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Sequence-Dependent Conformational Heterogeneity and Proton-Transfer Reactivity of the Fluorescent Guanine Analogue 6-Methyl Isoxanthopterin (6-MI) in DNA

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Abstract

The local conformations of individual nucleic acid bases in DNA are important components in processes fundamental to gene regulation. Fluorescent nucleic acid base analogues, which can be substituted for natural bases in DNA, can serve as useful spectroscopic probes of average local base conformation and conformational heterogeneity. Here we report excitation–emission peak shift (EES) measurements of the fluorescent guanine (G) analogue 6-methyl isoxanthopterin (6-MI), both as a ribonucleotide monophosphate (NMP) in solution and as a site-specific substituent for G in various DNA constructs. Changes in the peak positions of the fluorescence spectra as a function of excitation energy indicate that distinct subpopulations of conformational states exist in these samples on time scales longer than the fluorescence lifetime. Our pH-dependent measurements of the 6-MI NMP in solution show that these states can be identified as protonated and deprotonated forms of the 6-MI fluorescent probe. We implement a simple two-state model, which includes four vibrationally coupled electronic levels to estimate the free energy change, the free energy of activation, and the equilibrium constant for the proton transfer reaction. These parameters vary in single-stranded and duplex DNA constructs, and also depend on the sequence context of flanking bases. Our results suggest that proton transfer in 6-MI-substituted DNA constructs is coupled to conformational heterogeneity of the probe base, and can be interpreted to suggest that Watson–Crick base pairing between 6-MI and its complementary cytosine in duplex DNA involves a “low-barrier-hydrogenbond”. These findings may be important in using the 6-MI probe to understand local base conformational fluctuations, which likely play a central role in protein–DNA and ligand–DNA interactions.

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ASSOCIATED CONTENT

Supporting Information

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Derivation of the theory presented here, EES plots for all compounds and parameters for fitting EES plots, and CD spectra of 6-MI dimer in ss- and dsDNA (PDF)

The authors declare no competing financial interest.

Graphical Abstract

INTRODUCTION

A number of recent biophysical studies of DNA replication and transcription have relied on spectroscopic measurements that sensitively probe the local conformations and dynamics of site-specific positions within protein–nucleic acid complexes. Some of these experiments use fluorescent chromophores that are incorporated into the nucleic acid backbone, or that are attached to a base using a flexible linker.^{1–3} Another important class of fluorescently labeled nucleic acid constructs involves the site-specific substitution of one (or two) native base(s) within the nucleotide sequence by a fluorescent base “analogue”.^{4–9} Such base-substituted DNA constructs are useful probes of local conformation, since the chromophores themselves can experience Watson–Crick base-pairing and base-stacking interactions similar to those of native bases. Moreover, the base analogues absorb light at significantly lower energies than do the canonical bases and proteins, so that they can be selectively excited and detected within large macromolecular complexes.^{10–12}

Previous experiments performed on fluorescent-base-analogue-substituted DNA constructs have used UV peak absorbance, fluorescence, and circular dichroism (CD) to study local base conformation. However, little attention has been devoted to understanding the presence of inhomogeneous broadening of the absorption line-shape and its relationship to local base conformational heterogeneity. When a base analogue chromophore is dissolved in a low-viscosity polarizable liquid such as water, changes to its charge distribution upon electronic excitation induce rapid structural reorganization of the local solvent. These excited-state relaxations occur on picosecond time scales, which lead to emission from the lowest energy

level of the excited electronic state.¹³ For a fluorescent base analogue that is substituted within a DNA strand, flanking bases can shield the chromophore from the aqueous solvent, so that local structural rearrangements might occur very slowly, possibly exceeding the nanosecond time scale of fluorescence. It is also possible that the fluorescent base analogue substituted within a DNA construct can experience multiple local base conformations that are separated by relatively high activation barriers. If such conformational substates absorb at different energies, the resulting absorption spectrum is a weighted sum of substate contributions—a situation that leads to inhomogeneous broadening of the line shape.¹⁴ Because the intrinsic lineshapes of individual substates are themselves broad, the cumulative absorption spectrum is often featureless and does not directly reveal the presence of multiple distinct species.

Information about the dispersion of local chromophore conformations and their interconversions can be determined using “site-selective” spectroscopic methods.^{13–16} For example, when a spectrally narrow optical source is used to excite the system, the peak fluorescence energy can vary as the excitation energy is scanned across the absorption line.¹⁴ It is sometimes found that the narrow distribution of substates selected using either red-edge or blue-edge excitation do not relax to a single globally minimized excited electronic state on the time scale of fluorescence. In such systems, a photoselected substate undergoes relaxation to its *locally* minimized conformation, which has its own characteristic emission energy. Thus, the presence of multiple substates comprising a heterogeneous mixture can affect the peak position of the fluorescence, depending on whether the system is excited on the blue-edge or the red-edge of the absorption line.¹⁴

The heterogeneity of chromophores in solution has been studied in high-viscosity solvents using various site-selective spectroscopies.^{14,17–19} Such heterogeneity is most readily observed in polar solvents at low temperatures, for which the electronic transition dipole moment of the chromophore is strongly coupled to the slowly rearranging solvent shell. At elevated temperatures structural relaxations occur faster than the fluorescence lifetime, so that the system appears homogeneous on this time scale. When the environment relaxation is slower than fluorescence, site-selective measurements can provide information about the presence of different probe microenvironments and their dynamical behavior. In this way, site-selective experiments have provided rough estimates of the mobility of chromophores in membranes^{20,21} and in proteins.^{21,22}

In this work, we apply steady-state excitation–emission spectroscopy to study conformational heterogeneity of the fluorescent guanine (G) analogue 6-methyl isoxanthopterin (6-MI), which is site-specifically substituted for G in various DNA constructs. 6-MI was introduced as a fluorescent probe to study the local environments of G residues in DNA^{7,23–25} (see Figure 1A). The 6-MI:C base pair (bp) maintains the three Watson–Crick hydrogen bonds with C that are characteristic of G:C base pairs in duplex B-form DNA (dsDNA). The imino hydrogen at the N3 position of the 6-MI free base (6,8-dimethylisoxanthopterin) is reported to be slightly acidic, with $pK_a = 8.4$.²⁶ The behavior of 6-MI in DNA is similar to that of G with respect to its effect on the thermal stability of dsDNA, ligand access to the major groove, and template-dependent nucleotide addition by

DNA polymerase (although subsequent elongation *beyond* the newly formed bp 6-MI:C is inhibited).⁵

The lowest lying electronic transition ($S_1 \leftarrow S_0$) of the 6-MI chromophore in aqueous buffer is centered at ~340 nm, and its peak emission lies close to 425 nm.²⁷ The S_1 excited state is characterized by a 6 ns lifetime and a fluorescence quantum yield of ~0.7. Two faster population relaxation processes (~3 ns, ~1 ns) appear when 6-MI is substituted for G within single-stranded (ss) or double-stranded (ds) DNA.²³ The fluorescence intensity of 6-MI becomes progressively quenched in ss- and dsDNA constructs, which is thought to reflect effects of base stacking.^{5,23}

Here we examine the conformational heterogeneity of 6-MI in various sequence contexts of duplex and ssDNA constructs. Accessibility of the weakly acidic N3 hydrogen to potential hydrogen bonding acceptors depends on the local 6-MI environment, thus providing a spectroscopic signature to probe local base conformational heterogeneity. We find that conformational heterogeneities of 6-MI that are stable on time scales exceeding the fluorescence lifetime (nanoseconds or greater) can be observed in substituted DNA constructs in aqueous solution at room temperature. This local conformational heterogeneity of the 6-MI probe base depends significantly on DNA sequence context and strandedness.

MATERIALS AND METHODS

6-MI-substituted DNA constructs were purchased from Integrated DNA Technologies (Coralville, IA) and from Fidelity Systems (Gaithersburg, MD). 6-MI ribonucleotide monophosphate (NMP) was obtained from Fidelity Systems, and used without further purification. The sequences and nomenclatures of the DNA constructs are shown in Figure 1B. Oligonucleotide concentrations were determined using extinction coefficients provided by the manufacturer. Duplex DNA constructs were annealed by combining equimolar solutions of complementary strands, followed by heating the mixture to 90 °C and allowing it to gradually cool. Solutions were buffered using the following conditions: pH 7.6 (50 mM Tris, 150 mM NaCl); pH 6.2 (50 mM phosphate buffer, 150 mM NaCl). For titrations of 6-MI NMP, samples were prepared in 10 mM sodium pyrophosphate, pH 6–10.3, buffer conditions previously used to determine the pK_a of 6,8-dimethylisoxanthopterin.²⁶ Unless otherwise stated, experiments were carried out at 20 °C.

Fluorescence spectra and steady-state fluorescence anisotropy were measured using a Jobin–Yvon FluoroMax-3 spectrophotometer. The samples were excited over the wavelength range 300–400 nm, and the emission spectra were measured over the 300–375 nm wavelength range, or in some cases over the 380–500 nm wavelength range. All spectra were corrected for background contributions from the buffer solution itself. The peak emission wavelengths were determined by fitting the emission spectra to a polynomial or a Gaussian function. The error associated with the peak emission wavelength determined by repetition of experiments was ± 1 nm ($\sim \pm 100$ – 200 cm⁻¹).

CD spectra of 4 μ M oligonucleotide solutions were measured using a Jasco model J-720 CD spectrophotometer with a temperature-controlled cell holder, as described previously.⁵ The

CD spectra were measured over the 300–450 nm wavelength range. Each measurement was tested for reproducibility by repeating 8–20 times, and the resulting data were averaged together. CD spectra are defined in terms of the difference in extinction of the sample excited using left- and right-circularly polarized light: $\epsilon_L - \epsilon_R$ (in $M^{-1} \text{ cm}^{-1}$) per mole of 6-MI residue.

For DNA melting experiments, the UV absorbance was monitored at 260 nm using a Cary UV spectrophotometer equipped with a Peltier temperature controller. Samples were heated from 20–85 °C at a rate of 1 °C min^{-1} , and the absorbance was measured at 1 °C intervals.

RESULTS

Excitation–Emission Shift (EES)

We performed excitation–emission studies with the 6-MI substituted oligonucleotide constructs with sequences shown in Figure 1B. In some cases, the peak emission wavelength was relatively insensitive to the excitation energy. For example, when we excited the ssGXG sample over the 320–375 nm (26 666–31 250 cm^{-1}) wavelength range, the peak emission occurred at 425 nm (23 529 cm^{-1}), irrespective of the excitation wavelength (Figure 2A). We define the excitation–emission shift (EES) as the difference between the energy of the exciting optical source and that of the peak emission, and we plot this function versus excitation energy in Figure 2A (black filled circles). For this sample, the EES appeared to be a linear function of the excitation energy, with slope equal to approximately unity (0.97) over nearly the full range of excitation energies investigated. Deviation of the EES from linearity occurred only at the extreme red-edge of the absorption band.

In other cases, the plot of EES versus excitation energy exhibited a pronounced demarcation of behaviors. We illustrate this using the dsGXG construct (see Figure 2B). At excitation energies much greater than that of the peak absorbance ($\sim 350 \text{ nm}$, 28 571 cm^{-1}), the EES scaled linearly, similar to the behavior we observed for the ssGXG sample. However, as the excitation energy approached the absorption maximum, the emission peak began to shift so that the EES became a weaker function of excitation energy. Thus, for the dsGXG construct, it was possible to identify two distinct regimes of behavior. At high excitation energy the EES scaled linearly, while at intermediate energies a transition to nonlinear scaling was observed. These results suggest the presence of more than one conformational substate, each with distinct electronic properties that persist on time scales longer than the fluorescence lifetime.

Theoretical Considerations of the EES

To describe the relationship between the EES of 6-MI substituted DNA constructs and the presence of multiple conformational substates, we applied standard principles of molecular photochemistry.^{13,28} The mathematical details of the model are presented in the Supporting Information (SI).

We envision a multidimensional free energy hypersurface to characterize the ground and excited electronic states, which are coupled to nuclear motions (see Figure 3). It is useful to think about a one-dimensional projection of this surface onto a single generalized nuclear

coordinate Q , which serves as a reaction coordinate for state-to-state interconversion. The shape of the ground electronic surface, including the position of its minimum (defined at $Q = 0$), is determined by the equilibrium distribution of local environment configurations. It follows from thermodynamic principles that these surfaces can be well-approximated as parabolic functions near their minima.¹³ Upon optical excitation, the induced polarization leads to relaxation of the local environment, which occurs much faster than the fluorescence lifetime. The equilibrium distribution of local configurations in the excited state is different from that in the ground state, so that the minimum of the excited electronic surface is displaced at $Q = d_1$. The extent to which the excited state is stabilized by environment relaxation is characterized by the reorganization free energy λ_1 , which is the average optical energy that is absorbed and converted into heat before emission of fluorescence. The Stokes shift is the energy difference between the peaks of the absorption and fluorescence spectra. It can be understood from simple geometrical considerations that the Stokes shift is equal to twice the reorganization free energy $2\lambda_1$ (see SI).

For the ssGXG sample discussed above (see Figure 2A), local conformations experienced by the 6-MI probe must rapidly interconvert, so that the ground and excited free energy surfaces may be described as single parabolic wells (Figure 3A). This model is representative of an optically homogeneous system, for which the energy gap between ground and excited electronic surfaces is parametrized by the coordinate Q . The peak emission energy $E_{\text{em}}^{\text{max}}$ is the energy gap at $Q = d_1$, which for a homogeneous system is independent of excitation energy.

We define the EES as the difference between the excitation and peak emission energies, $Y \equiv E_{\text{ex}}(Q) - E_{\text{em}}^{\text{max}}$. The energy of the peak absorbance of the 6-MI-substituted DNA construct can vary depending on base sequence context and secondary structure.⁵ We therefore define the rescaled excitation energy, $X \equiv E_{\text{ex}}(Q) - E_{\text{ex}}(0)$, where $E_{\text{ex}}(0)$ is the peak absorption energy. It is straightforward to show that the EES plot (i.e., a graph of Y versus X) for an optically homogeneous system is a line with slope equal to unity, and y -intercept equal to the Stokes shift $2\lambda_1$ (see SI and Figure 3B).

Our EES experiments performed on dsGXG indicate that this system is optically heterogeneous (see Figure 2B). Molecules excited at the red-edge of the absorption band represent energetically selectable substates, which do not readily interconvert with substates that are excited using higher energy light. The observed shift in peak emission energy with changing excitation is due to the presence of distinct conformations or chemical species and local environments of the 6-MI probe, each with spectroscopically resolvable optical transition energies.

To explain these observations, we consider a two-state model, which captures the essential elements of the problem (see Figure 3C).²⁹ We assume that the 6-MI probe may adopt one of two possible conformations (labeled 1 and 2) in duplex DNA, each with ground and excited electronic free energy surfaces that depend on the generalized coordinate Q . As we shall see, these two conformations may be assigned to protonated and deprotonated forms of 6-MI. The ground-state surface on the left (shown in dark blue) represents substate 1 at $Q = 0$, while the surface on the right (shown in light blue) represents substate 2 at $Q = x$. Optical

excitation of the system on the blue-edge of the absorption band favors substate 1, while excitation on the red-edge favors substate 2. The free energy of activation, $G^\ddagger$, between the two substates occurs at the coordinate $Q = d_c$, where the ground-state surfaces cross. The effect of tuning the excitation energy across the absorption band is to adjust the weights of substates 1 and 2 that are electronically excited, and thus to tune the peak energy of the fluorescence. This model can explain the observed fluorescence peak shift as a function of excitation energy.

We simplify the model, and thus reduce the number of descriptive parameters, by making the approximation that each substate is characterized by the same reorganization free energy ($\lambda_1 = \lambda_2 = \lambda$), and by the same excited electronic state displacement ($d_1 = d_2 = d$). The EES can then be described by the equation

$$Y = X + 2\lambda \left[1 + P_2 \left(\frac{x}{d} \right) \right] \quad (1)$$

where we have used the same definitions for X and Y as above for the homogeneous one-state system (see SI for derivation). In eq 1, $P_2 = 1 - P_1$ is the Boltzmann population associated with substates 2. In the limit that only substate 1 is thermally populated ($P_2 = 0$), we see that eq 1 recovers the behavior of the homogeneous system. In the opposite limit ($P_2 = 1$), eq 1 reduces to a line with slope equal to unity and y -intercept $2\lambda(1 + x/d)$. These two limiting behaviors are depicted in Figure 3D as vertically displaced dashed gray lines. When both substates are thermally populated, there exists a narrow range of excitation energies over which the EES makes a transition from one linear regime to the other, as shown in Figure 3D. An inflection point occurs at the transition, which is specified by $Q = d_c$, and for which each substate is equally populated ($P_1 = P_2 = 1/2$). The coordinates of the inflection point are given by $X_c = -2(\lambda/d)d_c$ and $Y_c = X_c + \lambda[2 + (x/d)]$. The free energy of transition and the free energy of activation are given by the Marcus formulas^{13,28}

$$\Delta G^\circ = G_{g2} - G_{g1} = -\frac{1}{2}\alpha x^2 + \alpha x d_c \quad (2)$$

and

$$\Delta G^\ddagger = \frac{1}{2}\alpha d_c^2 \quad (3)$$

where $\alpha = 2\lambda/d^2$ is a “polarizability” that characterizes the curvature of the free energy surfaces. According to eq 2, the free energy of reaction increases linearly with respect to d_c . Thus, an equilibrium system that favors substate 1 ($G^\circ > 0$) will tend to exhibit an EES transition on the low-energy side (i.e., the red-edge) of the absorption band, while a system that favors substate 2 ($G^\circ < 0$) will tend to exhibit a transition on the blue-edge of the absorption band. An isoenergetic system ($G^\circ = 0$) will exhibit a transition on the red-edge of the absorption peak, at the coordinates $X_c = -(\lambda/d)x$ and $Y_c = 2\lambda$.

A quantitative fit of EES data to eq 1, or to more general versions of the model (e.g., with λ_1 , λ_2 and/or $d_1 = d_2$, see SI), can be used to obtain optimized values for the parameters d_c , x , $d_{1,2}$, $\lambda_{1,2}$, and, through eqs 2 and 3, values for G° and $G^\ddagger$. In the remainder of this work, we have specialized to the case $d_1 = d_2 = 1$, and specific values of the nuclear coordinate Q (i.e., d_c , and x) are determined relative to the excited-state displacement. As mentioned previously, this simplification serves to reduce the number of parameters needed to faithfully represent our experimental data, while retaining the essential elements of the two-state model.

EES of 6-MI Ribonucleotide Monophosphate (NMP)

As mentioned previously, the 6-MI chromophore is also a weak acid with ionizable hydrogen at the N3 (Watson–Crick) position (see Figure 1A), which is characterized by a pK_a value that is considerably lower than that of the equivalent position (N1, see Figure 1A) in guanine, and thus falls into the physiologically relevant pH range.²⁶ 6-MI NMP is soluble in aqueous solution, and the equilibrium concentrations of protonated and deprotonated forms depend on pH. To test whether the optical heterogeneity we observed in 6-MI substituted DNA systems is related to the acid–base properties of the 6-MI probe, we performed pH-dependent EES experiments on aqueous solutions of 6-MI NMP.

In Figure 4A, we present examples of our pH-dependent EES measurements at pH = 7, 8.5, and 9.5. These data were fit to eq 1, and the model curves are shown superimposed on the experimental data points. Details of the fitting procedure are provided in the SI. We see that, by increasing the pH through the vicinity of the pK_a (=8.4), the inflection points of the experimental EES curves move systematically toward increasing excitation energy, as predicted by the two-state model described above. We obtained excellent agreement between eq 1 and the experimental EES data over the full range of pH values we investigated (5–10.5). In Figure S1, we show additional EES data and model fits for 6-MI NMP taken at other pH values. We obtained identical results from these experiments using pyrophosphate buffer, and independently using 50 mM Tris, 150 mM NaCl. In Table S1, we list the optimized parameters that we determined from these experiments.

From the above analyses, we determined the reorganization free energy ($\lambda = 2850 \pm 40$ cm⁻¹) and the displacement of the ground electronic surface of the deprotonated state relative to the protonated one ($x/d = 0.21$). We found that the optimized values we obtained for λ and x/d were nearly the same for all of the pH conditions that we examined, while the optimized values for G° and $G^\ddagger$ varied systematically. We compared these values to those predicted by the principles of acid–base equilibria. For the acid–base reaction $HA \rightleftharpoons H^+ + A^-$, we may write the equilibrium condition $[A^-]/[HA] = 10^{(pH-pK_a)}$. This ratio is equal to the equilibrium constant K_{eq} for the reaction 6-MI (protonated) $\rightleftharpoons$ 6-MI⁻ (deprotonated). Thus, for a particular value of pK_a , we determined theoretical values for $G^\circ = -RT \ln K_{eq}$. In Figure 4B, we plot the experimental values we obtained for G° as a function of pH. The red line is the best fit of the function $G^\circ = 2.3RT(pK_a - pH)$ to these data, which yields the value $pK_a = 8.7 \pm 0.3$. We note that theory and experiment agree very well over the pH range 7–10.

The pK_a value we determined for 6-MI NMP ($= 8.7 \pm 0.3$) is higher than that previously reported for the 6-MI base ($= 8.4 \pm 0.1$, in the form of 6,8DMI, 6,8-dimethylisoxanthopterin).²⁶ The larger pK_a value of the 6-MI NMP may be due to the presence of the phosphate group, which can stabilize the uncharged protonated form. Furthermore, the phosphate group of 6-MI NMP is itself ionizable with $pK_a = 6-7$.³⁰ The presence of this second ionizable site in 6-MI NMP is likely the reason for the departure of the experimentally observed values of G° from theory at low pH (< 7 , see Figure 4B). This is because protonation of the phosphate group at low pH decreases its negative charge, which in turn stabilizes the deprotonated state of the 6-MI N3 site leading to a decrease in the observed value of G° . Our results suggest that the acid-base chemistry of 6-MI NMP in aqueous solution is responsible for the spectral heterogeneity apparent in the EES data for these samples.

EES of 6-MI Substituted DNA Constructs

In Figure 5, we present experimental EES data from single-stranded (Figure 5A) and duplex (Figure 5B) DNA samples containing the GXG sequence. Superimposed on these are theoretical curves derived from our analysis using the heterogeneous two-state model (see previous and SI). The resulting values of G° and $G^\ddagger$ for the proton-transfer reaction show that the anionic form of 6-MI is more stable in dsGXG ($K_{eq} = [6\text{-MI}^- \text{ (deprotonated)}]/[6\text{-MI (protonated)}] = 0.3 \pm 0.11$) than in ssGXG ($K_{eq} = 0.03 \pm 0.01$). We found that EES data for ssDNA samples were insensitive to temperature variation, which is very similar behavior to that we found for the 6-MI NMP samples. In Figure 5C, we show as an example EES data sets for the ssGXG sample taken over the 20–80 °C temperature range. On the other hand, our analyses of EES data for the duplex DNA samples did exhibit temperature-dependent changes in the values of K_{eq} . In Figure 5D, we plot the values of K_{eq} for the dsGXG sample as a function of temperature, which decrease with increasing temperature. Upon complete thermal denaturation of the DNA duplex, the value of K_{eq} for the dsGXG sample reaches that obtained for ssGXG.

The dsGXG sample undergoes cooperative thermal melting with midpoint transition temperature $T_m = 74$ °C. Because the value of K_{eq} also appears to undergo a cooperative change over this temperature range, we conclude that the enhanced stabilization of the deprotonated state we observed in room temperature duplex DNA is a consequence of secondary structure. We note that the ssGXG sample also unstacks over this temperature range, albeit noncooperatively and to a lesser extent than does dsDNA. If base stacking contributed to the temperature effects observed for the dsDNA samples, we might expect to observe a similar decreasing trend in the values of K_{eq} for the ssGXG sample over this temperature range. On the contrary, the value of K_{eq} for this sample appears to increase only very slightly over this range, and its behavior is indistinguishable from that of the 6-MI NMP.

To better understand the proton-transfer reactivity of 6-MI-substituted DNA constructs, we examined the behavior of the 6-MI probe in various sequence contexts (see Figure 1B). In Figure S3, we show comparisons between experimental data for all DNA constructs and model curves, which are based on our two-state analysis.

For the case of ssDNA constructs, we obtained best fits to our data when we set $\lambda_1 = \lambda_2$, and when the value of x was nearly the same for all samples (see Table S2). We thus found that the optimization conditions required to model 6-MI substituted ssDNA constructs were very similar to those for the 6-MI NMP samples previously discussed, although the displacement of the ground-state surface of the deprotonated state for ssDNA constructs ($x \simeq 0.31$) was somewhat larger than for 6-MI NMP ($x \simeq 0.21$). This result indicates that 6-MI in ssDNA requires a greater displacement of its proton along the reaction coordinate to achieve its deprotonated form, in comparison to 6-MI NMP in aqueous solution. The excellent agreement between theory and experiment for all of the ssDNA constructs indicates that the optical heterogeneity we observed in these systems can be explained using the two-state model. In contrast, in order to obtain agreement between theory and experiment for dsDNA, it was necessary to allow λ_1 and λ_2 to vary independently, and for the value of x to vary within the range 0.2–0.4 (see Table S3). The results of this analysis yielded values for G° , $G^\ddagger$, and K_{eq} characteristic of the equilibrium between protonated and deprotonated states of 6-MI substituted DNA with different sequence contexts. These values are compared graphically in Figure 6, and are reported in Tables S2 and S3.

To further test whether the effects we observed are specific to the 6-MI probe, we examined the EES for single-stranded and duplex DNA constructs that contained the fluorescent cytosine analogue pyrolocytosine (PC), and the fluorescent adenine analogue 2-aminopurine (2-AP). In Figure S2, we present EES data for single-stranded and dsDNA constructs containing the sequences C(PC)A or G(2-AP)G (see Figure 1B for complete sequences). In each case, the EES data appear to behave as an optically homogeneous one-state system. The $\text{p}K_{\text{a}}$ values of PC and 2-AP are less than 4,^{6,31} so that both of these analogues are expected to exist entirely in their deprotonated forms near neutral pH. Thus, our observations support the conjecture that a proton-transfer reaction plays a role in the optical heterogeneity we have observed in 6-MI-substituted DNA constructs.

We note that the local environment experienced by 6-MI in DNA is likely very different from that of 6-MI NMP in aqueous solution. The effects of stacking with neighboring bases in both single-stranded and duplex DNA constructs, in addition to the further screening from the solvent due to Watson–Crick base-pairing complementarity in the duplex constructs, are expected to limit accessibility of the aqueous solvent to the 6-MI probe. To test this conjecture, we performed EES experiments on 6-MI substituted single-stranded and duplex DNA at two different pH values (6 and 7.6). We found for nearly all of the DNA constructs we investigated, our results are experimentally indistinguishable at the two different pH values (see Figure 6, and Figures S2 and S3). In contrast, this same variation in pH shifts the equilibrium concentrations of 6-MI NMP between its protonated and deprotonated forms by nearly an order of magnitude (Figure 6 and Figure S1). This apparent insensitivity to pH of the EES data of 6-MI-substituted ss- and dsDNA constructs could be interpreted in multiple ways. For the case of duplex DNA constructs, variations in pH over this narrow range may not influence the local environment of the 6-MI probe since the cooperatively stabilized structure of the dsDNA in which the 6-MI probe is contained is also insensitive to pH over this range.³² On the other hand, it is possible that these results indicate that the participation of solvent water does not dominate the proton-transfer reaction for either ss or duplex DNA, which instead depends on the local proximity of adjacent or opposing DNA bases.

A notable exception to the general behavior described above occurs for the ssGXXG construct (Figure 6E). In this case, the change in pH shifts the equilibrium concentrations between protonated and deprotonated forms by a factor of 2.6. It is possible that the ssGXXG sequence somewhat destabilizes the local ssDNA stacking, thus increasing the exposure of the 6-MI dimer probe to the solvent environment, although much less so than 6-MI NMP in aqueous solution. To obtain some information about local base conformation within the single-stranded and duplex GXXG and TXXT constructs, we performed circular dichroism (CD) experiments at pH = 7.6 (see Figure S5). CD spectra for these constructs are sensitive to the exciton interactions between nearest neighbor 6-MI probes, which depend on the degree of base-stacking interactions. The CD spectra of the ssGXXG and ssTXXT constructs are nearly identical, and the signs and magnitudes of the CD indicate that the two 6-MI residues are stacked in a right-handed B-form conformation (Figure S5). We speculate that some previously unconsidered interaction between the two 6-MI probes (e.g., a minor pH-dependent conformation change) may render the ssGXXG sequence sensitive to pH.

Our findings allow us to make some general statements pertaining to the local environment experienced by the 6-MI probe in DNA. In 6-MI-substituted ssDNA constructs, the protonated form of the 6-MI probe can be stabilized relative to that of the 6-MI NMP in solution (see Figure 6E). The effect appears to be most pronounced when 6-MI is positioned adjacent to a T, and much less so when 6-MI is next to a G. Exceptions are the sequences GXXG (discussed above), TXG and TXTXG. The latter represent combinations of 6-MI next to flanking T and G, which may lead to an overall cancellation of effects. When 6-MI is positioned adjacent to a G (or to another 6-MI), the effect of hybridization in duplex DNA is to stabilize the deprotonated form of the probe relative to the equilibrium in its corresponding single-stranded construct (and in the NMP). This enhanced stability of the deprotonated 6-MI in dsDNA is absent for TXT and TXTXG sequences with probe residue flanked on both sides by a T (see Figure 6F). Sequences containing G or (6-MI):C base pairs are expected to be more stable than sequences containing A:T base pairs. These results indicate that $K_{eq} = [6\text{-MI}^- \text{ (deprotonated)}]/[6\text{-MI (protonated)}]$ increases in stable duplex structures.

DISCUSSION AND CONCLUSIONS

We have studied the optical heterogeneity of the fluorescent guanine analogue 6-MI, which we have shown to be sensitive to pH changes near neutrality when it is dissolved in aqueous solution, or to its local macromolecular environment when it is substituted for G in single-stranded and duplex DNA. We have quantified the degree of optical heterogeneity of 6-MI using steady-state excitation–emission shift (EES) measurements, in which the difference between the peak emission and excitation energies is recorded as a function of excitation energy. We demonstrated that the EES for 6-MI NMP in solution, and 6-MI-substituted DNA constructs, are well-described using a heterogeneous two-state model. On the basis of this approach, we determined the values of G° and $G^\ddagger$, the standard free energies of reaction and activation, to characterize the equilibrium distribution of the two substates. This approach can potentially be generalized to provide information about fluorescent probes of local macromolecular structure other than 6-MI, which may exist in multiple substates that are stable on the time scale of the fluorescence lifetime.

By performing EES experiments on 6-MI NMP in aqueous solution, we determined that the distribution of the two substates is pH-dependent. These data can be understood in terms of simple acid–base chemistry, in which the first substate was identified as the protonated species, and the second substate as the deprotonated species. As expected for two-state behavior, EES data obtained from the 6-MI NMP system could be self-consistently modeled for all values of pH using the same value of the parameter x , which represents the relative displacement of the protonated and deprotonated ground-state free energy surfaces. We obtained excellent agreement between theory and experiment under the constraint $\lambda_1 = \lambda_2$. We observed similar behavior for 6-MI-substituted ssDNA constructs (see below). We calculated the equilibrium constant $K_{eq} = [6\text{-MI}^- \text{ (deprotonated)}]/[6\text{-MI} \text{ (protonated)}]$ from the value of σ^2 , which we determined by fitting the EES data to the two-state model. The resulting value for the pK_a of 6-MI (8.7 ± 0.3 , see Figure 4B) agrees very well with that previously reported in the literature, where it was established that the ionizable proton resides at the N3 position of 6-MI²⁶ (see Figure 1). We note that the pK_a values determined from fluorescence measurements are the same as those determined by UV absorbance,²⁶ suggesting that the pH-dependent optical heterogeneity of 6-MI NMP reflects a ground-state equilibrium phenomenon.

Our EES experiments on 6-MI-substituted DNA also revealed the presence of optical heterogeneity in these systems. Here too, the heterogeneity could be understood using the same two-state model as was used to explain the 6-MI NMP samples. We interpret these observations to mean that the optical heterogeneity of 6-MI in DNA is due to an equilibrium distribution between protonated and deprotonated species. This assumption is supported by control experiments on single-stranded and duplex DNA constructs with nucleic acid base substitutions using the fluorescent probes pyrrolocytosine (PC) and 2-aminopurine (2-AP). Neither PC or 2-AP contain an ionizable proton, and we found that single-stranded and duplex DNA constructs containing these probes as substituents behave as homogeneous one-state systems, as expected (see Figure S2). The above findings lead us to conclude that the value for the equilibrium constant K_{eq} , which we determined from our two-state analysis of EES data, is a measure of the lability of the N3 proton of 6-MI. Our approach can thus be used to quantify the “acidity” of 6-MI within different macromolecular environments, such as DNA.

The existence of the deprotonated form of 6-MI was detected in all of the DNA constructs we investigated (see Figure 6 and Figure S3 and Tables S2 and S3). The value of the equilibrium constant of 6-MI substituted ssDNA constructs was generally less than or equal to that of the free probe in aqueous solvent at physiological pH, and varied between $K_{eq} = 0.01$ and 0.06 , depending on the probe sequence context. An exception was the sequence ssGXXG, which has two guanine bases flanking the 6-MI probes. Our EES data for ssDNA constructs were relatively insensitive to variation in pH ($=6, 7.6$, see Figure S4). In contrast, the value of K_{eq} for 6-MI NMP in aqueous solution was sensitive to pH ($=0.06 \pm 0.02$ at pH 7.6, $=0.02 \pm 0.01$ at pH 6, see Figure 5 and Figure S4 and Tables S1 and S2). Our results suggest that while the acid–base equilibrium of 6-MI NMP depends strongly on solution pH, a 6-MI probe residue embedded in ssDNA experiences a local environment that stabilizes the protonated form, and is at least partially shielded from the aqueous solvent.

We next consider how the secondary structure of ssDNA might perturb the acidity of the 6-MI N3 hydrogen. It is possible that base-stacking interactions, which play a dominant role in the stability of both single-stranded and duplex DNA, might severely restrict the accessibility of hydrogen acceptors that drive proton exchange with 6-MI. If base stacking were the predominant factor affecting proton-transfer reactivity, we might expect the equilibrium to shift toward the deprotonated state if the temperature is elevated to induce thermal unstacking of bases in ssDNA. On the contrary, we found that the value of K_{eq} was nearly equal to that of 6-MI NMP at room temperature, and this value only marginally increased over the temperature range (20–80 °C, see Figure 5D). Hence, base-stacking interactions do not appear to influence the acidity of 6-MI in ssDNA.

For DNA constructs in which the 6-MI probe residues were positioned adjacent to a G (or to another 6-MI), we found that the value of K_{eq} was generally larger in dsDNA than for the same sequences in ssDNA. For example, the value of K_{eq} is nearly 10-fold greater for dsGXG than for ssGXG. Exceptions to this behavior were observed for the sequences TXT and TXTXG, in which the 6-MI probe residues were positioned at sites adjacent on both sides to a T (see Figure 6 and Tables S2 and Table S3). Stacking interactions between 6-MI and adjacent T residues are expected to be much weaker than the interactions between 6-MI and an adjacent G (or to another 6-MI) due to the considerable difference in surface areas between G and T. These results suggest that mechanically rigid sequences of dsDNA, in which the probe residue was positioned adjacent to a G, resulted in stabilization of the deprotonated form of 6-MI. In contrast, more flexible sequences of dsDNA, in which the probe residue was positioned adjacent to a T, did not experience a significant change in acid–base equilibrium relative to the same sequence in ssDNA.

As we previously discussed, thermal unstacking of ssGXG does not significantly alter the value of K_{eq} (see Figure 5C). However, the enhanced value of K_{eq} in dsGXG does drop precipitously at elevated temperatures due to thermal denaturation, suggesting that the enhanced stability of the deprotonated form of 6-MI in duplex DNA is due to the cooperatively stabilized duplex structure. We note that the deprotonated form of 6-MI is generally more favored in duplex DNA than it is for the 6-MI NMP in solution, while the protonated form of 6-MI is more favored in ssDNA. Finally, the secondary structure associated with the duplex DNA environment is expected to decrease the accessibility of the 6-MI probe to solvent water relative to the 6-MI NMP and the 6-MI-substituted ssDNA constructs. Taken together, these observations suggest that the enhanced stability of the deprotonated form of 6-MI in duplex DNA is likely associated with the Watson–Crick hydrogen bond between the 6-MI N3 hydrogen and its complementary C on the opposite DNA strand. Unlike the hydrogen bonds between the canonical DNA bases G and C, in which the proton remains associated with the donor base G (see Figure 1A), the 6-MI:C hydrogen bond seems to involve significant proton transfer to the imino nitrogen of the acceptor base C. The latter situation is sometimes referred to as a low-barrier hydrogen bond (LBHB), in which the proton can be nearly equally shared between the hydrogen bond donor and acceptor sites. Such systems have been studied by determining hydrogen bond distances by X-ray crystallography, or through NMR measurements of ^1H – ^{15}N dipolar coupling.³³ The possible existence of LBHBs in proteins and small molecules has been an area of active interest and debate.^{34–38} The nuclear wave function of an LBHB in its ground vibrational

level is expected to have its maximal amplitude coincident with the position of the energy barrier, so that the ionizable proton might undergo quantum mechanical tunneling between protonated and deprotonated states.³⁴ The existence of this system would represent an interesting system for future spectroscopic investigations

The values of G° and $G^\ddagger$ that we have determined for the various DNA substrates can be used to construct projections of the free energy landscape onto the proton-transfer coordinate. Because these surfaces depend sensitively on base sequence context of the 6-MI probe, this information could be used to infer couplings between the proton-transfer coordinate and other nuclear degrees of freedom, such as base torsional motions and the disruption of Watson–Crick hydrogen bonds. As an example, we compared model free energy surfaces derived from our results for the duplex DNA constructs dsGXG and dsTXT, which we illustrate in Figure 7. The forward proton-transfer reaction is thermodynamically and kinetically more favorable for the dsGXG construct ($K_{eq} = 0.30$) than for the dsTXT construct ($K_{eq} = 0.01$, see Table S3). In the dsGXG construct, the 6-MI probe is expected to experience a mechanically more stable local environment than in the dsTXT construct. This leads to a free energy surface for the proton-transfer reaction with nearly isoenergetic end-states bridged by a relatively low activation barrier.

We note that hydrogen bond strength is greatest when the pK_a values of the donor and acceptor groups are similar to each other.³⁹ The lower pK_a value of 6-MI NMP (≈ 8.7 , see Figure 7) in comparison to dGMP (≈ 9.7 ⁴⁰) likely contributes to the enhanced lability of the 6-MI N3 proton in duplex DNA. Furthermore, this Watson–Crick hydrogen bond involves the directional interaction between the 6-MI N3 proton and the lone pair electronic orbital of the imino nitrogen of the opposing C. The weaker acidity of 6-MI probes with adjacent T residues (in comparison to G residues, see Figure 6 and Figure 7) may be due to the disruption of this orbital overlap resulting from the larger thermal fluctuations associated with T-rich sequences.

In this work, we have shown that the spectroscopic properties of the 6-MI probe in DNA can be used to obtain detailed information about local base conformations and conformational heterogeneity and fluctuations. Such information may be helpful in understanding detailed aspects of the mechanisms of specific and nonspecific protein–DNA interactions, including those involved in DNA replication, recombination, and repair. In work in progress, we are utilizing 6-MI substituted DNA constructs to understand the roles of thermal fluctuations of local base conformations in the assembly and functional pathways of protein–DNA complexes, and we expect that the improved spectroscopic understanding of the 6-MI probe obtained in this study will provide new insights into what “it sees” within DNA molecules.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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Figure 1.

(A) Chemical structure of the G:C and the 6-MI:C Watson–Crick base pair. The differences between the natural base G and the 6-MI analogue are indicated in red. Atom numbering systems for G and 6-MI are indicated. (B) The nomenclature and sequences of base-analogue-substituted ssDNA constructs used in this work. X indicates 6-MI, PC is pyrolocytosine, and 2-AP is 2-aminopurine. The regions that contain the 6-MI analogue and flanking bases are highlighted in red. Duplex DNAs are the same molecules annealed with a fully complementary strand.

Figure 2.

Excitation–emission shift (EES). Absorbance and emission spectra of (A) ssGXG and (B) dsGXG constructs, taken at 20 °C and pH = 7.6. Emission spectra were excited over the wavelength range 320–370 nm in 10 nm increments at the energies indicated by vertical arrows. Normalized emission spectra are shown in the corresponding color, and vertically displaced for clarity. Black circles are the “excitation–emission shift” (EES), i.e., the difference between the excitation and peak emission energies. Gray dashed lines are linear fits to the blue-edge of the EES data.

Figure 3.

Homogeneous one-state model and heterogeneous two-state model for excitation–emission shift (EES) experiments. Panels A and C show hypothetical free energy surfaces associated with ground and excited electronic states, both as a function of the generalized conformation parameter Q . Panels B and D show the expected EES, $Y \equiv E_{ex}(Q) - E_{em}^{max}(Q)$ (solid blue lines) as a function of the rescaled excitation energy [$X \equiv E_{ex}(Q) - E_{ex}(0)$], where $E_{ex}(0)$ is the energy of the peak absorbance. Panels A and B depict the situation for an optically homogeneous sample. Panels C and D depict an optically heterogeneous two-state sample with an activation barrier $G^\ddagger = G^\ddagger - G_{g1}$. For the heterogeneous two-state system, the EES undergoes an excitation energy-dependent transition between the two substates, as depicted in panel D for three different energetic conditions.

Figure 4.

EES data and model fits of 6-MI NMP in aqueous solutions for different pH conditions. (A) The EES [$Y \equiv E_{\text{ex}}(Q) - E_{\text{em}}^{\text{max}}(Q)$] for 6-MI NMP is plotted versus the rescaled excitation energy $X \equiv E_{\text{ex}}(Q) - E_{\text{ex}}(0)$, where $E_{\text{ex}}(0)$ is the energy of the peak absorbance. Solid curves are fits of eq 1 to the data. (B) The standard Gibbs free energy G° was determined from an analysis of similar data over the pH range 5–10.3 (see Figure S1). A fit of these data to the acid–base equilibrium equation $G^\circ = 2.3RT(\text{p}K_a - \text{pH})$ (shown in red) yields the value $\text{p}K_a = 8.7 \pm 0.3$.

Figure 5.

EES data for 6-MI substituted DNA constructs. Experimental EES data and their corresponding fits to the heterogeneous two-state model are shown for (A) ssGXG and (B) dsGXG, as a function of the rescaled excitation energy at 20 °C. X = 6-MI. (C) EES data for ssGXG at 20 °C (○), 35 °C (△), 50 °C (□), and 80 °C (×). (D) Temperature dependence of $K_{\text{eq}} = [\text{6-MI}^- \text{ (deprotonated)}] / [\text{6-MI (protonated)}]$ for the acid–base reaction of ssGXG, dsGXG, and 6-MI NMP. Oligonucleotide sequences are shown in Figure 1B.

Figure 6.

Standard free energy of reaction, free energy of activation, and equilibrium constant for proton transfer in 6-MI substituted DNA constructs. Standard free energies of reaction ΔG° (A, B), free energies of activation $\Delta G^\ddagger$ (C, D), and deprotonation reaction equilibrium constant K_{eq} (E, F) of ssDNA (left) and dsDNA (right) at pH = 7.6 (blue) and pH = 6 (red). Horizontal dashed lines indicate the values for 6-MI NMP at pH = 7.6. Representative fits of EES curves for all single-stranded and duplex DNA constructs are shown in Figure S2. The optimized values of the fitting parameters are presented in Table S2 and Table S3.

Figure 7.

Reaction coordinates for proton exchange dsGXXG and dsTXT. The absolute positions of the curves on the *Y*-axis are arbitrary, and they have been offset for clarity.