Differential Stabilities and Sequence-Dependent Base Pair Opening Dynamics of Watson–Crick Base Pairs with 5-Hydroxymethylcytosine, 5-Formylcytosine, or 5-Carboxylcytosine

Marta W. Szulik,‡ Pradeep S. Pallan‡ Boguslaw Nocek,§ Markus Voehler,† Surajit Banerjee,|| Sonja Brooks,⊥ Andrzej Joachimiak,§ Martin Egli,‡ Brandt F. Eichman,⊥ and Michael P. Stone‡∗

†Department of Chemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt Ingram Cancer Center, and Center for Structural Biology, Vanderbilt University, Nashville, Tennessee 37235, United States
‡Department of Biochemistry, Vanderbilt Institute of Chemical Biology, Vanderbilt Ingram Cancer Center, and Center for Structural Biology, School of Medicine, Vanderbilt University, Nashville, Tennessee 37232, United States
§Bioscience Division, Argonne National Laboratory, Argonne, Illinois 60439, United States
||Northeastern Collaborative Access Team and Department of Chemistry and Chemical Biology, Cornell University, Argonne National Laboratory, Argonne, Illinois 60439, United States
⊥Department of Biological Sciences, Vanderbilt Institute of Chemical Biology, and Center for Structural Biology, Vanderbilt University, Nashville, Tennessee 37235, United States

Supporting Information

ABSTRACT: 5-Hydroxymethylcytosine (5hmC), 5-formylcytosine (5fC), and 5-carboxylcytosine (5caC) form during active demethylation of 5-methylcytosine (5mC) and are implicated in epigenetic regulation of the genome. They are differentially processed by thymine DNA glycosylase (TDG), an enzyme involved in active demethylation of 5mC. Three modified Dickerson–Drew dodecamer (DDD) sequences, amenable to crystallographic and spectroscopic analyses and containing the 5′-CG-3′ sequence associated with genomic cytosine methylation, containing 5hmC, 5fC, or 5caC placed site-specifically into the 5′-TAXGC10-3′ sequence of the DDD, were compared. The presence of 5caC at the X9 base increased the stability of the DDD, whereas 5hmC or 5fC did not. Both 5hmC and 5fC increased imino proton exchange rates and calculated rate constants for base pair opening at the neighboring base pair A5:T8, whereas 5caC did not. At the oxidized base pair G4:X9, 5fC exhibited an increase in the imino proton exchange rate and the calculated kex. In all cases, minimal effects to imino proton exchange rates occurred at the neighboring base pair C3:G10. No evidence was observed for imino tautomerization, accompanied by wobble base pairing, for 5hmC, 5fC, or 5caC when positioned at the X9 base increased the stability of the DDD, whereas 5hmC or 5fC did not. Both 5hmC and 5fC increased imino proton exchange rates and calculated rate constants for base pair opening at the neighboring base pair A5:T8, whereas 5caC did not. At the oxidized base pair G4:X9, 5fC exhibited an increase in the imino proton exchange rate and the calculated kex. In all cases, minimal effects to imino proton exchange rates occurred at the neighboring base pair C3:G10. No evidence was observed for imino tautomerization, accompanied by wobble base pairing, for 5hmC, 5fC, or 5caC when positioned at the base pair G4:X9; each favored Watson–Crick base pairing. However, both 5fC and 5caC exhibited intranucleobase hydrogen bonding between their formyl or carboxyl oxygens, respectively, and the adjacent cytosine N4 exocyclic amines. The lesion-specific differences observed in the DDD may be implicated in recognition of 5hmC, 5fC, or 5caC in DNA by TDG. However, they do not correlate with differential excision of 5hmC, 5fC, or 5caC by TDG, which may be mediated by differences in transition states of the enzyme-bound complexes.

Received: December 17, 2014
Published: January 29, 2015

© 2015 American Chemical Society 1294
DOI: 10.1021/bi501534x
Biochemistry 2015, 54, 1294−1305
DNA glycosylases may also exploit differential base pair opening rates as a basis for substrate recognition. For example, enhanced base pair opening rates at A:U base pairs facilitate the recognition of uracil by uracil DNA glycosylase (UDG).\textsuperscript{35} It has also been hypothesized that TDG recognizes wobble base pairing geometry at oxidized cytosines,\textsuperscript{17,19,23,36,37} as the imino tautomers of 5caC or 5fC may adopt wobble-like base pairs with the complementary G.\textsuperscript{20,37} However, calculations of the stabilities of the amino and imino tautomers of 5fC and 5caC at the nucleobase level have suggested that, when paired with G, both 5fC and 5caC, which are substrates for TDG,\textsuperscript{14} preferentially form Watson–Crick pairs.\textsuperscript{23} Alternatively, as proposed by Maiti et al.,\textsuperscript{14} the differential processing by TDG could be mediated by differences in the corresponding transition state catalytic complexes involving 5fC, 5caC, or 5hmC. Maiti et al.\textsuperscript{14} proposed that the preferential excision of 5fC and 5caC by TDG is facilitated by the presence of electron-withdrawing substituents at the C5 carbon for these two oxidized cytosines. This electron-withdrawing effect\textsuperscript{14,23} would be anticipated to stabilize developing negative charge in the transition state complex for base excision.

Here, we have incorporated 5hmC, 5fC, or 5caC into the 5′-T8X9G10-3′ sequence of the self-complementary Dickerson–Drew dodecamer (DDD),\textsuperscript{38,39} which contains the 5′-CG-3′ sequence associated with genomic cytosine methylation, forming DDD\textsubscript{hm}, DDD\textsubscript{f}, and DDD\textsubscript{ca} duplexes (Chart 1), respectively. Importantly, the DDD is amenable to crystallographic\textsuperscript{38–43} and spectroscopic\textsuperscript{44–47} analyses. The characterization of the DDD\textsubscript{hm}, DDD\textsubscript{f}, and DDD\textsubscript{ca} duplexes by thermal melting studies, measurements of base pair opening dynamics, crystallography, and NMR reveals lesion- and sequence-specific differences among ShmC, SfC, or ScaC in the 5′-T8X9G10-3′ sequence, which may be relevant to their recognition by TDG. Relative to ShmC and SfC, incorporation of ScaC increases the stability of the DDD. This is reflected in reduced base pair opening dynamics for DDD\textsubscript{ca}, as compared to that for DDD\textsubscript{hm} and DDD\textsubscript{f}, at neighboring base pair A5:T8. Similar, but smaller, differences in base pair opening dynamics are observed at the oxidized base pair G4:X9, whereas minimal effects are observed at neighboring base pair C3:G10. No evidence for wobble base pairing interactions involving the oxidized cytosines is observed; each of these oxidized cytosines favors Watson–Crick base pairing. These sequence-specific differences in the DDD may be related to the recognition of these oxidized cytosines by TDG. However, they differ from the sequence-specific effects observed by Raiber et al.\textsuperscript{34} for an iterated CG repeat containing three 5fC sites. Moreover, they do not correlate with the differential ability of TDG to excise SfC and ScaC vs ShmC,\textsuperscript{14} which may be mediated by differences in the transition state complex for base excision.

**Experimental Procedures**

**Oligodeoxynucleotide Synthesis.** Oligodeoxynucleotides were synthesized by Midland Certified Reagent Co. (Midland, TX) and purified by anion-exchange HPLC. The DDD\textsubscript{hm} duplex was prepared with an Expedite 8909 DNA synthesizer (PerSeptive Biosystems) on a 1 μmol scale using ethylenediamine-protected 5-hydroxymethyl-dC, phenoxacyctyl-protected dA, 4-isopropyl-phenoxacyctyl-protected dG, acetyl-protected dC, and dT phosphoramidites and solid supports (Glen Research, Inc., Sterling, VA). The modified phosphoromidade was incorporated by removing the column from the synthesizer and sealing it with two syringes, one of which contained 250–300 μL of the manufacturer’s 1H-tetrazole activator solution (1.9–4.0% in CH\textsubscript{3}CN, v/v) and the other contained 250 μL of the modified phosphoromidade solution (15 mg in anhydrous CH\textsubscript{3}CN). The 1H-tetrazole and the phosphoromidade solutions
were sequentially drawn through the column (1H-tetrazole first), and this procedure was repeated over 30 min. The column was washed with anhydrous CH₃CN and returned to the synthesizer for capping, oxidation, and detritylation steps. The deprotection was accomplished with 30% NH₄OH for 17 h at 75 °C.

Oligodeoxynucleotide Purification and Characterization. Oligodeoxynucleotides were purified by semipreparative HPLC at 260 nm (Atlantis, Waters Corporation, C18, 5 µm, 250 mm × 10.0 mm). The column was equilibrated either with 30 mM sodium phosphate (pH 7.0) (for DDDm, DDDhm, DDDca) or 0.1 M ammonium formate (pH 6.5) (for DDDf). The gradient was 1−15% CH₃CN over 20 min, 15−80% CH₃CN over 5 min, and 1% CH₃CN over 5 min, at 4.5 mL/min. Oligodeoxynucleotides were desalted by passing over G-25 Sephadex (GE Healthcare, Little Chalfont, Buckinghamshire, UK). Oligodeoxynucleotides were characterized by MALDI-TOF mass spectrometry (calc for DDD [M − H]− m/z 3646.4, found m/z 3647.8; calc for DDDm [M − H]− m/z 3660.5, found 3663.4; calc for DDDhm [M − H]− m/z 3675.5, found 3679.7; calc for DDDca [M − H]− m/z 3674.4, found 3673.2; calc for DDDf [M − H]− m/z 3690.4, found 3693.1). Oligodeoxynucleotides were prepared in 100 mM NaCl, 50 µM Na₂EDTA, in 10 mM sodium phosphate (pH 7.0), heated at 85 °C for 15 min, and annealed by cooling to room temperature. Duplex concentrations were determined by UV absorbance, using extinction coefficients calculated at 260 nm.

Thermal Denaturation. The concentration of DNA was 1.2 µM. Measurements were conducted in 100 mM NaCl, 50 µM Na₂EDTA, in 10 mM sodium phosphate (pH 7.0). The temperature was increased from 10 to 80 °C at 1 °C/min. Tm values were calculated from first-order derivatives of 260 nm absorbance vs temperature profiles.

NMR. Spectra were obtained at 900 MHz using a 5 mm cryogenic probe (Bruker Biospin Inc., Billerica, MA). Oligodeoxynucleotides were prepared at a duplex concentration of 0.25 mM in 180 µL of 100 mM NaCl, 50 µM Na₂EDTA, 11 mM Na₂SO₄, in 10 mM sodium phosphate (pH 7.0). The solutions were exchanged with D₂O and dissolved in 180 µL of 99.996% D₂O to observe nonexchangeable protons. NOESY spectra were collected in 99.996% D₂O to observe nonexchangeable protons. NOESY spectra were collected at 500 MHz using 2k matrix. Chemical shifts were referenced to the chemical shift of water at the corresponding temperature, with respect to 4,4-dimethyl-4-silapentane-1-sulfonic acid (DSS). To observe exchangeable protons, samples were prepared in 9:1 H₂O/D₂O. For observation of imino protons, spectra were recorded at S, 15, 25, 35, 45, and 55 °C. NOESY spectra were collected at 5 °C with 70 or 250 ms mixing times and relaxation delay of 2.0 s. Water suppression was achieved by the Watergate pulse sequence. Data were processed with TOPSPIN (2.0.b.6, Bruker Biospin Inc., Billerica, MA).

Base Pair Opening. NMR data were collected at 500 MHz using a 5 mm cryogenic probe, at 15 °C. Samples were in 180 µL of 9:1 H₂O/D₂O containing 100 mM NaCl, 50 µM Na₂EDTA, 11 mM Na₂SO₄, 1 mM triethanolamine, in 10 mM sodium phosphate (pH 8.9). The presence of triethanolamine enabled the pH of the sample to be monitored during the titration, in situ, by measuring the chemical shift difference between the two methylene groups. Magnetization transfer from water to the imino protons was followed by observation of the imino proton resonances after variable mixing times. Selective spin inversion of the water protons was achieved with a 2 ms 180° sinc pulse with 1000 points. To minimize effects of radiation damping during the mixing time, a 0.1 G cm⁻¹ gradient was used. Water suppression was achieved by a binomial 1−1 echo sequence, jump and return, with flanking 1 ms smooth square shape gradients, 15 G cm⁻¹. Sixteen values of the delay ranging form 1 ms to 15 s were used. Data were processed in TOPSPIN. Ammonia, pKₐ of 9.2 at 15 °C, was the proton acceptor. Data analyses were performed using PRISM (v. 6.0b, GraphPad Software, Inc., La Jolla, CA). Exchange rates were calculated using established methods. In order to determine rates of base pair opening, exchange rates were plotted against concentrations of the active form of the ammonia base catalyst. Equilibrium constants for base pair opening were calculated by fitting exchange rate data as a function of ammonia concentration.

Crystallization and X-ray Diffraction. Crystals were grown at 18 °C over 8 to 16 days by hanging-drop vapor diffusion, using the nucleic acid mini-screen (Hampton Research, Aliso Viejo, CA). Droplets of 2 µL containing 1.2 mM duplex in precipitant solution were equilibrated against 0.75 µL of 35% MPD. The solution compositions are summarized in Table S1 in the Supporting Information. Single crystals were mounted in nylon loops and flash-frozen in liquid nitrogen.

For DDDhm, data were collected on the 19-ID beamline of the Structural Biology Center at the Advanced Photon Source (APS) of Argonne National Laboratory (ANL, Argonne, IL). The wavelength was 0.9794 Å. Initial indexing and scaling of diffraction images and further reflection merging was done using HKL3000. To ensure completeness of the data, two passes were collected. For DDDca, data were collected on the 24-IDC beamline of the Northeastern Collaborative Access Team (NE-CAT) at the APS (ANL). The wavelength was 0.9792 Å. Initial indexing and scaling of diffraction images, together with reflection merging, were done using XDS, and SCALA in the CCP4 suite as part of the RAPD data collection strategy at NE-CAT. For DDDf, data were collected on the 21-IDD beamline of the Life-Sciences Collaborative Access Team (LS-CAT) at the APS (ANL). The wavelength was 1.00 Å. Initial indexing and scaling of diffraction images, together with reflection merging were done in HKL2000. Details are shown in Table 1.

Crystal Structure Determination and Refinement. Structures were determined by molecular replacement using the DDD as the search model (PDB ID code 436D). Molecular replacement searches were completed with MOLREP in the CCP4 suite. An initial model was checked and rebuilt in COOT. The model was rebuilt and further refined using REFMAC. Final models were refined against all reflections, except for 5% randomly selected reflections used for monitoring Rfree. The refinement statistics are presented in Table 1.

Data Deposition. Complete structure factors and data coordinates were deposited in the Protein Data Bank (http://pdbe.org); PDB ID code 419V for DDDhm, 4QC7 for DDDf, and 4PWM for DDDca.
Table 1. Crystal Data, Data Collection, and Refinement Statistics for the DDD\textsuperscript{hm}, DDD\textsuperscript{f}, and DDD\textsuperscript{ca} Duplexes

| Parameter                  | DDD\textsuperscript{hm} | DDD\textsuperscript{f} | DDD\textsuperscript{ca} |
|----------------------------|--------------------------|-------------------------|--------------------------|
| Crystal Data               |                          |                         |                          |
| space group                | P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} | P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} | P2\textsubscript{1}2\textsubscript{1}2\textsubscript{1} |
| Unit Cell                  |                          |                         |                          |
| a (Å)                      | 25.61                    | 25.09                   | 24.25                    |
| b (Å)                      | 41.34                    | 41.47                   | 41.34                    |
| c (Å)                      | 64.32                    | 65.69                   | 66.41                    |
| Data Collection            |                          |                         |                          |
| resolution range (Å)       | 40–1.02                  | 35–1.90                 | 26–1.95                  |
| no. of unique reflections  | 37,637                   | 5801                    | 5113                     |
| completeness (%)           | 99.6                     | 99.3                    | 99.5                     |
| in the outer shell (%)     | 98.5                     | 100                     | 97.5                     |
| R\textsubscript{merge} \(^{a}\) | 0.044                    | 0.064                   | 0.045                    |
| in the outer shell (%)     | 0.979                    | 0.738                   | 0.619                    |
| I/σ(I)                    | 52                       | 60                      | 16                       |
| in the outer shell (%)     | 1.7                      | 3.3                     | 2.8                      |
| Structure Refinement       |                          |                         |                          |
| resolution range (Å)       | 40–1.02                  | 35–1.90                 | 26–1.95                  |
| R\textsubscript{work}      | 0.156                    | 0.226                   | 0.221                    |
| R\textsubscript{free}      | 0.178                    | 0.245                   | 0.267                    |
| RMS Deviation              |                          |                         |                          |
| bond lengths (Å)           | 0.014                    | 0.011                   | 0.009                    |
| angle distances (deg)      | 2.4                      | 1.3                     | 2.2                      |
| no. of ions                | 1 Mg\textsuperscript{2+} |                         |                          |
| no. of ligands             | 3                        |                         |                          |
| no. of water molecules     | 178                      | 17                      | 13                       |

\(^{a}\)R\textsubscript{merge} = \sum_{hkl} \sum |I_{hkl} − ⟨I⟩| / \sum_{hkl} \sum |I_{hkl}|, where I is the intensity for the hklth measurement of an equivalent reflection with indices h, k, and l.

**RESULTS**

Stabilities of the Duplexes Containing Oxidized Cytosines. The impact of placing 5hmC, 5fC, or 5caC site specifically into the 5'CG-3' sequence was investigated by incorporating each oxidized cytosine into the 5'-T\textsuperscript{X}X\textsuperscript{10}G\textsuperscript{10}-3' sequence of the DDD.\textsuperscript{38,39} The T\textsubscript{m} values of the duplexes were obtained in 100 mM NaCl at pH 7. They were compared to both the unmodified DDD and also to the DDD containing SmC in the 5'-T\textsuperscript{X}X\textsuperscript{10}G\textsuperscript{10}-3' sequence (DDD\textsuperscript{m}). The T\textsubscript{m} of the DDD duplex was 48 °C, the T\textsubscript{m} of the DDD\textsuperscript{m} duplex was 46 °C, the T\textsubscript{m} of the DDD\textsuperscript{hm} duplex was 48 °C, and the T\textsubscript{m} of the DDD\textsuperscript{ca} duplex was 46 °C. These small differences in T\textsubscript{m} suggested that the presence of SmC or of the oxidized cytosines ShmC or SfC in the 5'-T\textsuperscript{X}X\textsuperscript{10}G\textsuperscript{10}-3' sequence did not greatly affect the T\textsubscript{m} of the DDD. In contrast, the T\textsubscript{m} of the DDD\textsuperscript{ca} duplex increased to 54 °C. NMR spectra of the exchangeable guanine N1H and thymine N3H imino protons were recorded from 5–55 °C (Figure 1). The resonances were assigned using standard methods.\textsuperscript{34} For the DDD\textsuperscript{ca} duplex, the G\textsuperscript{4} N1H proton remained sharp at 55 °C, consistent with the increased T\textsubscript{m} value associated with the ScaC nucleobase in the 5'-T\textsuperscript{X}X\textsuperscript{10}G\textsuperscript{10}-3' sequence. At the neighbor A\textsuperscript{5}:T\textsuperscript{8} base pair, the T\textsuperscript{8} N3H resonance remained detectable at 55 °C, although it exhibited broadening. At the neighbor C\textsuperscript{1}:G\textsuperscript{10} base pair, the G\textsuperscript{3} N1H resonance remained detectable at 55 °C, also exhibiting broadening. The stabilizing effect extended two base pairs in each direction, also including the imino protons of base pairs G\textsuperscript{2}:C\textsuperscript{11} and A\textsuperscript{6}:T\textsuperscript{7}. In contrast, for the DDD\textsuperscript{hm} duplex at the oxidized G\textsuperscript{1}:X\textsuperscript{8} base pair, the G\textsuperscript{4} N1H resonance was severely broadened at 55 °C. Likewise, the corresponding resonance in the DDD\textsuperscript{f} duplex was severely broadened at 55 °C. At the neighboring base pair C\textsuperscript{1}:G\textsuperscript{10}, the G\textsuperscript{9} N1H resonance in the DDD\textsuperscript{f} duplex broadened at 35 °C. The T\textsuperscript{8} N3H resonances broadened at 45 °C in the DDD\textsuperscript{f} duplex and at 55 °C in the DDD\textsuperscript{ca} duplex. The temperature dependence of line widths of the imino resonances is shown in Figure S1 of the Supporting Information.

**Base Pair Opening Dynamics.** Magnetization transfer from water after variable times was followed by observation of the guanine N1H and thymine N3H resonances, at 15 °C. The imino proton exchange rates were measured in the absence and the presence of added ammonia base catalyst.\textsuperscript{45,52,57,58,61,75} The exchange with water follows a two-state model, where the base pair undergoes a conformational change from the closed to the open state, from which proton exchange occurs.\textsuperscript{57,75} The open base pair is exchange-competent because the proton is accessible to acceptors in solution. As described by Russu and co-workers,\textsuperscript{75,77} in the EX1 regime, the concentration of

![Figure 1](image-url)
acceptors is sufficient for rapid exchange from the open state \((k_{\text{ex,open}} \gg k_{\text{cl}})\), so exchange occurs at each opening event and \(k_{\text{ex}} = k_{\text{op}}\). In the EX2 regime, where the concentration of base is low \((k_{\text{ex,open}} \ll k_{\text{cl}})\), the rate of exchange from the open state is proportional to the exchange rate and the concentration of the acceptor.\(^{73-77}\)

Figure 2 shows the results for the C\(^3\):G\(^10\), G\(^4\):X\(^9\), A\(^5\):T\(^8\), and A\(^6\):T\(^7\) base pairs of the DDD\(^{hm}\), DDD\(^{f}\), and DDD\(^{ca}\) duplexes.
They were compared to both the unmodified DDD and to the DDDm duplexes. Plots of exchange rates as a function of ammonia concentration suggested that the EX1 regime was attained. Consistent with the results of Moe et al., the rates of imino proton exchange were lower for G:C base pairs C3:G10 and G4:X9 and greater for A:T base pairs A5:T8 and A6:T7. At 15 °C, the oxidized cytosines differentially altered exchange rates of the imino protons of the C3:G10, G4:X9, A5:T8, and A6:T7 base pairs. The greatest effects were observed at the neighbor A5:T8 base pair. For the DDDhm and DDDf duplexes, the exchange rate of the A5:T8 base pair imino proton increased at all concentrations of ammonia (Figure 2). There was a 3-fold increased rate of base pair opening in the DDDhm duplex and a 5-fold increased rate of base pair opening in the DDDf duplex, with respect to the DDD duplex (Table 2). In contrast, for the DDDca duplex, the exchange rate of the A5:T8 imino proton was similar to those of the DDD and DDDm duplexes at all concentrations of ammonia. These differences were reflected in measurements of the respective equilibrium constants for base pair opening. For the DDDhm duplex, the equilibrium constant for base pair opening (k_eq) at base pair A5:T8 was 7.3 × 10^6, and for the DDDf duplex, the equilibrium constant for base pair opening at A5:T8 was 1.1 × 10^9, differing from the DDD and DDDm duplexes (3.4 × 10^8 and 3.5 × 10^8, respectively). In contrast, for the DDDca duplex, the equilibrium constant for base pair opening of A5:T8 was 4.1 × 10^6, similar to that of the DDD and DDDm duplexes. The neighbor effect did not extend beyond the A5:T8 base pair. At base pair A5:T8, exchange rates as a function of ammonia concentration were comparable for all duplexes.

A smaller effect on base pair opening dynamics was observed at the G4:X9 base pair. For the DDDf duplex, the exchange rate of G4:X9 was greater at all concentrations of ammonia than that for the DDDhm, DDDca, and DDDm duplexes (k_eq = 26 s⁻¹ in DDDf vs k_eq = 16, 4, 7, and 8 s⁻¹ for DDDhm, DDDca, and DDDm, respectively). For the DDDf duplex, the equilibrium constant for base pair opening increased 3-fold, calculated as 2.8 × 10^7 vs 1.2 × 10^7, 7.5 × 10^6, 1.2 × 10^6, and 6.0 × 10^6, respectively, for the DDDf, DDDm, DDDhm, and DDDca duplexes.

In contrast, base pair opening dynamics at the neighboring C3:G10 base pair were not affected by the presence of the oxidized cytosines in the DDDhm, DDDf, or DDDca duplex. Thus, the differences in base pair opening dynamics for the ShmC, SfC, and ScaC bases in the S′-T′X′G10-3′ sequence of the DDD exhibit a pronounced sequence dependence, with the greatest effects being evident at the neighboring A5:T8 base pair. This is the base pair located in the S′-direction with respect to the oxidized cytosine X′. The overall results are summarized in Table 2.

Structures of the DDDhm, DDDf, and DDDca Duplexes. The modified DDDhm, DDDf, and DDDca duplexes yielded diffraction-quality crystals. Crystals belonged to the orthorhombic P2₁2₁2₁ space group. The crystal structures were determined using the unmodified DDD (PDB ID code 436D) as a search model for molecular replacement. Structures were refined using anisotropic B factors to a resolution of 1.02 Å for DDDhm and isotropic B factors to resolutions of 1.90 and 1.95 Å for DDDf and DDDca, respectively. Each of the structures was compared to that of the DDD. Overall, the structures were similar to the DDD as indicated by comparative rmsd analyses, with rmsd values of 0.67, 0.46, and 0.49 Å for DDDhm, DDDf, and DDDca, respectively. Classical features of the DDD, including waters forming the minor groove spine of hydration, were conserved. The data and refinement statistics are provided in Table 1.

Figure 3 shows electron density and base pairing arrangements for the ShmC:G, SfC:C, and ScaC:G base pairs in the DDDhm, DDDf, and DDDca duplexes, respectively. Watson–Crick base pairing was evident, and the hydroxymethyl, formyl, or carboxyl moieties of the oxidized cytosines were oriented into the major groove. The formyl group of SfC and the carboxyl group of ScaC were within hydrogen-bonding range of the N4 exocyclic amines of the oxidized cytosines. For the DDDhm duplex, electron density associated with the hydroxymethyl moiety of ShmC suggested partial occupancy of two conformations. The major conformation refined with occupancy 0.8, and the minor conformation refined with occupancy 0.2. In the major conformation, the hydroxyl group hydrogen bonded with the terminal N1 ammonium moiety of a spermine and with G10 O6 via an ordered water molecule (Figure S2 of the Supporting Information). In the minor conformation, the hydroxyl group was oriented toward the backbone phosphate and formed interactions with neighboring waters (Figure S3 of the Supporting Information). A hydrogen bond was also observed between the hydroxyl group at the modified cytosine X21 and an axially coordinated water (HOH 12) at a distance of 2.7 Å, with a further interaction to G22 N7 (2.8 Å). An additional hydrogen bond was observed between the X21 hydroxyl and G22 O6 via water HOH 11 (3.0 Å distance from X21 to HOH 11 and 2.7 Å from HOH 11 to G22 O6) (Figures S2 and S3 of the Supporting Information). Base stacking patterns for the DDDhm, DDDf, and DDDca duplexes were similar (Figure 4).

The DDDhm, DDDf, and DDDca duplexes were also examined by NMR, using standard methods. The sequential base aromatic → deoxyribose anomic NOEs were...
identified from C1 → G12 (Figure S4 of the Supporting Information). For the DDDhm, DDDf, and DDDca duplexes (and as well for the DDD and DDDm duplexes), the intensities of NOE cross-peaks between the purine H8 and pyrimidine H6 protons and the deoxyribose H1′ protons were of the same relative magnitudes as those between other bases in the sequence, indicating that the glycosyl bonds maintained the anti conformations. In all instances, the NOE connectivity of the purine N1H and pyrimidine N3H protons was obtained from G2:C11 → C3:G10 → G4:X9 → A5:T8 → A6:T7 (Figure 5). NOE cross-peaks from the oxidized base X9 N4H1 and N4H2 protons to the complementary base G4 N1H proton were observed, as well as interactions to neighbor bases T8 N3H and G10 N1H, consistent with Watson−Crick geometry being favored, corroborating the crystallographic data (Figure 3). Significantly, evidence for intranucleotide hydrogen bonding involving the formyl group of 5fC or the carboxyl group of 5caC and the N4 exocyclic amine of 5fC or 5caC was evident in NMR spectra of the DDDf and DDDca duplexes, for which both of the X9 N4 amino proton resonances shift downfield into the 7.8−8.8 ppm spectral range (Figure 5). The effect was most pronounced for the DDDca duplex. In contrast, for the DDD, DDDm, and DDDhm duplexes, one of the N4 amino protons shifts downfield, consistent with the maintenance of a Watson−Crick base pair, whereas the other remains in the 6.5−7.0 ppm spectral range, which is the anticipated result given that cytosine, 5mC, and 5hmC cannot form this hydrogen bond. Overall, the NMR data corroborated the crystallographic data, giving no indication of the presence of imino tautomers and suggesting that each of the oxidized cytosines participated in normal Watson−Crick base pairing when placed opposite guanine.

**DISCUSSION**

The 5hmC, 5fC, 5caC oxidation products of 5mC are intermediates in active demethylation and have potential roles in epigenetic regulation of cellular function. It has been reported that 5fC and 5caC, but not 5hmC, are substrates for thymine DNA glycosylase (TDG). Accordingly, it was of interest to determine whether these oxidized cytosines differentially alter duplex DNA and how such differences correlate with differences in excision of 5hmC, 5fC, and 5caC by TDG. The Dickerson−Drew dodecamer (DDD) provided a platform for conducting these studies. It
contains the 5'-CG-3' sequence associated with genomic cytosine methylation, and, most importantly, it is simultaneously amenable to crystallographic and spectroscopic analyses.

**Stabilization of the DDD by ScaC.** The presence of ScaC in the 5'-T'XG-3' sequence stabilizes the DDD, as evidenced by the 6–8 °C increase in $T_m$ for the DDD$^{3a}$ as compared to the $T_m$ values of the DDD and of the DDD$^{3a}$ under the same conditions. NMR data for the base paired guanine N1H and thymine N3H imino protons (Figure 1) confirm this conclusion. For the DDD$^{3a}$ duplex, at temperatures as high as 35 °C, the imino proton resonances of base pairs C$^5$-G$^{10}$, G$^3$-X$^4$, and A$^3$:T$^8$ remain detectable (Figure 1E). In contrast, for the DDD$^{3a}$ duplex and DDD$^{3b}$ duplexes, the imino proton resonances of base pairs C$^5$-G$^{10}$, G$^3$-X$^4$, and A$^3$:T$^8$ broaden at temperatures above 35 °C (Figure 1C,D). While the inclusion of ScaC into the 5'-T'XG-3' sequence in the DDD provides only a single data point for thermodynamic comparison, the observation that ScaC stabilizes the DDD$^{3a}$ is consistent with calculations performed by Sumino et al.$^{31}$ It also corroborates data obtained by the same group for the stabilities of 13-mers and 14-mers containing ScaC. This stabilization of the DDD$^{3a}$ is not attributable to improved base stacking geometry of ScaC in DDD$^{3a}$ because ScaC exhibits a base stacking geometry in the DDD that is similar to both ShmC and Sfc (Figure 4). However, electronic dipole–dipole interactions associated with ScaC$^{14,23}$ might enhance the thermodynamic stability of the DDD$^{3a}$ duplex without disturbing the stacking geometry.

**Sequence-Specific Base Pair Opening Dynamics of the Oxidized Duplexes.** The imino proton exchange rates at base pairs C$^5$:G$^{10}$, G$^3$:X$^4$, and A$^3$:T$^8$ depend upon the identity of the cytotoxic oxidant product and exhibit sequence dependence. The greatest effects are observed for the neighbor base pair A$^3$:T$^8$, with a smaller effect at G$^3$:X$^4$ and minimal effect at the neighbor base pair C$^5$:G$^{10}$. Base pair A$^3$:T$^8$ is the 5'-neighbor with respect to the oxidized cytosine at position X$^4$, whereas base pair C$^5$:G$^{10}$ is the 3'-neighbor with respect to the oxidized cytosine at position X$^4$ (Chart 1). While the A$^3$:T$^8$ base pair of the DDD intrinsically exhibits enhanced exchange kinetics,$^{45}$ the presence of either ShmC or Sfc further enhances imino proton exchange rates at A$^3$:T$^8$, whereas the presence of ScaC does not (Figure 2), an observation that is consistent with the thermal stabilization of the duplex by ScaC as opposed to Sfc or ShmC. Thus, for DDD$^{3a}$, base pair A$^3$:T$^8$ base pair opens with the frequency of $k_{op} = 222$ s$^{-1}$, five times faster than in the DDD. For the DDD$^{3a}$ duplex, base pair A$^3$:T$^8$ opens three times faster than in the DDD ($k_{op} = 110$ s$^{-1}$ vs $k_{op} = 40$ s$^{-1}$ in DDD$^{3a}$ and DDD, respectively).

**Structures of Duplexes Containing ShmC, Sfc, or ScaC.** Evidence for wobble base pairing geometry at oxidized cytosines,$^{7,19,23,36,35}$ arising from imino tautomers of ScaC or Sfc,$^{20,37}$ is not observed. The results are consistent with calculations of the stabilities of the amino and imino tautomers of Sfc and ScaC at the nucleobase level, which have suggested that, when paired with G, both Sfc and ScaC preferentially form Watson–Crick pairs. Instead, each of the ShmC, Sfc, and ScaC oxidation products favors Watson–Crick hydrogen-bonding interactions when located in the 5'-T'XG-3' sequence (Figures 3–5).

A common structural feature of Sfc and ScaC in the DDD is formation of intranucleobase hydrogen bonds between the carbonyl oxygens of the formyl or carboxyl groups, respectively, and a cytosine N$^3$H amino proton. This hydrogen bond has been observed between the exocyclic N$^4$ amino group and the formyl oxygen at CS of Sfc at the nucleoside level.$^{27,82}$ The downfield shifts of both of the X$^9$ N$^6$ amino proton resonances into the 7.8–8.8 ppm spectral range is evident in NMR spectra of the DDD$^3$ and DDD$^{3a}$ duplexes (Figure 5) and is consistent with the formation of these hydrogen bonds. The NMR data corroborates the crystallographic structure data (Figure 3), which shows that the carbonyl oxygens of the formyl or carboxyl groups of Sfc or ScaC, respectively, and a cytosine N$^3$H amino proton are within hydrogen-bonding distance. The effect in the NMR data is most pronounced for the DDD$^{3a}$ duplex (Figure 5). In the crystallographic structure of the DDD$^{3a}$ duplex (Figure 3), this hydrogen bond keeps the carboxyl group in plane with the oxidized cytosine. For the DDD$^{3b}$ and DDD$^{3m}$ duplexes, one of the N$^6$ amino protons shifts downfield, consistent with the maintenance of a Watson–Crick base pair, whereas the other remains in the 6.5–7.0 ppm spectral range, which is the anticipated result given that cytosine, 5mC, and 5hmC cannot form this hydrogen bond.}

**Structure–Activity Relationships.** DNA glycosylases typically employ an extrahelical base-flipping mechanism to position substrates for catalysis. Differences in the ability of TDG to excise Sfc, ScaC, or ShmC from DNA$^{14}$ could be mediated by differential recognition of these oxidized cytosine bases in DNA. Stivers et al.$^{61,90}$ demonstrated that damage recognition by a different glycosylase, uracil DNA glycosylase (UDG), is facilitated by enhanced base open rates for destabilized A:U base pairs.$^{35}$ The present data reveal that site- and sequence-specific differences with regard to duplex stability and base pair opening dynamics are observed when the ShmC, Sfc, and ScaC are placed into the DDD$^{3m}$, DDD$^3$, and DDD$^{3a}$ dodecamers within the 5'-T'XG-3' sequence. Neither the stabilization of the DDD by ScaC nor the differences in base pair opening dynamics correlate with differences in the excision of ShmC, Sfc, and ScaC by TDG, as reported by Maiti et al.$^{14}$ Both ShmC and Sfc exhibit increased base pair opening rates at the neighboring A$^3$:T$^8$ base pair. However, only Sfc is excised by TDG. Moreover, ScaC, which also is excised by TDG, thermally stabilizes the DDD$^{3a}$ and does not exhibit increased base pair opening kinetics at the 5'-neighbor A$^3$:T$^8$ base pair (Figure 2).

It has been proposed that the imino tautomers of ScaC or Sfc adopt wobble-like base pairing geometry with the complementary G, which might provide a basis for recognition by TDG.$^{20,37}$ The present crystallographic and NMR data indicate that the ShmC, Sfc, and ScaC bases each favor Watson–Crick base pairing in the DDD duplex. This argues against wobble base pairing involving imino tautomers of these oxidized cytosines as a primary mode of recognition by TDG. However, the presence of small amounts of the imino tautomers cannot be ruled out, nor can a shift from Watson–Crick base pairing to wobble pairing subsequent to enzyme binding. It has been proposed that the hydrogen bond between the exocyclic N$^4$ amino and the formyl or carboxyl oxygen at CS of the Sfc or the ScaC base might shift the equilibrium toward the imino tautomer$^{49,92}$ and lead to protonation at N3 of the oxidized cytosine.$^{37,96}$ Additionally, other factors such as electrostatic and steric contributions, which remain to be examined, might modulate the differential recognition of these oxidized cytosines by TDG.

Alternatively, differences in the ability of TDG to excise Sfc, ScaC, or ShmC from DNA could be controlled by differences.
in the catalytic step of base excision, once the oxidized cytosine bases have been inserted into the active site of the glycosylase. Maiti et al.\textsuperscript{97} implied a role of the conserved Asn\textsuperscript{140} in the chemical step and of the conserved Arg\textsuperscript{75} in nucleotide flipping into the active site. In additional studies, Maiti et al.\textsuperscript{13} accounted for the differential excision ability of TDG with respect to ShmC, Sfc, and ScaC by arguing that activity is greatest for oxidized cytosines possessing electron-withdrawing substituents at the C5 carbon, which stabilize developing negative charge in the transition state complex for base excision. Following their argument, Sfc is a good substrate and ShmC is not.\textsuperscript{14} At neutral pH, ScaC exists as an anion with pK\textsubscript{a} values of 2.4 for the carboxyl and 4.3 for the N3 position,\textsuperscript{75} and catalysis is facilitated because the ionized carboxyl group lowers the pK\textsubscript{a} of cytosine and stabilization of the carboxyl by the exocyclic amine of cytosine creates an electron-withdrawing effect.\textsuperscript{14,23} Maiti et al.\textsuperscript{23} demonstrated that the excision ability of TDG with respect to Sfc is pH-independent but that the excision of ScaC is acid-catalyzed. Moreover, Zhang et al.\textsuperscript{25} found that TDG binds to ScaC with greater affinity than to Sfc, U, or T and proposed that residues Asn\textsuperscript{157}, His\textsuperscript{151}, and Tyr\textsuperscript{152} are involved in hydrogen bonds with the ScaC carboxyl group. Finally, the structure of TDG in complex with DNA containing a G:ShmU mismatch showed that TDG engages in hydrogen-bonding interactions with both ShmU and ScaC.\textsuperscript{19}

The present results are consistent with the proposal by Maiti et al.,\textsuperscript{14} in which the excision specificity of TDG for Sfc and ScaC vs ShmC is dictated by differences in the enzyme—substrate complex transition state. Both Sfc and ScaC form hydrogen bonds between the carbonyl oxygens of their formyl or carboxyl groups, respectively, and a cytosine exocyclic N\textsuperscript{4}H amino proton. The electron-withdrawing effect of the Sfc and ScaC substituents\textsuperscript{14,23} should be enhanced by hydrogen bonding between the carbonyl oxygens of their formyl or carboxyl groups, respectively, and a cytosine exocyclic N\textsuperscript{4}H amino proton. This would be anticipated to stabilize developing negative charge in the transition state complex for base excision.

**Summary.** The cytosine oxidation products ShmC, Sfc, and ScaC exhibit differences in thermodynamics and base pair opening dynamics when placed into the S\textsuperscript{7}‘-T\textsuperscript{8}X\textsuperscript{9}G\textsuperscript{10}-3\textsuperscript{‘} sequence of the DDD, but these do not correlate with differences in the ability of TDG to excise these cytosine oxidation products.\textsuperscript{14} While TDG may exploit thermodynamic and base pair opening dynamics in the recognition of oxidized cytosines in DNA, differences in the transition state complexes for the base excision step may be rate-limiting with respect to the chemical step of base excision. Of course, the S\textsuperscript{7}‘-T\textsuperscript{8}X\textsuperscript{9}G\textsuperscript{10}-3\textsuperscript{‘} sequence is just one sequence, and it will be of interest to further examine the sequence dependence of these effects, particularly in light of the recent report from Raiber et al.\textsuperscript{54} showing that the presence of three Sfc sites in an iterated CG repeat sequence changes the geometry of the DNA grooves and base pairs containing the Sfc oxidation product. DNA glycosylases may exploit different mechanistic pathways toward base excision. The recognition of uracil by uracil DNA glycosylase (UDG) is reported to be facilitated by enhanced base pair opening rates at A:U base pairs.\textsuperscript{55} Interestingly, the SmC DNA glycosylase DEMETER (DME) removes SmC, ShmC, and ScaC but has no activity for Sfc.\textsuperscript{99,98} Its inactivity toward Sfc also does not seem to be correlated with Sfc base pair opening rates, and it does not seem to correlate with the electron-withdrawing effect of the Sfc and ScaC substituents.\textsuperscript{14,23}
(4) Okano, M., Bell, D. W., Haber, D. A., and Li, E. (1999) DNA methyltransferases Dnmt3a and Dnmt3b are essential for de novo methylation and mammalian development. Cell 99, 247–257.

(5) Wyatt, G. R. (1950) Occurrence of 5-methylcytosine in nucleic acids. Nature 166, 237–238.

(6) Meissner, A. (2010) Epigenetic modifications in pluripotent and differentiated cells. Nat. Biotechnol. 28, 1079–1088.

(7) Feng, S., Jacobsen, S. E., and Reik, W. (2010) Epigenetic reprogramming in plant and animal development. Science 330, 622–627.

(8) Wu, S. C., and Zhang, Y. (2010) Active DNA demethylation: many roads lead to Rome. Nat. Rev. Mol. Cell Biol. 11, 607–620.

(9) Globisch, D., Munzel, M., Muller, M., Michalakis, S., Wagner, M., Koch, S., Bruckl, T., Biel, M., and Carell, T. (2010) Tissue distribution of 5-hydroxymethylcytosine and search for active demethylation intermediates. PLoS One 5, e15367.

(10) Munzel, M., Globisch, D., and Carell, T. (2011) 5-Hydroxymethylcytosine, the sixth base of the genome. Angew. Chem., Int. Ed. 50, 6460–6468.

(11) Gu, T. P., Guo, F., Yang, H., Wu, H. P., Xu, G. F., Liu, W., Xie, Z. G., Shi, L., He, Y., Jin, S. G., Iqbal, K., Shi, Y. G., Deng, Z., Szabo, P. E., Pfeifer, G. P., Li, J., and Xu, G. L. (2011) The role of Tet3 DNA dioxygenase in epigenetic reprogramming by oocytes. Nature 477, 606–610.

(12) Iqbal, K., Jin, S. G., Pfeifer, G. P., and Szabo, P. E. (2011) Reprogramming of the paternal genome upon fertilization involves genome-wide oxidation of 5-methylcytosine. Proc. Natl. Acad. Sci. U.S.A. 108, 3642–3647.

(13) Wossidlo, M., Nakamura, T., Lepikhov, K., Marques, C. J., Zakhartchenko, V., Boiani, M., Arand, J., Nakano, T., Reik, W., and Walter, J. (2011) 5-Hydroxymethylcytosine in the mammalian zygote is linked with epigenetic reprogramming. Nat. Commun. 2, 241.

(14) Matti, A., and Drohat, A. C. (2011) Thymine DNA glycosylase can rapidly excise 5-formylcytosine and 5-carboxylcytosine: Potential implications for active demethylation of CpG sites. J. Biol. Chem. 286, 35334–35338.

(15) He, Y. F., Li, B. Z., Li, Z., Liu, P., Wang, Y., Yang, H., Ding, J., Jia, Y., Chen, Z., Li, L., Sun, Y., Li, X., Dai, Q., Song, C. X., Zhang, K., He, C., and Xu, G. L. (2011) Tet-mediated formation of 5-hydroxymethylcytosine is present in Purkinje neurons and the brain. Science 333, 1303–1307.

(16) Matti, A., and Drohat, A. C. (2011) Dependence of substrate binding and catalysis on pH, ionic strength, and temperature for thymine DNA glycosylase: insights into recognition and processing of G-T mismaps. DNA Repair 10, 545–553.

(17) Cortellino, S., Xu, J., Sannai, M., Moore, R., Caretti, E., Cigliano, A., Le Coz, M., Devarajan, K., Wessels, A., Sopranzo, D., Abramowizt, L. K., Bartolomei, M. S., Rambow, F., Bassi, M. R., Bruno, T., Fanciulli, M., Renner, C., Klein-Szanto, A. J., Matsumoto, Y., Kobi, D., Davidson, L., Alberti, C., Larue, L., and Bellacosa, A. (2011) Active DNA demethylation by thymine DNA glycosylase. Environ. Mol. Mutagen. 52, S14–S14.

(18) Cortellino, S., Xu, J. F., Sannai, M., Moore, R., Caretti, E., Cigliano, A., Le Coz, M., Devarajan, K., Wessels, A., Sopranzo, D., Abramowizt, L. K., Bartolomei, M. S., Rambow, F., Bassi, M. R., Bruno, T., Fanciulli, M., Renner, C., Klein-Szanto, A. J., Matsumoto, Y., Kobi, D., Davidson, L., Alberti, C., Larue, L., and Bellacosa, A. (2011) Active DNA demethylation by thymine DNA glycosylase. Environ. Mol. Mutagen. 52, S14–S14.

(19) Hashimoto, H., Hong, S., Bhagwat, A. S., Zhang, X., and Cheng, X. (2012) Excision of 5-hydroxymethyluracil and 5-carboxylcytosine by the thymine DNA glycosylase domain: its structural basis and implications for active DNA demethylation. Nucleic Acids Res. 40, 10203–10214.

(20) Hashimoto, H., Zhang, X., and Cheng, X. (2012) Excision of thymine and 5-hydroxymethyluracil by the MBD4 DNA glycosylase domain: structural basis and implications for active DNA demethylation. Nucleic Acids Res. 40, 8276–8284.
(39) Drew, H. R., Wing, R. M., Takano, T., Broka, C., Tanaka, S., Itakura, K., and Dickerson, R. E. (1981) Structure of a B-DNA dodecamer: conformation and dynamics. Proc. Natl. Acad. Sci. U.S.A. 78, 2179–2183.

(40) Tereshko, V., Minasov, G., and Egl, M. (1999) The Dickerson–Drew B-DNA dodecamer revisited at atomic resolution. J. Am. Chem. Soc. 121, 470–471.

(41) Howerton, S. B., Sines, C. C., VanDerveer, D., and Williams, L. D. (2001) Locating monovalent cations in the grooves of B-DNA. Biochemistry 40, 10023–10031.

(42) Kowal, E. A., Ganguly, M., Pallan, P. S., Marky, L. A., Gold, B., Egl, M., and Stone, M. P. (2011) Altering the electrostatic potential in the major groove: thermodynamic and structural characterization of 7-deaza-2′-deoxyadenosine: dT base pairing in DNA. J. Phys. Chem. B 115, 13923–13934.

(43) Kowal, E. A., Lad, R. R., Pallan, P. S., Dhummakupt, E., Wawrzak, Z., Egl, M., Sturla, S. J., and Stone, M. P. (2013) Recognition of O5′-benzyl-2′-deoxyguanosine by a perimidinone-derived synthetic nucleoside: a DNA interstrand stacking interaction. Nucleic Acids Res. 41, 7566–7576.

(44) Hare, D. R., Wenmer, D. E., Chou, S. H., Drobny, G., and Reid, B. R. (1983) Assignment of the non-exchangeable proton resonances of d(C-G-C-G-A-A-T-T-C-G-C-G) using two-dimensional nuclear magnetic resonance methods. J. Mol. Biol. 171, 319–336.

(45) Moe, J. G., and Russu, I. M. (1990) Proton exchange and base-pair opening kinetics in 5′-d(CGCGAATTCGCG)-3′ and related dodecamers. Nucleic Acids Res. 18, 821–827.

(46) Tjandra, N., Tate, S.-I., Ono, A., Kainosho, M., and Bax, A. (2000) The NMR structure of a DNA dodecamer in an aqueous dilute liquid crystalline phase. J. Am. Chem. Soc. 122, 6190–6200.

(47) Singh, S. K., Szulik, M. W., Ganguly, M., Khaitshivili, L., Stone, M. P., Marky, L. A., and Gold, B. (2011) Characterization of DNA with an 8-oxoguanine modification. Nucleic Acids Res. 39, 6789–6801.

(48) Cavalluzi, M. J., and Borer, P. N. (2004) Revised UV extinction coefficients for nucleoside-5′-monophosphates and unpaired DNA and RNA. Nucleic Acids Res. 32, e13.

(49) Marky, L. A., and Breslauer, K. J. (1987) Calculating thermodynamic data for transitions of any molecularity from equilibrium melting curves. Biopolymers 26, 1601–1620.

(50) Bodenhausen, G., Wagner, G., Rance, M., Sorensen, O. W., Wuthrich, K., and Ernst, R. R. (1984) Longitudinal two-spin order 2D exchange spectroscopy (NOESY). J. Magn. Reson. 59, 542–550.

(51) Piotto, M., Saudak, V., and Sklenar, V. (1992) Gradient-tailored excitation for single-quantum NMR spectroscopy of aqueous solutions. J. Biomol. NMR 6, 661–665.

(52) Chen, C., and Russu, I. M. (2004) Sequence-dependence of the energetics of opening of at basepairs in DNA. Biophys. J. 87, 2545–2551.

(53) Chen, C., Jiang, L., Michalczyn, R., and Russu, I. M. (2006) Structural energetics and base-pair opening dynamics in sarcin-rinic domain RNA. Biochemistry 45, 13606–13613.

(54) Huang, Y., Chen, C., and Russu, I. M. (2009) Dynamics and stability of individual base pairs in two homologous RNA–DNA hybrids. Biochemistry 48, 3988–3997.

(55) Huang, Y., Weng, X., and Russu, I. M. (2010) Structural energetics of the adenine tract from an intrinsic transcription terminator. J. Mol. Biol. 397, 677–688.

(56) Huang, Y., Weng, X., and Russu, I. M. (2011) Enhanced base-pair opening in the adenine tract of a RNA double helix. Biochemistry 50, 1857–1863.

(57) Gueron, M., and Leroy, J. L. (1995) Studies of base pair kinetics by NMR measurement of proton exchange. Methods Enzymol. 261, 383–413.

(58) Szulik, M. W., Voehler, M., and Stone, M. P. (2014) NMR analysis of base-pair opening kinetics in DNA. Curr. Protoc. Nucleic Acid Chem. 59, 7.20.1–7.20.18 DOI: 10.1002/0471142700.nc0702s09.

(59) Plateau, P., and Gueron, M. (1982) Exchangeable proton NMR without base-line distortion, using new strong-pulse sequences. J. Am. Chem. Soc. 104, 7310–7311.
(81) Sumino, M., Ohkubo, A., Taguchi, H., Seio, K., and Sekine, M. (2008) Synthesis and properties of oligodeoxynucleotides containing 5-carboxy-2′-deoxycytidines. Bioorg. Med. Chem. Lett. 18, 274–277.

(82) Burdzy, A., Noyes, K. T., Valinluck, V., and Sowers, L. C. (2002) Synthesis of stable-isotope enriched 5-methylpyrimidines and their use as probes of base reactivity in DNA. Nucleic Acids Res. 30, 4068–4074.

(83) Lau, A. Y., Scharer, O. D., Samson, L., Verdine, G. L., and Ellenberger, T. (1998) Crystal structure of a human alkylbase-DNA repair enzyme complexed to DNA: mechanisms for nucleotide flipping and base excision. Cell 95, 249–258.

(84) Parikh, S. S., Mol, C. D., Hosfield, D. J., and Tainer, J. A. (1999) Envisioning the molecular choreography of DNA base excision repair. Curr. Opin. Struct. Biol. 9, 37–47.

(85) Bruner, S. D., Norman, D. P., and Verdine, G. L. (2000) Structural basis for recognition and repair of the endogenous mutagen 8-oxoguanine in DNA. Nature 403, 859–866.

(86) Hollis, T., Ichikawa, Y., and Ellenberger, T. (2000) DNA bending and a flip-out mechanism for base excision by the helix-hairpin-helix DNA glycosylase, Escherichia coli AlkA. EMBO J. 19, 758–766.

(87) Tainer, J. A. (2001) Structural implications of BER enzymes: dragons dancing—the structural biology of DNA base excision repair. Prog. Nucleic Acid Res. Mol. Biol. 68, 299–304.

(88) Fromme, J. C., Banerjee, A., and Verdine, G. L. (2004) DNA glycosylase recognition and catalysis. Curr. Opin. Struct. Biol. 14, 43–49.

(89) Fromme, J. C., and Verdine, G. L. (2004) Base excision repair. Adv. Protein Chem. 69, 1–41.

(90) Cao, C., Jiang, Y. L., Stivers, J. T., and Song, F. (2004) Dynamic opening of DNA during the enzymatic search for a damaged base. Nat. Struct. Mol. Biol. 11, 1230–1236.

(91) Krosky, D. J., Song, F., and Stivers, J. T. (2005) The origins of high-affinity enzyme binding to an extrahelical DNA base. Biochemistry 44, 5949–5959.

(92) Parker, J. B., Bianchet, M. A., Krosky, D. J., Friedman, J. I., Amzel, L. M., and Stivers, J. T. (2007) Enzymatic capture of an extrahelical thymine in the search for uracil in DNA. Nature 449, 433–437.

(93) Friedman, J. I., and Stivers, J. T. (2010) Detection of damaged DNA bases by DNA glycosylase enzymes. Biochemistry 49, 4957–4967.

(94) Karino, N., Ueno, Y., and Matsuda, A. (2001) Synthesis and properties of oligonucleotides containing 5-formyl-2′-deoxycytidine: in vitro DNA polymerase reactions on DNA templates containing 5-formyl-2′-deoxycytidine. Nucleic Acids Res. 29, 2456–2463.

(95) Kamiya, H., Tsuchiya, H., Karino, N., Ueno, Y., Matsuda, A., and Harashima, H. (2002) Mutagenicity of 5-formylcytosine, an oxidation product of 5-methylcytosine, in DNA in mammalian cells. J. Biochem. 132, 551–555.

(96) Hashimoto, H., Liu, Y., Upadhyay, A. K., Chang, Y., Howerton, S. B., Vertino, P. M., Zhang, X., and Cheng, X. (2012) Recognition and potential mechanisms for replication and erasure of cytosine hydroxymethylation. Nucleic Acids Res. 40, 4841–4849.

(97) Maiti, A., Morgan, M. T., and Drohat, A. C. (2009) Role of two strictly conserved residues in nucleotide flipping and N-glycosyl bond cleavage by human thymine DNA glycosylase. J. Biol. Chem. 284, 36680–36688.

(98) Jang, H., Shin, H., Eichman, B. F., and Huh, J. H. (2014) Excision of 5-hydroxymethylcytosine by DEMETER family DNA glycosylases. Biochem. Biophys. Res. Commun. 446, 1067–1072.

(99) Brooks, S. C., Fischer, R. L., Huh, J. H., and Eichman, B. F. (2014) 5-Methylcytosine recognition by Arabidopsis thaliana DNA glycosylases DEMETER and DML3. Biochemistry 53, 2525–2532.