% H2O/10% D2O. The C3′-H3′ and C4′-H4′ resonances were assigned using 2D [1H, 1H] Total Correlation Spectroscopy (TOCSY) and 2D [13C, 1H] HSQC spectra of samples in 100% D2O. Spectra of GT-CGcisoG and GT-GGCcisoG were collected at a lower temperature (pH 6.9, 10 °C) to minimize alternative tautomeric forms of isoG, which are known to be populated at higher temperatures.

Off-resonance 13C/15N R1p RD measurements

Off-resonance 13C/15N R1p measurements were performed using 1D R1p schemes employing selective Hartman-Hahn magnetization transfers as described previously (Hansen et al. 2009; Korzhnev et al. 2005; Nikolova et al. 2012). Weak matched 1H and 13C/15N RF fields were used to transfer magnetization selectively from protons to the 13C/15N nucleus of interest. The longitudinal 13C/15N magnetization thus produced was allowed to relax for 5 ms to allow equilibration of ground state and excited state populations and then tilted along the appropriate effective field direction. Then a 13C/15N spin-lock was applied for a maximal delay time using NMRPipe (Delaglio et al. 1995) and then tilted along the appropriate effective field direction thus produced was allowed to relax for 5 ms to allow equilibration of ground state and excited state populations. Then a 13C/15N spin-lock was applied for a maximal delay time using NMRPipe (Delaglio et al. 1995) and then tilted along the appropriate effective field direction. The fitted exchange parameters for the 2-state individual fits were pb, kexAB, R1, R2 and Δω, while those for the 3-state individual fits were pb, pca, kexAB, kexAC, R1, R2, ΔωAB and ΔωAC. Global fits were performed by sharing pb/pca and kexAB/kexAC across multiple nuclei.

Fitting of R1p RD data

R1p values for a given spin-lock power and offset were obtained by extracting the peak intensities as a function of delay time using NMRPipe (Delaglio et al. 1995) and then fitting them to a mono-exponential function (Kimsey et al. 2015). Errors in R1p (σR1p) were estimated using a Monte-Carlo scheme as described previously (Rangadurai et al. 2019). Numerical integration of the Bloch–McConnell (B-M) equations were used to fit R1p values as a function of spin-lock power and offset to 2-state or 3-state exchange models, by minimizing $\chi^2 = \sum \frac{R_{1p,\text{meas}} - R_{1p,\text{calc}}}{R_{1p}}$, using least squares minimization as described previously (Rangadurai et al. 2019). R1p,meas and R1p,calc are the measured and calculated R1p values, respectively, and the summation is over all spin-lock power offset combinations. The fitted exchange parameters for the 2-state individual fits were pb, kexAB, R1, R2 and Δω, while those for the 3-state individual fits were pb, pca, kexAB, kexAC, R1, R2, ΔωAB and ΔωAC. Global fits were performed by sharing pb/pca and kexAB/kexAC across multiple nuclei.

It has been shown that base carbon and imino nitrogen RD measurements on G-T mismatches can passively sense the WC to Hoogsteen exchange of neighboring bps (Kimsey et al. 2015), a phenomenon that will hereafter be referred to as “Hoogsteen blowback”, in addition to the exchange of the mismatch itself into a WC-like species. In principle, G-T mismatches in GT-CGC and GT-GGC (Fig. 1c, Supplementary Figure S4) can experience Hoogsteen blowback due to both of the neighboring G-C bps, thereby introducing extraneous contributions to the measured RD data. The Hoogsteen blowback contribution from the G6-C14 bp is expected to be significant for T5-C3′ in the G-T mismatch in GT-CGC. This is because formation of Hoogsteen bps is accompanied by sizeable (~2 ppm) downfield shifts in the C3′ carbon of the nucleotide 5′ to the purine (Shi et al. 2018). Hoogsteen blowback contributions are expected to be smaller for GT-GGC since none of the mismatched nucleotides are 5′ to the purines of the neighboring WC bps. The WC to Hoogsteen exchange at the G6-C14 bp was quantified by measuring 13C R1p RD on G6-C1′ in fGT-CGC (Supplementary Figs. S4 and S6). The G6-C1′ R1p RD data could be directly fit to the Bloch–McConnell equations assuming a two-state exchange process to characterize the WC to Hoogsteen exchange at the G6-C14 bp. It should be noted that G6-C1′ is not expected to passively sense the transition between wobble and WC-like G-T as ΔωG6-C1′, estimated as the difference in chemical shifts of G6-C1′ between GT-CGC and GC-CGC or AT-CAC is <~0.3 ppm (Supplementary Figure S5).

Including a shared Hoogsteen blowback exchange process when individually fitting 13C/15N R1p RD profiles of the G-T mismatch in sGT-CGC to a 3-state exchange model in a star-like (Trott and Palmer 2004) topology resulted in exchange parameters for the wobble to WC-like exchange that are more similar across different nuclei relative to parameters obtained from 2-state fits which do not account for Hoogsteen blowback (Supplementary Tables S3 and S4, Supplementary Figure S7). Moreover, the 12C and 15N R1p RD data for multiple nuclei of the G-T mismatch could also be globally fitted to a 3-state exchange model sharing pb and kex for the exchange to a WC-like species with the inclusion of a shared Hoogsteen blowback exchange contribution in

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a star-like (Trott and Palmer 2004) topology (Supplementary Tables S3 and S4, Fig. 5a, Supplementary Figure S9a). In addition, the populations of the WC-like G-T mismatch obtained from RD measurements in 90% H₂O:10% D₂O (Supplementary Table S3) and 100% D₂O (Supplementary Table S4) were in better agreement with each other when globally fitting the data with the inclusion of a shared Hoogsteen blowback exchange process, relative to when blowback was not considered during fitting (Supplementary Figure S8a). Reasonable estimates of Δω for the atoms of the G-T mismatch on the formation of a G6-C14 Hoogsteen bp were also obtained from shared fitting of the RD data, that were similar to the difference in chemical shifts of the atoms between GT-CGC⁰⁶ and GT-CGC (Supplementary Tables S3 and S4, Supplementary Figure S8b). The poor agreement seen for T5-C6 is likely due to N⁰¹-methylation of G6 in GT-CGC⁰⁶. RD profiles for slGT-CGC obtained from a global fit with the inclusion of a shared blowback exchange are displayed in Fig. 5a and Supplementary Figs. 6 and 9a. Fits to RD profiles for slGT-GGC to a 2-state exchange model without inclusion of a shared blowback Hoogsteen exchange are shown in Fig. 5b and Supplementary Figure S9b.

Prior studies (Kimsey et al. 2018) have shown that at high pH > ~8.0, G-T mismatches can dynamically exchange with tautomeric and anionic states in a star-like or a triangular (Trott and Palmer 2004) exchange topology. The exchange topology for the exchange of the G-T mismatch in slGT-GGC at pH 8.8 and 10 °C was determined by fitting of the ¹⁵N R₁ρ data for G15-N1 and T5-N3. Based on the Akaike (Wagenmakers and Farrel 2004) and Bayesian (Burnham and Anderson 2004) information criterion weights, a statistically better fit was obtained for a triangular topology for the exchange of the G-T mismatch in slGT-GGC at pH 8.8 and 10 °C was determined by fitting of the ¹⁵N R₁ρ data for G15-N1 and T5-N3. Based on the Akaike (Wagenmakers and Farrel 2004) and Bayesian (Burnham and Anderson 2004) information criterion weights, a statistically better fit was obtained for a triangular topology, with populations of the tautomeric and anionic state equal to 0.075 ± 0.005% and 8.019 ± 1.007%, respectively. The pH = 9.9 for the ionization of the G·T mismatch thus obtained was inconsistent with prior estimations (Kimsey et al. 2018) of the pKa = 11 made based on ¹⁵N R₁ρ data for the G-T mismatch in slGT-GCC at 10 °C at pH values of 8.0 and 8.4, respectively. Thus, the ¹⁵N and ¹³C R₁ρ profiles for G15-N1, T5-N3 and T5-C4' in slGT-GGC at pH 8.8 and 10 °C were fit assuming 3-state exchange between wobble, tautomeric and anionic G·T mismatches in a star-like (Trott and Palmer 2004) exchange topology (Supplementary Figure S11a) instead to yield exchange parameters in Supplementary Table S7. The large error in the fitted ΔωT₅-C₄ for the tautomeric G-T excited state (Supplementary Table S7) is due its low population (0.065 ± 0.003%) and fast exchange rate (4029 ± 352 s⁻¹), which results in a small contribution to the observed RD profile, thereby making determination of Δω unreliable.

The uncertainties in the exchange parameters were computed using a Monte-Carlo scheme (Bothe et al. 2014). Alignment of magnetization during B-M fitting was performed as described previously (Rangadurai et al. 2019). Off-resonance R₁ρ profiles were generated by plotting (R₂ + R₂ex) = (R₁ρ−R₂cos²θ/2)/sin²θ, where θ is the angle between the effective field of the observed resonance and the z-axis, as a function of ω = ωobs−ωRF, where ωobs is the Larmor frequency of the observed resonance and ωRF is the angular frequency of the applied spin-lock.

Results and discussion

Wobble G-T mismatches have a distinct sugar-backbone conformation relative to canonical Watson–Crick base pairs

Before examining whether transitions between wobble and WC-like G-T mismatches entail changes in the sugar-backbone conformation, we first examined how and why the sugar-backbone conformation differs for wobble G-T relative to canonical WC G-C or A-T bps. If we were to start with a canonical WC G-C bp, the introduction of a G-T mismatch by replacing the C with a T would result in steric clashes between the imino hydrogen atoms. The steric clashes can be resolved through translation of the two bases by one hydrogen bond register to form a paired wobble G-T mismatch in which the T is displaced toward the major groove relative to the G. It is this movement of the bases to accommodate the wobble pair that will require changes in the sugar-backbone conformation (Allawi et al. 1998; Patel et al. 1982; Hare et al. 1986; Hunter et al. 1987).

To capture the changes in the sugar-backbone conformation required to accommodate the G-T wobble, we surveyed ("Materials and methods" section) existing crystal structures of DNA duplexes containing G-T mismatches. We find that relative to WC G-C and A-T bps, wobble G-T mismatches have sugar puckers that are biased away from C2'-endo towards puckers in between C2' and C3'-endo, and backbone torsion angles ξ and ζ that are biased away from the BII conformation for both G and T nucleotides (Fig. 2a, Supplementary Figure S1). While it is often assumed that the T (or U) base primarily moves to form a wobble (Westhof et al. 2019; Westhof 2014), the structures show both bases towards puckers in between C2' and C3'-endo, and backbone torsion angles ξ and ζ that are biased away from the BII conformation for both G and T nucleotides (Fig. 2a, Supplementary Figure S1). While it is often assumed that the T (or U) base primarily moves to form a wobble (Westhof et al. 2019; Westhof 2014), the structures show both bases move to form the wobble pair as noted previously for G-T mismatches in A-DNA (Rabinovich et al. 1988) (Fig. 2b).

To further evaluate the robustness of the above results, we carried out 0.5 μs long MD simulations using the parm99c6 forcefield (Cheatham et al. 1999; Perez et al. 2007) on DNA duplexes containing either G-T, G-C, or A-T bps (see "Materials and methods" section). In agreement with the crystal structure survey, the simulations show changes in the backbone torsion angles ξ and ζ away from the BII conformation at the G of the G-T mismatch, in addition to a bias away from the C2'-endo sugar pucker towards puckers...
in between C2′ and C3′-endo, at both the G and T of the G·T mismatch (Fig. 3, Supplementary Figure S3).

Sugar carbon chemical shifts for wobble G·T mismatches differ from canonical Watson–Crick base pairs

The differences in the sugar-backbone conformation, particularly the sugar pucker, between wobble G·T and canonical WC bps (Figs. 2 and 3) are expected to give rise to differences in sugar carbon chemical shifts. The C3′ chemical shift is highly sensitive to sugar pucker while C4′ is sensitive to both sugar pucker and the backbone angle γ (Santos et al. 1989; Fonville et al. 2012; Dejaegere and Case 1998; Xu and Au-Yeung 2000). If these changes in chemical shifts were sizeable (i.e. > 1 ppm), NMR RD measurements targeting sugar C3′/C4′ nuclei of the G·T mismatch could be used to examine whether transitions toward low-abundance short-lived WC-like conformations restore C3′/C4′ chemical shifts and sugar-backbone geometry typical of the canonical WC conformation. It should be noted that based on prior studies (Kimsey et al. 2015) it is not possible to infer the sugar-backbone geometry of WC-like G·T mismatches using the C1′ carbons as probes as they are not sensitive to the wobble to WC-like transition.

For two different DNA sequence contexts, we compared the sugar C3′ and C4′ chemical shifts of the wobble G·T mismatch with those of G-C and A-T WC bps. We used DNA duplexes containing either G-C or A-T bps in place of G·T (Fig. 1c, Supplementary Figure S4). Indeed, relative to either G-C or A-T bps, wobble G·T mismatches consistently have upfield shifted (∼1-3 ppm) sugar chemical shifts which are particularly sizeable for G-C3′, T-C3′, and T-C4′ (Fig. 4, Supplementary Figure S5). Such upfield shifted sugar chemical shifts observed for G·T mismatches are exactly as expected for a bias away from a C2′-endo conformation, in agreement with the structure survey (Fig. 2), MD simulations (Fig. 3), prior chemical shift measurements (Shi et al. 2018; Rangadurai et al. 2018; Santos et al. 1989) and Density Functional Theory (DFT) calculations (Fonville et al. 2012; Dejaegere and Case 1998). Additional measurements of 31P chemical shifts and $J_{\text{H3-P}}$ coupling constants (Gorenstein 1994) are required for characterizing mismatch induced changes in BI/BII phosphate conformations.

Furthermore, the observation of chemical shift perturbations for both the T and G (Fig. 4c) indicates that both residues experience a change in the sugar-backbone on formation of a wobble bp, as expected based on the comparison of crystal structures (Fig. 2b) (Rabinovich et al. 1988). The G·T wobble also induces sizeable chemical shift perturbations in the neighboring WC bps that are particularly large for the 3′ neighbor of the T and the 5′ neighbor of the G (Fig. 4b). These perturbations likely reflect additional potentially destabilizing conformational changes in neighboring bps that help to accommodate the movement of the T and G to form a wobble bp.
Tautomeric Watson–Crick-like G·T mismatches have sugar chemical shifts that are similar to Watson–Crick base pairs

Based on the above results, transitions between wobble and WC-like G·T mismatches are predicted to lead to large changes (> 1 ppm) in the G-C3′, T-C3′ and T-C4′ chemical shifts if they entail changes in the sugar-backbone to a WC-like conformation. These chemical shift perturbations are large enough to give rise to detectable NMR $R_1 \rho$ RD. In contrast, we do not expect sizeable RD for G-C4′ because the changes in chemical shifts are predicted to fall near or below the RD detection limit (< 1 ppm).

We tested the above predictions by measuring off-resonance $R_1 \rho$ RD profiles for G15-C3′, G15-C4′, T5-C3′ and T5-C4′ in the siGT-CGC and siGT-GGC DNA hairpins (Fig. 5, Supplementary Tables S4 and S6, “Materials and methods” section) at pH 6.9 and T = 25 °C in D2O. Under these pH conditions, any contribution to the RD profiles from the transient formation of the anionic G·T¯ mismatch ($p_B \sim 0.001\%$) can be safely ignored. Thus, RD profiles are expected to sense the two state exchange between wobble and tautomeric WC-like G·T mismatches. Experiments were performed in D2O to minimize interference from the water signal (“Materials and methods” section). To allow comparison with prior results (Szymanski et al. 2017; Kimsey et al.
we also measured 15N and 13C R1ρ RD profiles for G-N1, T-N3, G-C8 and T-C6 in water (Supplementary Figure S9, Supplementary Tables S3 and S5), as well as for G-C8 and T-C6 carbons, which are known probes for the wobble to WC-like transition, in D2O (Fig. 5, Supplementary Table S4).

As predicted, sizeable off-resonance R1ρ RD profiles were observed for G15-C3′ and T5-C3′ in slGT-CGC, and T5-C3′ in slGT-GGC whereas the profiles were weaker and in most cases undetectable for T5-C4′ and G15-C4′ (Fig. 5). The lack of observable RD for G15-C3′ in slGT-GGC is likely due to its small Δω (~ 1 ppm) coupled with its faster rate of exchange (1657 ± 189 s⁻¹) to form a WC-like species relative to slGT-CGC (909 ± 142 s⁻¹, Supplementary Tables S4 and S6). The lack of observable RD for T5-C4′ in both slGT-CGC and slGT-GGC is likely because of the small Δω (~ 1 ppm, see Fig. 4c).

The Δω values deduced from fitting the sugar C3′ RD data were in excellent agreement with those expected for a transition between the wobble and WC-like transition, in D2O (Fig. 5, Supplementary Figure S8a, Supplementary Tables S3–S6). and a sparsely populated excited state (ES) (Mulder et al. 2001) (Fig. 5, Supplementary Figs. S6–S8, “Materials and methods” section). The fits yielded exchange parameters of interest, including the ES population (pES), the GS ↔ ES exchange rate (kex = k⁺ + k⁻, where k⁺ and k⁻ are the forward and backward rate constants), and the chemical shift difference between the ES and GS (Δω = ωES−ωGS), which carries structural information.

The Δω values deduced from fitting the sugar C3′ RD data were in excellent agreement with those expected for a transition between the wobble and WC-like conformation, estimated as the difference between the chemical shifts of WC G-C/A-T bps and wobble G·T mismatches (Fig. 6). In addition, the ES populations were in very good agreement with values obtained independently from fitting base G-C8 and T-C6, and imino nitrogen G-N1 and T-N3 RD data in H2O (Kimsey et al. 2018; Kimsey et al. 2015) (Fig. 6, Supplementary Figure S8a, Supplementary Tables S3–S6).
However, the $k_{ex}$ measured in D$_2$O was ~3.5 fold slower than values measured in H$_2$O.

Based on DFT studies (Nomura et al. 2013; Brovarets et al. 2015), the transition state for conversion between wobble and tautomeric WC-like G·T entails movement of a proton from G-O6 to T-O4 (Supplementary Figure S10). If this were the case, we would expect a ~2-4 fold reduction in the rate of the reaction in D$_2$O versus H$_2$O (Anslyn 2005). Such isotope effects have previously been reported in studies of conformational changes involving proton transfer in proteins (Watt et al. 2007). The reduced $k_{ex}$ in D$_2$O versus H$_2$O therefore provides the first experimental evidence in support of a transition state for tautomerization involving proton transfer. While additional experiments including measurement of RD as a function of viscosity are needed to rule out that the observed change in $k_{ex}$ is not due to a change in viscosity between D$_2$O and H$_2$O (Watt et al. 2007), this highlights the general utility of kinetic isotope effects in the RD-based characterization of nucleic acid transition states. Taken together, the sugar RD data shows that the sugar-backbone adopts a WC-like conformation when forming the tautomeric WC-like G·T mismatch.

**Probing the sugar conformation of anionic G·T base pair**

We also used sugar RD to probe the sugar-backbone conformation of the anionic G·T ES observed at high pH (Kimsey et al. 2018; Kimsey et al. 2015). Ionization of T at high pH (> 8.0) was previously inferred based on the appearance of a second pH dependent ES with a large (~50 ppm) downfield shift in T-N3 (Kimsey et al. 2018; Kimsey et al. 2015). However, in addition to a WC-like conformation, a G·T mismatch can also form an ‘inverted wobble’ conformation in which the bases move by one additional hydrogen bond register past WC pairing (Fig. 7a). Inverted G·T wobble bps have previously been observed crystallographically (Johnson and Beese 2004; Xia and Konigsberg 2014) and more recently, G-U inverted wobbles have been observed in the context of modified bases at the wobble position of the tRNA-mRNA mini-helix in the ribosome (Rozov et al. 2016). The inverted wobble was not suggested to contribute significantly to the anionic G·T ES based on DFT calculations in a prior study (Kimsey et al. 2015). The measured $\Delta\omega$ (56 ppm) of T-N3 for the anionic ES was in good agreement with DFT calculations assuming a Watson–Crick like ES ($\Delta\omega = 54$ ppm), and was different from the calculated $\Delta\omega$ for an inverted wobble ($\Delta\omega = 46$ ppm). However, direct experimental data is needed to rule out the possibility that the ES observed at high pH is a WC-like conformation not an inverted wobble.

Given the greater movement of the bases in forming an inverted wobble relative to WC, we hypothesized that the inverted wobble has a unique sugar-backbone conformation, and in turn, unique sugar chemical shifts distinct from those of canonical WC bps. If this were the case, sugar RD data could be used to rule out an inverted anionic wobble G·T ES. We used G to isoguanosine (isoG) substitution.

![Fig. 7 a Chemical structures of G·T and isoG·T inverted wobble bps. Chemically modified sites in isoG are indicated in red. Arrows on the isoG·T bp denote characteristic NOE connectivities expected for the inverted wobble. b 2D [1H,1H] NOESY spectrum of the imino-imino and imino-amino region for GT-GGCisoG. c Comparison of $\Delta\omega$ of the sugar carbons for the formation of a WC-like G·T mismatch, estimated as the difference in chemical shift between hairpins containing G-C and G·T bps (brown) or A-T and G·T bps (red). Also shown is the $\Delta\omega$ for the formation of an inverted wobble G-T mismatch, estimated as the difference in chemical shift between hairpins containing isoG·T and G·T bps (blue). d Comparison of $\Delta\omega$ for T5-C4 for the formation of a WC-like (brown and red) or inverted wobble G-T mismatch (blue), with the experimental value for the G·T ES obtained from $R_1\rho$ measurements (yellow) on GT-GGC at pH 8.8 and 10 °C in 90% H$_2$O:10% D$_2$O (Materials and Methods). The uncertainty in $\Delta\omega$ denotes the fitting error estimated using a Monte-Carlo scheme as described previously (Bothe et al. 2014).](image-url)
(Robinson et al. 1998) to stabilize an inverted wobble conformation (Fig. 7a) and thereby deduce its sugar chemical shifts. While the chemical modification of the G and lack of ionization of the T would not be expected to faithfully reproduce the base chemical shifts of the inverted wobble G-T, the sugar chemical shifts should not be significantly affected by chemical modification of the base (Xu and Au-Yeung 2000).

2D $^{1}H$, $^{1}H$ NOESY spectra showed cross peaks between T-H3, isoG-H1 and isoG-H6 as expected for an inverted wobble isoG-T bp (Fig. 7b). Furthermore, 2D HSQC spectra (Supplementary Figure S5) revealed that the T-C3′ and the T-C4′ sugar carbon chemical shifts are more downfield shifted for the inverted wobble relative to the WC conformation. Therefore, transition of a wobble G-T to an inverted wobble G-T is expected to result in a larger downfield chemical shift for T-C3′ and T-C4′ relative to a transition toward the WC-like G-T′ conformation (Fig. 7c). In contrast, the other sugar carbon chemical shifts (T-C1′, G-C1′, G-C3′, G-C4′) were small (< 1 ppm) and/or similar to the shifts expected for a WC bp (Fig. 7c), and therefore were less suited for distinguishing inverted wobble versus WC-like G-T′ conformations using RD.

We therefore chose T-C3′ and T-C4′ as RD probes as they should in theory be able to distinguish between the inverted wobble and WC-like G-T′ conformation. In principle, one can obtain these sugar chemical shifts for G-T′ by fitting the sugar RD data measured at high pH. However, in practice, owing to the small magnitude of the exchange contributions in the RD profiles, fitting the sugar RD data to a 3-state exchange process involving the wobble, tautomeric and anionic species yields unreliable estimates for the exchange parameters, including the chemical shifts. The exchange parameters can be more reliably determined with the inclusion of RD data from G-N1 and T-N3 RD owing to their much larger exchange contributions in the RD profiles, which is a consequence of the large changes in chemical shifts during tautomeration and ionization (Kimsey et al. 2018). This in turn necessitates that the RD be measured in H2O. While this complicates C3′ RD measurements owing to the fact that the H3′ protons are close to the water resonance frequency, this is not an issue for the C4′ RD measurements as the H4′ protons are relatively farther away from water. Thus, our strategy to rule out the inverted wobble conformation for the G-T′ anionic ES involved performing imino nitrogen and T-C4′ RD measurements in H2O.

The imino G15-N1 and T5-N3, and sugar T5-C4′ RD profiles for slGT-GGC could be globally fitted together to a 3-state exchange process between wobble, tautomeric and anionic ES G-T′ bps in a star-like topology (Kimsey et al. 2018; Trott and Palmer 2004) (Supplementary Figure S11a, “Materials and methods” section) to obtain exchange parameters (Supplementary Table S7). The difference in chemical shifts between the GS and anionic ES for T-C4′ was found to be in better agreement with that expected for formation of a WC-like bp as compared to an inverted wobble (Fig. 7d). Moreover, the quality of the fit worsened when fixing the $\Delta \chi$ to be equal to the value expected for an inverted wobble G-T′ bp (Supplementary Figure S11b). These data indicate that the anionic G-T′ ES has a WC-like conformation and help to rule out the alternative inverted wobble conformation.

Discussion

Determining the structures of short-lived low-abundance excited conformational states of biomolecules, such as the WC-like G-T′ mismatches studied in this work presents a significant challenge to conventional biophysical methods. NMR RD experiments provide a rare opportunity to detect such ESs as well as to probe their conformations (Rangadurai et al. 2019; Sekhar and Kay 2013; Korzhnev and Kay 2008; Hansen et al. 2008; Xue et al. 2015; Zhao and Zhang 2015). In both proteins and nucleic acids, measurements of RD data for a variety of spins is crucial for accurately deducing the ES conformation (Shi et al. 2018; Sathyamoorthy et al. 2017; Hansen et al. 2008). Prior studies (Shi et al. 2018; Clay et al. 2017) have highlighted the utility of sugar RD data in characterizing ES Hoogsteen bps in DNA and ESs involving alternative secondary structures in RNA. In this study, the sugar RD data could be used to obtain new evidence in support of an ES that involves tautomeric and anionic WC-like G-T′ conformations. It should be noted that the lack of detectable RD on the C1′ carbons of the G-T mismatch, in spite of the known sensitivity of the C1′ chemical shift to changes in sugar pucker (Santos et al. 1989; Fonville et al. 2012; Dejaegere and Case 1998; Xu and Au-Yeung 2000) that accompany formation of a WC-like mismatch (Figs. 2 and 3) is likely due to compensatory changes in $\chi$ angle (Supplementary Figs. 1 and 3). Such compensation effects the $\chi$-angle and sugar pucker have previously been noted in the context of RNA (Zhou et al. 2016) and DNA (Shi et al. 2018).

While it is not surprising that tautomeric WC-like G-T′ mismatches adopt a WC-like sugar-backbone conformation, the results help to rule out an inverted wobble conformation for the case of anionic G-T′ mismatches. The WC-like sugar-backbone conformation further supports a mutagenic role for these fleeting WC-like states as this is likely to align functional groups for phosphodiester bond formation during catalysis by DNA polymerases. Based on these results, it is very likely that base modifications that stabilize ionization and promote miscoding such as 5-Bromo and Fluoro uridine (Yu et al. 1993), and Uridine 5-oxyacetic acid (Strebiteur et al. 2018) do so in part via formation of a WC-like ES conformation.
Our studies add to a growing view that in duplex DNA, non-canonical bps such as Hoogsteen and wobble G·T mismatches have sugar puckers that are biased away from the canonical C2′-endo conformation. In this regard, it is interesting to note that prior NMR studies indicated that the sugar pucker at wobble G·T mismatches is C2′-endo (Allawi and SantaLucia 1998; Hare et al. 1986) and not biased away from the C2′-endo conformation as inferred here based on analysis of chemical shifts and MD simulations. This discrepancy is likely due to the absence of J-coupling restraints for constraining the sugar pucker during structure refinement in the study by Hare et al. (Hare et al. 1986) and possibly due to differences in sequence context in the study by Allawi and SantaLucia (1998). It is also interesting to note that changes in backbone angles and movements of the bases required for the formation of wobble G·T mismatches in analogous environments such as RNA–DNA hybrids or A-RNA could shed light into the nature of the sugar-backbone changes required for formation of WC-like mismatches have sugar puckers that are biased away from the C2′-endo conformation of General Medical Sciences (P01GM0066275) Grants to H. M. A.

Compliance with ethical standards

Conflict of interest The authors declare that they have no conflict of interest.

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Acknowledgements We would like to thank Dr. Eric Westhof for helpful discussions pertaining to the inverted wobble and members of the Al-Hashimi lab for their critical input.

Funding This work was supported by the US National Institutes of Health (R01GM089846) and US National Institutes of Health.
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