

Fluorescence quenching of 2-aminopurine in dinucleotides

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Abstract

2-Aminopurine is a fluorescent base analog that probes local dynamics in DNA because its fluorescence is quenched by the surrounding bases. But, the underlying mechanisms are still under investigation. We have studied fluorescence decay of 2-aminopurine in dinucleotides and found a similar heterogeneity as was previously observed in larger oligonucleotides. This indicates that dinucleotides could function as model for local dynamics of intact DNA. We also found that fast (21–35 ps) quenching could be induced by all natural bases. Our results confirm that the product of this fast transition has the characteristics of a dark state.

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1. Introduction

The fluorescent base analog 2-aminopurine (2AP) has several properties that make it useful as a probe for local dynamics in DNA. Firstly, 2AP is an analog of adenine which it can replace in DNA with little effect on structure [1–5] and function [6–8]. In addition, 2AP is strongly fluorescent ($\Phi = 0.66$, $\tau \approx 10$ ns) [9,10] compared to the natural bases ($\Phi = 0.5 - 1.0 \times 10^{-4}$, $\tau = 290 - 720$ fs) [11,12]. Also, the excited state of 2AP is red-shifted with respect to the natural bases, which allows selective excitation even in large DNA fragments. Finally, when 2AP is part of a DNA fragment, its fluorescence is quenched strongly and multi-exponentially [1,3,13–16]. The various decay components can be used to monitor the neighbouring bases.

While several groups have studied 2AP in DNA, an unambiguous model of its fluorescence characteristics is not yet available. Fast decay of excited 2AP induced by a neighbouring guanine has been attributed to electron transfer [13,14,17,18], but a charge-separated product state has not been observed unambiguously [19,20].

Moreover, fast (30–40 ps) 2AP quenching by the other natural bases has also been observed [19]. Slower components may be due to gating, i.e., structural reorganization that precedes quenching [1,17]. Studies of DNA-protein interaction have shown the importance of conformational states [1,15,16,21]. But a detailed characterization of the conformations is yet to be obtained.

We have studied fluorescence decay of 2AP linked to a single DNA base, in order to investigate the underlying mechanisms. Previous studies involved transient absorption [19] and ab initio calculations [22] of these complexes. By studying the interaction with only one neighbouring base, we hope to clarify the interaction between 2AP and individual bases. This should help to use 2AP as a probe in intact DNA.

2. Materials and methods

Dinucleotides (5' 2AP-X 3' with X = guanine, adenine, cytosine, thymine or inosine) were purchased from Biologio (Malden, Netherlands, purity > 99%). Monomeric 2AP was purchased from Sigma-Aldrich (purity > 99%). All samples were dissolved to $OD_{305} = 0.1$ in 20 mM phosphate buffer (pH 7.0) with 100 mM NaCl. Steady-state spectra were recorded on Cary 5E

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(absorption) and Spex Fluorolog (fluorescence) spectrophotometers. We used $10 \times 4 \text{ mm}^2$ cuvettes (1.5 ml, Hellma).

Time-resolved fluorescence was recorded with time-correlated single-photon counting. Briefly, 0.2 ps pulses (290 nm, 3.8 MHz) were produced by a mode-locked titanium:sapphire laser (Coherent Inc., Mira 900-D), with pulse picker, frequency tripler and Glan-laser polarizer. Fluorescence was collected at 90° through a rotatable sheet type polarizer and emission filter (374.6 nm, FWHM = 7.8 nm). Individual photons were detected by a microchannel plate photomultiplier (Hamamatsu R3809U-50) with wide-band amplifier and a constant fraction discriminator. A reference pulse was also detected. Photon arrival times were determined by a time-to-amplitude converter (Tennelec Inc., Oak Ridge, TS) and stored in a multi-channel analyser (4096 channels). The channel spacing was 7.56 ps.

The excitation intensity was reduced with neutral density filters to obtain a count-rate below 30000 s^{-1} [23] and care was taken to minimize data distortion [24]. The samples were kept at 20°C . The instrument response function (50 ps FWHM) was obtained from *para*-terphenyl in a 1:1 (volume) mixture of cyclohexane and CCl_4 . A fresh sample was used for every recording. Home-written software was used for data-analysis [25,26].

3. Results

The steady-state spectra of the dinucleotides (Fig. 1) illustrate that 2AP behaves largely as an independent fluorophore. The absorption spectra show a similar red-most band at 305 nm, originating from 2AP. Also the fluorescence spectra of the five dinucleotides are similar, except for the reduced magnitude. Small shifts with respect to monomeric 2AP can be attributed to the ribo-side group [9,27]. Finally, the excitation spectra show limited excitation energy transfer. The 2AP–adenine dinucleotide shows a bump around 260 nm. We conclude that, upon excitation in the 305 nm band only 2AP fluorescence will be observed. The neighbouring base affects only the fluorescence strength.

The fluorescence yields confirm that the fluorescence of 2AP is strongly quenched by all natural bases, including inosine. These yields were obtained from the integrated fluorescence spectra and are listed in the first column of Table 1. Similar yields ($\pm 5\%$) were obtained for two batches. As shown previously, the fluorescence yield correlates with the driving force for electron transfer [19]. Indeed, the quenching is strongest for guanine. But even for inosine, which should be redox-inactive [17], strong quenching (80%) is observed. These results indicate that electron transfer is not the only relevant quenching mechanism.

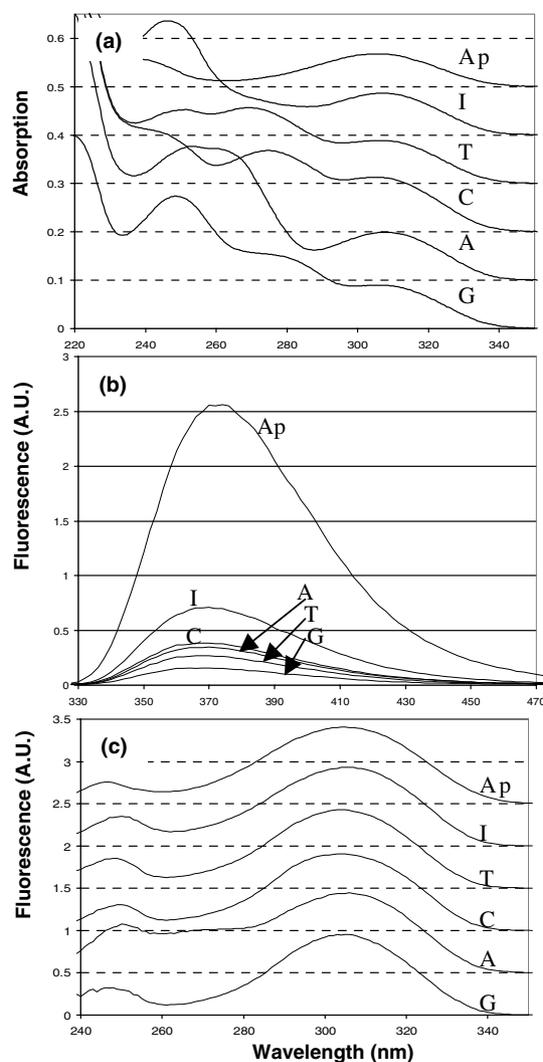


Fig. 1. Steady-state spectra of monomeric 2-aminopurine and the five dinucleotides in this study. (a) Absorption spectra. (b) Fluorescence emission spectra recorded at one scale and corrected for small absorption differences as in (a). Excitation wavelength: 320 nm. (c) Fluorescence excitation spectra. Emission wavelength: 380 nm. Labeling: G, 2AP–guanine; A, 2AP–adenine; C, 2AP–cytosine; T, 2AP–thymine; I, 2AP–inosine; Ap, monomeric 2-aminopurine.

The fluorescence decay curves (Fig. 2) show a multi-exponential fluorescence decay of 2AP in the dinucleotides. As expected, the dinucleotides decay faster than monomeric 2AP. But, while the fluorescence of 2AP can be fitted with a mono-exponential decay of 9.3 ns, four-exponential fits are necessary for the dinucleotides. The fit is good ($X^2 = 1.0 - 1.1$) although some non-random residual remains (see Fig. 2). The fits do not improve with an extra exponential, but three-exponential fits show clearly non-random residuals ($X^2 = 1.8 - 2.0$) [residues not shown].

The fitted fluorescence decay components of the five dinucleotides in our study occur in four distinct time-regions (7.6–8.5 ns, 1.7–3.1 ns, 0.3–0.85 ns and 21–35 ps). The fit parameters are shown in Table 1. The two slow

Table 1
Fluorescence quantum yields and decay components for the five dinucleotides in this study and monomeric 2-aminopurine

Sample	Φ_{rel}	τ_1/ns (a1)	τ_2/ns (a2)	τ_3/ns (a3)	τ_4/ns (a4)	Φ_{fit}
G–Ap	0.065	8.5(3)	1.7(17)	0.30(9)	0.021(71)	0.064
A–Ap	0.13	8.3(3.5)	2.1(23)	0.61(42)	0.035(31)	0.11
C–Ap	0.15	7.6(2)	2.4(37)	0.37(14)	0.029(47)	0.12
T–Ap	0.11	8.1(1.2)	2.4(33)	0.41(10)	0.031(56)	0.10
I–Ap	0.27	8.3(6)	3.1(43)	0.85(22)	0.027(29)	0.22
Ap	1	9.3				

The first column (Φ_{rel}) shows the fluorescence quantum yield of the five dinucleotides in this study, relative to monomeric 2-aminopurine. The yields were obtained from the integrated fluorescence bands. The next four columns show the decay times (in ns) obtained by fitting the fluorescence decay curves (see text). The numbers between brackets are the relative amplitude of each component. The final column (Φ_{fit}) shows the quantum yield calculated from the fit parameters (see text). Experimental uncertainties (estimated by comparing two batches): τ_1 (2%), τ_2 (3%), τ_3 (10%), τ_4 (15%), amplitudes and quantum yields (10%).

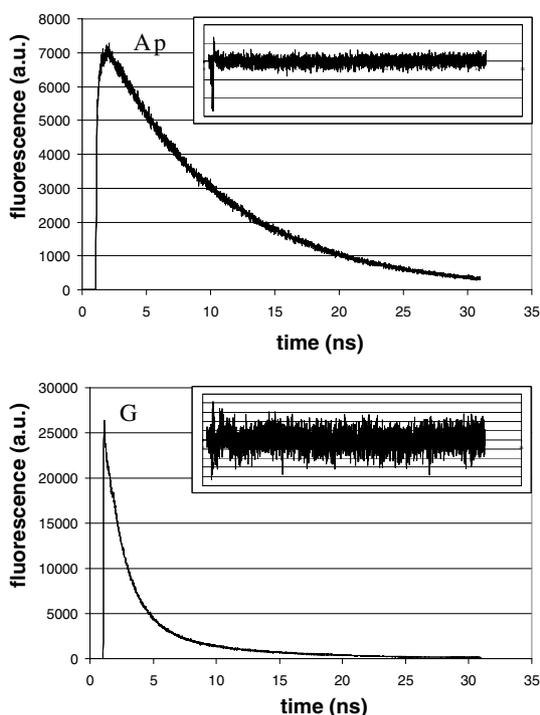


Fig. 2. Typical fluorescence decay curves with a one-exponential (monomeric 2-aminopurine) or four-exponential (dinucleotide) fit: excitation wavelength, 290 nm; emission wavelength, 374 nm. Insets: residual of the fit over the full time range. Labeling as in Fig. 1.

components were also obtained in three-exponential fits, with slightly shorter decay times (3–10%). The 8 ns component is essentially unquenched. Its amplitude is small (1–6%), but larger than the specified sample impurity. Therefore, it is not due to monomeric 2AP. For each dinucleotide, a considerable fraction of the fluorescence decays in the fastest time region (21–35 ps).

The steady-state fluorescence is lowest for guanine–2AP, highest for inosine–2AP and intermediate for the other three dinucleotides. The same order is observed for the fitted time-constants and (with one notable exception for adenine–2AP) the amplitudes of the intermediate components. The amplitudes of the fastest com-

ponent change in reverse order. All these trends agree with a population shift from the fast component towards the intermediate components and an increased lifetime of these components. The amplitude of the slowest component shows a strong variation, but no correlation with the steady-state fluorescence. The other parameters do not change much.

The fluorescence yield (relative to monomeric 2AP) can be estimated from the fit parameters

$$\Phi_{\text{fit}} = \sum a_i \tau_i / \tau_0.$$

Here, τ_i and a_i are the decay time and relative amplitude of component i while τ_0 is the decay time of monomeric 2AP. The estimate should equal the steady-state result, provided that the radiative rate of 2AP has not changed in the dinucleotide. The values in Table 1 show a good agreement for all dinucleotides. This demonstrates that the fluorescence of our samples has no significant contribution from decay components much shorter than ~ 30 ps.

4. Discussion

The observation of a multi-exponential fluorescence decay indicates structural heterogeneity. The specified melting temperature is less than 5 °C for these small DNA-fragments and all fractions are larger than the sample impurity. Thus, the excited dinucleotide itself must occur in several states, each with a different fluorescence decay time. These states may originate before or after the excitation. The observed decay times correspond either to the quenching rate in a particular state or to the conversion rate to the efficiently quenched state. Each decay component could correspond to a distribution of sub-states, but an overlap between these distributions is unlikely since the decay time regions are well separated. Note that the two ns-components are slow with respect to the time-scale of conformational motions [28]. This indicates that the corresponding states are separated by energy barriers.

The fit parameters correlate with the steady-state fluorescence. This can be interpreted in various ways. In a ‘static’ interpretation we could assume that each decay component corresponds to a ground state conformation of the dinucleotide. In a ‘dynamic’ interpretation we could assume that one or both of the intermediate components originates after the excitation. In the ‘static’ interpretation, the approximately fourfold increase of the steady-state fluorescence yield (from guanine–2AP to inosine–2AP) would be caused by an approximately two-fold population increase of the ‘intermediate’ conformations and a similar increase of their decay time. Most notably, the quenching rate in the ‘fastest’ conformation would not play a role. In the ‘dynamic’ interpretation, a decrease of this fastest quenching rate can explain both the higher amplitude and the longer decay time of the intermediate components. More research is needed to decide between the two interpretations. Moreover, the exceptions in the aforementioned correlations indicate that more than one parameter plays a role.

Multi-exponential fluorescence decay has also been observed in larger DNA fragments [1,3,15–17,21]. Measurements into the ns range show similar decay time regions to the ones in our study [15,30]. The slow component was attributed to a flipped conformation with 2AP twisted out of the helix. In some studies the amplitude of this component could be systematically altered [30]. The overall similarity of the decay time regions indicates that the dinucleotide states in our study are also relevant for understanding the dynamics of intact DNA. A discrepancy is that the fastest component in the above studies was 100–200 ps, rather than the 21–35 ps component found by us. Other studies find fast (10–40 ps) components even in large DNA fragments [1,17]. Rachofski et al. [30] report a discrepancy between the fluorescence yield in steady state measurements and the estimate derived from the fit, and attribute this to static quenching. Since we do not find such a discrepancy, we propose that this static quenching is in fact the 21–35 ps component observed by us and by others [1,17].

Fluorescence decay curves of nucleotide trimers have recently been published by Jean and Hall [31]. The 21–35 ps component could not be resolved in that study due to the limited time resolution. More strikingly, also the 8 ns component was not observed in this study. Possibly, the amplitude of this component is too small because 2AP needs to unstack from both neighbouring bases. The authors propose that the multi-exponential fluorescence decay is due to fast quenching in one conformation combined with rapid transitions between conformations. This agrees with the ‘gating’ model proposed earlier for 2AP-labeled DNA [17] and the ‘dynamic’ interpretation discussed above.

With regards to the quenching mechanism itself, the groups of Zewail and Barton find that fast decay of ex-

cited 2AP is induced only by a neighbouring guanine, and propose that this is due to electron transfer [13,14,17,18]. This was found in transient absorption studies of 35 bp DNA [17] as well as in mixtures of 2AP with various nucleotides [13]. In contrast, we find fast (21–35 ps) quenching of 2AP fluorescence by all nucleotides. This has also been found by others [19,29]. This discrepancy could be due to the fact that the aforementioned studies were done with transient absorption at one wavelength. If the states before and after the fast transition have a similar absorption spectrum, the transition could go undetected at particular wavelengths.

In a recent transient absorption study of 2AP-substituted dinucleotides the fastest decay component of the excited state absorption was 30–40 ps, depending on the neighbouring base [19]. This agrees with our results. However, the decrease of the excited state absorption, on that time-scale, was 20–30% while the fastest component in our results corresponds to 30–70% of the population (depending on the dinucleotide). Apparently, the product of the 21–35 ps transition has a strong excited state absorption. This could explain the aforementioned discrepancy that the fast component is not always detected. It also indicates that the product is electronically excited but non-fluorescent, i.e., it has characteristics of a dark state.

A dark state was indeed predicted by ab initio calculations on dinucleotides [22]. In some conformations the dinucleotides show a state with strongly reduced transition strength on the red side of the main excited state. After excitation in the main band, the 21–35 ps quenching component could reflect internal conversion to this red-shifted dark state. However, the aforementioned calculations also predicted a strong red-shift of the absorption of the natural base as well as that of 2AP. Such a shift is not observed in our steady-state spectra. In summary, our time-resolved data indicates that excited 2-aminopurine could be quenched by transition to a dark state, rather than by electron transfer. Further investigation should reveal the nature of this dark state.

5. Summary

The fluorescence of 2AP is quenched strongly and multi-exponentially in dinucleotides with the natural bases and inosine. The decay times form distinct regions with the slowest around 8 ns, and the fastest 21–35 ps. This is confirmed by a comparison to the steady-state yield. These results indicate a structural heterogeneity similar to what has been observed in larger DNA fragments [15,30]. Thus, dinucleotides may serve as a model to study local dynamics of intact DNA. The heterogeneity could be ‘static’ during the decay of the excited state or ‘dynamic’ as proposed for larger DNA fragments [17]

and nucleotide trimers [31]. By comparing our results to transient absorption [19] we find that the quenching of 2AP has the characteristics of transition to a dark state, but a previously predicted dark state [22] does not exactly match with our results.

References

- [1] O.F.A. Larsen, I.H.M. van Stokkum, B. Gobets, R. van Grondelle, H. van Amerongen, *Biophys. J.* (2001) 1115.
- [2] S.M. Law, R. Eritja, M.F. Goodman, K.J. Breslauer, *Biochemistry* 35 (1996) 12329.
- [3] T.M. Nordlund, S. Andersson, L. Nilsson, R. Rigler, *Biochemistry* 28 (1989) 9095.
- [4] L.C. Sowers, G.V. Fazakerley, R. Eritja, B.E. Kaplan, M.F. Goodman, *Proc. Natl. Acad. Sci.* 83 (1986) 5434.
- [5] R. Eritja, B.E. Kaplan, D. Mhaskar, L.C. Sowers, J. Petruska, M.F. Goodman, *Nucleic Acids Res.* 14 (1986) 5869.
- [6] A. Újvári, C.T. Martin, *Biochemistry* 35 (1996) 14574.
- [7] K.D. Raney, L.C. Sowers, D.P. Millar, S.J. Benkovich, *PNAS* 91 (1994) 6644.
- [8] L.W. McLaughlin, F. Benseler, E. Graeser, N. Piel, S. Scholtissek, *Biochemistry* 26 (1987) 7238.
- [9] A. Holmén, B. Nordén, B. Albinsson, *J. Am. Chem. Soc.* 119 (1997) 3114.
- [10] D.C. Ward, E. Reich, L. Stryer, *J. Biol. Chem.* 244 (1969) 1228.
- [11] J. Peon, A.H. Zewail, *Chem. Phys. Lett.* 348 (2001) 255.
- [12] T. Gustavsson, A. Sharonov, D. Onidas, D. Markovitsi, *Chem. Phys. Lett.* 356 (2002) 49.
- [13] T. Fiebig, C. Wan, A.H. Zewail, *ChemPhysChem* 3 (2002) 781.
- [14] S.A. Kelley, J.K. Barton, *Science* 283 (1999) 375.
- [15] R.A. Hochstrasser, T.E. Carver, L.C. Sowers, D.P. Millar, *Biochemistry* 33 (1994) 11971.
- [16] C.R. Guest, R.A. Hochstrasser, L.C. Sowers, D.P. Millar, *Biochemistry* 30 (1991) 3271.
- [17] M.A. O’Neil, H.-C. Becker, C. Wan, J.K. Barton, A.H. Zewail, *Angew. Chem.* 42 (2003) 5896.
- [18] C. Wan, T. Fiebig, O. Schiemann, J.K. Barton, A.H. Zewail, *PNAS* 97 (2000) 14052.
- [19] O.F.A. Larsen, I.H.M. van Stokkum, F.L. de Weerd, M. Vengris, C.T. Aravindakumar, R. van Grondelle, N.E. Geacintov, H. van Amerongen, *Phys. Chem. Chem. Phys.* 6 (2004) 154.
- [20] M.A. O’Neill, C. Dohno, J.K. Barton, *J. Am. Chem. Soc.* 126 (2004) 1316.
- [21] B. Holz, S. Klimasauskas, S. Serva, E. weinhold, *Nucleic Acids Res.* 26 (1998) 1076.
- [22] J.M. Jean, K.B. Hall, *PNAS* 98 (2001) 37.
- [23] K. Vos, A. van Hoek, A.J.W.G. Visser, *Eur. J. Biochem.* 165 (1987) 55.
- [24] A. van Hoek, A.J.W.G. Visser, *Anal. Instrum.* 14 (1985) 359.
- [25] E.G. Novikov, A. van Hoek, A.J.W.G. Visser, J.W. Hofstraat, *Opt. Commun.* 166 (1999) 189.
- [26] A.V. Digris, V.V. Skakun, E.G. Novikov, A. van Hoek, A. Claiborne, A.J.W.G. Visser, *Eur. Biophys. J.* 28 (1999) 526.
- [27] O.F.A. Larsen, *Ultrafast dynamics of DNA and a tryptophan-containing peptide*, Ph.D. Thesis, 2002.
- [28] J.M. Schurr, B.S. Fujimotoe, P. Wu, L. Song, in: J.R. Lakowicz (Ed.), *Topics in Fluorescence Spectroscopy*, vol. 3, Plenum Press, New York, 1992, pp. 137.
- [29] E.L. Rachofsky, R. Osman, J.B.A. Ross, *Biochemistry* 40 (2001) 946.
- [30] E.L. Rachofsky, E. Seibert, J.T. Stivers, R. Osman, J.B.A. Ross, *Biochemistry* 40 (2001) 957.
- [31] J.M. Jean, K.B. Hall, *Biochemistry* 43 (2004) 10277.