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Cytosine excited state dynamics studied by femtosecond fluorescence upconversion and transient absorption spectroscopy

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Abstract

We report a femtosecond spectroscopic study of the DNA base cytosine in aqueous solution at room temperature. Two different experimental techniques were used, fluorescence upconversion and transient absorption, providing complementary information on the excited state relaxation. While the fluorescence decay is clearly bi-exponential, with an ultrafast (0.2 ps) and a slower (1.3 ps) component, the decay of the transient absorption signal is mono-exponential with a 1.1 ps characteristic time. In addition, the fluorescence anisotropy is also found to decay in a bi-exponential manner. The results are discussed in terms of possible non-radiative relaxation processes that may intervene in the deactivation of the excited state.

1. Introduction

Understanding the excited state properties of DNA is essential for the comprehension of UV induced damage of living matter. In order to characterise the fundamental processes occurring in the excited DNA molecule after the initial energy deposition, a precise knowledge of the photophysical properties of the excited states of its constituents (nucleobases, nucleosides and nucleotides) is needed. In particular, the excited state lifetimes are of uttermost importance but paradoxically, these have for long evaded a precise determination due to their extreme briefness.

Already from steady-state spectroscopy and measurements of fluorescence quantum yields it was deduced that the excited state lifetimes of the nucleobases in aqueous solution are very short, on

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the order of a picosecond or less [1]. The subpicosecond character of the excited states of nucleobases was subsequently confirmed by femtosecond transient absorption measurements in the UV region [2–4]. For cytosine an excited S_1-state lifetime of (1.1 ± 0.2) ps was measured.

Kohler and co-workers [5,6] reported transient absorption measurements performed for four DNA nucleosides in aqueous solution. After exciting at 266 nm the transient absorption spectra were recorded in the whole UV–Vis region. Interestingly, they observed different characteristic decay times for the induced absorption observed in the UV (close to the excitation wavelength) and that observed in the visible region. They interpreted the former to be 'hot' vibrational levels in the ground state and the latter to the first excited singlet state. All excited state decay times were found to be significantly shorter than 1 ps. Very recently, the same authors reported lifetimes of the S_1 electronic states of cytosine and some of its derivatives [7]. Interestingly, they found the same lifetime, (1.0 ± 0.2) ps for the base and the nucleoside. It is worth mentioning that the transient absorption bands observed in the visible region overlap partly with the absorption band of the solvated electron, produced by two-photon absorption of water. For this reason, a detailed analysis is needed to disentangle the dynamics of the nucleic acid from that of the solvated electron, as discussed by Kohler and co-workers [7]. Direct measurements of the excited state lifetimes of four nucleosides and four nucleotides in aqueous solution were measured using fluorescence upconversion by Peon and Zewail [8]. The lifetimes given for the nucleosides ranged between 0.5 and 1.0 ps, close to the values given in [6].

We have recently also reported excited state lifetimes for several DNA constituents in aqueous solution obtained by the fluorescence upconversion technique; the thymine homologous series [9], the adenine homologous series [10] and a comparative study of eight DNA nucleosides and nucleotides [11]. A common conclusion in all our works is that the fluorescence decays in general cannot be described by single exponentials, implying complex excited state relaxation mechanisms.

In this Letter, we extend our ongoing study of DNA constituents to the nucleobase cytosine in aqueous solution. We compare fluorescence upconversion and transient absorption data, with excitation at 266 nm and probing around 330–320 nm, with the aim to shed new light on the nature of the excited/fluorescent state.

2. Experimental

Cytosine was purchased from Sigma–Aldrich and ICN Biochemicals GmbH and used without further purification. Ultra-pure water was obtained from a Millipore (Milli-Q Synthesis) purification system. For the transient absorption experiments, about 200 ml of 5×10^{-4} M cytosine aqueous solution was used, while for the fluorescence upconversion measurements, about 20 ml of 2×10^{-3} M cytosine aqueous solution was used. Different concentration were used for the transient absorption and fluorescence measurements due to the different thickness of the flow-cells used. In both cases, the solutions were kept flowing during the measurements as described below.

Absorption spectra were recorded with a Perkin–Elmer Lamda 900 spectrophotometer using 1 mm quartz cells (QZS). Fluorescence spectra were recorded with a SPEX Fluorolog-2 spectrofluorometer equipped with a 450 W arc xenon lamp.

2.1. Transient absorption measurements

The pump–probe setup is a standard two-colour scheme, based on a 20 Hz amplified Ti:S laser system. A small fraction of the fundamental output at 800 nm was focused into a 1 mm watercell generating a white light continuum. The white light was subsequently amplified in four-stage dye amplifier (DCM) after spectral selection. For excitation (the 'pump') the frequency tripled output from the Ti:S laser was used. The pump wavelength was 267 nm, the temporal width about 100 fs and the typical energy 20 μJ per pulse. By defocusing the sample cell this gave a power density of about 66 GW/cm^2 at the sample. The 'probe' pulse was obtained by frequency-doubling

the output from the dye amplifier. The probe wavelength was fixed to 320 nm, corresponding to the cytosine fluorescence maximum, and the typical energy was $\ll 1$ μJ per pulse. The sample cell was a moving 1 mm thick flow-cell of quartz through which the solution was kept flowing during experiments. The temporal apparatus function of the setup was found to be about 700 fs (FWHM) due to the relatively thick doubling and tripling crystals and sample cell used.

2.2. Time-resolved fluorescence measurements

The fluorescence upconversion setup has been described elsewhere [9,10]. Briefly, the third harmonic from an unamplified Ti:S laser was used for excitation. The average power at 267 nm was 50 mW and the peak power density at the sample was 0.2 GW/cm^2. The sum-frequency was generated in a 1 mm type I BBO crystal, filtered by a small monochromator and detected by a photon counting system. Parallel (I_{par}) and perpendicular (I_{perp}) kinetic traces were recorded by controlling the polarisation of the exciting beam with a half-wave plate. The cytosine solution was kept flowing through a moving 0.4 mm flow-cell of quartz during experiments. The apparatus function was determined from the rise of the cytosine fluorescence, ranging between 400 and 450 fs (FWHM) for different runs. This value was corroborated by measurements on the dominant Raman line of water at 296 nm. We judge that the time resolution of our setup is 100 fs after deconvolution. Fluorescence decays were recorded every 10 nm between 310 and 380 nm with 4 nm resolution. Typical scans were made in a 8 ps time window with 33.3 fs steps in both parallel and perpendicular configurations.

All measurements, both absorption and fluorescence, were performed at room temperature (21 ± 1 °C) under aerated conditions.

3. Results and data analysis

The steady-state absorption spectrum of cytosine is shown in Fig. 1. It features a first absorption band centered at 267 nm. The relatively

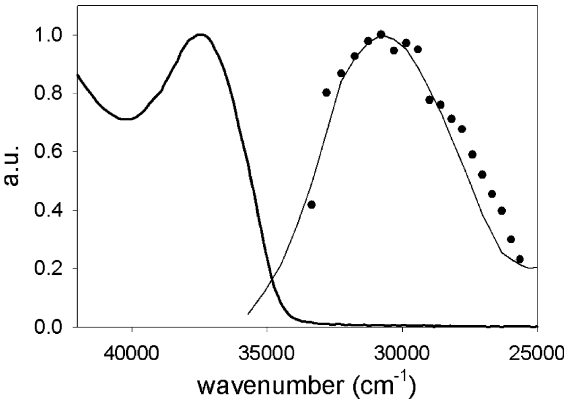

Fig. 1. Steady-state absorption and fluorescence spectra (solid lines) of cytosine; fluorescence spectrum is taken from [12]. The circles represent the time-integrated fluorescence spectrum as determined from upconversion data. Both fluorescence spectra are λ^2-scaled.

stronger absorption at shorter wavelengths indicates the presence of several close-lying transitions, but to what extent these higher bands overlap with the first band at 267 nm is not clear.

The steady-state fluorescence spectrum shows a main band centred at 325 nm, but also a spurious band between 350 and 380 nm (this spurious band is not shown in Fig. 1, instead the fluorescence spectrum from [12] is shown). We attribute this extra band to the presence of small traces of a fluorescent impurity. The frequent presence of impurities in commercial cytosine samples has already been noted in earlier studies [12]. However, comparing with a time-gated fluorescence spectrum obtained with our upconversion setup, we judge that the presence of this impurity does not influence the results on the probed time scale. Indeed, this time-gated fluorescence spectrum, integrated over the 8 ps time-interval, and corrected for the spectral response of the detection system, compares well with the steady-state fluorescence spectrum taken from [12] (Fig. 1).

Fig. 2 shows the induced absorption probed at 320 nm for pure water (full circles) and a 5×10^{-4} M aqueous solution of cytosine (open circles) after excitation at 267 nm. Two different excitation energies were used, (15 ± 5) μJ in the upper panel (a) and (20 ± 5) μJ in the lower panel (b), corresponding to intensities of $(50 \pm 2) \times 10^1$ and $(66 \pm 2) \times 10^1$ GW/cm^2 at the sample (assuming

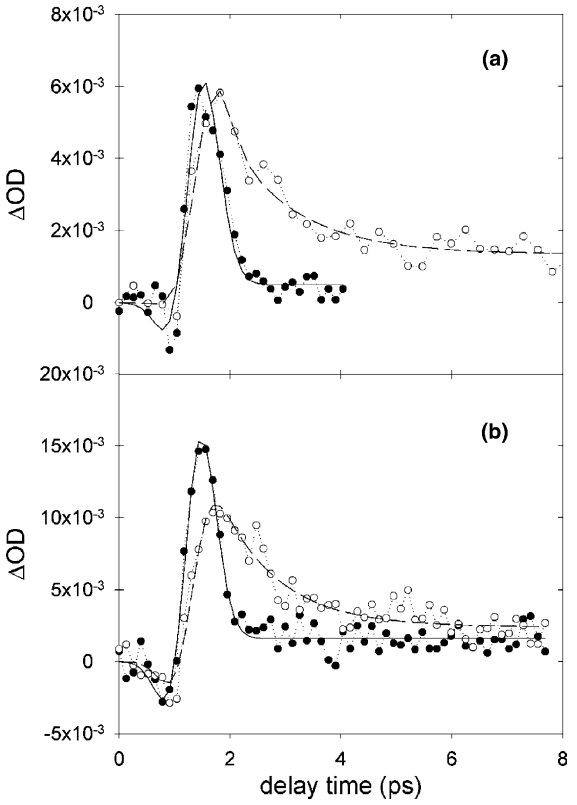

Fig. 2. The induced absorption probed at 320 nm for pure water (full circles) and a 5×10^{-4} M aqueous solution of cytosine (open circles) after excitation at 267 nm with (a) (15 ± 5) μJ and (b) (20 ± 5) μJ. The solid lines show the fits with bi-modal model functions as explained in the text.

100 fs pulses and a spot diameter of 0.3 mm at the cell).

The very rapid signal observed in neat water stems from two-photon absorption (267 + 320 nm) of water, leading to dissociation and ionisation [13,14]. Since this is a non-resonant process, the width of the observed signal can be taken as a measure of the apparatus function of the detection system (700 fs FWHM). Data were analysed by a merged non-linear fitting/deconvolution process simultaneously on the two traces, the neat water and the cytosine solution, for each excitation energy. The non-convoluted model function for the neat water signal was described by an infinitely narrow delta function plus a constant term for positive times, while that for the cytosine signal was described by an infinitely narrow delta function, a mono-exponential decay plus a constant term for positive times. The need for a delta function also in the cytosine signal was judged by the improvement in the rms-error this caused; a 8% better fit was obtained in the high energy case with a delta function. However, the presence of the delta function in the cytosine signal is not surprising; at the relatively high pump intensities used, two-photon absorption of water cannot be excluded. The characteristic decay time for cytosine as obtained from the mono-exponential term was determined to (1.1 ± 0.2) ps. It is worth mentioning here that although the probe wavelength, 320 nm, is close to the maximum of fluorescence, no stimulated emission was observed.

Regarding the signal amplitudes, they are in line with the expected energy dependencies. Increasing the excitation energy by roughly a factor of 1.3 leads to an increase of the neat water signal with a factor of 2.5, implying a non-linear process. The cytosine signal, on the other hand, increases only by about a factor of 1.7. This is higher than a pure linear dependency, but it should be kept in mind that the cytosine signal also contains some contribution from two-photon absorption of water. These considerations should of course be taken with some caution, not only due to the uncertainty in the excitation energy, but also due to the fact that they concern the non-deconvoluted amplitudes.

Fig. 3 shows the fluorescence decays recorded at 330 nm for a 2×10^{-3} M aqueous solution of cytosine with parallel and perpendicular excitation/detection configurations. Similar traces were obtained at other wavelengths. After subtraction of the baseline, the total fluorescence (I_{tot}) kinetics were constructed from the parallel and perpendicular signals according to the following equation:

$$I_{\mathrm{tot}}(t) = I_{\mathrm{par}}(t) + 2I_{\mathrm{perp}}(t). \quad (1)$$

The total fluorescence decay signal obtained for cytosine at 330 nm is presented on a semi-log scale in Fig. 4. It can be observed that the major part of the fluorescence decays in less than 1 ps but a longer tail persists for several picoseconds. It is clear that the fluorescence decay is far from mono-exponential and is dominated by an ultrafast component.

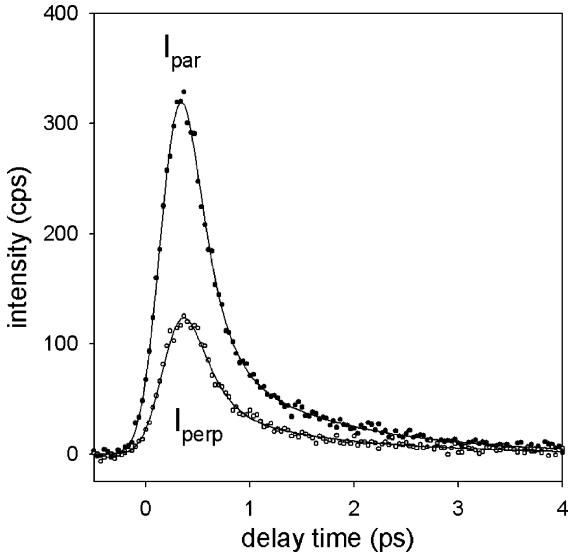

Fig. 3. Fluorescence decay observed for a 2×10^{-3} M aqueous solution of cytosine at 330 nm after excitation at 267 nm with parallel (solid circles) and perpendicular (open circles) excitation/detection configurations. The solid lines show the fits with bi-exponential model functions.

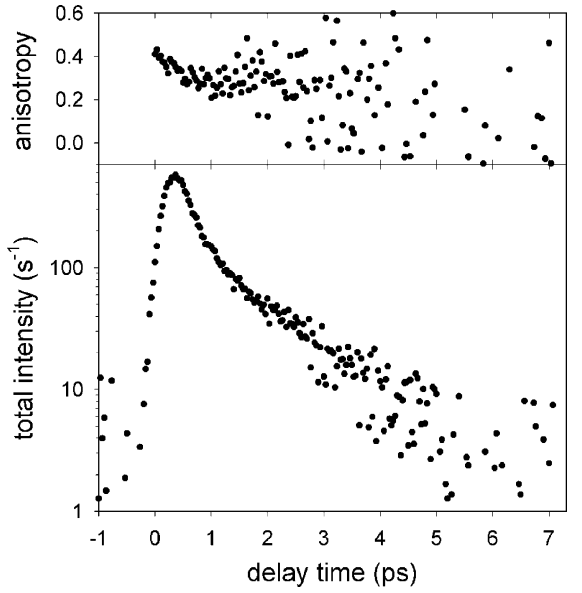

Fig. 4. The fluorescence anisotropy (upper panel) and the total fluorescence (lower panel) decays determined for a 2×10^{-3} M aqueous solution of cytosine at 330 nm after excitation at 267 nm.

Our recordings with parallel and perpendicular excitation/detection configuration allowed us also to determine the fluorescence anisotropy $r(t)$ defined as

$$r(t) = \frac{I_{\mathrm{par}}(t) - I_{\mathrm{perp}}(t)}{I_{\mathrm{par}}(t) + 2I_{\mathrm{perp}}(t)}. \tag{2}$$

The raw anisotropy is also indicated in Fig. 4. As can be seen, it decays rapidly during the fluorescence lifetime. Since both the total fluorescence and the fluorescence anisotropy display fast decays on the same timescale as the apparatus function, we performed a merged non-linear fitting/deconvolution process simultaneously on the parallel (I_{par}) and perpendicular (I_{perp}) signals, using the model functions:

$$i_{\mathrm{par}}(t) = (1 + 2r(t))f(t), \tag{3}$$

$$i_{\mathrm{perp}}(t) = (1 - r(t))f(t). \tag{4}$$

Bi-exponential functions were used for both $f(t)$ and $r(t)$. For the fluorescence, this yields one very fast component (τ_1), (0.20 ± 0.02) ps with an 85% amplitude, and a longer one (τ_2), (1.30 ± 0.07) ps with a 15% amplitude. The briefness of the fluorescence prevents the fitting to produce accurate time constants for the anisotropy decay. However, the use of a bi-exponential model function for the anisotropy was judged necessary since it improved the rms-error of the fit by 8%. The time-constants were therefore fixed arbitrarily to 0.2 ps, equal the fast fluorescence component, and 25 ps, corresponding roughly to the rotational diffusion time for a molecule of this size in water at 20 °C [10]. This yields an initial fluorescence anisotropy r_0 of 0.40 ± 0.05, with amplitudes 34% and 66% for the fast and the slow component, respectively.

Results from the model fittings of both transient absorption and fluorescence upconversion data for cytosine are given in Table 1. In this table are also shown, for comparison, the fluorescence lifetimes of cytidine (dC), and cytidine monophosphate (dCMP) at 330 nm, taken from [11].

We have observed a slight increase of the fluorescence lifetime of cytosine with the wavelength. For example, the long decay time τ_2 at 310 nm is

Table 1
Characteristic times (ps) of the transient absorption (at 320 nm) and fluorescence (at 330 nm) decays of cytosine after excitation at 267 nm. Also shown are the characteristic times of the fluorescence decays of cytidine (dC), and cytidine monophosphate (dCMP) at 330 nm[a]

Compound	Technique	τ_0	rms_2/rms_1[b]	τ_1	τ_2	α	p[c]	$\langle\tau\rangle$[d]
C	Abs			Δ[e]	1.1 ± 0.2			
C	Fluo	0.50 ± 0.02	0.54	0.20 ± 0.02	1.30 ± 0.07	0.85 ± 0.02	0.46 ± 0.02	0.37 ± 0.02
dC[a]	Fluo	0.40 ± 0.01	0.58	0.18 ± 0.02	0.92 ± 0.06	0.83 ± 0.02	0.51 ± 0.02	0.30 ± 0.02
dCMP[a]	Fluo	0.53 ± 0.02	0.66	0.27 ± 0.02	1.4 ± 0.2	0.84 ± 0.02	0.50 ± 0.02	0.45 ± 0.02

The values given are those resulting from fits with mono-exponential ($\exp(-t/\tau_0)$) and bi-exponential functions ($\alpha\exp(-t/\tau_1) + (1-\alpha)\exp(-t/\tau_2)$). Uncertainties given correspond to one standard deviation as obtained from the fits.

[a] Values taken from [11].

[b] The ratio of the root-mean-square errors obtained for fits with mono-exponential (rms_1) and bi-exponential functions (rms_2).

[c] $p = (1-\alpha)\tau_2/(\alpha\tau_1 + (1-\alpha)\tau_2)$ corresponds to the relative contribution of the slow component to the time-integrated total fluorescence.

[d] $\langle\tau\rangle = \alpha\tau_1 + (1-\alpha)\tau_2$.

[e] Δ means a infinitely narrow delta function.

1.1 ± 0.2 ps whereas it becomes is 1.5 ± 0.2 ps at 370 nm.

4. Discussion

It is interesting to compare the fluorescence decay observed for cytosine with those obtained for the nucleoside and the nucleotide as shown in Table 1. Since the chromophore is the same, no drastic changes are expected and this is indeed what is observed, the mean characteristic time $\langle\tau\rangle = (0.37 \pm 0.02)$ ps for cytosine is slightly reduced to (0.30 ± 0.02) ps for cytidine while it becomes somewhat longer, (0.45 ± 0.02) ps, for cytidine monophosphate. However, the fluorescence decay of cytosine is best described by a bi-exponential function. The fast component observed in the fluorescence decay (0.2 ps) is close to but slightly longer than the lower limit of our time resolution after deconvolution (100 fs). The large amplitude, about 85%, leaves no doubt of its importance.

It is important to note that the ultrafast component observed by us in the fluorescence decay not only of cytosine but also the other DNA bases, nucleosides and nucleotides has not been observed in other transient absorption studies [6,7]. On the other hand, the bi-exponential behaviour of the fluorescence decays of these compounds has also been discussed by Pal et al. [15]. The presence of this ultrafast component in our fluorescence measurements is in general independent of concentration (2×10^{-3} M and lower by a factor of five), excitation intensity (0.2 GW/cm^2 and lower by a factor of five) and laser repetition rate (76 MHz and lower by a factor of 16), implying an intramolecular origin.

Regarding the slow fluorescence component ($\tau_2 = 1.3$ ps), it is interesting to note its similarity to the transient absorption value (1.1 ps), which is in line with a common origin. However, for the DNA nucleosides, and notably cytidine, Kohler and co-workers [6] have shown that the induced absorption observed in the UV decays more slowly than a second induced absorption observed in the visible. They tentatively explained this by assigning the visible signal to the 'true' excited state absorption, while the UV signal rather has its origin in 'hot' vibrational levels in the ground state, in line with [4]. From this reasoning one would expect to observe a rise in the UV signal, but this has neither been reported in the literature nor observed in our experiments. Indeed, the induced absorption observed at 320 nm in our experiments seems to appear instantaneously (within the apparatus function), implying that the excited state responsible for the induced absorption is populated directly by the photoexcitation at 267 nm. In this context, it should be underlined that no stimulated emission was observed in the transient absorption measurements at 320 nm in spite of the probe

wavelength being close to the fluorescence maximum. This is surprising and can only be explained by the induced absorption from the probed transient species being much larger than the probability for stimulated emission from the photo-excited state.

To explain the bi-exponential fluorescence decay one may of course assume the existence of two fluorescent excited states, one responsible for the ultrafast 200 fs component, the other for the slower 1.3 ps component. If these two states are excited simultaneously, their deactivation may take place independently along different paths. The ultrafast decay may be internal conversion of one state to the ground state, while the slower decay may correspond to another photophysical or photochemical process affecting the other state. These two hypothetical excited states may be of different origin, either coexisting already in the ground state or being the result of a branching from the initially excited Franck–Condon state.

Regarding the first possibility, it has been proposed that there are several thermally populated ground state tautomers of cytosine both in the gas phase [16,17] and in aqueous solution [18]. This has also been corroborated by theoretical calculations [19–22]. For the second possibility, phototautomerisation may in principle occur in the excited state, but, at least for the amino-oxo → imino-oxo reaction, calculations have shown it to be energetically unfavourable [23].

Regarding the excited state relaxation, let us recall that the steady-state Stokes shift is very large, about 7000 cm^{-1}, indicating a very important stabilisation of the fluorescent state after excitation. Moreover, the fluorescence anisotropy decays with a characteristic time which is close to the fast fluorescence decay component (0.2 ps). It is tempting to take these two observations as signs of an ultrafast electronic relaxation. Assuming that several electronic states are involved in the excited state relaxation, it may be that the transition moments for absorption and emission for theses states differ radically, thus explaining the observed differences between the induced absorption and the fluorescence signals.

Indeed, two different theoretical studies of DNA bases and cytosine in particular predict a relaxation from the photo-excited π,π^* state to another close-lying excited electronic state, either of π,σ^* or n,π^* character, leading to an ultrafast internal conversion to the ground state through a conical intersection [24,25]. Evidently, more experimental data, for example on substituted bases [7] or using other solvents than water, with different polarity and hydrogen-bonding character is needed to get a better understanding of the mechanism responsible for the ultrafast non-radiative deactivation of excited DNA bases. Such work is in progress in our laboratory.

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