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Neely, Robert; Jones, Anita

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2-Aminopurine as a fluorescent probe of DNA conformation and the DNA-enzyme interface

Anita C. Jones[1*] and Robert K. Neely[2]

[1] EaStCHEM School of Chemistry, King's Buildings, The University of Edinburgh, Edinburgh, EH9 3JJ, UK.

[2] School of Chemistry, The University of Birmingham, Edgbaston, Birmingham, B15 2TT, UK.

*E-mail: a.c.jones@ed.ac.uk; Tel: +44 131 6506449; Fax: +44 131 6506453

Abstract

Nearly 50 years since its potential as a fluorescent base analogue was first recognised, 2-aminopurine (2AP) continues to be the most widely used fluorescent probe of DNA structure and the perturbation of that structure by interaction with enzymes and other molecules. In this review, we begin by considering the origin of the dramatic and intriguing difference in photophysical properties between 2AP and its structural isomer, adenine; although 2AP differs from the natural base only in the position of the exocyclic amine group, its fluorescence intensity is one thousand times greater. We then discuss the mechanism of interbase quenching of 2AP fluorescence in DNA, which is the basis of its use as a conformational probe but remains imperfectly understood. There are hundreds of examples in the literature of the use of changes in the fluorescence intensity of 2AP as the basis of assays of conformational change; however, in this review we will consider in detail only a few intensity-based studies. Our primary aim is to highlight the use of time-resolved fluorescence measurements, and the interpretation of fluorescence decay parameters, to explore the structure and dynamics of DNA. We discuss the salient features of the fluorescence decay of 2AP

when incorporated in DNA and review the use of decay measurements in studying duplexes, single strands and other structures. We survey the use of 2AP as a probe of DNA-enzyme interaction and enzyme-induced distortion, focusing particularly on its use to study base flipping and the enhanced mechanistic insights that can be gained by a detailed analysis of the decay parameters, rather than merely monitoring changes in fluorescence intensity. Finally we reflect on the merits and shortcomings of 2AP and the prospects for its wider adoption as a fluorescence-decay-based probe.

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1. Introduction

The genetic code is protected from photo-induced damage by the extremely short, sub-picosecond excited state lifetimes of the nucelobases. While this is highly advantageous to life, it is a considerable inconvenience to biophysical science because it has the inevitable consequence that DNA is essentially non-fluorescent. This means that fluorescence techniques, amongst the most powerful for the study of biomolecular systems, can only be applied to nucleic acids by the introduction of an extrinsic fluorophore into the molecular structure. 2-Aminopurine (2AP) is one such fluorophore and is unique in its structural similarity to a natural nucleobase. 2AP differs from adenine (6-aminopurine) only in the position of the exocyclic amine group, and yet its fluorescence intensity is one thousand times that of adenine.

It is important at this point to make the distinction between a fluorescent label and a fluorescent probe. A fluorescent label serves to confer detectable fluorescence on an otherwise non-fluorescent species, or to provide fluorescence in a spectral region that differs from the intrinsic fluorescence of the species. A fluorescent probe, on the other hand, reports on its local molecular environment through a change in its fluorescence properties (intensity/lifetime/wavelength) in response to interaction with this environment. 2AP is not valuable as a fluorescent label because its fluorescence is highly quenched by stacking with the natural bases, when it is inserted in an oligonucleotide. However, it is this very susceptibility to interbase quenching that makes 2AP an exquisitely sensitive fluorescent probe of nucleic acid structure. Fluorescent base analogues that are environmentally insensitive labels, valuable in techniques such as Förster resonance energy transfer (FRET) and fluorescence

anisotropy, have been reviewed recently (Wilhelmsson, 2010) and will not be discussed here.

Using solid-phase synthesis and phosphoramidite chemistry, 2AP can be substituted for a natural base at any selected position in an oligonucleotide. The introduction of 2AP into DNA, in this fashion, causes minimal disturbance to the duplex structure. Melting temperatures of single-2AP-substituted duplexes are typically 1-3 ^{o}C less than the unsubstituted duplex (Eritja et al., 1986; Nordlund et al., 1989; Xu et al., 1994). 2AP forms a Watson-Crick base pair with thymine (T), as shown by NMR spectroscopy (Sowers et al., 1986) and revealed clearly by X-ray crystallography, as illustrated by the structure in Figure 1. It forms a wobble base pair with cytosine (C) (Neely et al., 2005; Sowers et al., 2000) or adenine (A) (Fazakerley et al., 1987) and can form a Hoogsteen-type base pair with guanine (G), as shown by the X-ray structure in Figure 2 (Neely et al., 2005). Spectroscopic and calorimetric measurements indicate the base pair stability to be 2AP-T>2AP-C>2AP-A>2AP-G (Law et al., 1996). The effects of substitution of adenine by 2AP on duplex structure and base pair dynamics have been examined in detail in a recent NMR study (Dallmann et al., 2010). It was found that substitution of adenine by 2AP in the central base pair of 13mer duplex did not affect the overall helical structure; slight local conformational perturbations at the substitution site were seen but these are weak compared with those reported for other base or base-pair analogues (Engman et al., 2004; Guckian et al., 1998; Guckian et al., 2000; Smirnov, 2002). In agreement with previous studies (Lycksell et al., 1987) the lifetime (opening times) of the 2AP-T base pair was found to be significantly shorter that of the A-T base pair, 1.6 ms compared with 8 ms. Moreover, a cooperative effect on base dynamics was observed,

with lifetimes of three adjacent base pairs in either direction reduced by around 30% by the substitution of 2AP for A. Although introduction of 2AP is not entirely without structural repercussions, an over-riding, crucially important fact is that 2AP-substituted oligonucleotides are biologically active as substrates for enzymes that modify DNA.

In this review, we begin by considering the dramatic difference in photophysical properties between 2AP and its structural isomer, adenine, the origin of which has intrigued physical chemists and been widely studied by both experimentalists and theorists. Even if it had proved useless as a fluorescent probe, 2AP would still stand out as a paradigm of molecular photophysics! We then turn to the mechanism of interbase quenching of 2AP fluorescence in DNA which is the basis of its use as a conformational probe, but remains imperfectly understood.

There are hundreds of examples in the literature of the use of changes in the fluorescence intensity of 2AP as the basis of assays of conformational change in studies of DNA, and numerous, although fewer, examples of its use in studies of RNA. However, in this review we will consider in detail only a few intensity-based studies, in the context of base (nucleotide) flipping. In considering the applications of 2AP as a fluorescent probe, our main aim is to highlight the use of time-resolved fluorescence measurements, and the interpretation of fluorescence decay parameters, to explore the structure and dynamics of DNA and the perturbation and manipulation of the duplex structure by enzymes. In Section 3, we discuss the salient features of the fluorescence decay of 2AP when incorporated in DNA and review the use of decay measurements in studying duplexes, single strands and other structures. In Section 4, we survey the use of 2AP as a probe of DNA-enzyme interaction and enzyme-induced

distortion. We focus particularly on its use to study base flipping by methyltransferases (and other enzymes), an area in which it has found extensive application. We highlight the enhanced mechanistic insights that can be gained by a detailed analysis of the decay parameters, rather than merely monitoring changes in fluorescence intensity.

The fluorescence decay measurements discussed in this review have been conducted mainly using the technique of time-correlated single photon counting, which is ideal for measurements in the range of ns down to 10's of ps. However, there have been a few reports of ultrafast, pump-probe fluorescence decay measurements which can detect sub-picosecond lifetimes. These are discussed briefly in Section 5.

Finally, in conclusion, we reflect on the merits and shortcoming of 2AP, the prospects for its wider adoption as a fluorescence decay-based probe and whether ultrafast measurements offer significant added value.

2. Fluorescence properties and photophysics of 2-aminopurine

2.1 Intrinsic properties of the fluorophore

The fluorescence properties of 2AP (and a number of its derivatives) were first reported by Stryer and coworkers (Ward et al., 1969). 2AP has a quantum yield of 0.68 in aqueous solution, compared with ~ 10^{-4} for adenine, and an emission maximum at ~370nm. Importantly, its excitation maximum, at ~303nm, lies to the red of the absorption of the nucleic bases and aromatic amino acids, allowing it to be excited selectively in DNA or RNA, and in the presence of proteins.

The fluorescence quantum yield of 2AP decreases markedly with decreasing polarity of the solvent environment (a five-fold reduction in dioxane relative to water),

accompanied by shifts in the emission spectrum to shorter wavelength (Evans et al., 1992; Rachofsky et al., 2001a; Ward et al., 1969). However, as shown by Rachofsky et al, specific hydrogen bonding with water has negligible effect on the fluorescence quantum yield or emission wavelength. The sensitivity of fluorescence quantum yield to solvent polarity was suggested to be due to the participation of an nπ* state in the non-radiative decay of the emitting ππ* state, with the nπ* lying higher in energy in water, but being stabilised relative to the ππ* state in non-polar solvents. (Rachofsky et al., 2001b).

The striking difference in photophysical properties between 2AP and adenine has been the subject of numerous theoretical studies, as summarised by Serrano Andres and coworkers (Serrano-Andres et al., 2006). The currently accepted explanation of the non-fluorescence of adenine, proposed by these authors and others, is that ultrafast excited state decay occurs by internal conversion to the ground state, facilitated by barrierless relaxation from the initially excited $^1\pi\pi^*$ state to a conical intersection between the excited and ground state potential energy surfaces. (A conical intersection can be thought of as an energy funnel between two electronic states where the probability of non-adiabatic, non-radiative jumps is high.) Initial calculations of Serrano Andres et al. on the isolated molecule *(*Serrano-Andres et al., 2006*)* predicted that, although such a conical intersection exists in 2AP, it cannot be accessed rapidly from the initially excited state because of an intervening potential energy barrier; the ultrafast decay pathway is, therefore blocked, resulting in a relatively high fluorescence quantum yield. A subsequent computational study examined the effects of aqueous solvation (Ludwig et al., 2008) and predicted that hydration increases the height of the barrier to the conical intersection, in line with the

experimentally observed increase in quantum yield in polar solutions. It was concluded that the photophysics of 2AP was governed by the accessibility of the conical intersection with the ground state and, contrary to previous proposals, the $^1n\pi^*$ state did not play an important role, since it was found to be higher in energy than the $^1\pi\pi^*$ state. However, this picture was soon to be challenged by new experimental data.

The important influence of solvation on the fluorescence of 2AP was emphatically demonstrated by the recent revelation that 2AP is barely fluorescent in the gas phase (Feng et al., 2009; Lobsiger et al., 2011). These high-resolution spectroscopic studies of 2AP molecules, free from collisions and cooled to a few Kelvin in supersonic molecular beams, showed the fluorescence lifetime of the isolated molecule to be 77 ps, corresponding to a quantum yield of about 0.005. They also provided clear evidence that the main non-radiative channel is the decay to a close-lying $^1n\pi^*$ dark state (a state which is neither emissive nor accessible by direct optical excitation), followed by intersystem crossing to the long-lived lowest triplet ($^3\pi\pi^*$) state. This led to reiteration of the proposal that the fluorescence quantum yield is high in polar solvents because the $^1n\pi^*$ state is shifted to higher energy, and a re-examination of the computational predictions. As discussed in detail by Lobsiger et al., the predicted relative energies of the $^1\pi\pi^*$ and $^1n\pi^*$ states depend on the computational method used (Feng et al., 2009; Lobsiger et al., 2011); by adopting a method different from those used in previous studies, they achieved good agreement between the calculated and experimental excited state energies for the isolated molecule. Definitive experimental evidence for the effect of solvation on the relative energies of $^1\pi\pi^*$ and

$^1n\pi^*$ states can be obtained, in principle, from high resolution spectroscopy of 2AP-solvent complexes in supersonic molecular beams. Although recent experiments (Lobsiger et al., 2013; Sinha et al., 2011) have demonstrated the ability to produce 2AP-H_2O clusters, containing up to three water molecules, and identify their different isomeric forms, a detailed analysis of their energy level structure has yet to be achieved.

Very recent experiments (Reichardt et al., 2013) have demonstrated for the first time the significant role of intersystem crossing in the fluorescence quenching of 2AP in solution. The triplet yield was found to depend sensitively on the hydrogen-bonding ability and polarity of the solvent, with a value of 0.4 in acetonitrile, decreasing to 0.2 in ethanol and 0.08 in aqueous buffer. This correlates with the increasing trend in fluorescence quantum yield: 0.26 in acetonitrile, 0.47 in ethanol and 0.68 in buffer. This lends strong support to the mechanism of $n\pi^*$-mediated intersystem crossing indicated by gas-phase studies. It can be inferred from the fluorescence and triplet quantum yields that there is a second solvent-independent non-radiative channel, with a yield of about 0.3; this is most likely internal conversion from the $^1\pi\pi^*$ state to the ground state.

The fluorescence decay of the 2AP base in aqueous solution is often reported to be monoexponential, with a lifetime around 11.5 ns (Holmén et al., 1997; Lakowicz, 2006; Rachofsky et al., 2001a; Rachofsky et al., 1998), and this is indeed the case for an individual decay curve measured at a particular excitation and emission wavelength. However, measurement over a range of excitation and emission wavelengths revealed that the decay is, in fact, biexponential, as a result of the presence of both the (minor) 7H and (major) 9H tautomers which differ slightly in

their lifetimes and their spectra. (He et al., 2006; Liang & Matsika, 2011; Neely et al., 2004). The fluorescence decay of 2AP riboside, in which tautomerism is absent, is genuinely monoexponential with a lifetime of 10.6 ns in water (Neely et al., 2004). In line with the observed decrease in quantum yield, the lifetime decreases in less polar solvents; in ethanol, for example, it is 5.8 ns. The substitution of hydrogen at the N(9) position by ribose or deoxyribose has negligible effect on the photophysics of the 2AP fluorophore (Reichardt et al., 2013). The small discrepancies in the values of quantum yields and lifetimes between the free base and the nucleosides, found in the literature, can be accounted for by the presence of the 7H tautomer in the former case. Although tautomerism is not directly relevant to the photophysics of 2AP as a fluorescent probe, since it does not occur in the nucleoside, it reinforces the close analogy between 2AP and adenine.

Under conditions where there is a strong π-stacking interaction between a pair of 2AP molecules, or between 2AP and a natural base, a second longer-wavelength emission band is observed at around 450 nm, in addition to the familiar short-wavelength spectrum at 370 nm. This is due to formation of an electronically coupled ground-state dimer, which has lower excitation energy than the 2AP monomer. The long-wavelength emission was first identified by Rist et al. in a DNA duplex containing four pairs of adjacent 2AP bases (Rist et al., 2002); they observed a weak, red-shifted emission band, in addition to the 'normal' 2AP fluorescence spectrum, and a corresponding red-shifted excitation spectrum. In a study of the X-ray structure and fluorescence of crystalline 2AP, we showed that interaction between 2AP molecules in the π-stacked structure of the crystal lattice gave rise to analogous long-wavelength emission (Neely et al., 2007). Subsequent examination of a variety of 2AP-containing

oligodeoxynucleotide duplexes and single strands showed that dual fluorescence is a general property of 2AP in DNA (Bonnist & Jones, 2008). We suggested that red-shifted fluorescence arises from conformational states of the duplex in which 2AP forms a highly eclipsed, π-stacked structure with one of its neighbouring bases. The long-wavelength fluorescence is usually much weaker than the short-wavelength band and escapes observation at the typically used excitation wavelengths of 300-320 nm; however, it is revealed by selective excitation at around 360 nm. The low intensity of the long-wavelength fluorescence means that it is unlikely to add to the utility of 2AP as a fluorescent probe of DNA structure and has not been explored in this regard, although it is another manifestation of the sensitivity of 2AP to base stacking interactions. Excitonic coupling between stacked pairs of 2AP molecules in DNA has, however, been exploited in low energy (> 300 nm) circular dichroism spectroscopy to probe DNA conformation (Finger et al., 2013; Johnson et al., 2004; Jose et al., 2009).

2.2. Quenching of 2-aminopurine fluorescence by stacking with the natural bases

As well as identifying 2AP as a fluorescent analogue of adenine, the Stryer group made the crucial observation that its fluorescence was quenched in polynucleotides and recognized its potential for probing enzyme-induced disruption of nucleic acid structure (Ward et al., 1969). When 2AP is substituted for a natural base in DNA, it displays an emission band with a maximum at ~370 nm that is essentially unchanged in spectral profile from that the of the free nucleoside, but is reduced in intensity by a factor of 10 to 100. Several early studies showed that the fluorescence is quenched by stacking interactions with neighbouring bases and the quenching efficiency is highly

sensitive to the local duplex conformation (Guest et al., 1991; Nordlund et al., 1989; Rachofsky et al., 2001c; Xu et al., 1994).

Barton and coworkers have presented a substantial body of evidence that excited 2AP is quenched efficiently by electron transfer from guanine and have exploited this effect in studying the mechanism of charge transfer in DNA (see, for example, (Kelley, 1999; O'Neil & Barton, 2002; O'Neill & Barton, 2002; O'Neill & Barton, 2004a; O'Neill & Barton, 2004b; O'Neill et al., 2003; O'Neill et al., 2004; Wan et al., 2000). The following observations have emerged from their work. Intrastrand electron transfer is more efficient than interstrand transfer, though both occur. The electron transfer efficiency (from guanine to excited 2AP) is greater in the 3'-5' direction and is less dependent on distance than in the 5'-3' direction. Electron transfer is possible between guanine and excited 2AP over distance of up to ~14Å. The transfer is most efficient when the bridging bases, between G and 2AP, are adenines. Pyrimidine bases attenuate the transfer efficiency significantly. A full understanding of the mechanisms of charge transport in the duplex has yet to be achieved (see, for example, (Genereux & Barton, 2010)), but an established feature, that is relevant to the use of 2AP as a probe, is conformational gating, whereby conformational fluctuations of the duplex facilitate charge transfer by allowing the formation of charge-transfer-active states. Indeed, in frozen duplexes, the quenching of 2AP fluorescence is dramatically reduced (Neely & Jones, 2006; O'Neill & Barton, 2004a) and, if there is no guanine in close proximity, virtually eliminated. Although the role of guanine is well-established, the extent to which excited 2AP in DNA is quenched by charge transfer (CT) with the other natural bases, and the contribution of other, non-CT processes remain matters of debate.

Excited 2AP cannot be quenched by electronic energy transfer to the natural bases, since its excitation energy is lower than theirs. 2AP acts as an acceptor for energy transfer from the excited natural bases and has been used as an energy trap in studies of interbase energy transfer, as described in a review by Nordlund (Nordlund, 2007). Although this is an interesting application of 2AP, it is outside the scope of the present review and will not be discussed further.

2AP-containing deoxydinucleotides have been the subject of several studies to explore the quenching interaction between the natural bases and 2AP. Larsen *et al* reported steady-state fluorescence measurements of 2AP in dinucleotides with each of the natural DNA bases, and with inosine (I), the riboside of hypoxanthine (Larsen et al., 2004). Inosine is deemed to be redox-inactive towards excited 2AP and was adopted as a non-CT control in the electron transfer studies of Barton *et al*. (*vide supra*). 2AP fluorescence was found to be quenched in all of the dinucleotides and the quenching efficiency, G>T~A>C>I, could be correlated with the driving force for interbase charge transfer. In addition to CT-quenching, they invoked the existence of a non-fluorescent excited state of 2AP (supported by transient absorption measurements) to account for quenching by inosine. The relationship between quenching efficiency and the driving force for charge transfer between the natural base and excited 2AP, greatest for G and least for C, was reinforced by a recent study of photo-induced electron transfer between 2AP and the natural monophosphate nucleotides in aqueous solution (Narayanan et al., 2010). This study included a re-assessment of the redox potentials of the natural bases and excited 2AP, using cyclic voltammetry in aprotic solvents to avoid complications introduced by proton transfer. It was concluded that photoinduced electron transfer could occur between all of the

natural bases and 2AP, with excited 2AP being reduced by G or A, and oxidised by T. (For C, the free energies for oxidation and reduction were equivalent, within experimental error). Time-resolved fluorescence studies (Somsen et al., 2005a; Somsen et al., 2005b; Somsen et al., 2006) confirmed that fluorescence of 2AP is quenched strongly in dinucleotides by the natural bases and inosine, and inferred the involvement of a dark state. An ultrafast (time resolution of ~200 fs) spectroscopic study of 2AP-G and 2AP-A dinucleotides provided further insight into the quenching process (Wan et al., 2005). A important and unusual feature of this work was the use of both fluorescence upconversion, to probe the ultrafast decay of the initial, optically excited state, and transient absorption spectroscopy, to reveal the existence of dark states formed in the decay process. Two significant observations emerged from this work. Firstly, a discrepancy between the decay time measured by fluorescence upconversion and that from transient absorption indicated the existence of a dark state to which the initially excited, emissive state decays. An emission decay time on the 10's of ps timescale was attributed to charge transfer (to form the dark state) while the decay time of the dark state, also on the 10's of ps timescale was attributed to charge recombination. Secondly, 50% of the initial fluorescence intensity of 2AP-G and 2AP-A was lost in less than 200 fs, faster than the time resolution of the measurement. This extremely rapid non-radiative decay was attributed to barrierless charge transfer by vibrationally hot molecules prior to vibrational relaxation. Unfortunately this study did not include 2AP-inosine as a CT-free control system. Further evidence of ultrafast inter-base quenching of 2AP fluorescence comes from observed discrepancies between the fluorescence quantum yields determined from steady state intensity measurements and those from the measurement of time-resolved

decay parameters (discussed in more detail below); the latter are higher than the former, indicating the occurrence of non-radiative decay processes that are too fast to be observed in typical decay measurements, that is to say with lifetimes less than about 10 ps. Such discrepancies have been observed for 2AP in duplexes and single strands (Avilov et al., 2008; Godet et al., 2011; Neely et al., 2009; Rachofsky et al., 2001c). Ultrafast fluorescence measurements on 2AP-containing DNA duplexes and single strands have shown fluorescence decay times of < 5 ps, with values as low as 400 fs when 2AP is closely stacked with G (Gelot et al., 2012; Manoj et al., 2008). The base-sequence-dependence of the decay times observed by Manoj et al. implies that ultrafast quenching on the sub-ps to ps timecale (as well as quenching on the 10-100 ps timescale) is due to charge-transfer processes. Gelot et al. found good agreement between decay-derived and intensity derived quantum yields showing the absence of unresolved, faster (< 300 fs) decay processes, contrary to the findings of Wan et al. for dinucleotides (Wan et al., 2005). Similarly, the experiments of Manoj et al found no decay time faster than 420 fs, although their time resolution should have allowed observation of decay components < 100 fs.

The nature of the excited states that may be involved in interbase quenching of 2AP have been explored in computational studies of gas-phase 2AP-nucleobase dimers (and trimers), in which 2AP is stacked with a DNA base in a B-form structure, but there is no covalent link between them. Early calculations, using time-dependent density functional theory (TDDFT), attributed quenching to internal conversion to dark charge-transfer states, energetically below the initially excited 2AP-like transition.(Jean & Hall, 2001; Jean & Hall, 2002). However, it is now known that the TDDFT methods fail to compute CT states accurately and later studies using

configuration interaction singles (CIS) predicted that CT states are higher in energy than the locally excited 2AP ππ* state (Hardman & Thompson, 2006; Hardman & Thompson, 2007). Two recent computational studies (CIS with second order perturbation theory) by Matsika and coworkers, on 2AP-pyrimidine dimers (Liang & Matsika, 2011) and 2AP-purine dimers (Liang et al., 2013), represent a significant advance over previous studies by exploring the fate of the excited state population after absorption. Excited states were calculated for a typical B-DNA conformation and relaxation along the S_1 surface was then examined. Ground state structures with 2AP in either the 5' or 3' positions were considered; these differed in the extent of π-overlap and other, specific interactions. From the initially excited (Franck-Condon) S_1 state, which was a ππ* state localised on 2AP, different quenching pathways were found, depending on whether 2AP was in the 5' or 3' position, as well as the identity of the partner base. Some quenching pathways involved charge transfer, but pathways involving conical intersections or dark states localised on 2AP were also found. The important outcome of these studies was the prediction that different interbase interactions, in the different initially excited conformations, can lead to alternative CT or non-CT quenching mechanisms. However, even this latter, relatively sophisticated computational scenario is far-removed from experimental conditions; it did not include the sugar-phosphate backbone or solvent. A full understanding of the photophysical mechanism(s) of inter-base quenching awaits the development of computational methods to successfully include the complex influence of solvation and conformational dynamics, in both the ground and excited states. A tall order, indeed!

3. The fluorescence decay of 2-aminopurine in DNA as a reporter of conformational properties

The interbase interactions that quench 2AP fluorescence in DNA not only have the effect of dramatically shortening the fluorescence decay time (Figure 3), but also result in a complex, multi-exponential decay function. This complex decay behaviour is observed for systems that are apparently homogeneous, such as a solution of identical oligonucleotides each of which contains a single 2AP in the same sequence position, and reflects the conformational heterogeneity of DNA that arises from thermal motion of the bases (DNA breathing). The excited 2AP population is partitioned between several different local conformational environments that provide distinctly different quenching efficiencies, resulting in a number of different fluorescence lifetimes. Time-resolved fluorescence studies of 2AP-containing DNA have been conducted mainly using the technique of time-correlated single-photon counting (Becker, 2005; Lakowicz, 2006); with a time-resolution of around 30 ps, and it is the results of these experiments that will be considered in this section. The relatively few ultrafast measurements (time resolution <1 ps) will be discussed separately in section 5. It should be also be mentioned that, although multi-exponential fitting of the 2AP fluorescence decay is the conventional approach, other descriptions of the decay function have been proposed, such as lifetime distributions (Fogarty et al., 2011), stretched exponential (Somsen et al., 2006) and phasor diagrams (Buscaglia et al., 2012).

3.1 2-aminopurine in duplex oligonucleotides

2AP-containing DNA duplexes generally show fluorescence decays that can be described by four exponential components, as expressed by Equation (1), with typical lifetimes of

<100 ps, ~0.5 ns, ~2 ns, and ~10 ns (Avilov et al., 2008; Guest et al., 1991; Hochstrasser et al., 1994; Neely et al., 2005; Neely & Jones, 2006; Nordlund et al., 1989; Rachofsky et al., 2001c; Ramreddy et al., 2009; Ramreddy et al., 2007; Sabir et al., 2012).

$$I(t) = \sum_{i=1}^{4} A_i \exp\left(\frac{-t}{\tau_i}\right) \qquad (1)$$

where I is the fluorescence intensity, measured as a function of time, t; τ_i is the fluorescence lifetime of the i^{th} decay component and A_i is fractional amplitude of that component.

In a simple interpretation, each lifetime, τ_i, can be considered to represent a distinct conformational state and its fractional amplitude (pre-exponential factor or A factor), A_i, indicates the fraction of the population occupying that state. More realistically, each lifetime is likely to represent a distribution of conformations in which 2AP experiences similar quenching rates.

Although the precise values of the lifetimes and A factors vary to some extent, depending on the sequence context of 2AP, their general magnitudes in the unperturbed duplex show a common pattern, with an accepted, common interpretation. The very short lifetime component, τ_1, <100ps, is attributed to a highly stacked conformation, in which excited 2AP is rapidly quenched by interbase interaction, primarily electron transfer from guanine bases in close proximity. This is the dominant conformation, typically accounting for more than 70% of the emitting population. The long, ~10ns, lifetime, τ_4, is attributed to an unstacked conformation in which 2AP is extrahelical and experiences a solvated environment, free from quenching interactions. The value of this decay time is comparable with that of free 2AP-riboside in solution. This is a minor conformation, typically accounting for <5%

of the emitting population. The intermediate lifetimes, τ_2 and τ_3, ~500 ps and ~2 ns, respectively, correspond to imperfectly or partially stacked structures, in which 2AP is intrahelical, but is not subject to rapid charge-transfer quenching. The magnitude of the A factors (fractional populations) almost invariably follow the trend $A_1 >> A_2 > A_3 > A_4$.

When interpreting the values of the decay parameters of 2AP obtained from fitting multi-exponential functions, it is important to be aware of the problem of correlation between A factors and lifetimes (Lakowicz, 2006). In the fitting process, the lifetime can be varied to compensate for the A factor and vice versa, so that equally good fits (within experimental error) can be obtained with different combinations of A_i and τ_i. This problem can be minimized by the use of global analysis in which a set of decays is recorded as a function of emission wavelength (or another suitable variable) and fitted simultaneously, with the lifetimes, τ_i, as common parameters (Beechem et al., 2002).

Many studies of DNA-enzyme interaction are based on measurement of the fluorescence intensity of 2AP and it may be informative at this point, to consider the relationship between the intensity (quantum yield) of fluorescence and the decay parameters. The quantum yield (and hence intensity) of fluorescence of the emitting species that contribute to the observed fluorescence decay is proportional to the number-average lifetime, $\langle \tau \rangle$, of the multi-component decay. The decay-derived value that is normally quoted is the quantum yield relative to the free riboside, Φ_{rel}, as defined by Equation (2).

$$\Phi_{rel} = \frac{\sum A_i \tau_i}{\sum A_i} \frac{1}{\tau_{2\mathrm{APr}}} = \frac{\langle \tau \rangle}{\tau_{2\mathrm{APr}}} \qquad (2)$$

where τ_i is the fluorescence lifetime of the i^{th} decay component, A_i is fractional amplitude of the i^{th} component and $\tau_{2\mathrm{APr}}$ is the fluorescence lifetime of 2AP riboside. The fractional contribution of each decay component to the steady-state fluorescence intensity, F_i, is determined by the product of the A-factor and lifetime for that component, as expressed by Equation (3). Thus, the steady-state fluorescence spectrum of 2AP in DNA arises mainly from the long-lifetime components (τ_3 and τ_4), although they constitute only a small minority if the emitting population.

$$F_i = \frac{A_i \tau_i}{\sum A_i \tau_i} \qquad (3)$$

The decay parameters shown in Table 1, for a single 2AP at two different positions in a 50-base-pair duplex (Sabir et al., 2012) are typical of those measured for 2AP-containing duplexes. Each duplex has an identical sequence, apart from the position at which 2AP is substituted in place of adenine. The effect of sequence context on the two shortest lifetimes, $\tau_1$and τ_2, and their amplitudes, $A_1$and A_2, is apparent. In sequence context CPT (where 2AP is denoted as P), a larger fraction of the 2AP population is highly stacked (84% compared with 70% in TPG), and in this highly stacked state, 2AP experiences faster quenching (shorter τ_1). These characteristics can be correlated with the proximity of multiple G:C base pairs to 2AP in the CPT position, resulting in a more constrained, tightly stacked local structure. The fractional intensity values show that the unquenched, extrahelical component (F_4) accounts for about 50% of the steady state intensity for each duplex, although it constitutes only few percent of the conformational population (A_4).

5′GTCGGATCCTCT**P**GATATCTCC**P**TGCTCACTGGTTATAGGAGAATCCGGG 3′
3′CAGCCTAGGAGATCTATAGAGGTACGAGTGACCAATATCCTCTTAGGCCC 5′

Context	τ_1/ns	τ_2/ns	τ_3/ns	τ_4/ns	A_1	A_2	A_3	A_4	F_1	F_2	F_3	F_4
TPG	0.08	0.50	2.9	10.2	0.70	0.17	0.09	0.04	0.07	0.11	0.32	0.50
CPT	0.05	0.37	2.7	10.6	0.87	0.08	0.03	0.02	0.12	0.08	0.22	0.57

Table 1. Fluorescence lifetimes, τ_i, and corresponding fractional amplitudes, A_i, for 2AP-containing duplexes in which 2AP, denoted as P, is substituted at two alternative positions within the same sequence (Sabir et al., 2012). The fractional contribution of each component to the steady state intensity, F_i, is also shown.

Early time-resolved fluorescence studies of 2AP in duplexes (Guest et al., 1991; Nordlund et al., 1989) demonstrated the non-exponentiality of the decay and that four lifetime components were needed give a satisfactory fit. In both studies, the temperature-dependence of the decay parameters was found to be consistent with the assignment of the shortest lifetime (τ_1) to intra-helical, stacked 2AP and the longest lifetime (τ_4) to extrahelical 2AP. Guest et al also found that the temperature-dependence of the stacked population (A_1) was correlated with the strength of the base-pair (decreasing with increasing temperature for the weaker base-pairs 2AP-A and 2AP-C). Both studies inferred a relationship between the distribution of lifetimes and stacking-unstacking motions of the duplex.

As part of a study of polymerase-induced melting of a DNA helix terminus, Hochstrasser et al used time-resolved fluorescence to analyze the effect of base-pairing interactions on the fluorescence decay of an end-terminal 2AP base in DNA primer/templates (Hochstrasser et al., 1994). They used global analysis and, for the first time, gave a detailed interpretation of the four-exponential fluorescence decay behaviour in terms of distinct, ground-state conformational states and their

populations. The variation of the apparent fraction of paired (highly stacked) 2AP bases with temperature was found to be in accord with optical melting data, and the extent of base-pairing observed in each duplex was consistent with the base-pairing preferences of 2AP established in previous studies.

A study by Rachofsky et al. of duplexes in which 2AP was paired with an abasic site in different sequence contexts provided further evidence of 4-component decays and also identified discrepancies between steady-state quantum yield values and those derived from decay parameters (Rachofsky et al., 2001c). However, a detailed interpretation of the decay parameters was not attempted. Instead, the discussion concentrated on the amplitude of the extrahelical component and attributed the other three components to a single conformation represented by a number average lifetime. This provides little insight into the conformational properties beyond that obtainable from the accompanying steady-state data.

During a study of nucleotide flipping by M.HhaI, discussed in Section 4.3, below, we investigated the decays of two crystalline 2AP-containing duplexes with known x-ray structures (Neely, 2005; Neely et al., 2005). The duplexes were co-crystallised as ternary complexes with the enzyme and cofactor, but 2AP was located several base-pairs from the protein binding site, so that the local duplex structure was not appreciably perturbed, as illustrated by the crystal structures in Figure 4. As shown in Figure 5 the two duplexes differ only in the identity of base opposite the 2AP, thymine and guanine, respectively, but 2AP occupies distinctly different conformations in each. In duplex PT (Figure 5(a), 2AP, in an *anti* conformation, forms a Watson-Crick base-pair with thymine, whereas in duplex PG (Figure 5(b), 2AP adopts a *syn* conformation, forming a Hoogsteen-type base pair with the guanine.

The consequent difference in stacking interactions between 2AP and the two neighbouring guanines (Figure 5(c) and (d)) is reflected in the decay parameters (Table 2) which suggest that 2AP is more effectively quenched in PG than in PT, consistent with increased π-overlap in the former.

Duplex	τ_1/ns	τ_2/ns	τ_3/ns	τ_4/ns	A_1	A_2	A_3	A_4
PT (crystal)	0.09	0.67	2.3	7.3	0.69	0.19	0.18	0.05
PG (crystal)	0.07	0.53	2.1	7.4	0.62	0.21	0.14	0.03
PT (solution)	0.06	0.64	3.1	10.1	0.60	0.15	0.12	0.13
PG (solution)	0.03	0.47	2.8	10.2	0.70	0.12	0.08	0.10

Table 2. Fluorescence lifetimes, τ_i, and corresponding fractional amplitudes, A_i, for two 2AP-containing duplexes in the crystalline state and in solution.(Neely, 2005; Neely et al., 2005).

As shown in Table 2, the fluorescence decays of the crystalline duplexes show four components with similar lifetimes and A-factors to those found in the equivalent solution-phase systems (enzyme-bound duplexes), and thus reveal conformational heterogeneity in the crystals at room temperature that is not apparent in the low-temperature x-ray structure. The fluorescence decay can detect the existence of species that constitute only a few percent of the excited state population and is sensitive to transient conformational states that exist on the timescale of the excited state lifetime. The crystal structure, on the other hand, shows the average or dominant conformational geometry at low temperature (90 K), within the limits of the available structural resolution. Small differences between crystalline and solution-phase decay parameters reflect the greater conformational mobility in solution; the lifetime of the highly quenched conformations (τ_1) is significantly shorter in solution than in the

crystal, consistent with greater dynamic freedom and enhanced charge-transfer quenching. However, comparison with the behaviour of duplexes rendered rigid in a frozen matrix at 77 K (*vide infra*) suggests that in the crystal, at room temperature, the base motions that facilitate quenching of 2AP remain largely uninhibited.

Our decay measurements on rigid duplexes in frozen solution at 77 K (Neely & Jones, 2006) threw light on nature of the conformational states that give rise to the heterogeneous decay of 2AP and the role of base dynamics in populating these states. The shortest decay component, τ_1, was eliminated in frozen duplexes, even when 2AP was stacked directly G, demonstrating the importance of base dynamics in facilitating rapid charge-transfer quenching. It was inferred that the highly stacked geometry that gives rise to the very short decay time can be attained only through thermal motion of the bases. On the other hand, the persistence of three longer decay components at 77 K implies the existence of a number of discrete, equilibrium conformational states that can be characterized by three distinguishable intrinsic decay times.

Rao, Krishnamoorthy and coworkers have probed site-specific dynamics in several DNA systems (Goel et al., 2010; Ramreddy et al., 2009; Ramreddy et al., 2007) by substituting 2AP at different positions relative to the ends and employing repeats of adenine to avoid sequence-specific quenching effects. In 30-nucleotide, poly(A-T) duplexes (Ramreddy et al., 2007), it was found that the value of the short lifetime component was dependent on the position of 2AP. The lifetime was longer when 2AP was placed at the end of the duplex, compared with the centre of the duplex, indicating weaker base stacking interactions as a result of base-pair fraying. In double-stranded DNA with singe-strand overhangs, base-pair fraying at the ends of the duplex region was largely absent. It was proposed that wrapping by the single-

strand region around the end base-pairs formed three-centred hydrogen-bonds and inhibited fraying. An unexpected finding was that the level of stacking interaction of 2AP with near neighbours was weaker within the longer strand than within the shorter strand. It was suggested that the presence of single-strand overhangs in the longer strand could be the cause of higher flexibility in the duplex region. It was speculated that DNA enzymes that discriminate between the long versus short strands might rely on such differences in dynamics as recognition cues for achieving specific binding during DNA repair events. In a later study, the same approach was used to investigate the effects of binding DNA to histone and micellar surfaces (Goel et al., 2010). It was observed that binding causes is an increase in rigidity of DNA backbone, and drastic dampening of the internal dynamics and fraying motions. A significant weakening of base-stacking interactions was also found.

In the interpretation of the decay parameters of 2AP in DNA, many authors use the terms ‘static’ and ‘dynamic’ to describe the quenching processes. However, the use of these terms in the context of this conformationally dynamic and heterogeneous system can be misleading. Conventionally, ‘dynamic’ quenching is used to describe processes such as collisions or conformational changes that occur following excitation of a fluorophore, and result in non-radiative decay within the excited state lifetime; quenching is described as ‘static’ when the observed fluorescence lifetime is intrinsic to the ground-state structure that was excited and, following excitation, the system remains static on the timescale of the excited state lifetime. The very short decay times (< 100 ps) exhibited by 2AP in DNA are often described as static quenching, because it is perceived that there is insufficient time for dynamic process to occur within the excited-state lifetime. However, population of the conformations that are

susceptible to very rapid (charge-transfer) quenching, and exhibit the shortest decay times, likely involves base dynamics in the ground state, prior to excitation. Indeed, there is clear evidence that these rapid decay mechanisms are inhibited or arrested by increasing the viscosity (Avilov et al., 2008; Gelot et al., 2012; Godet et al., 2011) or freezing the sample (Neely & Jones, 2006). It can be argued that all the 2AP decay components reflect dynamic processes; they are distinguished merely by the timescale of the dynamics and whether the dynamics occur before or after excitation (or both before and after).

3.2 2-aminopurine in single-strand oligonucleotides

The fluorescence decay of 2AP is not sensitive to hydrogen-bonding interactions per se (Guest et al., 1991; Rachofsky et al., 2001a) and so 2AP is not a direct probe of base pairing. However, the perturbation of base-stacking interactions that occurs as a result of local unpairing of the duplex gives rise to characteristic changes in the fluorescence decay of 2AP, as we showed in a systematic comparison of seven 2AP-containing single strands and their corresponding duplexes (Ma, 2012; Sabir et al., 2012). As illustrated in Figure 6(a), the decay of 2AP in a single strand is qualitatively longer than in the corresponding duplex, implying an overall decrease in quenching, as might be expected. The decay parameters shown in Table 3, for a single 2AP at two different positions in a 50-base sequence are typical of those measured in this study. The decay parameters for the corresponding duplexes are given in Table 1.

5′GTCGGATCCTCT**P**GATATCTCC**P**TGCTCACTGGTTATAGGAGAATCCGGG 3′

Context	τ_1/ns	τ_2/ns	τ_3/ns	τ_4/ns	A_1	A_2	A_3	A_4
T2G	0.07	0.66	2.5	7.4	0.69	0.13	0.13	0.05
C2T	0.07	0.58	2.2	6.7	0.67	0.16	0.14	0.03

Table 3. Fluorescence lifetimes, τ_i, and corresponding fractional amplitudes, A_i, for 2AP-containing single strands in which 2AP, denoted as P, is substituted at two alternative positions within the same sequence. (Sabir et al., 2012,).

The very short decay component (τ_1) persists in the single strands, indicating that highly stacked conformations exist in the absence of base-pairing and, moreover, remain highly populated (values of A_1 >0.6). The longest lifetime (τ_4) is noticeably shorter in the single strands, showing that 2AP does not tend to escape completely from stacking interactions with neighboring bases, whereas in duplexes this component is characteristic of extrahelical (solvent-exposed) 2AP. The population of this more-or-less destacked state remains low in single strands. The most characteristic difference between single strands and duplexes lies in the relative values of the A factors. In duplexes, the values of the A factors generally follow the trend: $A_1>>A_2>A_3>A_4$. In single strands, there is a transfer of population from well-stacked states ($A_1$and A_2) to weakly stacked states (A_3), so that the trend becomes $A_1>>A_2\approx A_3>>A_4$. This is illustrated by comparing the ratio A_3/A_2 for each single strand and duplex, as shown in Figure 6(b); in the single strands this ratio is around 1, but is consistently much smaller in the duplexes. This increase in the heterogeneity of the conformational population is consistent with greater base mobility in the absence of base-pairing. The extent to which decay parameters change between duplex and single strand depends on the sequence context of 2AP. For example, in CPT, the large decrease in A_1 in the single strand can be correlated with release from the constraints of local G-C base-pairs, and the significant increase in τ_1 reflects the loss of inter-strand charge-transfer quenching of 2AP by adjacent Gs in the complementary strand.

Our comparison of the decay parameters of three shorter, 18-nucleotide, single stands and duplexes (as part of our study of DNA unpairing by Flap Endonuclease-1) (Finger et al., 2013), yielded similar results to those described above, although unpairing resulted in a somewhat greater depopulation of the highly stacked states in these oligonucleotides.

Ramreddy et al. examined the position-dependence of the 2AP-decay in 30-nucleotide adenine repeats (Ramreddy et al., 2007) in comparison with the corresponding duplexes (already discussed above). They found that the short decay component was completely lost in the single strands, yielding a 3-component decay and a significant increase in average lifetime (quantum yield). They also noted a reduction in the longest lifetime in the single strands, consistent with that noted above. The decay parameters of 2AP showed little dependence on position in the poly-A strand. Absence of the short component can be attributed to the low efficiency of charge-transfer quenching of 2AP by A, rather than the absence of stacked states. In the poly(A-T) duplexes, inter-strand quenching of 2AP by T is probably a significant contributor to the short decay time.

Avilov *et al* demonstrated the role of base dynamics in the ultrafast quenching of 2AP in single-stranded DNA (Avilov et al., 2008). They examined the effect of viscosity on the decays of 2AP-containing hexanucleotides, with 2AP in four different sequence contexts, A**P**CGCC, AACG**P**C, A**P**TGCC and AATG**P**C. By carrying out complementary measurements of the steady state quantum yield, they quantified the fractional population of the ultrafast-decaying dark species, which they include explicitly as an additional amplitude parameter, α_0, in their tabulated decay parameters. The value of α_0 is sequence-dependent, being significantly higher when

2AP is stacked with G, and is dramatically decreased at high viscosity (77% glycerol). This is consistent with an ultrafast charge-transfer quenching mechanism that involves base dynamics. For 2AP in the G**P**C context, α_0 has the remarkably high value of 0.8; that is to say 80% of the 2AP population exists in conformations (presumably extremely highly stacked) that are invisible to conventional (not ultrafast) fluorescence detection. Consistent with other reports, this study also found the value of the longest lifetime component to be substantially shorter than that of unquenched 2AP.

3.3 2-aminopurine in other DNA structures

Time-resolved fluorescence of 2AP has been used in only a few studies of non-duplex DNA structures, but these have addressed a variety of systems, including looped domains, junctions, and G-quadruplexes. This approach is particularly valuable in exploring variations in conformational dynamics between base-paired and non-paired domains and investigating structural transitions.

Lee et al substituted 2AP for adenine in selected positions within the 18-base loop domain of a $(GC)_3(CAG)_6(GC)_3$ hairpin oligonucleotide, a model triple-repeat system (Lee et al., 2007). The 4-component exponential decays revealed differences in the global DNA conformation between duplex, hairpin and single strand and also position-dependent variations in conformation and dynamics within the repeat DNA domain. Since the 2AP probe was always in the same sequence environment in this model system, the values of the lifetimes showed little variation, but the conformational differences were apparent in the variations of the A factors which reflect changes in conformational populations. In particular, the conformational

distribution at the centre of the CAG repeat differed significantly from that close to the hairpin stem, showing substantially higher population of poorly stacked and destacked states.

More recently, Godet et al. investigated the position-dependence of the 2AP decay in the 5-nucloetide loop domain of a DNA hairpin, in the context of studying the effect of binding of HIV-1 nucleocapsid protein on loop dynamics (Godet et al., 2011). They continued the practice of this group to evaluate the fractional population of the 2AP dark species, as well as the decay parameters that are measured directly by TCSPC (see Section 3.2). The loop structure was found to restrict the inter-base quenching of 2AP within the loop, compared with single-strand oligonucleotides. Nevertheless, the dark species were again found to be dominant, representing 80-90% of the 2AP population, indicating efficient charge-transfer quenching by neighbouring guanine bases (in each position studied, 2AP was flanked by a G). The values of the longest lifetime (τ_4) were close to that of free 2AP indicating an extrahelical, fully destacked state; this differed from the shorter τ_4 value seen in single strands, confirming the limited flexibility of the loop. The low population of the extrahelical conformation (1-4%) showed the bases to be oriented towards the interior of the loop. 2AP placed at the 3' end of the loop, stacked on the 3' side with a G at the top of the stem, was quenched more efficiently than 2AP within the loop and showed the lowest destacked population. Interestingly, Gelot et al subsequently made ultrafast fluorescence measurements on the same 2AP-containing loop structures (Gelot et al., 2012). For 2AP within the loop, two ultrafast decay components, of 3-5 ps and 10-40 ps, were revealed, with combined amplitudes consistent with the fractional population of the dark species estimated by Godet et al. For the most highly quenched 2AP, at the 3-

end of the loop, a third, sub-picosecond component was detected. There was good agreement between the quantum yields derived from the ultrafast decay parameters and those from the fluorescence intensities, showing that there remained no undetected decay processes faster than the 0.3-ps time resolution of the measurements. In further agreement with the observations of Mély and coworkers, (Avilov et al., 2008), the sub-5 ps decay components were found to be entirely suppressed in the presence of 70% glycerol.

To assist in the development of a Holliday junction-based nanoswitch for detection of single-nucleotide mismatches in unlabelled targets, Campbell et al use the fluorescence decay of 2AP positioned at the branchpoint to probe the conformation (switch state) and to detect structural differences arising from hybridisation of matched and mis-matched targets (Campbell et al., 2009). The decay parameters reported enhanced base-stacking at the branchpoint on switch closure and could resolve variations in switch structure that enabled discrimination between target mutations that were indistinguishable from steady-state measurements.

We used 2AP time-resolved fluorescence was used to complement single-molecule, multi-parameter Förster resonance energy transfer (FRET) measurements to investigate branchpoint expansion in a three-way junction (Sabir et al., 2012). While single-molecule time-resolved FRET delivers the 3-dimensional, global structure (in solution), the 2AP decay reports the local conformation at the single-base level. Having established the response of 2AP decay parameters to unpairing (as discussed above), measurement of the decay of 2AP placed at strategic positions in the junction structure confirmed that bases adjacent to the branchpoint are unpaired (despite the full Watson−Crick complementarity of the molecule), as had been inferred from the

FRET-derived structure. As illustrated in Figure 7, the decay of 2AP located in the junction arms, distant from the branchpoint, closely resembled that of the corresponding duplex, whereas the decay of 2AP lying adjacent to the branchpoint tended towards that of the single-strand, signifying local unpairing.

Kimura et al. reported fluorescence lifetime measurements on 2AP-containing G-quadruplexes, to complement fluorescence intensity measurements (Kimura et al., 2004; Kimura et al., 2007). However the value of these measurements, made using a streak camera, is doubtful, since only two (sub-nanosecond) decay components are reported for duplexes and one component for G-quadruplexes. This is due, presumably, to the limited time range that can be covered, precluding the measurement of the longer decay components. The lifetime measurements provide no additional insight into the conformational change from duplex to quadruplex beyond that deduced from intensity changes. In a more recent study, Buscaglia et al employed (unusually for 2AP) frequency-domain measurements, rather than TCSPC (time-domain) to explore G-quadruplex structure and stability (Buscaglia et al., 2012). However, this paper is mainly concerned with promoting the use of phasor diagrams as model-free graphical representations of frequency-domain lifetime data, rather than an interpretation of decay parameters derived from exponential fitting. Phasor diagrams cannot provide high-resolution structural information but may be of value in monitoring changes in G-quadruplex conformation driven by changes in solution conditions.

Continuing their theme of utilising 2AP-containing poly-adenine strands to investigate site-specific dynamics, the Rao group turned their attention to TAT triple helices (Ramreddy et al., 2009). Significant findings of this work were the absence of

the very short (< 100 ps) decay component for 2AP in triplexes, implying a decrease in near-neighbour base-stacking interaction compared with duplexes, and an increase in the amplitude of the longest lifetime component, indicating a greater propensity for 2AP to occupy an extra-helical conformation. Strikingly, the decay data also revealed an asymmetry in the base dynamics at the two ends of the triplexes: the 5' end of the A strand showed a higher degree of base stacking (more duplex-like) than the 3' end. This was taken to reflect an asymmetry in the strength of the Hoogsteen base-pairing of the T strand with the A-T duplex., such that the triplex nature is more dominant towards the 3' end.

4. 2-aminopurine as a probe of the DNA-enzyme interface

The supreme sensitivity of the 2AP fluorescence response to changes in its environment is something of a 'double-edged sword'. Such sensitivity is exactly what we would hope for in a fluorescence reporter but the context of this reporting i.e. the highly dynamic DNA duplex makes quantitative interpretation of the signal extremely challenging. The magnitude of this challenge has been highlighted in the literature over several attempts to utilise changes in the fluorescence intensity of 2AP as a means to study enzyme-induced deformations of the DNA duplex. We discuss here the successful use and limitations of 2AP intensity as a probe of these systems and highlight the application of time-resolved fluorescence as a means for deriving greater insight and more quantitative information on the system of interest.

Towards the end of the nineteen-eighties the synthesis of DNA oligonucleotides was becoming increasingly routine such that (in principle) any laboratory could prepare

short DNA molecules to study. This evolution in DNA synthesis played a central role in the story of 2AP, which despite its characterisation in the nineteen-sixties, was not fully utilised as a probe of DNA structure until the early- to mid-nineties. These first studies utilized 2AP fluorescence intensity to report on the kinetics of DNA unwinding by a DNA helicase (Raney et al., 1994) and DNA melting and nucleotide incorporation by polymerase enzymes (Frey et al., 1995; Hochstrasser et al., 1994).

4.1 Base flipping: an extreme example of enzyme-induced distortion

Perhaps the first example of the use of 2AP to probe the DNA-enzyme interface was described in 1996 (Allan & Reich, 1996). This study focussed on the DNA methyltransferase enzyme, M.EcoRI, an enzyme that is naturally found in the ***E****schericia* ***co****li* bacterium, and set out to better understand the remarkable distortion of the DNA duplex, known as base (nucleotide) flipping, that is performed by this class of enzymes (amongst others). Here, we give a brief 'aside' on the DNA methyltransferase enzymes because of their critical role in the discovery of base flipping and the development of 2AP as a probe of base flipping enzymes.

The DNA methyltransferases are present in both pro- and eukaryotic organisms and though their functions at the atomic-level are similar in these hosts (i.e. methylation of the DNA bases) the impacts of these methylation events are quite distinct (Jeltsch, 2002)). In bacteria, for example, DNA methylation is a mechanism used by the bacterium to prevent digestion (cutting) of its own genome by the restriction enzymes it produces. Hence, both the DNA methyltransferases and restriction enzymes from the same host organism typically function symbiotically, targeting the same 4- to 8-base pair recognition sites for modification and cleavage. This is part of a defence mechanism in bacteria against viral invasion. In mammals, methylation plays a

critical role in health and development through the regulation of genes. Here, however, the methylation machinery is rather more involved, with multiple enzymes contributing to the maintenance of methylation at 5'-CG-3' sites across the genome. In bacteria, there are many hundreds of known DNA methyltransferase enzymes are and thousands have been predicted from genomic DNA sequences, largely thanks to their homology with other methyltransferases. They ordinarily methylate one of the bases *within* their target recognition sequence, either an adenine (at the N6 atom) or a cytosine (C5 or N4 atoms). For example, the M.EcoRI targets the central adenine of the six-base motif 5'-GA**A**TTC-3' for methylation. In both prokaryotic and eukaryotic methylation, the methyltransferases catalyse the transfer of a methyl-group from the small cofactor molecule, S-adenosyl-L-methionine, to a DNA base (adenine or cytosine).

Like most enzymes, the methyltransferases perform chemistry that is remarkable when compared with what is possible on the benchtop. The basis for the process of DNA methylation was revealed for the first time when crystallographers were able to trap the M.HhaI DNA methyltransferase in a ternary complex with DNA and a cofactor analogue, as illustrated in Figure 8. This amazing snapshot of methylation in action showed M.HhaI bound to its DNA target site with its target base for methylation flipped completely from the DNA duplex and into its catalytic pocket. This process is necessary because the enzyme needs to bring the base that it methylates into close proximity to the cofactor (S-adenosyl-L-methionine) from which it sources the methyl-group. Once flipping is complete, the enzyme is able to catalyse the methyl-transfer reaction.

The remarkable DNA distortion performed by M.HhaI (in the absence of any external source of chemical energy, such as ATP) changed the way in which scientists perceived the DNA duplex. DNA has two seemingly opposed functions, acting as a stable store for vast quantities of genetic information yet at the same time being an accessible, readable molecule that is able to interact with the multitude of proteins, enzymes and other molecules that our cell's rely upon to survive. The DNA duplex structure inferred by Watson and Crick satisfies the former function but not necessarily the latter. In order to be accessible, the duplex must be dynamic and the M.HhaI structure captured the most extreme distortion of DNA duplex conformation, for all to see. Of course, the immediate question, in the wake of the M.HhaI crystal structure was whether M.HhaI was a special case or if base flipping was a more general mechanism used not only by the methyltransferase enzymes to access their target bases but by all enzymes which might need to locally melt the DNA duplex in order to access its information, modify or repair it.

4.2 The fluorescence intensity of 2-aminopurine as a probe of base flipping

Since the creation of an enzymatic ternary complex in a crystal is very demanding, the search for other options to study this process in solution phase began, with 2AP being top of the list of possible probes for base-flipping enzymes. Allan and Reich (Allan & Reich, 1996)synthesised short DNA duplexes, containing the recognition sequence for M.EcoRI (GA<u>A</u>TTC, where the underlined adenine is the target for methylation), where 2AP was placed at the target site for methylation/ flipping, in place of adenine. Upon binding of M.EcoRI to this substrate (in the presence of a cofactor analogue) an increase in the fluorescence intensity of the 2AP of around 14-fold was observed. Hence, the authors rightly concluded that 2AP was shown to be a useful probe of the

disruption of the DNA duplex (likely base flipping) by M.EcoRI. However, the interpretation of their results was not entirely straightforward. The observed increase in intensity of the 2AP emission was around two-fold less than expected (compared with the free nucleoside) and it was accompanied by a significant (10 nm) hypsochromic shift of the 2AP emission spectrum. Allan and Reich tentatively attributed the low fluorescence enhancement to the fact that M.EcoRI can bind in two possible orientations to its recognition site and implied that only half of the available 2AP may be flipped from the duplex at any given time. However, there is some indication here (i.e. both the spectral shift and 'quenching' relative to the free nucleoside) that the interaction between 2AP and enzyme can be as critical in determining its photophysical properties as that between 2AP and the DNA duplex.

Subsequent studies using 2AP to monitor base flipping showed further unravelling of the complex relationship between the environment of 2AP and its photophysical behaviour. Holtz *et al* (Holz et al., 1998) applied similar methodology to Allan and Reich in order to compare base flipping by the prototypical M.HhaI with M.TaqI (an N6-adenine methyltransferase). Both enzymes show increases in 2AP fluorescence intensity upon enzyme binding and base flipping (of the 2AP) but the magnitude of the intensity increase is significantly different in each complex; 54-fold for M.HhaI and 14-fold for M.TaqI. A later study (Gowher & Jeltsch, 2000) using the same assay to probe DNA duplex disruption by M.EcoRV confirmed the unpredictable response of the 2AP fluorescence intensity to base flipping. This enzyme targets the six-base sequence 5'-G<u>A</u>TATC-3' for methylation. When 2AP was substituted for the target adenine base, no significant increase in fluorescence intensity was observed upon ternary (EcoRV+DNA+cofactor analogue) complex formation. Indeed, this enzyme,

known to bend the DNA duplex at its target binding site, gave a significant (11-fold) increase in 2AP fluorescence intensity upon ternary complex formation when the 2AP was substituted for the central (unflipped) adenine of the recognition sequence. Similar behaviour was observed for the EcoP151 enzyme (Reddy & Rao, 2000) which recognises the six-base target CAGCAG. 2AP was substituted for each of the adenine bases in the enzyme's recognition sequence and a fluorescence enhancement of similar proportions was observed for 2AP at either site, upon enzyme binding. Yet more complex photophysical behaviour from 2AP has been observed for the EcoKI DNA methyltransferase enzyme (Su et al., 2004). This enzyme gives a significant (4-fold) increase in 2AP intensity upon base flipping but also shows the formation (on the timescale of several minutes) of a new species with red-shifted excitation and emission spectra, relative to 2AP.

Taken together, these results are complex and confusing but the interesting question is from whence this confusion arises? As we now know from studies on the DNA duplex alone, the fluorescence response of 2AP is quite predictable; by and large stacking results in quenching. What cannot be easily predicted is the myriad of conformations that the DNA duplex can adopt, and the specific interactions that can be formed between the 2AP and its environment, especially when it is complexed by an enzyme. Even though base flipping appears a simple 'in' or 'out' question it involves a complex series of interactions between the 2AP probe, the DNA duplex and the amino acids that comprise the enzyme.

The studies highlighted here show that, whilst the steady-state fluorescence intensity of 2AP can often provide good evidence for a distortion of the DNA duplex it cannot be used reliably to identify nor quantify a specific rearrangement of the duplex. We

lose much of the useful information that is provided by the 2AP because we observe the steady-state fluorescence intensity, which is an average signal from the entire 2AP population. One way to access this information is to look at the time-resolved fluorescence response of 2AP.

4.3 The fluorescence decay of 2-aminopurine as a probe of base flipping

Following the lead of those groups pioneering the application of 2AP to study DNA duplex structure and dynamics, we employed time-correlated single-photon counting in order to study base flipping by a number of DNA methyltransferases, M.HhaI (Neely et al., 2005), M.TaqI (Lenz et al., 2007), M.EcoRV (Bonnist et al., 2012), M.EcoKI (Neely, unpublished work) and restriction enzymes, R.Ecl18kI, R.PspGI, R.EcoRII and PfoI (Neely et al., 2009), all of which were suspected or known base flippers. In short, we found that for every system, unlike the analogous steady-state measurements, there is a clear response to base flipping from the 2AP. Rather than deriving an overview of the average behaviour of the fluorophores within the ensemble (as seen in steady-state measurements) the fluorescence lifetimes reveal information about the heterogeneity of the ensemble, derived from the interaction between 2AP and enzyme and the dynamics of the flipping process.

The value of using a time-resolved measurement is clearly illustrated for the comparison of base flipping by M.HhaI and M.TaqI. In the fluorescence spectrometer, the fluorescence intensity arising from the free DNA duplexes containing 2AP is barely perceptible. Holz *et al* (Holz et al., 1998) showed that upon enzyme binding and base flipping of 2AP from these duplexes large increases in fluorescence intensity of 54-fold and 14-fold are observed for M.HhaI and M.TaqI, respectively. This disparate behaviour is puzzling since we naively expect that 2AP fluorescence is

simply quenched when the base is stacked in the duplex and not quenched when the base is unstacked. The fact that the increase in intensity is not so great for flipping by M.TaqI as it is for M.HhaI (despite using saturating enzymatic conditions) cannot be readily explained from steady-state data alone. When we observed the fluorescence decay of 2AP-labelled DNA bound and flipped by M.HhaI we saw a dramatic fluorescence response that could be readily rationalized from earlier studies of 2AP in the duplex. As shown by the decay parameters in Table 4, the 2AP fluorescence response changes dramatically upon formation of the ternary complex. Indeed, we

Sample	**A-Factor**				**Lifetime/ ns**			
M.HhaI	A_1	A_2	A_3	A_4	τ_1	τ_2	τ_3	τ_4
Free duplex	0.53	0.26	0.14	0.06	0.08	0.58	2.94	9.60
Ternary complex	0.22	0.26	0.23	0.29	0.12	0.95	4.23	10.49

Table 4. Fluorescence response of 2AP in an unbound DNA duplex and in ternary complex with wild-type M.HhaI and cofactor, with 2AP at the target site for flipping.

observe a significant increase in the fraction of 2AP molecules with lifetimes of around a nanosecond or longer (τ_2-τ_4) and a general increase of all of these lifetimes. Most noteworthy, however, is the significant reduction of A_1 on ternary complex formation, indicating a decrease in the amount of highly stacked 2AP in the DNA duplex. We confirmed the specificity of this result with a series of control experiments with 2AP at different locations in the DNA duplex, relative to the target site for flipping and concluded that, along with the corroborating biochemical and crystallographic data, the observed change in the 2AP response was indeed the fluorescence signature for base flipping.

We subsequently moved on to look at the analogous behaviour of the M.TaqI enzyme (target site TCGA) in the hope of better understanding the reasons for the discrepancy in the steady-state fluorescence response to base flipping of 2AP by each enzyme. As shown in Table 5, The response of 2AP to base flipping by M.TaqI is quite dissimilar

Sample	**A-Factor**				**Lifetime/ ns**			
M.TaqI	A_1	A_2	A_3	A_4	τ_1	τ_2	τ_3	τ_4
Free duplex	0.81	0.06	0.09	0.04	0.03	0.57	2.4	7.8
Ternary complex	-	0.54	0.38	0.08	-	0.55	1.9	7.3

Table 5. Fluorescence response of 2AP in an unbound DNA duplex and in ternary complex with wild-type M.TaqI and cofactor, with 2AP at the target site for flipping.

to that seen for base flipping by M.HhaI. The fluorescence response of the 2AP population complexed by M.HhaI is dominated by a slow ~11 ns component, whereas that of the 2AP population complexed by M.TaqI decays much more rapidly, with a lifetime of around 0.5 ns. Figure 9 summarises this contrasting photophysical behaviour.

Critically, the very short decay component, τ_1, characteristic of a stacked 2AP population is completely absent from the decay of the MTaqI-complexed DNA duplex. We have already noted that this very shortest component (typically around 100 ps) of the 2AP decay is the result of de-excitation via electron transfer along the DNA duplex. We observed a dramatic reduction in the contribution of this component to the fluorescence decay upon base flipping by both M.HhaI and M.TaqI, showing a significant reduction in the fraction of the 2AP population that is stacked in the DNA duplex. In other words, the suggestion here is that, in the ternary complexes with both enzymes most, or the vast majority of, the 2AP population is in an extrahelical

location. The differences that are observed in the time-resolved and steady-state fluorescence responses of these systems to base flipping are not related to base flipping or the lack of it but, rather, are derived from the fact that the 2AP is being flipped into quite distinct atomic environments in M.HhaI and M.TaqI.

We were able to dig a little further into the interpretation of the solution phase fluorescence response of 2AP in these complex molecular systems by recording the 2AP fluorescence response on single crystals formed from 2AP-modified DNA in ternary complexes with the M.HhaI and M.TaqI enzymes. In the case of M.HhaI, the ternary complex with the wild-type enzyme proved difficult to crystallize and so we resorted to studying a mutant (T250G) of this enzyme that is catalytically active and which shows a 100-fold increase in 2AP fluorescence intensity upon base flipping. The ternary complex with this enzyme crystallized readily, perhaps because of the pocket that is created in the enzyme by the T250G mutation, Figure 10.

Figure 11 shows the crystal structures of the M.HhaI and M.TaqI ternary complexes in the immediate vicinity of the flipped 2AP base.

Naively, we had anticipated that the 2AP population in the crystals would be both static and uniform and as a result, we would observe a simple, single exponential fluorescence decay from these samples that would allow us to accurately quantify the extent of base flipping in the solution phase. However, fluorescence responses from both crystals, shown in Table 6, require at least three decay components for a good fit, indicating a rather more complex, heterogeneous or dynamic crystal than imagined. This may, in part, be derived from the fact that the crystals from which we recorded

fluorescence decays were at room temperature, allowing some thermal motion of the complexes therein.

Sample	A-Factor				Lifetime/ ns			
Ternary complex	A_1	A_2	A_3	A_4	τ_1	τ_2	τ_3	τ_4
M.HhaI (T250G)	-	0.07	0.13	0.79	-	0.91	5.86	11.05
M.TaqI	-	0.46	0.51	0.03	-	0.93	2.3	8.6

Table 6. Fluorescence response of 2AP in DNA at the target site for base flipping in ternary complexes in the crystal phase with T250G M.HhaI or wild-type M.TaqI enzymes.

This highlights a very important feature of protein crystallography and our interpretation of the amazing images that can be derived; these images show only a snapshot of the multitude of conformations that the ensemble can adopt in the solution phase. In fact, they may not even represent the most populated state in the solution phase and as such, the achievable mechanistic insight for a complex enzymatic pathway, such as base flipping, is limited. This insight can be greatly extended by use of a complementary approach, such as time-resolved fluorescence, which derives information on the behaviour of an ensemble in the solution phase.

Indeed, we can clearly correlate some features of the fluorescence and structural data, thereby linking the static crystal snapshot with some of the behaviour we observe by time-resoled fluorescence studies in the solution phase. The complexes in both crystals display a 2AP fluorescence decay that can be fit using only three exponential components. The shortest lifetime component of the decays has been lost, indicating that, despite the apparent heterogeneity, there is no 2AP in the crystals that is stacked

in the DNA duplex. The distribution of the 2AP population is quite different in each complex from the lifetime data. This can be correlated with the structures shown in Figure 11 which indicate that the environment into which the 2AP is moved by the enzyme is quite distinct for M.HhaI (T250G mutant) and TaqI. Indeed, in the M.HhaI crystal structure the 2AP is surrounded by polar- or charged groups and interacts closely with the phosphate group 5'- of it and with arginine residues from the M.HhaI. In M.TaqI, however, the 2AP is stacked between tyrosine 108 (shown behind the 2AP in Figure 11) and valine 21. Its environment contains many apolar amino acids and it is π-stacked with an aromatic residue We know from studies made in solution on 2AP or its ribonucleoside that the fluorescence lifetime of 2AP is sensitive to polarity, dropping by about a factor of five when going from pure water to pure 1,4-dioxane. Moreover, 2AP fluorescence can be rapidly quenched by electron transfer from tyrosine (or tryptophan) (Harriman, 1987; Wan et al., 2005; Xia et al., 2003), in an analogous fashion to electron transfer quenching of intrahelical 2AP by guanine; this accounts for the observation of a sub-nanosecond decay component for the flipped 2AP in M.TaqI.. We observed subsequently (Bonnist & Jones, 2008; Bonnist, 2008) that the close stacking interaction of 2AP with tyrosine 108 in the M.TaqI active site also gives rise to red-shifted fluorescence, analogous to that seen for 2AP in DNA (Bonnist & Jones, 2008). This provided the explanation for the unusual emission reported previously for 2AP flipped into M. EcoKI (Su et al., 2004), where 2AP stacks closely with an aromatic residue in the active site, in this case phenyalanine.

These two studies, allowed us to link the fluorescence response of 2AP to specific molecular environments and have informed more recent work on a subset of base flipping restriction endonucleases (Neely et al., 2009), on the M.EcoRV and M.EcoRI

methyltransferases (Bonnist et al., 2012; Youngblood et al., 2008), the Type III EcoP15I restriction-modification system (Ma et al., 2014), the flap endonuclease-1 (Finger et al., 2013) and on the archaeal family-B DNA polymerase, *Pfu*-Pol (Richardson et al., 2013). In all of these systems, the basis of the interpretation of the 2AP signature is similar to that outlined using M.HhaI and M.TaqI. However, each system has its own idiosyncrasies and as the dataset expands, so does our ability to interpret the 2AP fluorescence response.

Our work on M.EcoRV (Bonnist et al., 2012) illustrates the ability of time-resolved fluorescence to clarify the different response to enzyme binding of 2AP placed at different positions within in the recognition sequence, thereby resolving the apparently anomalous observation from steady-state measurements that 2AP substituted for the non-target adenine in the recognition sequence showed a much greater intensity increase than 2AP at the target site (Gowher & Jeltsch, 2000). The fluorescence decay parameters showed that the target 2AP is indeed flipped by the enzyme, but its fluorescence is quenched by interaction with aromatic residues in the catalytic site, whereas bending of the duplex at the non-target site alleviates inter-base quenching and exposes the 2AP to solvent. Furthermore, information on the conformational populations that can be gleaned from the decay parameters supported previous evidence for specific and non-specific binding.

A nice example of two very different fluorescence responses from 2AP flipped into an enzymatic environment comes from the Ecl18kI and PfoI restriction enzymes. These enzymes recognise the sequences CCNGG and TCCNGGA, respectively and in nature, make a sequence-specific, double-stranded DNA break at these sites. A surprising, yet critical part of this cleavage mechanism is base flipping. The central

base of the recognition sequences ('N'= any base) was found, in the Ecl18kI crystal structure, to be flipped from the DNA duplex on both sides of the duplex. This leads to a collapse of the DNA duplex and an effective shortening of its length whilst bound to the enzyme (Bochtler et al., 2006). Steady-state measurements using 2AP at the target site for flipping confirmed the flipping in the solution phase and also identified the analogous EcoRII and PspGI enzymes (recognising the same sequence) as base flipping enzymes. Interestingly, the Ecl18kI enzyme forms a stacking interaction via tryptophan 61 to the base that is flipped from the duplex. When we examined the fluorescence response of 2AP flipped into these pockets of Ecl18kI we saw almost no perceptible change in the fluorescence response of the 2AP compared with the unbound DNA duplex, as shown in Table 7.

Sample	**A-Factor**				**Lifetime/ ns**			
	A_1	A_2	A_3	A_4	τ_1	τ_2	τ_3	τ_4
Free DNA	0.89	0.05	0.05	0.01	0.07	0.46	2.0	7.8
DNA + Ecl18kI	0.80	0.07	0.08	0.05	0.07	0.70	3.1	8.9
DNA + Y61A	0.38	0.12	0.24	0.26	0.16	1.3	4.6	9.0
DNA + PfoI	-	-	0.09	0.91	-	-	3.4	8.3

Table 7. Fluorescence response of free DNA containing 2AP and this same DNA duplex bound by either wild-type Ecl18kI, the W61A mutant of Ecl18kI or PfoI, where 2AP is flipped from the duplex into a pocket of the enzyme.

As we found with studies on M.HhaI and M.TaqI, the real power of the time-resolved measurements is realised when combined with either biochemical or structural information that leads to new understanding about the enzyme-DNA complex. In the case of Ecl18kI, the wild-type enzyme was mutated to remove the replace the

tryptophan residue that stacks with 2AP in the flipping pocket with an alanine residue. The fluorescence response of 2AP bound by this W61A mutant far more closely resembles that of the familiar flipped 2AP in the complex with M.HhaI. This observation confirms the efficient quenching of 2AP fluorescence via stacking with the tryptophan 61 residue, resulting in a short lifetime component for 2AP flipped into the wild-type active site, comparable to that seen for intrahelical 2AP quenched by G. By contrast, base flipping by PfoI could barely be more simple to interpret. The 2AP decay becomes almost mono-exponential with 90% of the 2AP population having a lifetime similar to that of the free 2AP ribonucleoside. Hence, in this enzyme, 2AP is efficiently flipped into a hydrophilic or solvated environment and is effectively prevented from returning to the DNA duplex.

The seemingly similar Ecl18kI and PfoI enzymes act to stabilise their flipped bases using completely different mechanisms. Using time-resolved fluorescence to study this process not only reveals the dramatic extent of their disparate behaviour but allows us to gain some insight into what causes it. The extra information that the fluorescence decay yields enables greater understanding of (in this case) the base flipping process and when this is combined with complementary structural or biochemical studies, entirely new insight can be derived.

4.4 The fluorescence decay of 2-aminopurine as a probe of other enzyme-induced distortions

The use of 2AP time-resolved fluorescence to elucidate DNA-protein interactions is not limited to the study of base flipping. Other, more subtle binding-induced deformations can be addressed, as exemplified by the following studies.

Reha-Krantz and coworkers have used 2AP decay measurements in a series of investigations of the interactions of Bacteriophage T4 DNA polymerase with DNA, see for example (Hariharan & Reha-Krantz, 2005; Reha-Krantz, 2009; Subuddhi et al., 2008; Tleugabulova & Reha-Krantz, 2007) These measurements have been made using a stroboscopic optical boxcar technique (James et al., 1992) which delivers lower time resolution and lower quality data than the other studies (employing TCSPC) considered in this review. This prevents the detection of short-lifetime components (less than about 200 ps) and compromises the reliability of multi-exponential fitting, precluding a detailed interpretation of the conformational behaviour. Nevertheless the time-resolved measurements have proved a valuable supplement to intensity data. In particular they have enabled the empirical identification of functionally different complexes, based on the dominance of different 2AP decay times.

Mély and coworkers have used 2AP in mechanistic studies of the role of nucleocapsid protein (NC) in the lifecycle of human immunodeficiency virus type 1 (HIV-1). (Avilov et al., 2009; Avilov et al., 2008; Godet et al., 2011). NC is a 55 amino acid protein which contains two highly conserved zinc fingers. In an initial study (Avilov et al., 2008), they demonstrated the ability of 2AP time-resolved fluorescence to report on the dynamics of target hexanucleotides in complex with NC. Site-specific changes in oligonucleotide dynamics were observed during NC binding and correlated with the known 3-D structure of the complex. The 2AP decay parameters were interpreted to show that NC strongly restricts the oligonucleotide flexibility. This conformational ‘freezing’ seemed to be mainly supported by the folded zinc finger domain of the NC. Use of NC mutants allowed the restriction of local base dynamics

to be more specifically attributed to the hydrophobic platform at the top of the folded fingers and to be identified with base interaction with specific aromatic residues. They then examined site-specific binding of NC to 2AP-substituted dodecanucleotides containing two binding sites (TG motifs) (Avilov et al., 2009). Lifetime measurements again showed that NC-binding inhibits conformational fluctuations of the oligonucleotides and strongly decreases the population of fully stacked conformations. 2AP at a given position was found to respond mainly to the binding of NC to its closest TG motif, enabling site-specific investigations of NC binding to oligonucleotides with multiple binding sites. In a subsequent study (Godet et al., 2011), they investigated the role of the nucleopcapsic zinc fingers in the mechanism of synthesis of HIV-1 viral DNA, a complex multi-step process catalysed by the viral reverse transcriptase (RT). The mechanism involves two obligatory strand transfer reactions, the second of which relies on the annealing of two primer binding site (PBS) DNA stem loops, which is chaperoned by NC. By exploiting sequence-specific insertion of 2AP into PBS loop sequences and site-specific mutations of NC, Godet et al. were able to show that NC can freeze PBS conformations competent for annealing via the loops and, moreover, the modifications to the loop structure and dynamics that govern the annealing reaction are dependent on the integrity of the zinc finger hydrophobic platform.

As discussed above (Section 3.2), the fluorescence decay of 2AP is sensitive to local disruption of base pairing. We have exploited this to investigate two cases in which enzyme-induced duplex unpairing had been postulated to be mechanistically essential, but for which there was no direct evidence: unwinding of primer-templates by an

archaeal DNA polymerase (Richardson et al., 2013) and unpairing of substrate by a flap endonuclease (Finger et al., 2013).

Archaeal family-B DNA polymerases bind tightly to deaminated bases and stall replication on encountering uracil in template strands, four bases ahead of the primer-template junction (+4 position). Should the polymerase progress further towards the uracil, for example to a position where uracil is only two bases in front of the junction (+2 position), 3'-5' proof-reading endonuclease activity is stimulated to trim back the elongating primer and re-sets uracil to the +4 position. This uracil-sensing process serves to prevent the inappropriate copying of the deaminated base and the introduction of mutations into the genome. The polymerase and exonuclease domains of DNA polymerase are well separated and unwinding of the primer to expose a short, single-stranded region is required to position the 3-terminal base of the primer in the the exonuclease active site. The aim of our study (Richardson et al., 2013) was to test the hypothesis that if the polymerase approaches closer than four bases to uracil, the primer strand starts to unravel. The fluorescence decay parameters of 2AP showed clearly that binding of the polymerase caused pronounced unwinding of primer-template with uracil at the +2 position, whereas, with uracil at the +4 position, only minor distortion of the duplex structure occurred.

Flap endonuclease-1 (FEN-1) is a structure- and strand-specific phosphodiesterase that catalyzes the essential removal of 5'-single-stranded flaps during DNA replication and repair. FEN-1 achieves this by selectively catalyzing hydrolysis one nucleotide into the duplex region of the substrate, always targeting the 5' strand. This specificity had been proposed to arise by unpairing of the 5'end of the duplex to permit the scissile phosphate diester to contact catalytic divalent metal ions in the

active site. Using 2AP decay data, in conjunction with low-energy CD, enabled us (Finger et al., 2013) to provide the first direct evidence of unpaired conformations in DNA substrates bound to FEN-1 and to elucidate the role of 5'-nuclease superfamily conserved (K93, R100) and semi-conserved (Y40) residues in unpairing and stabilisation of unpaired bases in the substrate and product complexes.

5. Extension of fluorescence decay measurements to ultrafast timescales

Fluorescence lifetime measurements can be extended to the ps and sub-picosecond timescale, beyond the resolution of TCSPC, by the use of the ultrafast, pump-probe technique of fluorescence up-conversion (or down-conversion). There are few groups equipped to carry out such measurements at the ultraviolet wavelengths required for 2AP and activity in this area has been dominated by Xia and coworkers who have focussed mainly on the study of 2AP in RNA. Their work is the subject of a mini-review (Xia, 2008) and will not be discussed further here.

To our knowledge there is only one example in the literature of directly comparable TCSPC and ultrafast fluorescence decay measurements on the same DNA system, the studies of Godet et al. (Godet et al., 2011) and Gelot et al. (Gelot et al., 2012), respectively, on 2AP-containg DNA hairpins. The TCSPC study reports typical 4-component decays with lifetimes of ~ 100 ps, ~700 ps, ~ 3 ns and ~8 ns, together with an unresolvable dark species which accounts for about 80% of the 2AP population (as discussed in Section 3.3 above). The decays measured by femtosecond fluorescence down-conversion are also fitted by 4 exponential components, with lifetimes of ~ 3 ps, ~ 20 ps, ~ 100 ps and ~ 3 ns. The former two ultrashort lifetimes

correspond to the dark species, undetectable by TCSPC, while the latter two longer lifetimes can be identified with two of the components measured by TCSPC. The limited time-range (3 ns) of the ultrafast measurements and the impossibility of extracting more than four decay components, means that the four longer decay times measured by TCSPC are compressed into two components in the ultrafast decay measurement. A complete picture of the decay properties requires the use of both techniques. At present, this is a far-from-practical proposition.

6. Conclusions

Over 40 years since its remarkable fluorescence properties were first reported (Ward et al., 1969) 2AP remains unsurpassed as the most widely used fluorescent base analogue and probe of DNA conformation, because of its structural similarity to a natural base, its exquisite sensitivity to interbase interactions and its commercial availability. In spite of its success, 2AP is certainly not without its disadvantages and we will now reflect on these shortcomings and the possibility of improving upon them.

The short, UV excitation wavelength of 2AP is disadvantageous (particularly in the context of time-resolved fluorescence measurements, as discussed below), but is a consequence of its isomeric relationship to adenine. A shift to longer excitation wavelength requires a more delocalised electronic structure and results in greater structural deviation from a natural base.

The complex fluorescence decay of 2AP in DNA may seem disadvantageous, but is a consequence of the highly desirable sensitivity of the fluorophore to interbase

quenching; a simpler decay function would be the signature of a less responsive probe. The shortness of its fluorescence lifetime (< 100 ps), and consequently low quantum yield, in a fully stacked state is indeed disadvantageous, demanding the use of ultrafast lasers and fast-response detectors to measure it fluorescence decay, and often requiring long data acquisition times because of the low fluorescence intensity. The combination of low quantum yield and the need for UV excitation also makes it unpromising as a probe for single-molecule studies, although we note that detection of 2AP at the single-molecule level in surface-immobilised oligonucleotides has been reported recently (Aleman et al., 2014).

The short lifetime is, in fact, essential to the wide dynamic range of lifetimes that 2AP exhibits in DNA, which makes its complex mutli-component decay tractable to reproducible and reliable analysis and interpretation. A fluorophore with similar responsiveness but a slower decay time would indeed be welcome in facilitating lifetime measurements, although, to maintain the same dynamic range of lifetimes would inevitably lead to the same low quantum yield for the highly quenched component. Aromatic fluorophores rarely have radiative lifetimes longer than about 100 ns, so an order of magnitude increase in decay times is the best that could be hoped for.

The susceptibility of 2AP to ultrafast quenching, resulting in species which are invisible to all but the most advanced, sub-picosecond measurements, is a cause for concern. This does not prevent the use of 2AP as a very effective probe, but the notion that a vast proportion of the conformational population evades observation is certainly disquieting. The use of ultrafast spectroscopy to provide a window on the ‘dark’ conformations that are invisible to TCSPC is undoubtedly valuable in increasing our

understanding of base dynamics and the mechanism of quenching of 2AP in DNA. However, there is no evidence at present to indicate that ultrafast measurements would significantly enhance the utility of 2AP as a probe of DNA-enzyme interaction, even if they were practically feasible. An important, unanswered question is to what extent the dark conformational population(s) might behave differently, in response to enzyme binding, from the populations detectable by TCSPC. More specifically, would changes in the values of the ultrafast lifetimes themselves provide useful additional information or is it the fractional population that conveys the more valuable information? If, as our experience suggests, it is changes in the A factors that are often more informative, then the most essential information can be gained by the combination of TCSPC and quantitative intensity measurements.

For photophysicists, 2AP is an object lesson in the subtle interplay of electronic states that controls fluorescence properties, and exposes the inadequacy of the current understanding of the excited state properties of complex molecules. We cannot yet fully explain the fluorescence properties of 2AP, let alone predict precisely how to go about changing them for the better. Although considerable efforts are being devoted to the development of new, environmentally sensitive, fluorescent, isomorphic base analogues, see, for example, (Ben Gaied et al., 2005; Nadler et al., 2011; Noé et al., 2013; Sinkeldam et al., 2010; Wilhelmsson, 2010), there appears to be no immediate prospect of a definitively superior successor to 2AP.

In this review we have endeavoured to convey the detailed insight into DNA structure and dynamics, at the single-base level, that can be obtained from time-resolved fluorescence measurements of 2AP. At present, such measurements are restricted to a rather small number of groups because of the relatively sophisticated and costly

apparatus required. However recent and ongoing technological advances promise to make TCSPC measurements with UV excitation and sub-100 ps time resolution much more widely accessible. Compact, easy-to-use and relatively low-cost photonic-crystal-fibre-based supercontinuum ("white light") lasers have already started to replace mode-locked Ti:sapphire lasers as the excitation source of choice for TCSPC in the visible wavelength range, and are now beginning to make in-roads into the UV range. On the detection side, single-photon avalanche photodiodes (SPAD) and hybrid photomultiplier tube detectors (combing a photomultiplier tube front-end with an avalanche photodiode amplification stage) are now available as lower cost alternatives to expensive microchannel plate photomultipliers which were previously the only option for TSCPC with high time-resolution. We anticipate that, almost half-a-century since its potential as a fluorescent base analogue was first recognised, 2AP may be set for a revival as a fluorescence-lifetime probe.

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8. References

ALEMAN, E. A., DE SILVA, C., PATRICK, E. M., MUSIER-FORSYTH, K. & RUEDA, D. (2014). Single-Molecule Fluorescence Using Nucleotide Analogs: A Proof-of-Principle. J Phys Chem Lett, 5(5), 777-781.

ALLAN, B. W. & REICH, N. O. (1996). Targeted Base Stacking Disruption by the EcoRI DNA Methyltransferase†. Biochemistry, 35, 14757-14762.

AVILOV, S. V., GODET, J., PIEMONT, E. & MELY, Y. (2009). Site-Specific Characterization of HIV-1 Nucleocapsid Protein Binding to Oligonucleotides with Two Binding Sites. Biochemistry, 48(11), 2422-2430.

AVILOV, S. V., PIEMONT, E., SHVADCHAK, V., DE ROCQUIGNY, H. & MELY, Y. (2008). Probing dynamics of HIV-1 nucleocapsid protein/target hexanucleotide complexes by 2-aminopurine. Nucleic Acids Res, 36(3), 885-896.

BECKER, W. (2005). Advanced Time-Correlated Single Photon Counting: Springer.

BEECHEM, J., GRATTON, E., AMELOOT, M., KNUTSON, J. & BRAND, L. (2002). The Global Analysis of Fluorescence Intensity and Anisotropy Decay Data: Second-Generation Theory and Programs. In Topics in Fluorescence Spectroscopy, vol. 2 (ed. J. Lakowicz), pp. 241-305: Springer US.

BEN GAIED, N., GLASSER, N., RAMALANJAONA, N., BELTZ, H., WOLFF, P., MARQUET, R., BURGER, A. & MELY, Y. (2005). 8-vinyl-deoxyadenosine, an alternative fluorescent nucleoside analog to 2'-deoxyribosyl-2-aminopurine with improved properties. Nucleic Acids Res, 33(3), 1031-1039.

BOCHTLER, M., SZCZEPANOWSKI, R. H., TAMULAITIS, G., GRAZULIS, S., CZAPINSKA, H., MANAKOVA, E. & SIKSNYS, V. (2006). Nucleotide flips determine the specificity of the Ecl18kI restriction endonuclease. EMBO J, 25, 2219-2229.

BONNIST, E. Y. & JONES, A. C. (2008). Long-wavelength fluorescence from 2-aminopurine-nucleobase dimers in DNA. Chemphyschem, 9(8), 1121-1129.

BONNIST, E. Y. M. (2008). Ph.D. thesis, University of Edinburgh.

BONNIST, E. Y. M., LIEBERT, K., DRYDEN, D. T. F., JELTSCH, A. & JONES, A. C. (2012). Using the fluorescence decay of 2-aminopurine to investigate conformational change in the recognition sequence of the EcoRV DNA-(adenine-N6)-methyltransferase on enzyme binding. Biophysical Chemistry, 160, 28-34.

BUSCAGLIA, R., JAMESON, D. M. & CHAIRES, J. B. (2012). G-quadruplex structure and stability illuminated by 2-aminopurine phasor plots. Nucleic Acids Res, 40(9), 4203-4215.

CAMPBELL, C. J., MOUNTFORD, C. P., STOQUERT, H. C., BUCK, A. H., DICKINSON, P., FERAPONTOVA, E., TERRY, J. G., BEATTIE, J. S., WALTON, A. J., CRAIN, J., GHAZAL, P. & MOUNT, A. R. (2009). A DNA nanoswitch incorporating the fluorescent base analogue 2-aminopurine detects single nucleotide mismatches in unlabelled targets. Analyst, 134(9), 1873-1879.

DALLMANN, A., DEHMEL, L., PETERS, T., MÜGGE, C., GRIESINGER, C., TUMA, J. & ERNSTING, N. P. (2010). 2-Aminopurine Incorporation Perturbs the Dynamics and Structure of DNA. Angewandte Chemie International Edition, 49(34), 5989-5992.

ENGMAN, K. C., SANDIN, P., OSBORNE, S., BROWN, T., BILLETER, M., LINCOLN, P., NORDEN, B., ALBINSSON, B. & WILHELMSSON, L. M.

(2004). DNA adopts normal B-form upon incorporation of highly fluorescent DNA base analogue tC: NMR structure and UV-Vis spectroscopy characterization. Nucleic Acids Res, 32(17), 5087-5095.
ERITJA, R., KAPLAN, B. E., MHASKAR, D., SOWERS, L. C., PETRUSKA, J. & GOODMAN, M. F. (1986). Synthesis and properties of defined DNA oligomers containing base mispairs involving 2-aminopurine. Nucleic Acids Res, 14(14), 5869-5884.
EVANS, K., XU, D., KIM, Y. & NORDLUND, T. M. (1992). 2-Aminopurine optical spectra: Solvent, pentose ring, and DNA helix melting dependence. J Fluoresc, 2(4), 209-216.
FAZAKERLEY, G. V., SOWERS, L. C., ERITJA, R., KAPLAN, B. E. & GOODMAN, M. F. (1987). NMR studies on an oligodeoxynucleotide containing 2-aminopurine opposite adenine. Biochemistry, 26(18), 5641-5646.
FENG, K., ENGLER, G., SEEFELD, K. & KLEINERMANNS, K. (2009). Dispersed fluorescence and delayed ionization of jet-cooled 2-aminopurine: relaxation to a dark state causes weak fluorescence. Chemphyschem, 10(6), 886-889.
FINGER, L. D., PATEL, N., BEDDOWS, A., MA, L., EXELL, J. C., JARDINE, E., JONES, A. C. & GRASBY, J. A. (2013). Observation of unpaired substrate DNA in the flap endonuclease-1 active site. Nucleic Acids Res, 41(21), 9839-9847.
FOGARTY, A. C., JONES, A. C. & CAMP, P. J. (2011). Extraction of lifetime distributions from fluorescence decays with application to DNA-base analogues. Phys Chem Chem Phys, 13(9), 3819-3830.
FREY, M. W., SOWERS, L. C., MILLAR, D. P. & BENKOVIC, S. J. (1995). The nucleotide analog 2-aminopurine as a spectroscopic probe of nucleotide incorporation by the Klenow fragment of Escherichia coli polymerase I and bacteriophage T4 DNA polymerase. Biochemistry, 34(28), 9185-9192.
GELOT, T., TOURON-TOUCEDA, P., CREGUT, O., LEONARD, J. & HAACKE, S. (2012). Ultrafast site-specific fluorescence quenching of 2-aminopurine in a DNA hairpin studied by femtosecond down-conversion. J Phys Chem A, 116(11), 2819-2825.
GENEREUX, J. C. & BARTON, J. K. (2010). Mechanisms for DNA charge transport. Chem Rev, 110(3), 1642-1662.
GODET, J., RAMALANJAONA, N., SHARMA, K. K., RICHERT, L., DE ROCQUIGNY, H., DARLIX, J. L., DUPORTAIL, G. & MELY, Y. (2011). Specific implications of the HIV-1 nucleocapsid zinc fingers in the annealing of the primer binding site complementary sequences during the obligatory plus strand transfer. Nucleic Acids Res, 39(15), 6633-6645.
GOEL, T., MUKHERJEE, T., RAO, B. J. & KRISHNAMOORTHY, G. (2010). Fluorescence dynamics of double- and single-stranded DNA bound to histone and micellar surfaces. J Phys Chem B, 114(27), 8986-8993.
GOWHER, H. & JELTSCH, A. (2000). Molecular enzymology of the Eco RV DNA-(adenine-N6)-methyltransferase: kinetics of DNA binding and bending, kinetic mechanism and linear diffusion of the enzyme on DNA. Journal of Molecular Biology, 303, 93-110.
GUCKIAN, K. M., KRUGH, T. R. & KOOL, E. T. (1998). Solution structure of a DNA duplex containing a replicable difluorotoluene-adenine pair. Nat Struct Biol, 5(11), 954-959.

GUCKIAN, K. M., KRUGH, T. R. & KOOL, E. T. (2000). Solution Structure of a Nonpolar, Non-Hydrogen-Bonded Base Pair Surrogate in DNA. J Am Chem Soc, 122(29), 6841-6847.
GUEST, C. R., HOCHSTRASSER, R. A., SOWERS, L. C. & MILLAR, D. P. (1991). Dynamics of mismatched base pairs in DNA. Biochemistry, 30(13), 3271-3279.
HARDMAN, S. J. & THOMPSON, K. C. (2006). Influence of base stacking and hydrogen bonding on the fluorescence of 2-aminopurine and pyrrolocytosine in nucleic acids. Biochemistry, 45(30), 9145-9155.
HARDMAN, S. J. O. & THOMPSON, K. C. (2007). The fluorescence transition of 2-aminopurine in double- and single-stranded DNA. International Journal of Quantum Chemistry, 107(11), 2092-2099.
HARIHARAN, C. & REHA-KRANTZ, L. J. (2005). Using 2-aminopurine fluorescence to detect bacteriophage T4 DNA polymerase-DNA complexes that are important for primer extension and proofreading reactions. Biochemistry, 44(48), 15674-15684.
HARRIMAN, A. (1987). Further comments on the redox potentials of tryptophan and tyrosine. The Journal of Physical Chemistry, 91(24), 6102-6104.
HE, R. X., DUAN, X. H. & LI, X. Y. (2006). Theoretical investigation of spectral properties and tautomerization mechanism of 2-aminopurine. Phys Chem Chem Phys, 8(5), 587-591.
HOCHSTRASSER, R. A., CARVER, T. E., SOWERS, L. C. & MILLAR, D. P. (1994). Melting of a DNA helix terminus within the active site of a DNA polymerase. Biochemistry, 33(39), 11971-11979.
HOLMÉN, A., NORDÉN, B. & ALBINSSON, B. (1997). Electronic Transition Moments of 2-Aminopurine. J Am Chem Soc, 119(13), 3114-3121.
HOLZ, B., KLIMASAUSKAS, S., SERVA, S. & WEINHOLD, E. (1998). 2-Aminopurine as a fluorescent probe for DNA base flipping by methyltransferases. Nucl. Acids Res., 26, 1076-1083.
JAMES, D. R., SIEMIARCZUK, A. & WARE, W. R. (1992). Stroboscopic optical boxcar technique for the determination of fluorescence lifetimes. Review of Scientific Instruments, 63(2), 1710.
JEAN, J. M. & HALL, K. B. (2001). 2-Aminopurine fluorescence quenching and lifetimes: Role of base stacking. Proceedings of the National Academy of Sciences, 98(1), 37-41.
JEAN, J. M. & HALL, K. B. (2002). 2-Aminopurine Electronic Structure and Fluorescence Properties in DNA†. Biochemistry, 41(44), 13152-13161.
JELTSCH, A. (2002). Beyond Watson and Crick: DNA Methylation and Molecular Enzymology of DNA Methyltransferases. ChemBioChem, 3(4), 274-293.
JOHNSON, N. P., BAASE, W. A. & VON HIPPEL, P. H. (2004). Low-energy circular dichroism of 2-aminopurine dinucleotide as a probe of local conformation of DNA and RNA. Proc Natl Acad Sci U S A, 101(10), 3426-3431.
JOSE, D., DATTA, K., JOHNSON, N. P. & VON HIPPEL, P. H. (2009). Spectroscopic studies of position-specific DNA "breathing" fluctuations at replication forks and primer-template junctions. Proc Natl Acad Sci U S A, 106(11), 4231-4236.

KELLEY, S. O. (1999). Electron Transfer Between Bases in Double Helical DNA. Science, 283(5400), 375-381.
KIMURA, T., KAWAI, K., FUJITSUKA, M. & MAJIMA, T. (2004). Fluorescence properties of 2-aminopurine in human telomeric DNA. Chem Commun (Camb), (12), 1438-1439.
KIMURA, T., KAWAI, K., FUJITSUKA, M. & MAJIMA, T. (2007). Monitoring G-quadruplex structures and G-quadruplex–ligand complex using 2-aminopurine modified oligonucleotides. Tetrahedron, 63(17), 3585-3590.
LAKOWICZ, J. R. (2006). Principles of Fluoresence Spectroscopy, 3rd edition: Springer.
LARSEN, O. F. A., VAN STOKKUM, I. H. M., DE WEERD, F. L., VENGRIS, M., ARAVINDAKUMAR, C. T., VAN GRONDELLE, R., GEACINTOV, N. E. & VAN AMERONGEN, H. (2004). Ultrafast transient-absorption and steady-state fluorescence measurements on 2-aminopurine substituted dinucleotides and 2-aminopurine substituted DNA duplexes. Physical Chemistry Chemical Physics, 6(1), 154.
LAW, S. M., ERITJA, R., GOODMAN, M. F. & BRESLAUER, K. J. (1996). Spectroscopic and calorimetric characterizations of DNA duplexes containing 2-aminopurine. Biochemistry, 35(38), 12329-12337.
LEE, B. J., BARCH, M., CASTNER, E. W., JR., VOLKER, J. & BRESLAUER, K. J. (2007). Structure and dynamics in DNA looped domains: CAG triplet repeat sequence dynamics probed by 2-aminopurine fluorescence. Biochemistry, 46(38), 10756-10766.
LENZ, T., BONNIST, E. Y. M., PLJEVALJČIĆ, G., NEELY, R. K., DRYDEN, D. T. F., SCHEIDIG, A. J., JONES, A. C. & WEINHOLD, E. (2007). 2-Aminopurine Flipped into the Active Site of the Adenine-Specific DNA Methyltransferase M.TaqI: Crystal Structures and Time-Resolved Fluorescence. Journal of the American Chemical Society, 129, 6240-6248.
LIANG, J. & MATSIKA, S. (2011). Pathways for fluorescence quenching in 2-aminopurine pi-stacked with pyrimidine nucleobases. J Am Chem Soc, 133(17), 6799-6808.
LIANG, J., NGUYEN, Q. L. & MATSIKA, S. (2013). Exciplexes and conical intersections lead to fluorescence quenching in pi-stacked dimers of 2-aminopurine with natural purine nucleobases. Photochem Photobiol Sci, 12(8), 1387-1400.
LOBSIGER, S., SINHA, R. K. & LEUTWYLER, S. (2013). Building up water-wire clusters: isomer-selective ultraviolet and infrared spectra of jet-cooled 2-aminopurine (H2O)n, n = 2 and 3. J Phys Chem B, 117(41), 12410-12421.
LOBSIGER, S., SINHA, R. K., TRACHSEL, M. & LEUTWYLER, S. (2011). Low-lying excited states and nonradiative processes of the adenine analogues 7H- and 9H-2-aminopurine. J Chem Phys, 134(11), 114307.
LUDWIG, V., AMARAL, M. S. D., DA COSTA, Z. M., BORIN, A. C., CANUTO, S. & SERRANO-ANDRÉS, L. (2008). 2-Aminopurine non-radiative decay and emission in aqueous solution: A theoretical study. Chemical Physics Letters, 463(1-3), 201-205.
LYCKSELL, P. O., GRÄSLUND, A., CLAESENS, F., MCLAUGHLIN, L. W., LARSSON, U. & RIGLER, R. (1987). Base pair opening dynamics of a 2-

aminopurine substituted Eco RI restricion sequence and its unsubstituted counterpart in oligonucleotides. Nucleic Acids Res, 15(21), 9011-9025.
MA, L. (2012). Ph.D. thesis, University of Edinburgh.
MA, L., WU, X., WILSON, G. G., JONES, A. C. & DRYDEN, D. T. (2014). Time-resolved fluorescence of 2-aminopurine in DNA duplexes in the presence of the EcoP15I Type III restriction-modification enzyme. Biochem Biophys Res Commun, 449(1), 120-125.
MANOJ, P., MIN, C.-K., ARAVINDAKUMAR, C. T. & JOO, T. (2008). Ultrafast charge transfer dynamics in 2-aminopurine modified double helical DNA. Chemical Physics, 352(1-3), 333-338.
NADLER, A., STROHMEIER, J. & DIEDERICHSEN, U. (2011). 8-Vinyl-2'-deoxyguanosine as a fluorescent 2'-deoxyguanosine mimic for investigating DNA hybridization and topology. Angew Chem Int Ed Engl, 50(23), 5392-5396.
NARAYANAN, M., KODALI, G., XING, Y. & STANLEY, R. J. (2010). Photoinduced Electron Transfer Occurs between 2-Aminopurine and the DNA Nucleic Acid Monophosphates: Results from Cyclic Voltammetry and Fluorescence Quenching. The Journal of Physical Chemistry B, 114(32), 10573-10580.
NEELY, R. K. (2005). Ph.D. thesis, University of Edinburgh.
NEELY, R. K., DAUJOTYTE, D., GRAZULIS, S., MAGENNIS, S. W., DRYDEN, D. T., KLIMASAUSKAS, S. & JONES, A. C. (2005). Time-resolved fluorescence of 2-aminopurine as a probe of base flipping in M.HhaI-DNA complexes. Nucleic Acids Res, 33(22), 6953-6960.
NEELY, R. K. & JONES, A. C. (2006). Influence of base dynamics on the conformational properties of DNA: Observation of static conformational states in rigid duplexes at 77 K. J Am Chem Soc, 128(50), 15952-15953.
NEELY, R. K., MAGENNIS, S. W., DRYDEN, D. T. F. & JONES, A. C. (2004). Evidence of Tautomerism in 2-Aminopurine from Fluorescence Lifetime Measurements. The Journal of Physical Chemistry B, 108(45), 17606-17610.
NEELY, R. K., MAGENNIS, S. W., PARSONS, S. & JONES, A. C. (2007). Photophysics and X-ray structure of crystalline 2-aminopurine. Chemphyschem, 8(7), 1095-1102.
NEELY, R. K., TAMULAITIS, G., CHEN, K., KUBALA, M., SIKSNYS, V. & JONES, A. C. (2009). Time-resolved fluorescence studies of nucleotide flipping by restriction enzymes. Nucleic Acids Res, 37(20), 6859-6870.
NOÉ, M. S., SINKELDAM, R. W. & TOR, Y. (2013). Oligodeoxynucleotides Containing Multiple Thiophene-Modified Isomorphic Fluorescent Nucleosides. The Journal of Organic Chemistry, 78(16), 8123-8128.
NORDLUND, T. M. (2007). Sequence, Structure and Energy Transfer in DNA†. Photochemistry and Photobiology, 83(3), 625-636.
NORDLUND, T. M., ANDERSSON, S., NILSSON, L., RIGLER, R., GRAESLUND, A. & MCLAUGHLIN, L. W. (1989). Structure and dynamics of a fluorescent DNA oligomer containing the EcoRI recognition sequence: fluorescence, molecular dynamics, and NMR studies. Biochemistry, 28(23), 9095-9103.
O'NEIL, M. A. & BARTON, J. K. (2002). 2-Aminopurine: A Probe of Structural Dynamics and Charge Transfer in DNA and DNA:RNA Hybrids. J Am Chem Soc, 124(44), 13053-13066.

O'NEILL, M. A. & BARTON, J. K. (2002). Effects of strand and directional asymmetry on base-base coupling and charge transfer in double-helical DNA. Proc Natl Acad Sci U S A, 99(26), 16543-16550.
O'NEILL, M. A. & BARTON, J. K. (2004a). DNA-mediated charge transport requires conformational motion of the DNA bases: elimination of charge transport in rigid glasses at 77 K. J Am Chem Soc, 126(41), 13234-13235.
O'NEILL, M. A. & BARTON, J. K. (2004b). DNA charge transport: conformationally gated hopping through stacked domains. J Am Chem Soc, 126(37), 11471-11483.
O'NEILL, M. A., BECKER, H. C., WAN, C., BARTON, J. K. & ZEWAIL, A. H. (2003). Ultrafast dynamics in DNA-mediated electron transfer: base gating and the role of temperature. Angew Chem Int Ed Engl, 42(47), 5896-5900.
O'NEILL, M. A., DOHNO, C. & BARTON, J. K. (2004). Direct chemical evidence for charge transfer between photoexcited 2-aminopurine and guanine in duplex DNA. J Am Chem Soc, 126(5), 1316-1317.
RACHOFSKY, E. L., OSMAN, R. & ROSS, J. B. A. (2001a). Probing Structure and Dynamics of DNA with 2-Aminopurine: Effects of Local Environment on Fluorescence†. Biochemistry, 40(4), 946-956.
RACHOFSKY, E. L., ROSS, J. B. A., KRAUSS, M. & OSMAN, R. (2001b). CASSCF Investigation of Electronic Excited States of 2-Aminopurine. The Journal of Physical Chemistry A, 105(1), 190-197.
RACHOFSKY, E. L., SEIBERT, E., STIVERS, J. T., OSMAN, R. & ROSS, J. B. A. (2001c). Conformation and Dynamics of Abasic Sites in DNA Investigated by Time-Resolved Fluorescence of 2-Aminopurine†. Biochemistry, 40(4), 957-967.
RACHOFSKY, E. L., SOWERS, L. C., HAWKINS, M. E., BALIS, F. M., LAWS, W. R. & ROSS, J. B. A. (1998). Emission kinetics of fluorescent nucleoside analogs, vol. 3256, pp. 68-75.
RAMREDDY, T., KOMBRABAIL, M., KRISHNAMOORTHY, G. & RAO, B. J. (2009). Site-specific dynamics in TAT triplex DNA as revealed by time-domain fluorescence of 2-aminopurine. J Phys Chem B, 113(19), 6840-6846.
RAMREDDY, T., RAO, B. J. & KRISHNAMOORTHY, G. (2007). Site-specific dynamics of strands in ss- and dsDNA as revealed by time-domain fluorescence of 2-aminopurine. J Phys Chem B, 111(20), 5757-5766.
RANEY, K. D., SOWERS, L. C., MILLAR, D. P. & BENKOVIC, S. J. (1994). A fluorescence-based assay for monitoring helicase activity. Proceedings of the National Academy of Sciences, 91(14), 6644-6648.
REDDY, Y. V. & RAO, D. N. (2000). Binding of EcoP15I DNA methyltransferase to DNA reveals a large structural distortion within the recognition sequence. J Mol Biol, 298(4), 597-610.
REHA-KRANTZ, L. J. (2009). The use of 2-aminopurine fluorescence to study DNA polymerase function. Methods Mol. Biol. (Totowa, NJ, U. S.), 521(DNA Replication), 381-396.
REICHARDT, C., WEN, C., VOGT, R. A. & CRESPO-HERNANDEZ, C. E. (2013). Role of intersystem crossing in the fluorescence quenching of 2-aminopurine 2'-deoxyriboside in solution. Photochem Photobiol Sci, 12(8), 1341-1350.
RICHARDSON, T. T., WU, X., KEITH, B. J., HESLOP, P., JONES, A. C. & CONNOLLY, B. A. (2013). Unwinding of primer-templates by archaeal

family-B DNA polymerases in response to template-strand uracil. Nucleic Acids Res, 41(4), 2466-2478.
RIST, M., WAGENKNECHT, H.-A. & FIEBIG, T. (2002). Exciton and Excimer Formation in DNA at Room Temperature. ChemPhysChem, 3(8), 704-707.
SABIR, T., TOULMIN, A., MA, L., JONES, A. C., MCGLYNN, P., SCHRODER, G. F. & MAGENNIS, S. W. (2012). Branchpoint expansion in a fully complementary three-way DNA junction. J Am Chem Soc, 134(14), 6280-6285.
SERRANO-ANDRES, L., MERCHAN, M. & BORIN, A. C. (2006). Adenine and 2-aminopurine: paradigms of modern theoretical photochemistry. Proc Natl Acad Sci U S A, 103(23), 8691-8696.
SINHA, R. K., LOBSIGER, S., TRACHSEL, M. & LEUTWYLER, S. (2011). Vibronic spectra of jet-cooled 2-aminopurine.H2O clusters studied by UV resonant two-photon ionization spectroscopy and quantum chemical calculations. J Phys Chem A, 115(23), 6208-6217.
SINKELDAM, R. W., GRECO, N. J. & TOR, Y. (2010). Fluorescent analogs of biomolecular building blocks: design, properties, and applications. Chem Rev, 110(5), 2579-2619.
SMIRNOV, S. (2002). Integrity of duplex structures without hydrogen bonding: DNA with pyrene paired at abasic sites. Nucleic Acids Res, 30(24), 5561-5569.
SOMSEN, O. J., KEUKENS, L. B., DE KEIJZER, M. N., VAN HOEK, A. & VAN AMERONGEN, H. (2005a). Structural heterogeneity in DNA: temperature dependence of 2-aminopurine fluorescence in dinucleotides. Chemphyschem, 6(8), 1622-1627.
SOMSEN, O. J. G., HOEK, V. A. & AMERONGEN, V. H. (2005b). Fluorescence quenching of 2-aminopurine in dinucleotides. Chemical Physics Letters, 402(1-3), 61-65.
SOMSEN, O. J. G., TRINKUNAS, G., KEIJZER, M. N. D., HOEK, A. V. & AMERONGEN, H. V. (2006). Local diffusive dynamics in DNA. Journal of Luminescence, 119-120, 100-104.
SOWERS, L. C., BOULARD, Y. & FAZAKERLEY, G. V. (2000). Multiple Structures for the 2-Aminopurine−Cytosine Mispair†. Biochemistry, 39(25), 7613-7620.
SOWERS, L. C., FAZAKERLEY, G. V., ERITJA, R., KAPLAN, B. E. & GOODMAN, M. F. (1986). Base pairing and mutagenesis: observation of a protonated base pair between 2-aminopurine and cytosine in an oligonucleotide by proton NMR. Proc Natl Acad Sci U S A, 83(15), 5434-5438.
SU, T.-J., CONNOLLY, B. A., DARLINGTON, C., MALLIN, R. & DRYDEN, D. T. F. (2004). Unusual 2-aminopurine fluorescence from a complex of DNA and the EcoKI methyltransferase. Nucl. Acids Res., 32, 2223-2230.
SUBUDDHI, U., HOGG, M. & REHA-KRANTZ, L. J. (2008). Use of 2-aminopurine fluorescence to study the role of the beta hairpin in the proofreading pathway catalyzed by the phage T4 and RB69 DNA polymerases. Biochemistry, 47(23), 6130-6137.
TLEUGABULOVA, D. & REHA-KRANTZ, L. J. (2007). Probing DNA polymerase-DNA interactions: examining the template strand in exonuclease complexes

using 2-aminopurine fluorescence and acrylamide quenching. Biochemistry, 46(22), 6559-6569.
WAN, C., FIEBIG, T., SCHIEMANN, O., BARTON, J. K. & ZEWAIL, A. H. (2000). Femtosecond direct observation of charge transfer between bases in DNA. Proc Natl Acad Sci U S A, 97(26), 14052-14055.
WAN, C., XIA, T., BECKER, H.-C. & ZEWAIL, A. H. (2005). Ultrafast unequilibrated charge transfer: A new channel in the quenching of fluorescent biological probes. Chemical Physics Letters, 412(1-3), 158-163.
WARD, D. C., REICH, E. & STRYER, L. (1969). Fluorescence Studies of Nucleotides and Polynucleotides: I. FORMYCIN, 2-AMINOPURINE RIBOSIDE, 2,6-DIAMINOPURINE RIBOSIDE, AND THEIR DERIVATIVES. Journal of Biological Chemistry, 244(5), 1228-1237.
WILHELMSSON, L. M. (2010). Fluorescent nucleic acid base analogues. Q Rev Biophys, 43(2), 159-183.
XIA, T. (2008). Taking femtosecond snapshots of RNA conformational dynamics and complexity. Curr Opin Chem Biol, 12(6), 604-611.
XIA, T., BECKER, H. C., WAN, C., FRANKEL, A., ROBERTS, R. W. & ZEWAIL, A. H. (2003). The RNA-protein complex: direct probing of the interfacial recognition dynamics and its correlation with biological functions. Proc Natl Acad Sci U S A, 100(14), 8119-8123.
XU, D., EVANS, K. O. & NORDLUND, T. M. (1994). Melting and Premelting Transitions of an Oligomer Measured by DNA Base Fluorescence and Absorption. Biochemistry, 33(32), 9592-9599.
YOUNGBLOOD, B., BONNIST, E., DRYDEN, D. T. F., JONES, A. C. & REICH, N. O. (2008). Differential stabilization of reaction intermediates: specificity checkpoints for M.EcoRI revealed by transient fluorescence and fluorescence lifetime studies. Nucleic Acids Research, 36, 2917-2925.

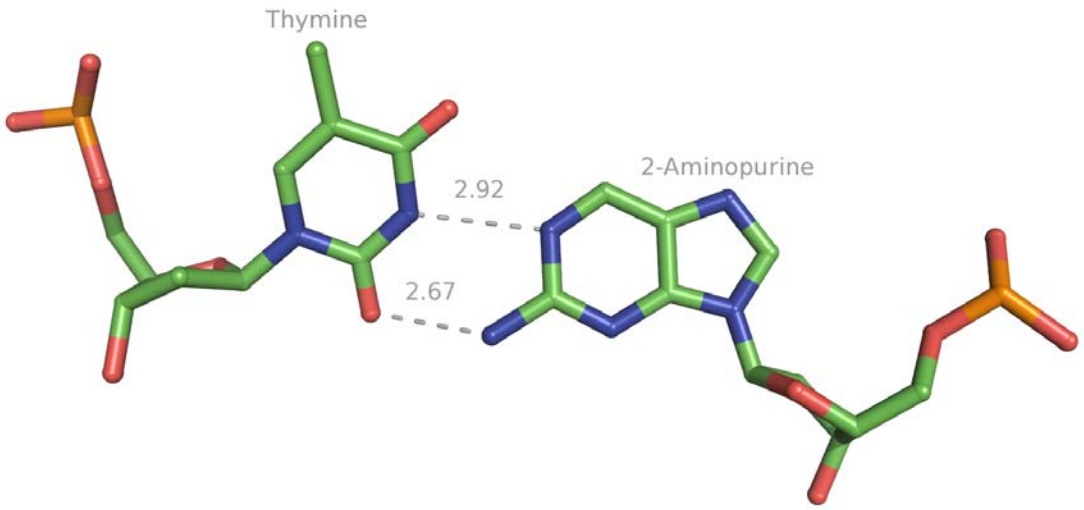

Figure 1. Detail from the crystal structure of a duplex (PDB ID: 4IHX) showing the Watson-Crick base-pair formed between 2AP and T. (The lone oxygen atoms (red) in the image indicate the presence of water molecules). Figure generated using Pymol 0.99.

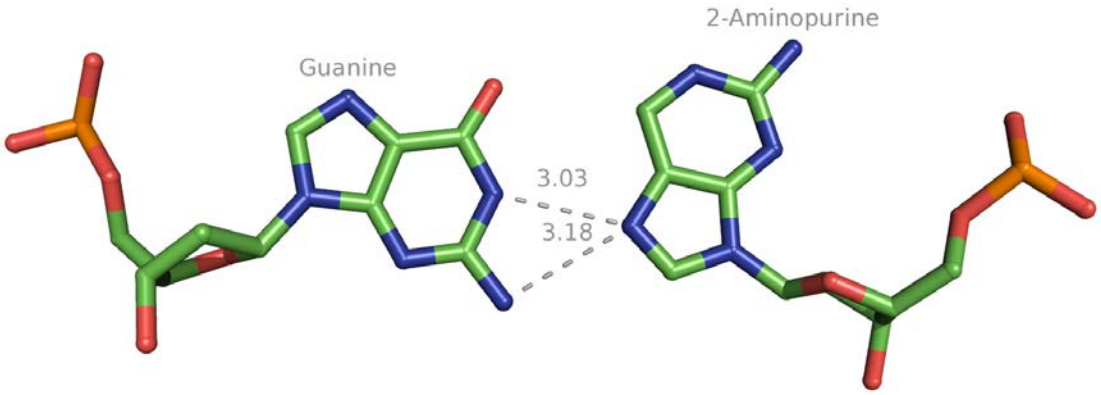

Figure 2. Detail from the crystal structure of a duplex (PDB ID: 2C7Q) in which 2AP is base-paired with G, showing the hydrogen bonds between the paired bases. 2AP forms a Hoogsteen-type base pair with G. (The lone oxygen atom (red) in the image indicates the presence of a water molecule). Figure generated using Pymol 0.99.

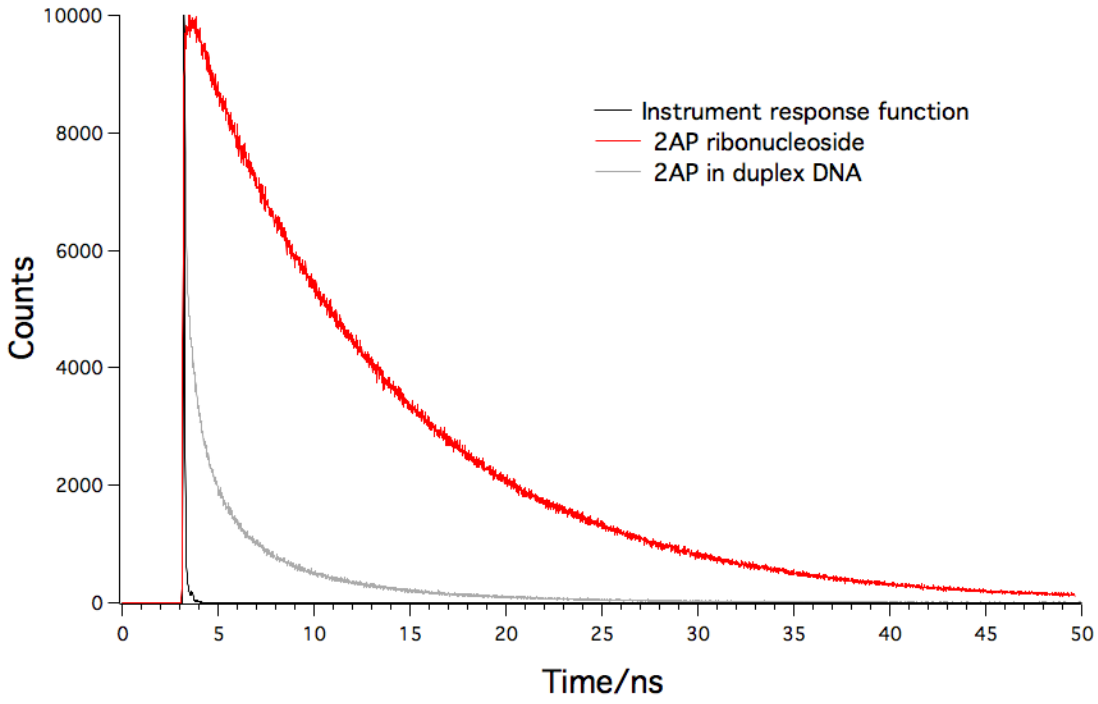

Figure 3. A comparison of the decay curves of the free 2AP-ribonuscleoside (red) and a 2AP-containing DNA duplex (grey). The instrument response function is shown in black.

5'-GACAGTATCAGGCGCCTCCCCACAA _-3'
3'-_ TGTCATAGTCCGMGGPGGGGTGTTG-5'

5'-GACAGTATCAGGCGCCGCCCCACAA _-3'
3'-_ TGTCATAGTCCGMGGPGGGGTGTTG-5'

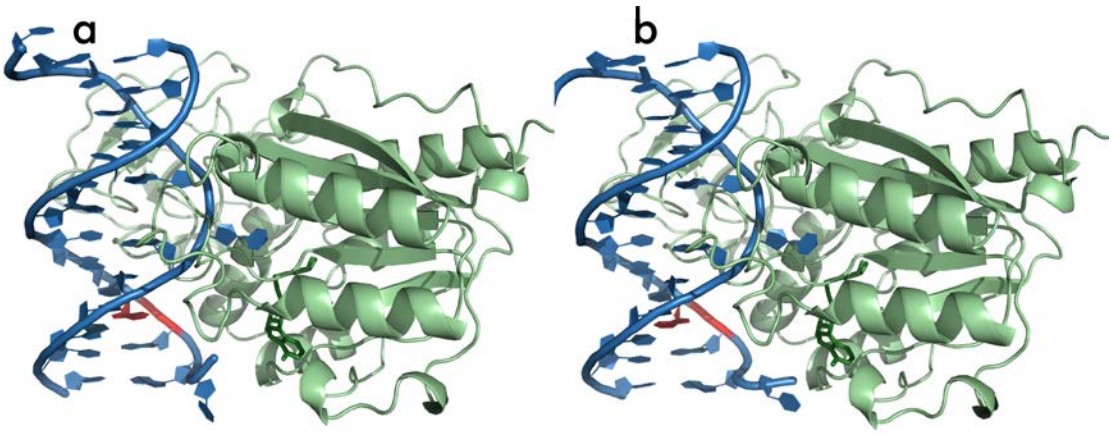

Figure 4. The crystal structure of (a) the PG (PDB ID: 2C7Q) and (b) the PT duplex (blue) (unpublished work) in ternary complex with M.HhaI (green ribbons) and the AdoHcy cofactor (dark green sticks). The 2AP base is shown in red.

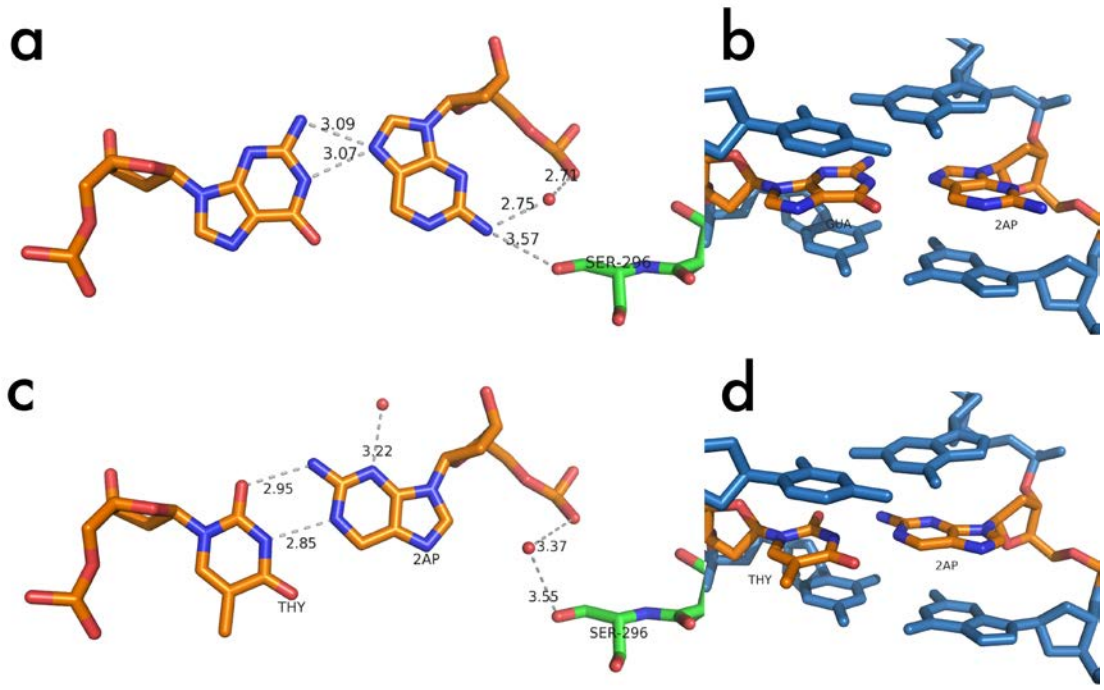

Figure 5. The immediate environment of 2AP in the crystalline (a and b) PG duplex (PDB ID: 2C7Q) and (c and d) PT duplex (unpublished work). Water molecules are shown as lone oxygen atoms (red). Distances shown are for donor-acceptor interactions between hydrogen bonding atoms. For the 2AP-containing oligonucleotide strands (b and d), the bases are shown 3' to 5' going from the top to bottom of the Figure.

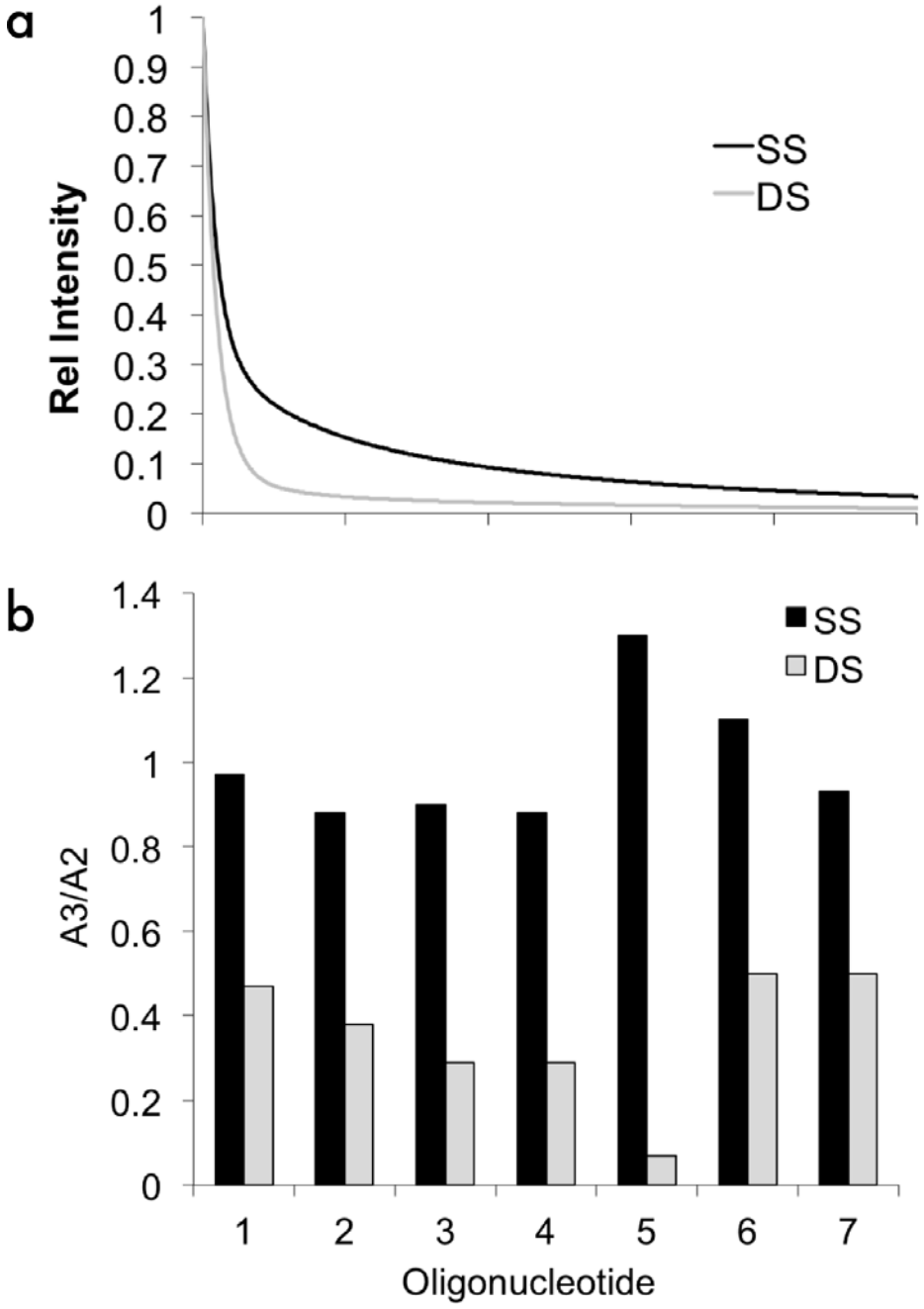

Figure 6. (a) Comparison of the fluorescence decay curve of a 2AP-containing single strand (black) with that of the corresponding duplex (black). (b) Comparison of the ratio of the A factors, A3 and A2, for 2AP in each of seven single-strand (black) and duplex (grey) oligonucleotides.

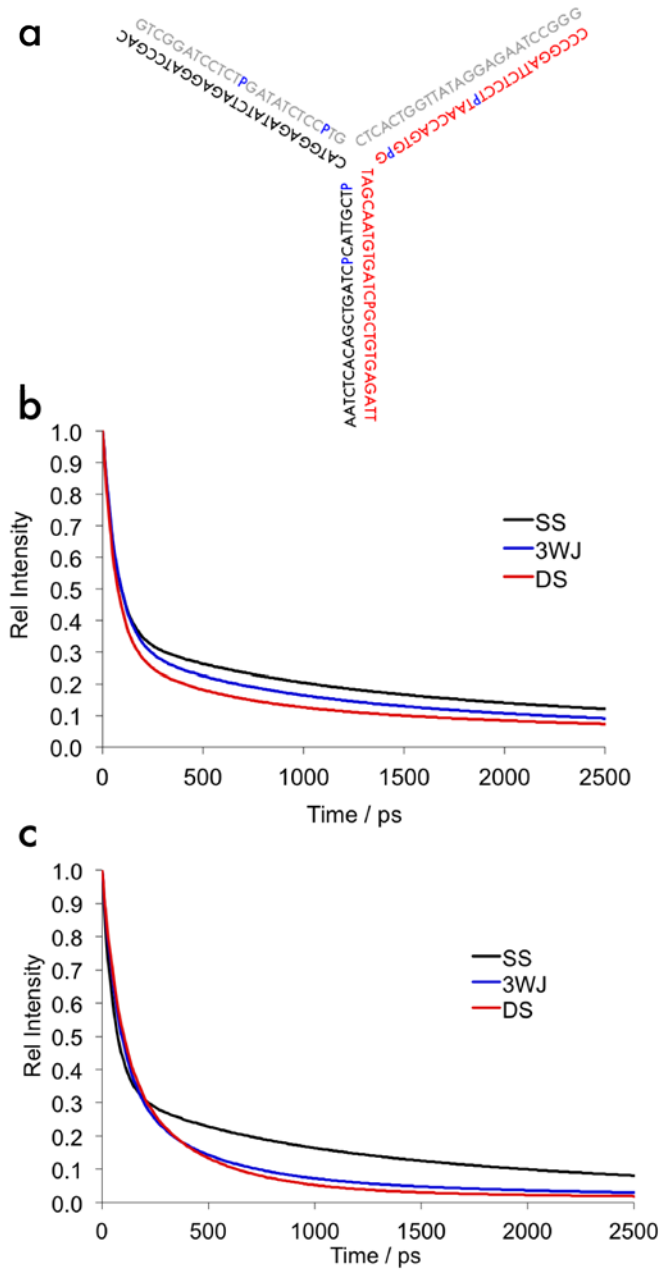

Figure 7. Comparison of the 2AP fluorescence decay in a three-way DNA junction (3WJ) to the corresponding decays for single-stranded (SS) and duplex (DS) DNA. (a) The three-way junction sequence and positions at which the 2AP base (blue P) was placed. (b) The fluorescence decays of a 3WJ (blue), with 2AP distant from the branchpoint, and of the respective SS (black) and DS (red). (c) The fluorescence decays of a 3WJ (blue), with 2AP adjacent to the branchpoint, and of the respective SS (black) and DS (red).

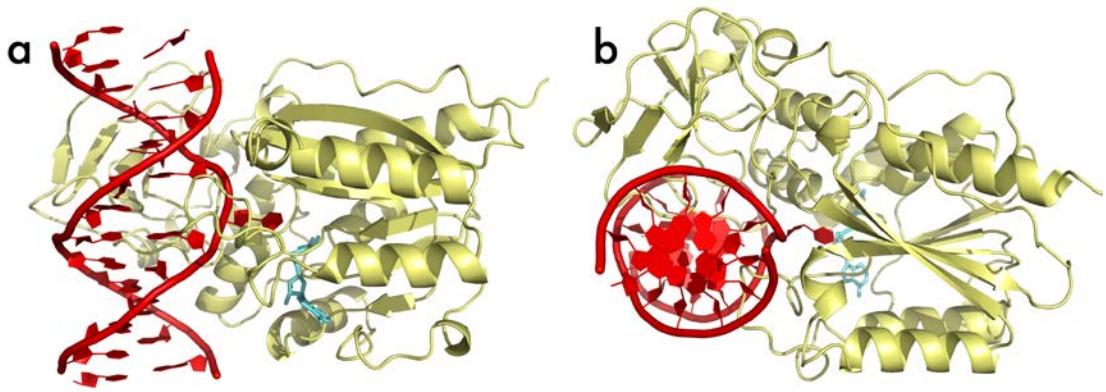

Figure 8. Views from a crystal structure (PDB ID: 3MHT) showing base flipping of cytosine by M.HhaI (yellow), (a) looking from the side of the duplex and (b) looking down the axis of the duplex. DNA is shown in red with the S-adenosyl-L-homocysteine cofactor analogue in pale blue.

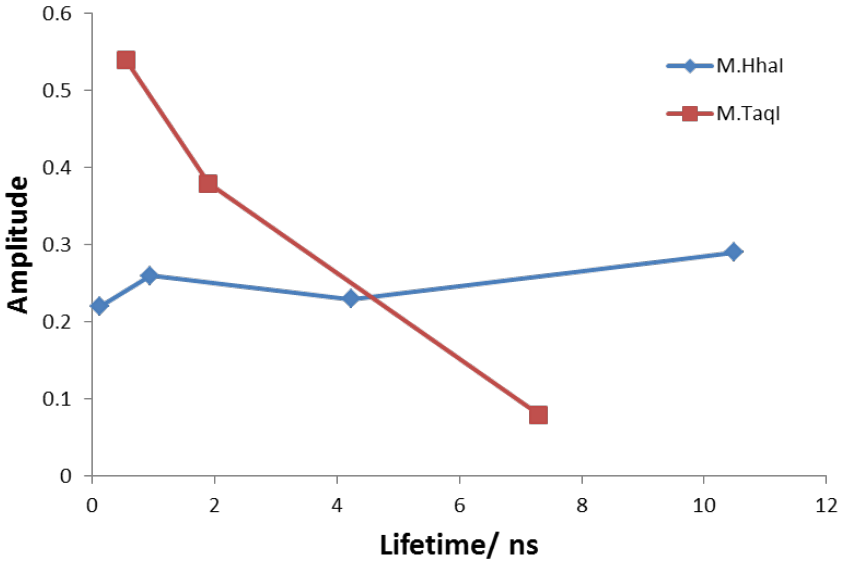

Figure 9. Plot of amplitudes (A factors) versus lifetimes for DNA duplexes containing 2AP at the target site for flipping, in ternary complexes with either M.HhaI (blue diamonds) or M.TaqI (red squares) and cofactor (S-adenosyl-L-methionine).

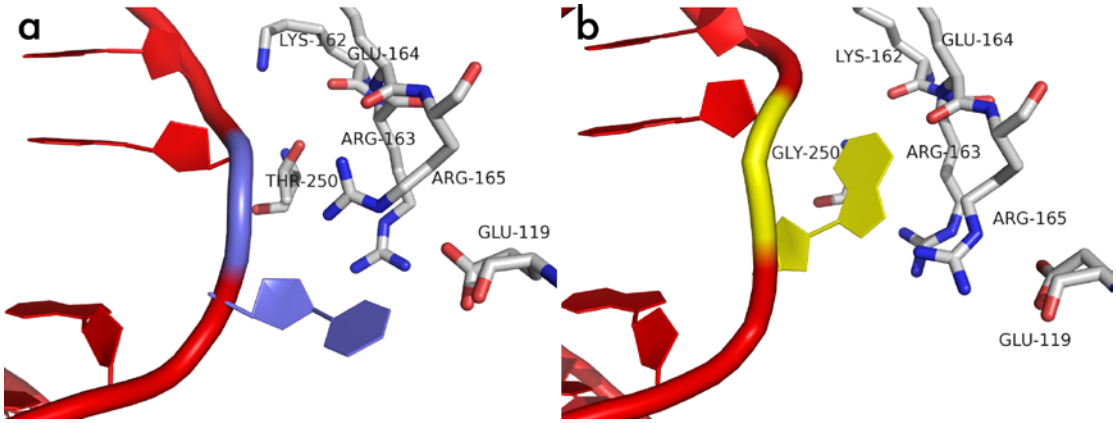

Figure 10. Structures of M.HhaI in the region around residue 250. (a) Wild-type enzyme with flipped cytosine base in the enzyme active site (PDB ID: 3MHT); (b) T250G mutant with flipped 2AP base occupying the void created by the T250G mutation (PDB ID:2C7R). Note that the R165 residue has apparently been displaced into the region defined as the enzyme 'active site' for the wild-type M.HhaI.

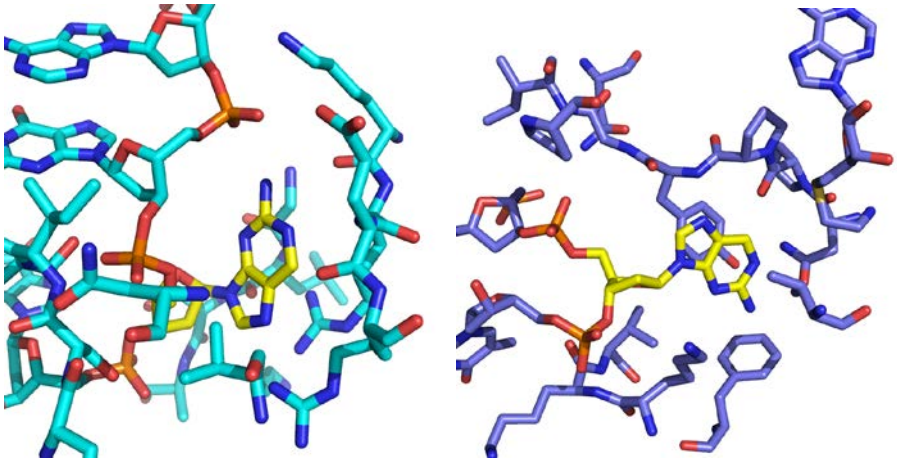

Figure 11. The immediate environment of 2AP (yellow) in crystalline ternary complexes of the T250G M.HhaI mutant (cyan) (PDB ID: 2C7R) and the wild-type M.TaqI (blue) enzyme (PDB ID: 2IBS).