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A Selective, Noncovalent Assay for Base Flipping in DNA

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Base flipping, i.e., the conformational change of a nucleobase from its intrahelical position to a solvent-exposed, extrahelical position, is a key step in many DNA repair reactions and is thus crucial for maintaining the genetic integrity of the living cell. While a nucleobase is held in its regular position inside the duplex through a combination of base stacking and hydrogen bonding to its complementary base (Watson−Crick pairing), DNA damage through mismatched or abasic sites as well as oxidative or photodamage increases the propensity for base flipping. Many DNA repair enzymes, including C5-cytosine DNA methyltransferase, uracil DNA glycosylases, and CPD photolyase, exploit base flipping for the selective recognition of the DNA lesion.[1]

The study of base flipping in DNA requires considerable experimental effort because the conformational equilibrium is often too far on the flipped-in side to allow direct detection. Although site-directed mutagenesis and X-ray crystallography have been successfully used, they are experimentally demanding and yield structural information on the DNA in complex with the protein but not on pathways, relative energies, or base flipping in the unbound species. By far the most commonly employed direct method is the use of 2-aminopurine (2-AP) as a probe for base flipping.[2] When 2-AP is base stacked in the DNA helix, its fluorescence is strongly quenched, but the fluorescence increases and shifts when 2-AP is in an extrahelical, flipped-out position.[3] The ease and sensitivity of the fluorescence detection and the possibility of detection of base-flipping of the 2-AP into an enzyme-bound position make this an attractive method. However, the use of the 2-AP probe is not without disadvantages. Conceptually, it is not a natural base or DNA lesion, but a probe-modified DNA that is detected. The covalent incorporation of 2-AP requires a synthetic effort and, however subtle, perturbs the native DNA strand. It would therefore be desirable to develop a simple assay that does not require the covalent modification of DNA.

Here, we report the design, synthesis, and evaluation of a DNA base-flipping assay that exploits specific, noncovalent interactions with pyrimidine nucleobases. Figure 1 summarizes the design of this assay. The native DNA duplex is in equilibrium with the duplex in which one of the bases is in a flipped-out orientation. A binding moiety (red) selectively recognizes the flipped-out base and binds to it. This change in the environment from bulk water to the highly charged DNA duplex causes a change in the fluorescence of the reporter unit (green).

To implement this design, there must be two components to the designed molecule, the binding and reporter units. While the two may be combined into one, the use of such a molecule in this assay is not practical. The signal from the reporter unit is generally detected as a change in the electronic properties, such as shifting in the UV−visible absorption or fluorescence emission, and reporters of this type are generally large aromatic molecules which are capable of intercalation. Therefore, separate binding and reporter units were used. For the binding unit we chose a zinc-cyclen complex that selectively recognizes pyrimidines by a combination

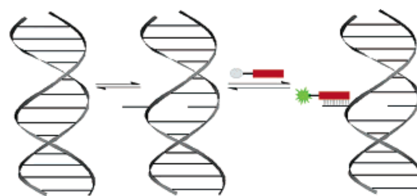


Figure 1. Schematic representation of the base-flipping assay.

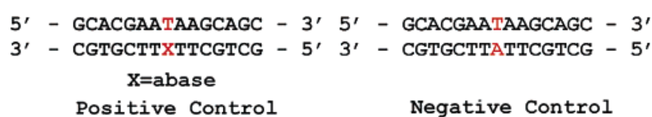
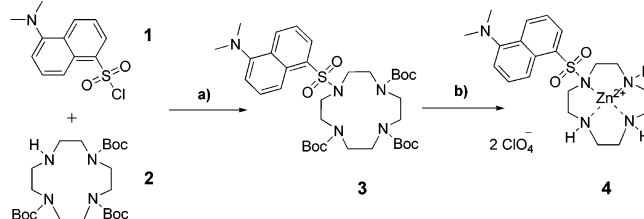


Figure 2. Sequences of positive and negative control DNAs.

Scheme 1. Synthesis of Dansyl-Cyclen[a]



[a] Reagents and conditions: (a) K_2CO_3, CH_3CN, RT, 4 days, 48%; (b) 1. TFA, CH_2Cl_2, RT, 3 h; 2. $Zn(ClO_4)_2$, MeOH, RT, 1 h, 86% from 3.

of hydrogen bonding and complexation of the divalent zinc cation to the deprotonated N3 nitrogen of the pyrimidine base.[4] For the reporter unit we chose the widely used dansyl group based upon the solvatochromic properties of this fluorescent dye. The change in environment of the solvatochromic dye from bulk solution to DNA bound will allow the binding of the zinc-cyclen complex to the DNA to be monitored. The resulting compound **4** was prepared in 41% yield by coupling of tri-Boc cyclen **2**[4] to the dansyl chloride **1** as shown in Scheme 1. Deprotection and complexation of the zinc ion completed the synthesis.

To detect binding of the dansyl-cyclen to the base-flipped thymine residue, the fluorescence emission of the dansyl moiety upon excitation at 335 nm was monitored as a function of the concentration of the two 15-mer oligonucleotides shown in Figure 2. The positive control DNA sequence contains an abasic unit, which has been previously shown to induce flipping of the opposite base.[5] As a negative control, the corresponding nondamaged duplex DNA, which should show very little base flipping and therefore not bind to **4**, was used.

To detect the wavelength at which there was the maximum fluorescence difference between abasic and duplex DNA, the emission spectra at the highest DNA concentration used, 9 μM, were subtracted (Figure 3). The wavelength at which the dansyl-cyclen **4** emission in the presence of abasic and duplex DNAs shows the greatest difference was found to be 531 ± 11 nm (average of five trials). To ensure that this response was indeed due to specific

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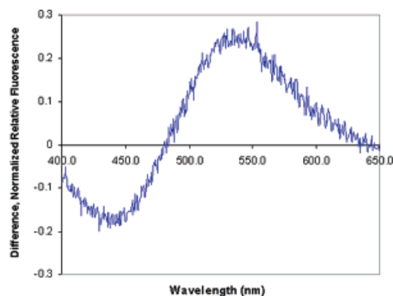

Figure 3. Difference plot of the normalized relative fluorescence of 1 μM dansyl-cyclen **4** (λ_{exc} = 335 nm) in the presence of 9 μM abasic and duplex DNAs in 100 mM phosphate buffer, pH 7.0.

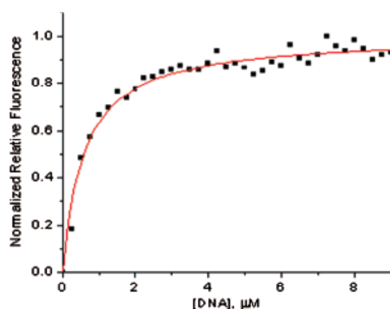

Figure 4. Normalized relative fluorescence emission of 1 μM dansyl-cyclen **4** (λ_{exc} = 335 nm, λ_{em} = 533 nm) in the presence of increasing concentrations of abasic DNA in 100 mM phosphate buffer, pH 7.0.

Table 1. Fitting Parameters and Binding Constants

trial no.	R^2	F_{max}	K (μM)
1 (shown)	0.9687	1.00 ± 0.01	0.58 ± 0.04
2	0.9664	0.99 ± 0.02	1.01 ± 0.08
3	0.9361	0.99 ± 0.02	0.58 ± 0.07

binding of the cyclen to a flipped-out pyrimidine and not due to the intercalation of the dansyl moiety into the DNA strands, the methylamino-derivatized dansyl **5** was prepared using coupling conditions similar to those used to prepare **4** (see Supporting Information). The difference plot of the methylamino-dansyl **5** in the presence of 9 μM abasic and duplex DNAs shows no appreciable fluorescence signal, allowing the conclusion that the signal observed for the dansyl-cyclen **4** is not due to intercalation (see Supporting Information).

The fluorescence emission of **4** was monitored at 533 ± 5 nm as 1 μL aliquots of 250 μM abasic and duplex DNAs were titrated into the solution. The results (Figure 4) show that there is saturation of the abasic DNA while the response from the duplex DNA is scattered (see Supporting Information). The data were fitted to a one-site binding model using Origin 6.1,[6] and the results are shown in Table 1.

As shown in Figure 4 and Table 1, the constant-wavelength analysis of the abasic DNA shows saturation and the correlation coefficients for the three trials performed are quite acceptable. Data fitting gave constants for the equilibrium between the complex of abasic DNA **4** and free abasic DNA of 10^{-6} M or less. The accuracy within a measurement was, at the maximum, ±12%, while the accuracy between measurements was 57%. This difference in equilibrium constants corresponds to an energy difference of ~0.3 kcal/mol. Because binding of **4** requires the flipping of the thymine opposite the abasic site into an extrahelical position, the measured equilibrium constant is a combination of the equilibrium constants for the flipping and binding. It could thus be used to calculate the equilibrium constant for base flipping in DNA, although an accurate determination of the binding constant of **4** to a thymine in the DNA environment would need to be obtained.[7] More importantly, this assay could be used to obtain relative measurements of base flipping of various sequences and damage types in DNA.

In summary, we have developed a noncovalent assay to detect base flipping in DNA. Base flipping of a thymine base in a DNA duplex in which an adenine base was replaced by an abase was detected using a combination of fluorescence titrations and monitoring the fluorescence response at a constant wavelength. The abasic DNA showed saturation, from which an equilibrium constant could be obtained using single-site fitting. Future studies will apply this new assay to the investigation of base flipping in different types of damaged DNA and its dependence on the sequence.

Acknowledgment. We are grateful for a gift of **2** by Prof. B. König (Regensburg) and to Prof. B. Smith (Notre Dame) for the permission to use the fluorimeter. L.L.O. is the recipient of a Schmitt Fellowship by the University of Notre Dame.

Supporting Information Available: Synthesis and characterization of **3**−**5**, UV and fluorescence spectra of **4** and **5**, as well as experimental procedures for the assay. This material is available free of charge via the Internet at http://pubs.acs.org.

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