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Distinguishing “Looped-Out” and “Stacked-In” DNA Bulge Conformation Using Fluorescent 2-Aminopurine Replacing a Purine Base

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Abstract

The conformation of a bulged DNA base, whether looped-out of the DNA helix or stacked-in between the flanking bases, can be distinguished using fluorescence spectroscopy of an inserted fluorescent base. If 2-aminopurine, a structural analog of adenine and guanine, is placed in duplex DNA as the bulged base replacing an adenine or guanine, it loops out of the DNA helix into solution. This is determined by the decrease or increase of 2-aminopurine fluorescence during DNA thermomelting: if the 2-aminopurine base stacks into the helix, its fluorescence increases or remains about the same during DNA duplex melting, but if the 2-aminopurine base loops out of the helix, its fluorescence decreases upon melting of the DNA duplex.

Introduction

An extra, unpaired base in one strand of a nucleic acid duplex is termed a “bulged” base. Nucleic acid bulges have been involved in a number of biological processes such as frameshift mutagenesis, RNA splicing, and RNA-protein interactions (1–7). The structure and conformation of nucleic acid bulges have been under intensive investigation (8–23). It is believed that the conformation of the bulge is important for protein recognition (24–25). Studies using Nuclear Magnetic Resonance (NMR) spectroscopy (8–16), X-ray crystallography (17), gel electrophoresis (18–22), and molecular dynamics simulation (23) have revealed that the bulged base can adopt either a “stacked-in” conformation, if the unpaired base stacks between the two flanking bases in the helix, or a “looped-out” conformation, if the unpaired base faces away from the helix. Whether the bulged base adopts the looped-out or stacked-in conformation depends on the nature of the bulged base itself and its flanking bases (8–11, 22, 23). While the crystal structure of a DNA bulge does not necessarily represent its conformation in solution, NMR studies have provided structural information, including the identification of a looped-out or stacked-in conformation (8–16). However, unlike spectroscopic methods, the NMR technique is usually restricted to solve structures of relatively short DNA strands and the interpretation of NMR spectra is laborious.

Fluorescence spectroscopy has been used as a sensitive technique to probe local structural variations of DNA using fluorescent molecules incorporated at specific sites of DNA. 2-Aminopurine (^{2a}P), a highly fluorescent isomer of adenine, is one of the most widely used probes (26–31). It has been incorporated into DNA via automated DNA synthesis (32, 33) and its fluorescence and thermodynamic properties are well investigated (28–31, 34–40). There are two unique characteristics about ^{2a}P: (1) it can replace an adenine in DNA and

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introduces minimal perturbation to the local structure (32, 33, 36–39) and (2) the fluorescence of ^{25}P is extremely sensitive to environmental changes such as base stacking, hydrogen bonding, and solvent exposure (30, 31, 34–36). The fluorescence emission intensity for ^{25}P in a free nucleoside decreases thirty folds upon incorporation into single strand (ss) DNA, and decreases two more folds upon hybridization with its complementary strand to form double strand (ds) DNA (28–31). Quenching of ^{25}P fluorescence in DNA has been mainly attributed to base stacking and hydrogen bonding (28).

In this study, ^{25}P is incorporated into DNA as the bulged base. The increase or decrease of the fluorescence emission for ^{25}P during DNA duplex thermomelting may verify whether it adopts a stacked-in or a looped-out conformation. As illustrated in Figure 1, if the bulged ^{25}P is in a stacked-in conformation (left), its environment in terms of base stacking and hydrogen bonding changes very little upon duplex melting to become ss-DNA (middle). The mere change is from a more rigid ds-DNA to a more flexible ss-DNA environment. The fluorescence of ^{25}P should remain the same or increase slightly upon duplex melting. In contrast, if the bulged ^{25}P loops out of the helix (right), it does not stack with any bases. However, it does stack between flanking bases in ss-DNA upon duplex melting. Thus, a decrease of ^{25}P fluorescence should be observed during this transition because its fluorescence is quenched more by base stacking in ss-DNA than in the looped-out bulged duplex. A third scenario that is not illustrated here is if ^{25}P is in a normal duplex DNA paired with a T or C through two or one hydrogen bonds, respectively. In comparison with the stacked-in conformation (Figure 1, left), melting of the ^{25}P paired with a C or T in the duplex will cause stronger fluorescence increase since hydrogen bond(s) is broken due to DNA melting.

Materials and Methods

Preparation of 2'-deoxy oligonucleotides containing bulged 2-aminopurine

All chemicals were obtained from Sigma-Aldrich Chemical Company (St Louis, MO) and used without further purification. DNA oligonucleotides were synthesized through standard procedures at Gemini Biotech (Alachua, Florida), who also provided the 2-aminopurine phosphoramidite. The oligonucleotide single strands were desalted and purified by reverse-phase HPLC. Upon receiving, the oligonucleotides were dissolved in 1× TBE buffer (89 mM Tris base, 89 mM boric acid, and 0.1 mM EDTA, pH=8.14) with 200 mM NaCl made with deionized water (18 M ultra pure water) to make approximately 1 mM solution of the single strand oligonucleotide. Then it was diluted with the same buffer to make a solution of approximately 20 μM (in base pairs). The exact concentration of the oligonucleotides was determined by its absorbance using Beer's Law: $C = A_{260} / (\epsilon \cdot l)$, where A_{260} is the absorbance of the oligomer at 260 nm; ϵ is the molar absorption extinction coefficient at 260 nm and l is the cell path length (1 cm). The ϵ was calculated based upon the following equation: $\epsilon = 0.89 [(A \times 15480) + (C \times 7340) + (G \times 11760) + (T \times 8850)]$, where A, C, G, T = number of A, C, G, and T bases in the oligomer. The molar absorption coefficient value for ^{25}P was assumed to be the absorbance value for guanine at 260 nm as Fagan et al did in their study (38). Then the two complimentary single strand oligonucleotides were matched equimolar and annealed by heating in a water bath to 75°C followed by slow cooling. The final concentration of oligonucleotide duplex solution is around 10 μM in base pairs.

DNA thermomelting monitored by UV absorption and fluorescence

A Varian Cary 3E UV-Vis Spectrophotometer was used to follow the absorption change of all the duplexes at 260 nm during temperature increase. Sample solutions (1 mL) in quartz cuvettes (1.0 × 0.4 cm) were put in the temperature control block with dry air blowing through the cuvette holder. A Peltier temperature controller was used to increase the

temperature of the solution at a rate of 0.5–1°C/min in the temperature range of 10–75°C. DNA melting temperature, T_m , was determined by using the Bio package software provided for the spectrophotometer by Varian. All experiments were in triplicates. The average error for T_m values were ± 2 degrees. The output data was taken to be plotted using the Excel program.

Fluorescence change of oligonucleotides containing ^{25}P upon heating was obtained by measuring the fluorescence intensity at various temperatures. Fluorescence intensity of DNA duplex solutions (about 3 μM in base pairs) in a quartz cuvette (1 $\times$ 1 cm) was measured with a FluoroMax-2 spectrofluorometer (Juben-Yvonne-Spex, Inc.). The excitation and emission wavelengths were 300 nm and 370 nm, respectively. The temperature was controlled by an external water bath, and the actual temperature of the solution in the cuvette was measured with a temperature probe. Dry air blowing into the sample holder chamber prevented condensation of water on the surface of the cuvette.

Results and Discussion

Preparation of bulged DNA duplexes containing 2-aminopurine

All six 2'-deoxy oligonucleotides (Oligo 1–6) were synthesized and their extinction coefficient were calculated and listed in Table 1. Bulge-1 and Bulge-2 were prepared by equimolar matching of Oligo-1 with Oligo-2 and Oligo-4 with Oligo-5, respectively, followed by annealing. In both bulged duplexes, ^{25}P should be the bulged base as shown in the right column of Table 1. Duplex-1 and Duplex-2 were made by matching Oligo-1 with Oligo-3 and Oligo-4 with Oligo-6, respectively. In these duplexes, ^{25}P is paired with a T or C. The ^{25}P -T pair has two hydrogen bonds similar to an A-T pair (32, 33), while the ^{25}P -C pair mostly forms one hydrogen bond, but can form two hydrogen bonds under acidic conditions (38, 39). Oligo-4, the top strand of Bulge-2, is partially self-complementary. It can pair with itself to form Duplex-3 with one base on each end of the duplex unpaired. In this duplex, ^{25}P is paired with a T. Therefore, if Oligo-4 and Oligo-5 are mixed, Bulge-2 and Duplex-3 coexist or are in equilibrium, and if Oligo-4 and Oligo-6 are mixed, Duplex-2 and Duplex-3 coexist or are in equilibrium.

Thermomelting of duplexes monitored by UV absorption

Thermomelting curves of all five duplexes are shown in Figure 2 by plotting the absorbance of DNA at 260 nm versus temperature. All these curves do not have a sharp transition, but spreads to a broader temperature range. Other authors also obtained such melting curves for similar ^{25}P containing DNA duplexes under similar conditions (28, 34, 35). In addition to broad melting transitions, the melting curves for Duplex-2 and Bulge-2 overlap in the temperature range of 52–58°C. The overlap could be due to the fact that both Duplex-2 and Bulge-2 coexist with Duplex-3 since the top strand of both Duplex-2 and Bulge-2 is self-complementary. The evidences for the coexistence of Duplex-2 or Bulge-2 with Duplex-3 will be shown later in the section discussing fluorescence monitored DNA melting. The T_m values for Bulge-1, Duplex-1, Bulge-2, Duplex-2 and Duplex-3 (10 μM of DNA in base pairs in 1 $\times$ TBE buffer with 200 mM NaCl), obtained directly using the Bio package software provided for the spectrophotometer by Varian, are 46, 52, 43, 48, and 53°C, respectively. The bulged duplexes Bulge-1 and Bulge-2 have lower melting temperature than the non-bulged Duplex-1 and Duplex-2, respectively, each by 5–6°C. The lower melting temperature for the bulges versus the duplexes may due to two factors: 1) the duplexes are longer than the bulges because of an extra base pair; 2) the bulged base is not hydrogen bonded with any other bases. Both of these factors would make the bulged duplexes to dissociate at a lower temperature than the “normal” duplexes.

Thermomelting of duplexes monitored by fluorescence emission

The fluorescence emission intensity for ^{2a}P at 370 nm versus temperature plot for all the duplexes was obtained on a FluoroMax-2 Spectrofluorometer with an excitation wavelength of 300 nm. Figure 3 depicts the duplex melting curve for Bulge-1 and Duplex-1. The fluorescence of ^{2a}P in Duplex-1 is nearly constant (slight decrease) in the temperature range of 0–40°C and thereafter increases until 60°C before it levels off. This fluorescence increase is due to breaking of the two ^{2a}P -T hydrogen bonds during duplex melting, resulting in less fluorescence quenching by hydrogen bonding. Other authors also observed such increase of ^{2a}P fluorescence upon duplex melting (34, 37). The transition looks like a typical DNA duplex melting curve with an estimated T_m of 52°C, consistent with the UV measurement. For Bulge-1, the fluorescence of the bulged ^{2a}P base decreases gradually in the temperature range of 0–30°C before a sharper decrease from 30°C to 53°C appears. The first gradual fluorescence decrease observed is due to the temperature dependent quenching of ^{2a}P fluorescence, also observed by Law et al (37). As a control, temperature dependent fluorescence quenching for single-stranded 5'-CGCXGAATCTCGT-3' was carried out. Its fluorescence decreased steadily as temperature decreased in the entire temperature range with no melting transition (data not shown). The sharper fluorescence decrease is due to the melting of the bulged duplex with an estimated T_m of 42°C, very close to the T_m value obtained from the UV measurement. As discussed earlier, the fluorescence decrease upon duplex melting indicates that the bulged ^{2a}P loops out of the helix. As the bulged duplex melts, the looped-out ^{2a}P base is forced to stack with the flanking bases in ss-DNA, thus causing fluorescence decrease due to base-stacking quenching. Figure 3 further reveals that the fluorescence intensities of ^{2a}P for both Bulge-1 and Duplex-1 have become the same within error after melting since the ^{2a}P concentration is the same in both duplexes.

In order to further confirm the above observation that a bulged ^{2a}P base loops out of the helix, Bulge-2 was specially designed so that it may be in equilibrium with Duplex-3, formed by the top strand of Bulge-2 alone because it is self-complementary (except for one base on the end). While ^{2a}P is a bulged base in Bulge-2, it is paired with a T in Duplex-3. If the equilibrium is true, two opposite transitions should be observed during thermomelting of the annealed mixture of Oligo-4 and Oligo-5. As seen in Figure 4, there are two transitions monitored by fluorescence of ^{2a}P . The first transition at about 42°C with a decrease in ^{2a}P fluorescence reflects the melting of the bulged duplex Bulge-2, where ^{2a}P is in a looped-out conformation. The second transition at the 48–65°C region with an increase of ^{2a}P fluorescence must be from the melting of Duplex-3, where ^{2a}P is paired with a T. In comparison, the ^{2a}P fluorescence for pure Duplex-3, formed by annealing Oligo-4 alone, increases in the temperature range of 40–65°C with one transition. This melting curve nearly overlaps the melting curve for the Bulge-2 and Duplex-3 mixture. The melting temperature for Duplex-3 is 52°C, similar to the 53°C determined from the UV measurement. If Oligo-4 and Oligo-6 are annealed equimolar, Duplex-2 and Duplex-3 suppose to be in equilibrium in which ^{2a}P is paired with a T or C, respectively. The fluorescence of ^{2a}P increases upon temperature increase with seemingly one transition in the temperature range of 40–65°C, very similar to the melting of Duplex-3. This may be because that ^{2a}P is paired with an opposing base, T or C in Duplex-2 or Duplex-3, respectively, and both duplexes have the similar melting temperature. At the end of duplex melting, the melting curves for Bulge-2, Duplex-2, and Duplex-3 all merged within error.

Quenching of the bulged 2-aminopurine by potassium iodide

An additional evidence that the bulged ^{2a}P loops out of the helix in Bulge-1 comes from a fluorescence quenching experiment. If ^{2a}P stacks into the helix, it is protected from collisional fluorescence quenchers such as KI. However, if ^{2a}P loops out of the duplex, it is accessible by KI and thus its fluorescence can be quenched. As expected, the Stern-Volmer

quenching constant for ^{2a}P by KI in the bulged duplex Bulge-1 is 8.8 M^{-1} , whereas quenching of the fluorescence of ^{2a}P in Duplex-2 by KI was negligible.

Conclusions

In conclusion, the bulged ^{2a}P , a type of purine base, adopts a looped-out conformation in the DNA duplexes examined. This observation confirms the results obtained by NMR experiments (8–16). However, this method is simpler and uses much less samples. This method may also be used to determine the conformation of bulged purines in longer DNA duplexes. What conformation would a pyrimidine base adopt or how flanking bases affect the bulge conformation needs further investigation.

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Figure 1. Conformational change for a “looped-out” or “stacked-in” bulged 2-aminopurine base (dark block) during DNA duplex thermomelting.

Figure 2.

DNA thermomelting profile monitored by UV-absorbance at 260 nm in 1× TBE buffer in the presence of 0.2 M NaCl at pH 8.14: Bulge-1 (▲), Duplex-1 (◆), Bulge-2 (◇), Duplex-2 (●), and Duplex-3 (○).

Figure 3. Plot of normalized fluorescence intensity of ^{25}P at 370 nm (F_{370}) versus temperature for Bulge-1 (●): 5'-CGCXGAATCTCGT-3' / 3'-GCGCTTAGAGCA-5' and Duplex-1 (○): 5'-CGCXGAATCTCGT-3' / 3'-GCGTCTTAGAGCA-5' ($3\ \mu\text{M}$ in base pairs) in $1\times$ TBE buffer with $0.2\ \text{M}$ NaCl.

Figure 4.

Plot of normalized fluorescence intensity of ^{2a}P at 370 nm (F_{370}) versus temperature for Bulge-2 (●): 5'-CGCXGAATTCTGC-3' / 3'-GCGCTTAAGACG-5', Duplex-2 (□): 5'-CGCXGAATTCTGC-3' / 3'-GCGCCTTAAGACG-5', and Duplex-3 (○): 5'-CGCXGAATTCTGC-5' / 3'-CGTCTTAAGXCGC-5' (3 μ M in base pairs) in 1 $\times$ TBE buffer with 0.2 M NaCl.

Table 1

Sequence for ss-DNA oligomers, bulged and normal duplexes (X = 2ap) and their T_m.^a

ss-DNA	ϵ_{260} (mol ⁻¹) ^b	Bulged and Duplex DNA		T _m (°C) ^b
Oligo-1: 5'-CGCXGAAATCTCGT-3'	151923	Bulge-1:	X 5'-CGC GAATCTCGT-3' 3'-GCG CTTAGAGCA-5'	46
Oligo-2: 3'-GCGCTTAGAGCA-5'	138146	Duplex-1:	5'-CGCXGAAATCTCGT-3' 3'-GCGTCTTAGAGCA-5'	52
Oligo-3: 3'-GCGTCTTAGAGCA-5'	144678			
Oligo-4: 5'-CGCXGAAATCTCGC-3'	151923	Bulge-2:	X 5'-CGC GAATCTTGC-3' 3'-GCG CTTAAGACG-5'	43
Oligo-5: 3'-GCGCTTAAGACG-5'	138146	Duplex-2:	5'-CGCXGAAATCTTGC-3' 3'-GCGCCTTAAGACG-5'	48
Oligo-6: 3'-GCGCCTTAAGACG-5'	144678	Duplex-3:	5'-CGCXGAAATCTTGC-5' 3'-CGTCTTAAGXC GC-5'	53

^aAll the DNA melting temperatures, T_m, were obtained from 3 repeats. The average errors were ±2 degrees.

^bThe ϵ_{260} are for single strand DNA in per moles of the single strand.