Femtosecond Fluorescence Upconversion Studies of Excited-State Proton Transfer Dynamics in 2-(2'-hydroxyphenyl)benzoxazole (HBO) in Liquid Solution and DNA

ARTICLE *in* CHEMICAL PHYSICS LETTERS · JANUARY 2003

Impact Factor: 1.9 · DOI: 10.1016/S0009-2614(02)01741-4

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Chemical Physics Letters 367 (2003) 599–608
CHEMICAL PHYSICS LETTERS
www.elsevier.com/locate/cplett

Femtosecond fluorescence upconversion studies of excited-state proton-transfer dynamics in 2-(2′-hydroxyphenyl)benzoxazole (HBO) in liquid solution and DNA

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Received 28 June 2002; in final form 15 October 2002

Abstract

A femtosecond fluorescence upconversion study is reported for HBO in solution, as well as for HBO incorporated in DNA. The typical time for the excited-state intramolecular proton-transfer reaction of the *syn*-enol tautomer in solution and in DNA has been determined to be 150 fs. In addition, the lifetimes of the keto, the *anti*-enol and the 'solvated enol' tautomer forms were determined in protic solvents, aprotic solvents and DNA. Picosecond rise and decay components in the fluorescence transients with characteristic times between 3 and 25 ps are also observed and attributed to the effects of vibrational cooling.

1. Introduction

Excited-state proton transfer plays an important role in a large variety of photoinduced chemical and biological processes. Of great interest is the photoreaction in DNA in which the equilibrium between keto-amine and enol-imine forms of the base pairs is established by double proton transfer [1]. The photoreaction in DNA may also involve rare tautomers that may be difficult to detect by conventional methods due to the fast time scale of the reactions and minimal heavy atom motion, as well as the reversibility inherent to the proton-transfer reactions. On the other hand, rapid interbase proton-transfer reactions are important because they may contribute to the physical properties of DNA, including duplex flexibility, protein recognition, and genetic mutations [1,2].

The tautomerization in DNA has still not been well characterized experimentally because, in addition to the difficulties inherent to studying the

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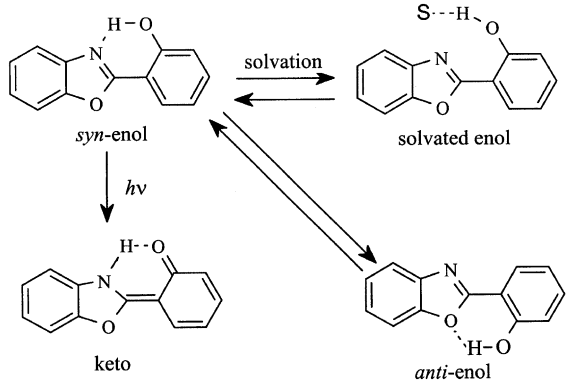
0009-2614/02/$ - see front matter © 2002 Published by Elsevier Science B.V.
PII: S0009-2614(02)01741-4

ultrafast and reversible proton transfer, it has not been possible to selectively initiate proton transfer within a specific base pair. Alternatively, some prototypical molecules have been extensively studied as a simplified model of hydrogen-bond base pairs in DNA, such as 7-azaindole [3,4]. Recently, Romesberg and co-workers [5,6] reported that 2-(2′-hydroxyphenyl)benzoxazole (HBO), when incorporated into DNA, is a valuable mimic of a natural Watson–Crick base pair. The tautomerization of HBO may be selectively and efficiently induced by photoexcitation, thus leading to excited-state intramolecular proton transfer (ESIPT) and allowing for the spectroscopic study of tautomerization in DNA.

The ESIPT process in HBO is known to occur on a subpicosecond time scale [7,8]. Subpicosecond ESIPT dynamics has also been found for analogous compounds, such as 2-(2′-hydroxyphenyl)benzothiazole (HBT) and 2-(2′-hydroxyphenyl)benzimidazole (HBI) [9–11]. For HBO, the tautomeric and conformational equilibrium, in several solvents and both in ground and excited states, have been studied by means of steady-state absorption and fluorescence spectroscopy [8,12–14]. In general, two tautomeric forms, the keto and enol forms, coexist. In the ground state, the enol-imine tautomer pair is more stable, whereas in the first excited-state the keto-amine tautomer is favoured. Depending on the solvent, several enol isomers have been identified: *syn*-enol (intramolecular O–H–N hydrogen bond), *anti*-enol (intramolecular O–H–O hydrogen bond), and in polar protic solvents the 'strongly solvated enol' (intermolecular hydrogen bonding with solvent). Only the *syn*-enol can be converted into the keto form by an ESIPT process. Scheme 1 presents an overview of the tautomeric forms for HBO.



Scheme 1.

From time-resolved fluorescence measurements the excited-state lifetime of the *anti*-enol was determined to be about 1.5 ns, independent of the solvent. The lifetime of the strongly solvated enol in the excited state was found to vary from 510 to 970 ps, depending on the solvent [8]. The lifetime of the excited-state keto form of HBO is also found to be solvent dependent; it ranges from 57 to 413 ps, displaying an apparent inverse relationship with the solvent dielectric constant [8,15].

From transient absorption experiments it was concluded that the time constants for ESIPT range from about 100 to 200 fs [7–10]. These characteristic times are suggestive of a low barrier proton transfer. Rios and Rios [16] conceived an ESIPT process in which initially the oxygen and the respective heteroatoms approach each other, while the O–H distance practically remains constant. The proton transfer occurs as soon as the H atom comes within the chemical interaction range of the other electronegative atom. Proton transfer is then completed by the separation of the heavy atoms, this separation being associated with vibrational stretching modes (the 'transfer promoting modes') [9,17,18]. Recently, Romesberg and co-workers [8] reported, in addition to a proton transfer time of approximately 170 fs, two intermediate time constants of about 560 fs and 10 ps. The origin of these times remained unsolved, however.

To further investigate the excited-state dynamics of HBO we have undertaken a femtosecond fluorescence upconversion study of this probe molecule in liquid solution and incorporated in single- and double-stranded-DNA. As is well-known, in such upconversion experiments one selectively probes excited-state dynamics without interference of ground-state bleaching or excited-state absorption kinetics. In this Letter we report results of the first femtosecond fluorescence upconversion experiments performed for HBO dissolved in cyclohexane, acetonitrile, methanol and ethanol, as well as attached to DNA and compare these results with those obtained previously from pump–probe and

fluorescence experiments [6,8]. Recently, several femtosecond fluorescence upconversion studies of nucleosides in DNA and RNA have been reported [19–21]. Here we report the first such upconversion studies for ESIPT in DNA.

2. Experimental

HBO was purchased from Aldrich and purified by vacuum sublimation. The solvents cyclohexane, acetonitrile, methanol and ethanol are of spectrophotometric grade (Aldrich). Solutions of HBO with a concentration of 10^{-3}–10^{-4} M were used for the measurements. It was verified that the results of the kinetic measurements were not dependent on the solute concentrations used. For the DNA samples, the procedure for the sample preparation was as described in [5,6].

Femtosecond fluorescence upconversion transients were measured by means of the set-up described previously [22]. The system response time as measured from the cross-correlation signal of the excitation and gating pulses at 340 and 800 nm, was estimated to be approximately 280 fs (FWHM) (Fig. 2a, dashed curve). The measured transients were fitted to multiexponential functions convoluted with the system response function. The experimental time resolution (after deconvolution) was approximately 100 fs. In the upconversion experiments, the excitation wavelength was at 340 nm, and the transients were detected from 407 upto 550 nm. To remove contributions from rotational reorientation motions of the solute molecules, measurements were performed under magic angle conditions (with the laser-excitation polarization at an angle of 54.7° relative to the vertically polarized gating beam). In addition, fluorescence anisotropy measurements were performed, in which the polarization of the excitation beam was, respectively, parallel and perpendicular with respect to the polarized gating beam. For time windows longer than 200 ps, the fluorescence transients were detected using the time-correlated single-photon-counting (TCSPC) set-up described previously [23]. In these experiments the wavelength of the laser pulses was kept at 323 nm; the instrument response was about 16 ps (FWHM).

Steady-state absorption spectra were recorded by means of a conventional spectrophotometer (Shimadzu, UV-240). Steady-state fluorescence spectra were measured using the emission spectrometer described before [23]. In these experiments excitation was at 323 nm; the emission spectra were corrected for the wavelength-dependent sensitivity of the monochromator-photomultiplier detection system. All experiments were performed at room temperature.

3. Results

Steady-state absorption spectra of 2-(2′-hydroxyphenyl)benzoxazole (HBO) were measured in cyclohexane, acetonitrile, methanol and ethanol (Fig. 1). In the polar solvents acetonitrile, methanol and ethanol, the absorption spectra are almost the same, with bands peaking at 330 and 319 nm. In cyclohexane, the spectrum is somewhat shifted to the red with peak positions at 334 and 321 nm. The results are consistent with those reported previously [8,9]. The absorption around 330–334 nm has been assigned as due to the *syn*-enol tautomer and the absorption around 320 nm as due to the *anti*-enol tautomer [8].

The steady-state fluorescence spectrum of HBO in cyclohexane shows two bands peaking at 505 and

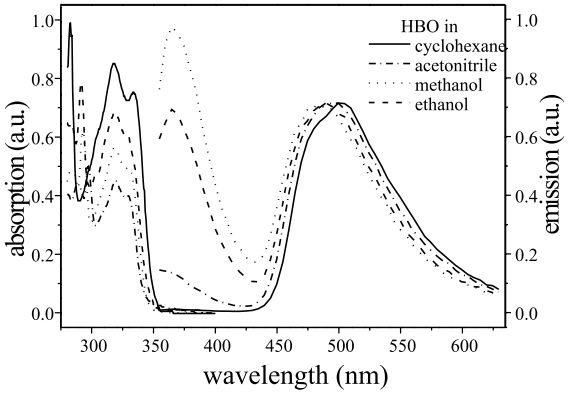


Fig. 1. Steady-state absorption and emission spectra of HBO in the solvents cyclohexane, acetonitrile, methanol and ethanol. Emission spectra were measured for excitation at 323 nm. The intensities of the emission spectra are normalized to the same value for the band maximum near 500 nm.

365 nm, respectively (Fig. 1). The 505 nm band is due to the keto tautomer formed by ESIPT in the *syn*-enol, while the 365 nm band originates in the *anti*-enol, the intensity being only about 2% of the keto emission.

The emission bands of the *anti*-enol and keto forms in acetonitrile peak near 360 and 500 nm, respectively. Compared to the emission in cyclohexane, the relative intensity of the *anti*-enol has increased to about 20%. In methanol, the emission of the keto form of HBO peaks at 485 nm, thus showing a blue shift compared to the corresponding emission in aprotic solvents. The emission band with its maximum at 365 nm is attributed to both the *anti*- and solvated enol forms. It is about 1.4 times the intensity of the keto emission.

The emission spectrum of HBO in ethanol is similar to that in methanol. It consists of two bands with maxima at 365 and 490 nm, respectively. The intensities of the bands due to *anti*- and solvated enol forms are approximately the same as that of the keto emission.

Fig. 2a displays the fluorescence upconversion transient of HBO in cyclohexane as detected at 407 nm, i.e., the shortest possible detection wavelength with our upconversion set-up. The transient was fitted to a three-exponential decay function convoluted with the signal response function. The signal is predominantly due to the *syn*-enol tautomer and exhibits for the most part (96%) a 150 fs decay component, followed by a 3 ps decay (2%) and a ~ns decay (2%). When detecting the upconversion transient for the keto tautomer at 500 nm for the same solution, a 150 fs rise is observed (vide infra). The 150 fs decay of the photoexcited *syn*-enol and the simultaneous rise of the keto form is evidence that the excited-state intramolecular proton transfer time for HBO in cyclohexane is 150 fs. The upconversion results thus nicely and independently confirm the approximately 170 fs proton-transfer time deduced previously from pump–probe experiments [8]. It should be added that the 150 fs decay component of the *syn*-enol species in the upconversion transient (Fig. 2a) could not be discerned in the pump–probe experiments and indeed was still missing in the latter experiments. The upconversion experiments, on the other hand, allow us to follow both the decay of the initial reactant and the rise of the final product in the ESIPT process.

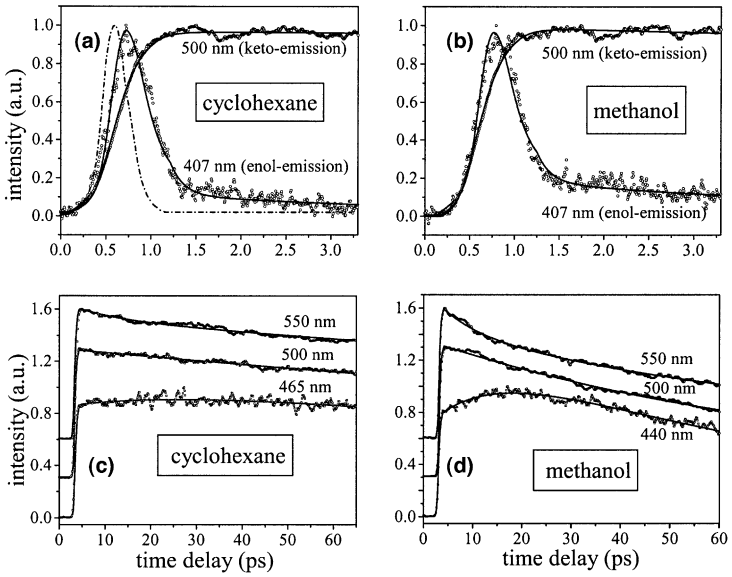


Fig. 2. Femtosecond enol/keto fluorescence upconversion transients of HBO in cyclohexane and methanol. Time windows and detection wavelengths are indicated. Excitation is at 340 nm. The dash-dotted curve presents the instrumental response function.

Table 1
Best-fit parameters of femtosecond fluorescence upconversion transients of HBO in cyclohexane, acetonitrile, methanol and ethanol and attached to single-strand-DNA and double-strand-DNA, after fitting to function $I \propto \sum_i A_i \exp(-t/\tau_i)$

	Fluorescence wavelength (nm)	τ_1 (ps)	τ_2 (ps)	τ_3 (ps)
Cyclohexane	407	0.15 (0.96)	3.2 (0.02)	~ns (0.02)
	420	0.26 (0.82)	3.2 (0.1)	~ns (0.08)
	440	0.89 (0.23)		157 (0.77)
	455	0.15 (−0.91)	12.0 (−0.09)	280 (1)
	465	0.16 (−0.81)	25.0 (−0.19)	280 (1)
	485	0.15 (−0.88)	9.5 (−0.12)	280 (1)
	500	0.15 (−1)		280 (1)
	525	0.13 (−1)		280 (1)
	550	0.18 (−1)	9.3 (0.08)	280 (0.92)
Acetonitrile	455	0.79 (0.11)		95 (0.89)
	500	0.18 (−1)		95 (1)
Methanol	407	0.15 (0.95)	2.5 (0.03)	~ns (0.02)
	440	0.15 (−0.57)	12.7 (−0.43)	90 (1)
	460	0.15 (−0.71)	10.7 (−0.29)	90 (1)
	485	0.15 (−0.88)	8.3 (−0.12)	90 (1)
	500	0.15 (−1)		90 (1)
	525	0.16 (−1)		90(1)
	550	0.15 (−1)	7.7 (0.20)	90 (0.80)
Ethanol	455	0.15 (−0.77)	18 (−0.23)	130 (1)
	500	0.19 (−1)		130 (1)
Single-strand-DNA	480	0.15 (−1)		
Double-strand-DNA	480	0.15 (−1)		

Relative weights (A_i) are given in brackets. Excitation is at 340 nm.

Upconversion transients were measured at a series of detection wavelengths for HBO in different solvents and fitted to multi-exponential functions convoluted with the system response function. Tables 1 and 2 include the fitting results for HBO in cyclohexane (see also Figs. 2a and c), acetonitrile, methanol (see also Figs. 2b and d) and ethanol. For detection wavelengths longer than 440 nm, the keto emission dominates. A fast rise with a time of 150 fs is observed at these (longer) detection wavelengths. As already mentioned, this rise reflects the formation of the keto component in the ESIPT from the *syn*-enol. The best-fit curves also show a decay characteristic of the lifetime of the HBO keto tautomer, with the lifetime being 280 ps in cyclohexane, 95 ps in acetonitrile, 90 ps in methanol and 130 ps in ethanol (τ_3, in Table 1).

These times are confirmed by fitting the fluorescence transients in a long-time window experiment performed for the same solutions with the TCSPC set-up (Fig. 3). For detection wavelengths between 360 and 430 nm, the TCSPC experiments for HBO in cyclohexane and acetonitrile yielded an initial fast decay of 450 fs (see τ_1 values in Table 2). It should be recalled that for this detection wavelength region the emission is mainly due to the *syn*-enol tautomer and thus the 450 fs decay time, as obtained with the TCSPC set-up with its limited time response, should be compared and identified with the actual decay time of 150 fs as determined in the upconversion experiment. For HBO in methanol and ethanol this fast decay component is not observed using the TCSPC set-up, however. This is understood as follows. When the excitation is at 323 nm (i.e., the excitation wavelength in the TCSPC experiments), the ratio of the intensities of the emission bands at 360 and 500 nm (Fig. 1) is appreciably enhanced when compared with the upconversion experiment. The reason is that in the protic solvents, the emission at 360 nm is by far dominated by the *anti*-enol and 'solvated enol' tautomers [8] and the 150 fs decay of the *syn*-enol cannot be resolved. On the other hand, for HBO in cyclohexane, excitation at 323 or

Table 2
Best-fit parameter values of picosecond TCSPC fluorescence transients of enol/keto emission of HBO in cyclohexane, acetonitrile, methanol, ethanol and attached to single-strand-DNA and double-strand-DNA, after fitting to function $I \propto \sum_i A_i \exp(-t/\tau_i)$

	Fluorescence wavelength (nm)	τ_1[a] (ps)	τ_2 (ps)	τ_3 (ps)
Cyclohexane	360	0.45 (0.98)		1300 (0.02)
	390	0.45 (0.96)	227 (0.01)	1310 (0.03)
	420	0.45 (0.92)	297 (0.06)	1320 (0.02)
	500	13 (−1)	280 (1)	
	580	4.7(0.05)	280 (0.95)	
Acetonitrile	355	0.45 (0.84)	95 (0.01)	1550 (0.15)
	390	0.45 (0.85)	98 (0.02)	1566 (0.13)
	420	0.45 (0.87)	95(0.07)	1579 (0.05)
	500	4.6 (−1)	95 (1)	
	580		95 (1)	
Methanol	365		520 (0.59)	1520 (0.41)
	380		540 (0.60)	1540 (0.40)
	430	90 (0.15)	584 (0.53)	1610 (0.31)
	500	3.7 (−1)	90 (1)	
	580	9.4 (0.20)	90 (0.80)	
Ethanol	365		545 (0.43)	1480 (0.57)
	380		600 (0.42)	1520 (0.58)
	430	135 (0.57)	620 (0.15)	1575 (0.28)
	500	7.2 (−1)	130 (1)	
	580	23 (0.23)	130 (0.73)	
Single-strand-DNA	360	15 (0.71)	162 (0.23)	1200 (0.06)
	380	15 (0.65)	159 (0.23)	1200 (0.08)
	480	200 (0.20)	1000 (0.35)	3500 (0.45)
	500	213 (0.18)	1000 (0.36)	3500 (0.46)
	580	178 (0.20)	1000 (0.35)	3500 (0.45)
Double-strand-DNA	360	5.6 (0.90)	173 (0.07)	1800 (0.03)
	380	24 (0.74)	286 (0.16)	2100 (0.10)
	480	130 (0.10)	1600 (0.10)	4700 (0.80)
	500	206 (0.08)	1600 (0.10)	4700 (0.82)
	580	302 (0.07)	1600 (0.09)	4700 (0.84)

Relative weights (A_i) are given in brackets.
[a] τ_1 (ps) for cyclohexane, acetonitrile is beyond instrument response of TCSPC set-up, see text.

340 nm does not significantly affect the ratio of the emission band intensities at 360 and 500 nm, and the dominant emission at 360 nm remains due to the *syn*-enol tautomer.

For detection wavelengths between 360 and 430 nm, we also observe a long decay component with a typical time of 1300 ps in cyclohexane, 1560 ps in acetonitrile, 1540 ps in methanol, and 1540 ps in ethanol (τ_3 values in Table 2). These decays, with relative weights as indicated in Table 2, are characteristic of the lifetime of the *anti*-enol component in the excited state in these solvents. Furthermore, for HBO in methanol and ethanol, in the same detection wavelength range, decay times of 540 and 600 ps, respectively, attributed to the 'solvated enol,' were found.

In addition to the already mentioned τ_1 and τ_3 components, the upconversion transients for HBO in cyclohexane also contain rise and decay components with a typical time of a few picoseconds (τ_2 values in Table 1). When the detection wavelength is varied from 455 to 485 nm, a 10–25 ps rise is observed, while for detection at 550 nm a ~9 ps (8%) decay is found. The rise and decay components also showed up in the TCSPC experiments (τ_1 values in Table 2 for 500 and 580 nm, respectively). These components become more pronounced, when performing the experiments for HBO in methanol or

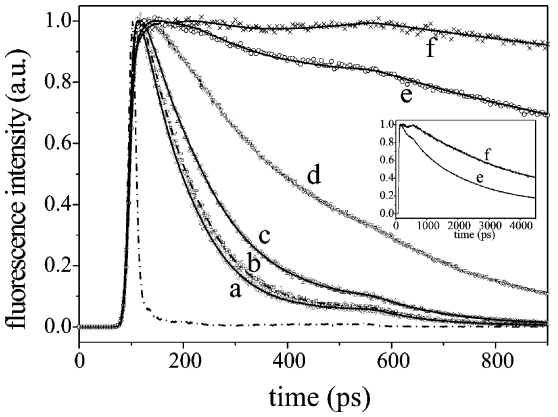


Fig. 3. Time dependence of keto emission of HBO, detected at 500 nm as measured with TCSPC set-up, in methanol (a), acetonitrile (b), ethanol (c), cyclohexane (d), single-strand-DNA (e), and double-strand-DNA (f). Inset shows long-time behavior of HBO/DNA transients. Excitation is at 323 nm. The hump in transient near 600 ps is due to the instrument response. The dash-dotted curve displays the instrument response.

ethanol (τ_2 values in Table 1 and τ_1 values in Table 2 for 500 and 580 nm).

Time-resolved measurements of the fluorescence anisotropy, $r(t)$, for the keto tautomer of HBO in the various solvents, with $r(t) = [I_{\parallel}(t) - I_{\perp}(t)]/[I_{\parallel}(t) + 2I_{\perp}(t)]$, were also performed. At those detection wavelengths, where the effects of vibrational cooling were minimal (vide infra), $r(t)$ showed a monoexponential decay with a decay time depending on the solvent (Table 3). The Table includes the initial fluorescence anisotropy values.

Table 3
Best-fit parameters for (bi)exponential decay of fluorescence anisotropy of HBO, at 500 nm, in cyclohexane, acetonitrile, methanol, ethanol and attached to single-strand-DNA and double-strand-DNA

	$r(0)$	τ_1 (ps)	τ_2 (ns)
Cyclohexane	0.31	30	
Acetonitrile	0.34	19	
Methanol	0.30	17	
Ethanol	0.31	27	
Single-strand-DNA	0.27	140	1.5
		(0.08)	(0.92)
Double-strand-DNA	0.30	160	3.6
		(0.03)	(0.97)

Fluorescence transient measurements have also been carried out for HBO in single- and double-stranded-DNA. The enol emission turned out to be too weak to be detected in the fluorescence upconversion experiment. On the other hand, the upconversion signal for the keto band emission (at 480 nm) could be obtained. The upconversion transient showed a 150 fs rise component and a very slow decay part for which the kinetics could only be accurately determined with the picosecond TCSPC fluorescence set-up using long time windows. In the latter experiments, for HBO attached to single- and double-stranded-DNA, the decay times were found as 200 ps (20%), 1 ns (35%), 3.5 ns (45%) and 130 ps (10%), 1.6 ns (10%), 4.7 ns (80%), respectively. For detection wavelengths between 360 and 380 nm, the TCSPC experiments for HBO in DNA yield decay times between 15 and 25 ps (cf τ_1 values in Table 2) that are of the same order of magnitude as the picosecond rise times (cf τ_2 values in Table 1) for HBO in liquid solution. We also observed long decay components with a typical time of 1200 and 1800 ps for HBO attached to single- and double-stranded-DNA, respectively (τ_3 values in Table 2). Finally, Table 3 includes the results of the time-resolved fluorescence anisotropy measurements for HBO incorporated in DNA.

4. Discussion

A major result of the fluorescence upconversion experiments of HBO is the 150 fs decay, when detection is near 407 nm, and the nearly 100% simultaneous rise, when detection is at 460 nm or above. The decay is characteristic of the *syn*-enol tautomer and the rise is typical of the keto tautomer. As already indicated above, these results directly reflect that the proton transfer time in the ESIPT process of HBO is 150 fs. The result confirms the time of ~170 fs obtained recently from transient absorption measurements [8]. Proton-transfer times of similar magnitude have also been reported for compounds analogous to HBO, such as HBI, HBT and 2,5-bis(2′-benzoxazoly)hydroquinone [9,10]; again, for the latter systems the results were obtained from transient absorption experiments.

In previous transient absorption measurements of HBO and analogs, vibrational modulation of the transients had been observed in a time window of several picoseconds [8–10]. It was proposed that the reaction coordinate characteristic of the enol-to-keto proton transfer is coupled to the normal coordinate of one or more low-frequency (~100–200 cm^{-1}) stretching (transfer promoting) mode(s) [17,18]. However, in our measured fluorescence upconversion transients of HBO we could not resolve any such oscillatory behavior and thus we find no additional evidence for a coupling between the proton transfer and transfer promoting modes.

Our results show that the lifetime of the *anti*-enol is about 1.5 ns; this time appears independent of the solvent. The latter result may indicate the existence of an intramolecular hydrogen bond, as also suggested elsewhere [8]. The lifetime of the solvated enol species in methanol and ethanol is on the order of a few hundred picoseconds and varies with the solvent (τ_2 values in Table 2). The sensitivity of the lifetime of this enol towards the solvent is probably due to hydrogen bond formation with neighboring solvent molecules. The excited-state lifetime of the keto tautomer is also solvent dependent (τ_3 in Table 1). The lifetime decreases with increasing solvent polarity and decreasing solvent viscosity, e.g., the keto lifetime is 280 ps for HBO in cyclohexane and 95 ps in acetonitrile. Similar results have been found elsewhere, the shortest lifetime being 57 ps for the keto tautomer in DMSO [8]. It is of interest to note that the keto lifetime in DNA is of the order of a few nanoseconds.

In addition to the aforementioned formation and lifetime components, the fluorescence upconversion transients also showed rise and decay components for the HBO keto tautomer, with a typical time between 3 and 25 ps (τ_2 values in Table 1 and τ_1 values in Table 2 for wavelengths above 500 nm). The amplitudes of these components were detection wavelength dependent and particularly pronounced for HBO in methanol and ethanol, but also clearly present for HBO dissolved in cyclohexane. The components are attributed to the effects of vibrational cooling of 'hot' keto in the excited state. The vibrational excess energy of the initially prepared keto tautomer is about 9000 cm^{-1}. Thermal equilibrium is achieved through intramolecular vibrational energy redistribution and energy dissipation to the bath of solvent molecules. Naturally, the redistribution of the population of the excited-state vibrational levels that accompanies vibrational cooling, will be a determining factor for the shape of the observed emission band. Typical vibrational cooling times on the order of picoseconds have also been concluded in previous transient absorption experiments of HBO [8], and also from fluorescence measurements of HPMO [24] and 10-hydroxybenzo[h]quinoline (HBQ) [25]; these molecules also exhibit fast ESIPT.

Fig. 4 shows the temporal dependence of the fluorescence band of HBO in methanol after spectral reconstruction [26,27], using the best-fit functions for the femtosecond fluorescence upconversion transients. The spectra show that vibrational cooling results in a blue shift (~300 cm^{-1}) and also a narrowing of the emission band by ~200 cm^{-1}. Similar phenomena, although of smaller magnitude, have been reported for the fluorescence of coumarine 153, in which case the dynamics of the band shift and narrowing was also attributed to vibrational cooling [28].

The initial fluorescence anisotropy value for the HBO keto product ($r(0) \approx 0.34$) is close to the maximum possible value of 0.4. The latter value would apply in case the direction of the transition dipole moment of the absorptive *syn*-enol species would be parallel to the radiative dipole of the

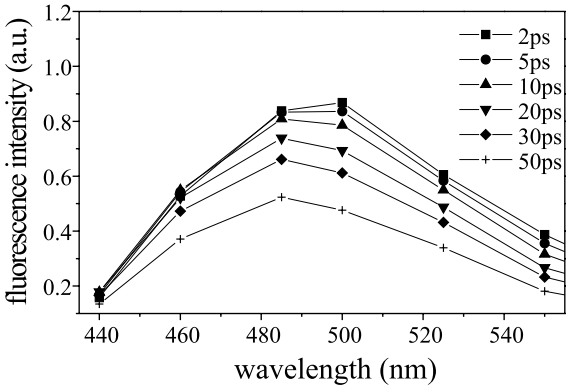


Fig. 4. Temporal dependence of reconstructed keto-emission band of HBO in methanol.

keto product [29]. So, the charge redistribution in HBO after ESIPT is such that it barely changes the directions of the enol absorption and keto emissive transition dipole moments.

Under conditions that vibrational cooling effects in the fluorescence transients are absent, the fluorescence anisotropy decay is single exponential. The decay times vary from 17 up to 30 ps, depending on the solvent (Table 3). It is very likely that the fluorescence anisotropy decay is due to the rotational diffusion motions of the HBO molecules. Hence the change of the characteristic anisotropy decay time with the viscosity of the solvent (compare e.g., the anisotropy decay times in ethanol and methanol).

Previous two-color transient absorption experiments showed the presence of a 560 fs rise component for HBO in hexane, methanol and DMSO [8]. This component could not be reproduced in the fluorescence upconversion measurements reported here. It is likely therefore that the 560 fs component refers to a dynamical process of HBO in the electronic ground state. We remark that a time constant of 600 fs was obtained in the 2-(2′-hydroxy-5′-methylphenyl)benzotriazole (TIN) and assigned to the proton back-transfer process in the electronic ground state [30].

The lifetime of the *anti*-enol for HBO, attached to single- and double-stranded-DNA, is about 1.2 and 1.8 ns (τ_3, for detection at 360–380 nm, in Table 2), respectively. These times are close to the lifetimes obtained for the *anti*-enol species in liquid solution. The emission intensity near 407 nm was too weak to allow for a femtosecond upconversion experiment at this wavelength. Thus, the decay of the *syn*-enol tautomer of HBO in DNA could not be measured with high-time resolution. In the TCSPC experiments for HBO in DNA, with detection between 360 and 380 nm, decay times between 15 and 25 ps (cf τ_1 values in Table 2) were obtained. These times are of the same order of magnitude as the picosecond rise components for the keto tautomer of HBO in liquid solution (cf τ_2 values in Table 1). We attribute these components to the effects of vibrational cooling. Likewise, the picosecond decay components measured for the *syn*-enol tautomer of HBO in DNA are manifestations of vibrational cooling.

The emission transient characteristic of the keto tautomer of the HBO/DNA system as detected at 480 nm contains several components. In this case the upconversion measurement could be realized and initially a 150 fs rise component is found, showing that, after ESIPT, the formation of the keto tautomer in DNA is on the same time scale as in solution. The keto-emission decay (measured with the picosecond fluorescence set-up) consists of three components ranging from a few hundred picoseconds up to nanoseconds (Table 2). The origin of this multi-exponential decay is not clear, but in general we find that the keto-emission lifetime is significantly longer (by at least an order of magnitude) than for the keto species in liquid solution in agreement with transient absorption results [6].

Finally we briefly discuss the fluorescence anisotropy decay for HBO incorporated in DNA (Table 3). The significant long anisotropy decay times (1.5 and 3.6 ns in single- and double-stranded-DNA, respectively) indicate the rigidity of the local structure of HBO attached to DNA. The decay times are about two orders of magnitude longer than those for HBO in liquid solution. It is probable that the loss of the fluorescence anisotropy is due to rotational diffusion of HBO–DNA complex as a whole in the phosphate buffer. The restricted motions of HBO in DNA further indicate that HBO is incorporated in a hydrophobic environment, well-shielded from the buffer solvent [5,6].

Acknowledgements

This research was financially supported in part by the Council for Chemical Sciences of the Netherlands Organization for Scientific Research (CW-NWO).

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