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Evidence of tautomerism in 2-aminopurine from fluorescence lifetime measurements**

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Abstract

The fluorescence decay characteristics of 2-aminopurine (2AP) and 2-aminopurine riboside (2APr) have been investigated as a function of excitation and emission wavelength in aqueous and ethanolic solutions. Global analysis of the decay data shows that 2AP exists as two emitting species, whereas 2APr exists as a single species. This is attributed to 9H/7H tautomerism of 2AP. The proportion of 7H tautomer is estimated to be 20% in ethanol and 40% in water.

Introduction

Fluorescence spectroscopy is finding increasing application for probing interactions of DNA with ligands and monitoring changes in DNA conformation, by introducing fluorescent probes into specific locations within the DNA chain. The natural bases of DNA are not useful as fluorescent probes because of their extremely low quantum yields, and thus the use of extrinsic probes is necessary. 2-aminopurine (2AP), a fluorescent analogue of adenine, is one such probe which, because of its structural similarity to adenine (6-aminopurine) can be introduced into DNA with minimal perturbation to the system[1]. The structures of 2AP and adenine are shown in Figure 1. The fluorescence properties of 2AP and a number of its derivatives were first reported by Ward *et al*[2]. It has a quantum yield of about 0.6 in aqueous solution, compared with ~10^{-3} for adenine, and an emission maximum at ~370nm. Its excitation maximum is at ~303nm, to the red of the naturally occurring DNA bases, allowing it to be excited selectively in DNA.

The aminopurines, adenine and 2AP, can, in principle, exist in several tautomeric forms. For both molecules, the 9H tautomer is the most stable. Adenine is known to tautomerise by proton transfer between N7 and N9 (Figure 1).

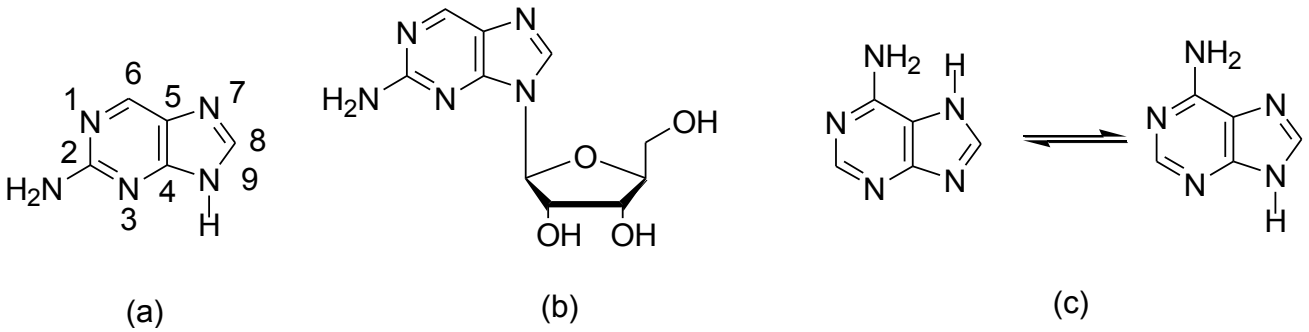

Figure 1. The structures of (a) 2AP (9-H tautomer), (b) 2AP riboside and (c) the 7H and 9H tautomers of adenine.

This process has been studied in solution phase by UV absorption and steady-state fluorescence spectroscopy (the 7H tautomer is fluorescent)[3] and temperature jump measurements in conjunction with UV absorption[4] and most recently by ultrafast transient absorption spectroscopy[5]; in the gas phase by microwave spectroscopy[6], infrared and high resolution electronic spectroscopy[7]; and in computational studies[8;9]. The 9H tautomer of adenine is predominant in the gas phase, but the two tautomers coexist in polar solution at room temperature, because of the greater dipole moment of the 7H tautomer. The fraction of the 7H isomer in solution has been reported as 6% in butanol[3] and 20% in aqueous solution[4,5]. For the parent molecule, purine, the 7H and 9H tautomers have been found to exist in approximately equal proportions in aqueous solution at room temperature [10-13].

For 2AP, *ab initio* computational studies[14] have shown that, like adenine, only the 9H and 7H tautomers have low enough energy to be observed at room temperature. As for adenine, the 7H tautomer was predicted to have a significantly greater dipole moment than the 9H tautomer, 4.46D compared with 3.41D. Thus, the tautomeric equilibrium is expected to shift towards the 7H form in polar solution. In aqueous solution (using a bulk solvation model) the population of 7H tautomers was predicted to be 3%[14]. There is very little experimental evidence for tautomerism in 2AP. In a study of transition moments, Holmen *et al.*[15] found that the linear dichroism (LD) spectrum of 2AP in stretched PVA films differed from that of related 9-substituted molecules (which cannot tautomerise). They attributed this to the presence of a small amount of the 7H tautomer. In an early time-resolved fluorescence study, using flash lamp excitation, Santhosh and Mishra[16] found the fluorescence response function of 2AP in water to consist of a rise time of 2.1ns and a decay time of 24.6ns. They assigned the two lifetime components to the 9H and 7H tautomers. However, the validity of this work is questionable, since subsequent measurements with superior time resolution (*vide infra*), including the present work, have found no evidence of a rise in the 2AP fluorescence response, nor a decay time as long as 25ns. Recently, an ultrafast transient absorption study of 2AP in aqueous solution reported the observation of a multi-exponential decay, implying heterogeneity of the chromopohore in the excited state[17]. In addition to a picosecond solvent relaxation time, two nanosecond decay components were observed with lifetimes of ~3.5 ns and ~14 ns (these values have low precision because of the short (5ns) time window of the experiments). However, assignment of the two lifetimes to the 7H and 9H tautomers was ruled out because similar behaviour was observed for 9-substituted 2AP in which tautomerism is prevented. Instead, the 3.5ns component was ascribed to a fluorescent state of the 9H tautomer and the 14ns component to a dark state of the same tautomer. This interpretation is inconsistent with results of time-resolved fluorescence measurements which find the fluorescence lifetime of 2AP in water to be ~12ns, as discussed below.

The fluorescence decay of 2AP in water is widely accepted to be monoexponential, with lifetime values of 11 to 12ns reported in the literature[15,18,19;20]. But, in previous studies, the fluorescence lifetime has generally been measured at only a single excitation wavelength and a single emission wavelength. In order to obtain a more complete picture of the photophysics of 2AP, and to explore the possibility of 9H-7H tautomerism, we have investigated the fluorescence decay as a function of both excitation and emission wavelengths in aqueous and

ethanolic solution, and made comparative measurements on 2AP riboside, in which the 7H tautomer is precluded.

Materials and Methods

2-aminopurine (>99%) was purchased from Aldrich and 2-aminopurine riboside (2APr) from Sigma; both were used as received. Water was freshly distilled and ethanol was of spectroscopic grade (Aldrich). Both solvents were checked for background fluorescence prior to use. 2AP and 2APr were dissolved to concentrations of 10^{-5}M in both solvents.

Fluorescence lifetimes were determined using the time-correlated single photon counting method. Fluorescence decay curves were recorded for sample solutions in fused silica cells of 1cm path length, using an Edinburgh Instruments spectrometer equipped with TCC900 photon counting electronics. The excitation source was a tuneable, mode-locked Ti-Sapphire laser system (Coherent Mira Ti-Sapphire laser pumped by Coherent 10W Verdi), producing ~200fs pulses at a repetition rate of 76MHz. The pulse repetition rate was reduced to 4.75MHz using a pulse picker (Coherent 9200) and the light was frequency tripled using a Coherent 5-050 harmonic generator. Fluorescence emission was detected orthogonal to the excitation beam through a polariser set at the magic angle with respect to the vertically polarised excitation. A bandpass of 10nm was used in the emission monochromator and photons were detected using a cooled microchannel plate detector (Hammamatsu R3809 series). The instrument response function, measured by scattering the excitation beam from a dilute suspension of colloidal silica (ludox), was 50ps FWHM.

Fluorescence decay curves were recorded on a timescale of 50ns, resolved into 4096 channels, to a total of 20000 counts in the peak channel. Decay curves were analysed using a standard iterative reconvolution method in the F900 (Edinburgh Instruments Ltd), Level 2 (Edinburgh Instruments Ltd) and FAST (Alango Ltd) software packages. A multiexponential decay function (Equation 1) was assumed.

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

where A_i is the fractional amplitude and τ_i is the fluorescence lifetime of the i^{th} decay component.

The quality of fit was judged on the basis of the reduced chi-square statistic, χ^2, and the randomness of residuals. Typically, we find that a χ^2 value <1.2 indicates an acceptable fit. In global analysis, a family of decay curves was fitted simultaneously, with lifetimes, τ_i, as common parameters.

Results

2AP in water

The fluorescence excitation and emission spectra of 2AP in water are shown in Figure 2.

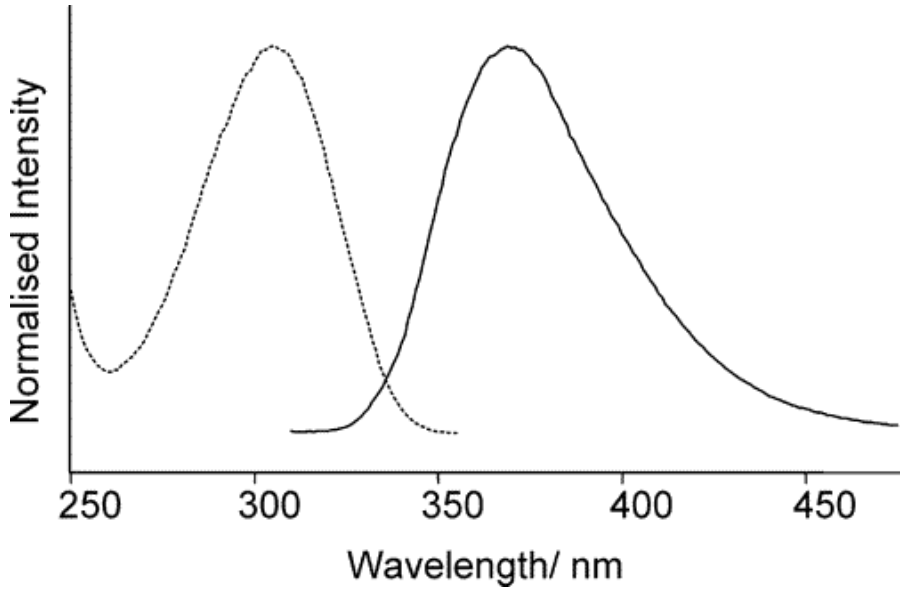

Figure 2. Fluorescence excitation (dotted line) and emission (solid line) spectra of 2AP in aqueous solution.

The fluorescence decay of 2AP in aqueous solution was measured at emission wavelengths of 370, 390 and 420 (or 410) nm, and at three excitation wavelengths, 280, 300 and 320nm. Each decay curve could be well fitted by a single exponential function and the lifetimes obtained are shown in Table 1.

Excitation Wavelength/ nm	**Emission Wavelength/ nm**	**τ/ ns**	**χ^2**
280	370	11.8	1.11
	390	12.0	1.12
	420	12.1	1.06
300	370	11.9	1.09
	390	12.1	1.05
	420	12.1	1.15
320	370	12.2	1.20
	390	12.3	1.12
	410	12.3	1.13

Table 1. Fluorescence lifetimes and reduced chi-square values for 2AP fluorescence decays measured in water, as a function of excitation wavelength and emission wavelength.

It can be seen that there is a significant variation in the lifetime with emission and excitation wavelength, ranging from 11.8ns (excitation at 280nm, emission at 370nm) to 12.3 ns (excitation at 320nm, emission at 390/410nm). On the basis of measurements of a standard fluorophore with a known single exponential decay, we would expect a statistical variation of <1% in the fluorescence lifetime. This wavelength-dependence suggests that there may be more than one emitting species present. Global analysis of the nine decays confirmed that they cannot be adequately described by a single fluorescence lifetime, as shown by the χ^2 values in Table 2. However, global analysis in terms of two common lifetimes yielded excellent fits, as shown in Table 3. Thus, there are two emitting species, with lifetimes of 11.0 and 13.5 ns, contributing to the fluorescence of 2AP in aqueous solution.

τ(global) = 12.1ns		**χ^2(global) = 1.39**
Excitation Wavelength/ nm	**Emission Wavelength/ nm**	**χ^2**
280	370	2.34
	390	1.52
	420	1.20
300	370	1.23
	390	1.05
	420	1.08
320	370	1.20
	390	1.48
	410	1.62

Table 2. For 2AP in water, results of global fitting of all decay curves to a monoexponential function with common lifetime. The global fluorescence lifetime, global chi-square and individual chi-square values for each decay are given.

		τ_1(global)/ns 11.0	**τ_2(global)/ns 13.5**	**χ^2(global) 1.06**
Excitation Wavelength/ nm	**Emission Wavelength/ nm**	**A_1**	**A_2**	**χ^2**
280	370	0.70	0.30	1.03
	390	0.65	0.35	1.04
	420	0.53	0.47	1.06
300	370	0.65	0.35	1.05
	390	0.59	0.41	1.02
	420	0.57	0.43	1.06
320	370	0.52	0.48	1.07
	390	0.50	0.50	1.04
	410	0.49	0.51	1.04

Table 3. For 2AP in water, results of global fitting of all decay curves to a biexponential function with common lifetimes. The global fluorescence lifetimes and global chi-square are given, followed by the A factors and chi-square values for each decay.

2AP in ethanol

The presence of two emitting species is also clearly apparent in ethanol. As for aqueous solutions, fluorescence decays were measured at three emission wavelengths and three excitation wavelengths. Individual fitting of each decay curve to a single exponential function yielded poor χ^2 values of about 1.5, and significant variation in lifetime with excitation and emission wavelength, ranging from 6.1ns (at 280nm excitation,370nm emission) to 6.5ns (at 320nm,410nm). As shown in Tables 4 and 5, global analysis confirmed that the decays could not be described by a single lifetime, but are well fitted by a biexponential decay with common lifetimes. The two emitting species have lifetimes of 5.8 and 7.5ns.

τ(global) = 12.1ns		**χ^2(global) = 1.39**
Excitation Wavelength/ nm	**Emission Wavelength/ nm**	**χ^2**
280	370	1.84
	390	1.56
	420	1.36
300	370	1.56
	390	1.25
	420	1.24
320	370	1.20
	390	1.67
	410	1.52

Table 4. For 2AP in ethanol, results of global fitting of all decay curves to a monoexponential function with common lifetime. The global fluorescence lifetime, global chi-square and individual chi-square values for each decay are given.

		τ_1(global)/ns 5.8	**τ_2(global)/ns 7.5**	**χ^2(global) 1.09**
Excitation Wavelength/ nm	Emission Wavelength/ nm	A_1	A_2	χ^2
280	370	0.87	0.13	1.08
	390	0.83	0.17	1.09
	420	0.80	0.20	1.04
300	370	0.84	0.16	1.09
	390	0.79	0.21	1.07
	420	0.79	0.21	1.08
320	370	0.72	0.28	1.02
	390	0.66	0.34	1.01
	410	0.65	0.35	1.07

Table 5. For 2AP in ethanol, results of global fitting of all decay curves to a biexponential function with common lifetimes. The global fluorescence lifetimes and global chi-square are given, followed by the A factors and chi-square values for each decay.

2AP riboside

To determine whether the two emitting species could be identified with the two tautomeric forms of 2AP, comparative measurements were made on 2AP riboside. As shown in Figure 1, the presence of the ribose substituent at N9 in 2APr prevents formation of the 7H tautomer.

The fluorescence decays of 2APr were measured in aqueous and ethanolic solutions at emission wavelengths of 370, 390 and 410 nm and excitation wavelengths of 300 and 320nm. In each solvent, global analysis showed that the fluorescence decays of 2APr across the entire excitation/emission space could be fitted by a single common fluorescence lifetime, with all chi-square values <1.2, indicating the presence of a single emitting species. The fluorescence lifetime of 2APr was found to be 10.6 ns in water and 5.8 ns in ethanol.

Discussion

Current understanding of the photophysics of 2AP in aqueous solution holds that its fluorescence decay is single exponential with a lifetime of around 11.5ns [15,18-20]. Our results show that, for an individual decay curve measured at a particular excitation and emission wavelength, this is indeed the case. However, measurement over a range of excitation and emission wavelengths reveals biexponential decay kinetics in both aqueous and ethanolic solutions of 2AP, indicating the presence of two emitting species. Comparison with 2APr, which demonstrably behaves as a single emitting species in both solvents, leads to the conclusion that the two species observed for 2AP are the 9H and 7H tautomers. In water, the 2APr lifetime of 10.6ns is very similar to the 11ns decay component of 2AP; therefore, we assign the latter to the 9H tautomer and the 13.5ns lifetime to the 7H tautomer. Similarly, in ethanol, the 5.8ns lifetime is assigned to the 9H tautomer (*cf* 5.8ns APr lifetime) and the 7.4ns lifetime to the 7H tautomer. The marked decrease in the fluorescence lifetimes in ethanol is consistent with the previously reported lifetime of 6ns for 2AP in ethanol and the observation of a decreasing trend in lifetime with decreasing solvent polarity [12]. Our observation of a biexponential decay for 2AP and a monoexponential decay for 2APr is analogous to the excited state decay behaviour of adenine and 9-methyladenine reported in a recent transient absorption study[5], which was also interpreted in terms of 9H/7H tautomerism.

The fractional contribution of the ith lifetime component to the total integrated (steady state) emission intensity, at a particular excitation and emission wavelength, is given by $\frac{A_i\tau_i}{\sum_i A_i\tau_i}$. Thus, measurement of the fluorescence decay of 2AP in water at a series of wavelengths across the emission envelope allowed the emission spectra of the two tautomers to be constructed, as shown in Figure 3.

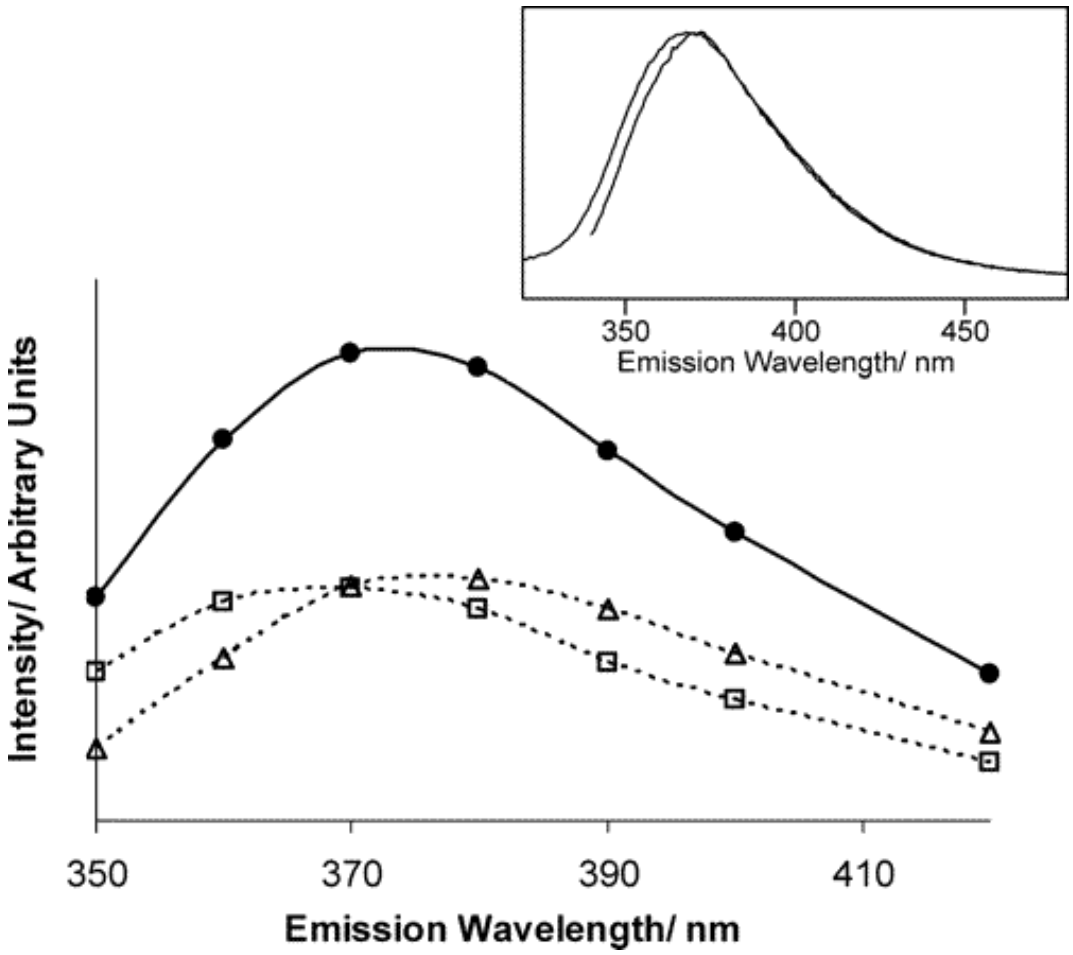

Figure 3. The emission spectra (at an excitation wavelength of 320nm) of the 9H tautomer (squares) and the 7H tautomer (triangles) of 2-AP in aqueous solution, derived from time-resolved fluorescence data. The total emission spectrum is shown by the solid line. The inset shows the red shift in the total emission spectrum when the excitation wavelength is shifted from 280nm (dotted line) to 330nm (solid line).

The spectra overlap closely; the maxima are separated by about 7 nm (~500 cm^{-1}), with the spectrum of the 7H tautomer lying at longer wavelength. The excitation wavelength dependence of the A factors in Tables 3 and 5 also suggests that the excitation spectrum of the 7H tautomer is red-shifted relative to the 9H. These observations are in agreement with the results of *ab initio* calculations by Broo[21] that predict both tautomers to be fluorescent (having lowest excited states of $\pi\pi^*$ character) and the emission maximum (for a vertical transition from the relaxed excited state) of the 7H tautomer to be about 1000 cm^{-1} below that of the 9H tautomer. The existence of the two tautomers should be apparent in the steady state emission of 2AP and, indeed, it was found that excitation on the red edge of the excitation spectrum gave an emission spectrum significantly red-shifted from that obtained with blue-edge excitation, as shown in the inset to Figure 3. For 2APr, the emission spectrum is independent of excitation wavelength.

If the radiative lifetimes of the two tautomers can be assumed to be similar, then the A factors indicate the fraction of the emitting population constituted by each tautomer. This appears to be a reasonable assumption, since the fluorescence lifetimes of the two tautomers are similar and their oscillator strengths are predicted to be similar[21], 0.37 for 9H and 0.25 for 7H. Examination of the A factors in Tables 3 and 5 indicates that the proportion of the 7H tautomer in both solvents is substantial, about 40% in water and about 20% in ethanol. These values are somewhat greater than those reported for adenine, 20% in water[4,5] and 6% in butanol[3], although the 40% value in water is similar to the observation of equal proportions of 7H and 9H tautomers for purine[11-13] in aqueous solution. The observation of a greater fraction of the 7H tautomer in water than in ethanol is consistent with the prediction of a larger dipole moment for this tautomer[14]. However, the magnitude of the 7H tautomer population is much greater than the 3% predicted for 2AP in water[14] in the same *ab intio* study. The calculations may significantly underestimate the effect of polar solvation in shifting the equilibrium towards the 7H tautomer, since they neglect specific solvent-solute interactions.

It is important to recognise that in these experiments we are observing the excited state tautomer population and to consider how this relates to the ground state population. *Ab initio* calculations by Broo[21] predict the 7H tautomer to be much closer in energy to the 9H tautomer in the excited state than in the ground state, for both 2AP and adenine in the gas phase. If the solvent effect on the relative excited state energies of the two tautomers were of the same magnitude as in the ground state, this would result in the two tautomers lying very close in energy, giving comparable proportions in the equilibrated excited state population. This might account for our observation of a relatively high proportion of 7H tautomer. However, we see no sign, in the fluorescence response functions, of any rise components (negative A factors) that would indicate a shift in the tautomer equilibrium following excitation. If the re-equilibration on excitation occurs faster than the time resolution of our measurements, i.e. on the picosecond timescale or less, we may be observing the fluorescence decay of the fully equilibrated excited state tautomer population. However, we see a definite dependence of the A factors (proportions of 7H and 9H tautomers in the emitting population) on excitation wavelength, indicating that memory of the ground state population is retained in the emitting population. It seems more likely, therefore, that the response of the tautomer equilibrium to excitation is slow compared with the lifetime of the excited state, and the excited state population that we observe reflects the composition of the ground state population.

We have found further evidence of tautomerism in 2AP from x-ray crystallography. The x-ray structure of 2AP crystals grown from ethanol solution shows the presence of both 9H and 7H tautomers in the crystal lattice, with the occupancy of the 7H form being about 15% [22]. This is in good agreement with the tautomer population in ethanol solution estimated from the fluorescence data. Further support for our derivation of ground state 2AP tautomer populations from the fluorescence decay amplitudes comes from the recent analogous study of adenine excited state decay by ultrafast transient absorption spectroscopy[5]. In the latter work, the fractional population of the 9H and 7H adenine tautomers was calculated from the decay amplitudes, giving a value of 22% for the 7H tautomer, in good agreement with previous measurements [4,13].

Conclusion

Fluorescence lifetime measurements have shown that 2AP exists as both the 9H and the 7H tautomer in aqueous and ethanolic solution at room temperature. There is a substantial proportion of the 7H isomer in the emitting population: about 40% in water and about 20% in ethanol. The two tautomers have closely overlapping fluorescence spectra but can be distinguished by their fluorescence lifetimes. The tautomeric composition of the emitting population depends on excitation wavelength and we see no evidence of a change in the tautomeric equilibrium in response to excitation. We conclude, therefore, that the observed excited state tautomer population arises directly from that in the ground state.

The observation of tautomerism in 2AP is further evidence of the similarity of this molecule to adenine and affirms its suitability as a fluorescent analogue of the nucleic base. It also offers the opportunity of applying sensitive fluorescence methods to the study of the tautomerisation process.

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