Single-Molecule Fluorescence Using Nucleotide Analogs: A Proof-of-Principle

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S *Supporting Information*

ABSTRACT: Fluorescent nucleotide analogues, such as 2-aminopurine (2AP) and pyrrolo-C (PyC), have been extensively used to study nucleic acid local conformational dynamics in bulk experiments. Here we present a proof-of-principle approach using 2AP and PyC fluorescence at the single-molecule level. Our data show that ssDNA, dsDNA, or RNA containing both 2AP and PyC can be monitored using single-molecule fluorescence and a click chemistry immobilization method. We demonstrate that this approach can be used to monitor DNA and RNA in real time. This is the first reported assay using fluorescent nucleotide analogs at the single-molecule level. We anticipate that single 2AP or PyC fluorescence will have numerous applications in studies of DNA and RNA, including protein-induced base-flipping dynamics in protein−nucleic acid complexes.
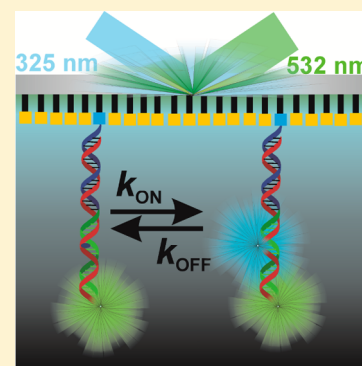
SECTION: Biophysical Chemistry and Biomolecules

For over two decades single-molecule fluorescence (SMF) techniques have provided unique opportunities to study the structural dynamics of proteins and nucleic acids otherwise hidden in ensemble-averaged experiments.[1−6] Traditionally, visible-range fluorophores have been used for these studies due to their high fluorescence quantum yield and photostability.[7] Expanding this range to the UV region would enable the use of naturally fluorescent amino acids and nucleotide analogues such as tryptophan, 2-aminopurine (2AP), and pyrrolo-C (PyC), thus eliminating the need to introduce artificial labels (Scheme 1). Single-molecule experiments in the UV region have been previously reported with cofactors, such as nicotinamide adenine dinucleotide (NAD), flavin adenine dinucleotide (FAD), and flavin mononucleotide (FMN),[2,8,9] but not with nucleotide analogues. Indeed, single-molecule imaging of 2AP and PyC incorporated in DNA or RNA has been considered to be essentially impossible due to their low quantum yield.[10]
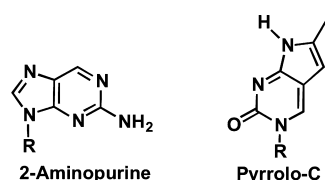
Unlike canonical nucleobases, 2AP fluoresces due to the presence of an energy barrier between a $\pi\pi^*$ excited state and the ground state.[11] This barrier may form a minimum well in the excited-state potential energy of 2AP that is below the intersection point with the ground-state potential energy surface and from where the fluorescence process can take place.[11] Here we report a new assay to measure 2AP and PyC fluorescence at the single-molecule level. We also apply this assay to measure the fluorescence of 2AP and PyC in single-stranded (ss) and double-stranded (ds) DNA and RNA. 2AP has been extensively used in bulk studies to characterize local nucleotide dynamics in DNA, RNA, and protein complexes[12−16] because their relative fluorescence yields depend dramatically on the environment (0.68 when solvent exposed, 0.04 to 0.18 in ssDNA and 0.02 in dsDNA, Figure S1 in the Supporting Information).[10,17] Although the exact mechanism for 2AP quenching in dsDNA is still controversial,[18−20] it is widely accepted that stacking interactions between 2AP and neighboring bases result in fluorescence quenching.[16,21,22]
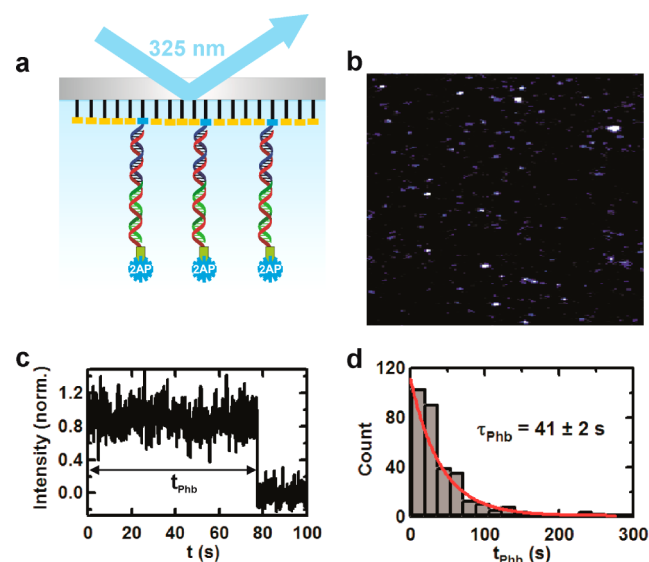
To characterize the fluorescence of 2AP at the single-molecule level, we surface-immobilized dsDNA on a quartz microscope slide (0.013 ± 0.001 molecules/μm^2) in standard

Scheme 1

2-Aminopurine   Pyrrolo-C

buffer (50 mM Tris-HCl, pH 7.5, 100 mM Na$^+$, 5 mM Mg^{2+}) using a recently developed click chemistry-based approach (Figure 1a).[23] The widely used biotin−streptavidin immobiliza-


Figure 1. Single-molecule 2AP fluorescence. (a) Prism-based, total internal reflection microscope with 325 nm excitation for 2AP fluorescence. 2AP is linked through a long, flexible linker to surface-immobilized DNA. (b) Fluorescence image of individual FREE-2AP molecules (bright spots). (c) Fluorescence trajectory of FREE-2AP reveals the absence of blinking and single-step photobleaching. (d) Histogram of photobleaching times (t_{Phb}).

tion approach cannot be used here due to the fluorescence background caused by the numerous tryptophan residues in streptavidin.[24] We linked 2AP at the 5′ terminus of a dsDNA through a triethylene glycol linker to mimic the behavior of free 2AP in solution (FREE-2AP). Following excitation of 2AP at 325 nm (3 mW) using a home-built, prism-based total internal reflection fluorescence microscope (Figure 1a), 2AP fluorescence was imaged onto a back-illuminated, electron-multiplied CCD camera (Figure 1b).

Characteristic single-molecule fluorescence trajectories (Figure 1c) reveal that 2AP fluoresces steadily over almost a minute prior to photobleaching (Phb) in a single step. A photobleaching-time analysis of 335 trajectories reveals that in the absence of an oxygen scavenging system 2AP photobleaching times are exponentially distributed with a lifetime $\tau_{Phb} = 41 \pm 2$ s (Figure 1d). Interestingly, <0.3% of molecules observed exhibit any fluorescence blinking at ∼30 frames per second acquisition time. We also find that ∼6% of the traces exhibit two-step photobleaching and <1% exhibit three steps or more (Figure S2 in the Supporting Information). The number of steps indicates the number of fluorescent molecules immobilized within a single diffraction-limited spot. When 2AP was substituted by a nonfluorescent adenine base, we did not observe any fluorescence, confirming that the observed fluorescence arises from 2AP.

In contrast, the single-molecule fluorescence trajectories of 2AP-incorporated eight nucleotides from the 5′ end of the ssDNA (2AP-ssDNA, Table S1 in the Supporting Information) exhibit random jumps between a bright (ON) and a dark (OFF) state (Figure 2a). Dwell-time analysis of the ON and OFF states (112 molecules) reveals distributions with lifetimes $\tau_{ON} = 2.3 \pm 0.3$ s and $\tau_{OFF} = 2.1 \pm 0.3$ s (Figure 2b). The exact
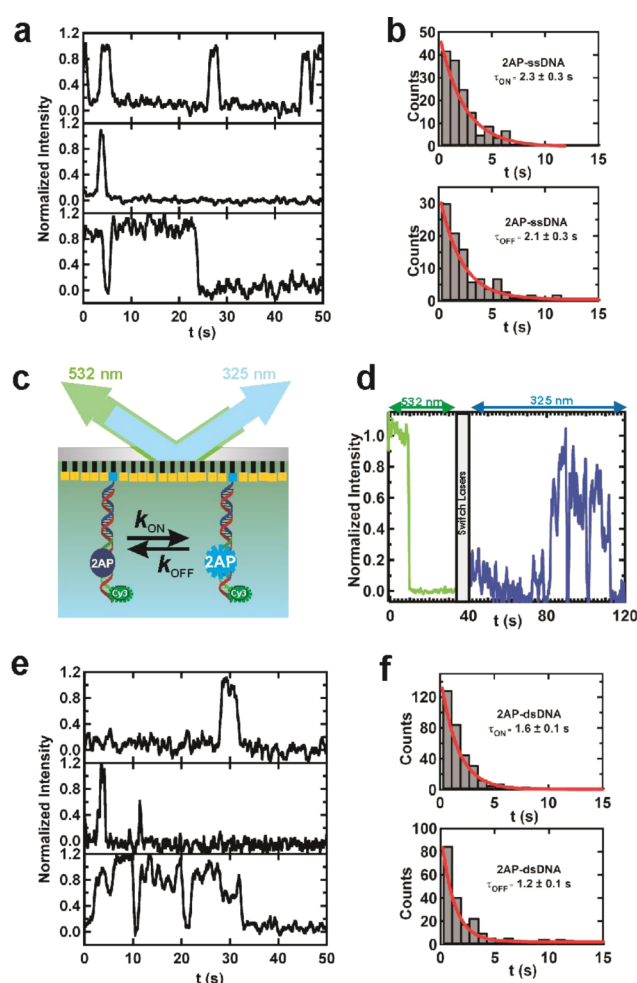

Figure 2. Fluorescence of single 2AP molecules incorporated into DNA. (a) Three fluorescence trajectories of 2AP in ssDNA. Fluorescence intensity jumps between a bright and a dark state. (b) Dwell-time histograms of 2AP-ssDNA ON (τ_{ON}) and OFF (τ_{OFF}) states (112 molecules). (c) Experimental setup combining 532 and 325 nm excitation for Cy3 and 2AP, respectively. (d) First, Cy3 is excited at 532 nm and fluorescence is monitored to localize dsDNA molecules. Next, 2AP is excited at 325 nm. (e) SM trajectories of 2AP in dsDNA reveal a small subpopulation of molecules with ON−OFF transitions. (f) Dwell time histograms of 2AP-dsDNA ON (τ_{ON}) and OFF (τ_{OFF}) states (182 molecules).

origin of these blinking dynamics is unclear. However, on the basis of prior bulk experiments and theoretical calculations of 2AP[21,22] as well as on the lack of fluorescence blinking of FREE-2AP, a possible explanation is that this blinking corresponds to changes in the fluorophore's local environment, for example, caused by 2AP stacking and unstacking with neighboring bases. Alternatively, the slow dynamics may simply represent nonspecific interactions between the ssDNA and the slide surface.[25] Although more experiments are needed to fully characterize what induces the ON-OFF transitions, these data show that this approach can be used to measure 2AP fluorescence at the single-molecule level and even report on different local environments. It is interesting to compare the brightness of FREE-2AP with 2AP in ssDNA. Because the on duty cycle is ∼50%, one would expect FREE-2AP to be significantly brighter than 2AP in ssDNA in ensemble-averaged experiments. Indeed, the fluorescence intensity for 2AP in ssDNA is about three times lower (Figure S1 in the Supporting

Information). In addition, a population of dark (or transiently bright) molecules that we cannot observe in our experiment may also contribute to decreasing the bulk intensity. These single-molecule data raise the interesting possibility that 2AP quantum yields measured in bulk experiments result from the time average of at least two dynamic populations with high (bright) and low (dark) quantum yields.

To characterize the fluorescence properties of 2AP in dsDNA, we annealed a 15-nucleotide complementary oligonucleotide to 2AP-ssDNA. To confirm hybridization, we labeled the complementary strand with 3′-Cy3 (Cy3cDNA, Figure 2c, Table S1 in the Supporting Information). The surface-immobilized dsDNAs were first localized by exciting Cy3 with a 532 nm laser, and we monitored its fluorescence until photobleaching (~40 s) (Figure 2d). Next, we switched to a 325 nm laser to monitor 2AP fluorescence (Figure 2d). Of the >200 dsDNA molecules observed, $89 \pm 2\%$ exhibited no 2AP fluorescence, as expected. Surprisingly, a small population molecules ($11 \pm 2\%$) behaved similarly to the ssDNA, with random jumps between ON and OFF states (Figure 2e), even though the complementary strand was present (as evidenced by Cy3 fluorescence). Interestingly, the presence of multiple populations is consistent with prior bulk fluorescence lifetime measurements of 2AP in nucleic acids, which also revealed a small population (~10%) of 2AP molecules with a fluorescence lifetime comparable to free 2AP.[26] A dwell time analysis (182 molecules) reveals that the lifetimes for this minor population ($\tau_{ON} = 1.6 \pm 0.1$ s and $\tau_{OFF} = 1.2 \pm 0.1$ s, Figure 2f) are similar to those of ssDNA (Figure 2a). Several factors may contribute to this blinking behavior, such as 2AP stacking and unstacking with neighboring bases, the influence of a strong π-stack of 6 GC base pairs neighboring the 2AP (Table S1 in the Supporting Information),[27] or even nonspecific interactions with the slide surface. Nonetheless, the observation that single 2AP molecules blink when incorporated into ssDNA or dsDNA suggests that this approach may be used to monitor local nucleotide dynamics in real time. Future experiments with different sequences and 2AP in different locations and in variable ionic strength buffers will help elucidate the molecular origin of this blinking behavior.

We also performed single-molecule experiments with 2AP across an abasic site on dsDNA (aDNA, Table S1 in the Supporting Information). In 10 mM MgCl₂, two characteristic behaviors are observed in the resulting time trajectories. The majority of molecules (79%) exhibit rapid transitions between ON and OFF states (Figure 3a), comparable to those observed with 2AP on ssDNA. A dwell-time analysis shows that the blinking rate constants for this population ($k_{ON} = 0.6$ s^{-1}, $k_{OFF} = 0.3$ s^{-1}, Figure S3 in the Supporting Information) are similar to those of 2AP-ssDNA. A minor population (~21%), exhibits constant fluorescence with single-step photobleaching (Figure 3b), similar to FREE-2AP. On the basis of the FREE-2AP and ssDNA data, it is possible that these two populations correspond to two distinct 2AP conformations or interactions with the DNA. It has been previously shown that binding of divalent cations to 2-aminopurine (2AP) opposite to an abasic site induces a conformational change that increases 2AP accessibility to solvent.[28,29] To test whether the minor population corresponds to solvent-exposed 2AP, we increased the [Mg²⁺] to 20 and 50 mM. The data show that, as expected, the static fraction increases to 35 and 49% (Figure 3c), respectively, while the rate constants of the blinking population remain unchanged (Figure S3 in the Supporting Information).
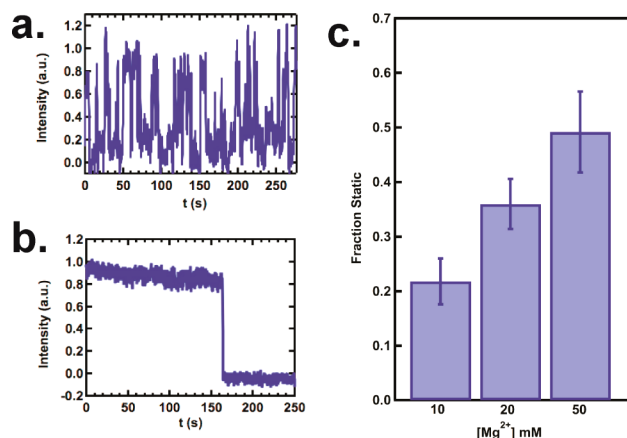
Figure 3. Single-molecule fluorescence of 2AP across an abasic site in dsDNA. Two populations are observed: (a) a major population exhibits ON−OFF (blinking) fluorescence transitions and (b) a minor population exhibits static fluorescence with single-step photobleaching. (c) Static population fraction increases with Mg²⁺ ion concentration.

These results are consistent with the assignment that the static population corresponds to solvent-exposed 2AP (similar to FREE-2AP), whereas the blinking population exhibits a local environment similar to ssDNA (such as an abasic nucleotide).

Next, we examined whether 2AP SMF can be used to study the formation of DNA−protein complexes (Figure S4a in the Supporting Information). The restriction enzyme *Psp*GI (a gift from Dr. A. Bhagwat, Wayne State University) binds the canonical sequence 5′-CCAGG in dsDNA and flips out the middle adenosine and its complementary thymine.[30] We substituted this adenine by 2AP and monitored its fluorescence in the absence and presence of 250 nM *Psp*GI. In presence of the enzyme, 4% of molecules exhibit a very long-lived ON state (Figure S4b in the Supporting Information), which is never observed in the absence of the enzyme or in experiments with only enzyme. It is possible that the low fraction of molecules in this long-lived bright state may reflect the weak binding affinity of *Psp*GI ($K_d = 0.5 \mu M$).[31] Unfortunately, higher *Psp*GI concentrations result in a significant tryptophan fluorescence background signal impeding these experiments. Nevertheless, the observation of a long-lived bright state, in principle, suggests that 2AP flipping by the enzyme may be directly monitored with this assay, and we will explore this possibility in future experiments.

A second fluorescent nucleotide analog PyC was also tested as a single-molecule probe. PyC was incorporated into a tRNA^Pro molecule[32] by substituting the base at position C74 with the fluorescent analog (Figure 4a). The tRNA^Pro construct is composed of a synthetic 16-mer RNA oligonucleotide with an internal PyC and a 5′-azide for surface immobilization[23] annealed to a transcribed 5′-RNA 57-mer (Figure 4a).

The 16-mer alone reveals steady PyC fluorescence with only 6% of all trajectories exhibiting any blinking (Figure 4b,c), similar to FREE-2AP. It is interesting to note that unlike 2AP in ssDNA PyC is not quenched in this 16-mer oligo. However, the quenching mechanism of PyC remains largely unexplored, and it may differ significantly from 2AP.[33] In contrast, most trajectories (~60%) obtained with the annealed PyC-tRNA^Pro construct exhibit blinking dynamics (Figure 2d and Figure S5 in the Supporting Information). Analysis of 100 fluorescent trajectories reveals an average of ~2 fluorescent bursts per trajectory with an average duration of ~5.5 s per burst. The
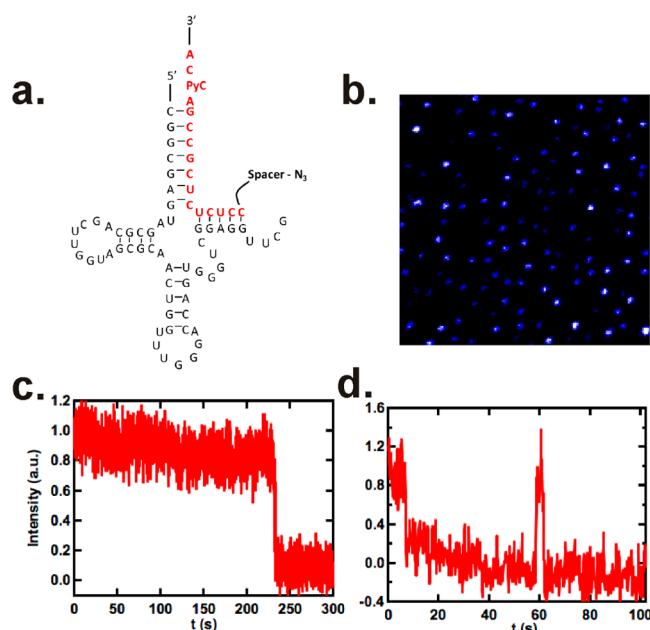
Figure 4. Single-molecule fluorescence of PyC in tRNA^{Pro}. (a) PyC-labeled tRNA^{Pro} is composed of a synthetic 3′-16-mer (red) annealed to a transcribed 5′-57-mer (black). The 16-mer strand includes PyC (C74) and 5′-N$_3$ for surface immobilization. (b) Fluorescence image of the surface-immobilized 16-mer strands. Each bright spot corresponds to an individual PyC-labeled 16-mer. (c) SM trajectory for a single PyC-16-mer molecule and (d) for an annealed/folded PyC-tRNA^{Pro}.

fraction of time during which the signal is above background is ~4.5%. It is possible that in the fully annealed tRNA^{Pro} PyC forms transient stacking interactions with neighboring bases,[32] leading to this blinking. But PyC may also be affected by other factors such as nonspecific interactions with the surface. Future experiments will address this issue directly.

In summary, we have shown that the fluorescent nucleotide analogs 2AP and PyC can be monitored at the single-molecule level and used to probe different local environments in DNA and RNA. Using 2AP single-molecule fluorescence, we show that 2AP in 2AP-ssDNA and -dsDNA jumps between bright and dark states, which may enable monitoring base-flipping dynamics in free nucleic acids or in complexes with proteins, such as PspGI. Similarly, PyC exhibits steady fluorescence in a short 16-mer, but its blinking increases when incorporated into tRNA^{Pro}. To the best of our knowledge, this study represents the first demonstration of nucleotide analog fluorescence at the single-molecule level. Future experiments will elucidate the molecular origin of the observed blinking, enabling the study of local nucleotide base flipping dynamics in DNA and RNA and protein−nucleic acid complexes at the single-molecule level. Further gains in experimental sensitivity will likely arise from the development of cameras with enhanced sensitivities in the ultraviolet region of the electromagnetic spectrum as well as the synthesis of novel fluorescent base analogs with higher quantum yields or with emission wavelengths in the visible range.

■ ASSOCIATED CONTENT

ⓈSupporting Information

Oligonucleotide sequences. Fluorescence spectra of FREE-2AP, 2AP-ssDNA, and 2AP-dsDNA. Single-molecule time trajecto-ries of 2AP-dsDNA-PspGI complexes. Methods and protocols. This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes
The authors declare no competing financial interest.

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