The Two-Photon Excitation Cross Section of 6MAP, a Fluorescent Adenine Analogue

Robert J. Stanley,*,† Zhanjia Hou,† Aiping Yang,† and Mary E. Hawkins‡

Department of Chemistry, Temple University, 201 Beury Hall, Philadelphia, Pennsylvania 19122, and National Cancer Institute, CRC 10/1-5742 MSC 1101, 10 Center Drive, Bethesda, Maryland 20892

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6MAP is a fluorescent analogue of adenine that undergoes Watson−Crick base pairing and base stacking in double-stranded DNA. The one-photon absorption spectrum of 6MAP is characterized by a maximum around 330 nm with moderate quantum yield fluorescence centered at about 420 nm. To take advantage of this probe for confocal and single-molecule microscopy, it would be advantageous to be able to excite the analogue via two photons. We report the first determination of the two-photon excitation cross section and spectrum for 6MAP from 614 to 700 nm. The power dependence of the fluorescence indicates that emission results from the absorption of two photons. The one-photon and two-photon emission line shapes are identical within experimental error. A study of the concentration dependence of the fluorescence yield for one-photon excitation shows no measurable quenching up to about 5 μM. The maximum in the two-photon excitation spectrum gives a two-photon cross section, δ_{TPE}, of 3.4 ± 0.1 Goeppert−Mayer (G.M.) at 659 nm, which correlates well with the one-photon absorption maximum. This compares quite favorably with cross sections of various naturally fluorescent biological molecules such as flavins and nicotiamide. In addition, we have also obtained the two-photon-induced fluorescence emission spectrum of quinine sulfate. It is approximately the same as that for one-photon excitation, suggesting that two-photon excitation of quinine sulfate may be used for calibration purposes.

Introduction

The nucleic acid bases of DNA and RNA are relatively unreactive to visible and near-ultraviolet light. This is due primarily to the high transition energies of the bases in the former case and very short excited state lifetimes of the bases in the latter case. It is only recently that the true excited-state lifetimes have been measured directly.[1,2] All of the bases have lifetimes shorter than 1 ps with pyrimidines living somewhat longer than purines. The excited state appears to be quenched by vibrational deactivation (internal conversion), possibly due to hydrogen-bond interactions between the bases and water.[2] Thus, the fluorescence quantum yields (Φ_f) of nucleic acid bases are very low, and steady-state or time-resolved fluorescence based on nucleic acid base emission has not been a useful probe of DNA dynamics or structure.

One of the earliest fluorescent base analogues used to study DNA structure and dynamics was 2-aminopurine (2AP).[3] 2AP is an adenosine analogue where the 6-amino group in adenine is shifted to the 2 position. This has the effect of extending the aromaticity of the heterocycle, which leads to a lowering of the transition energy. Whereas the canonical bases have their lowest energy transition of any oscillator strength around 260 nm, 2AP has its lowest energy transition around 306 nm. The fluorescence quantum yield for 2AP monomer is 0.66 with Stokes-shifted emission centered about 370 nm. The exact emission wavelength appears to depend on the polarity of the environment but does not appear to be sensitive to hydrogen bonding.[4]

Interestingly, 2AP fluorescence is quenched when incorporated into oligomeric DNA. Further quenching occurs upon annealing with complementary DNA. The source of the quenching is not well understood, although calculations[5] suggest that base stacking between 2AP and adjacent bases engenders low-lying charge-transfer (CT) states which become rapidly populated by ultrafast internal conversion.[6] These CT states would have little or no fluorescence. Recent experimental results suggest that true electron transfer between bases is probably not the source of fluorescence quenching for 2AP,[7] but this does not rule out a role for CT states in the internal conversion of excited state nonradiatively to the ground state. Efficient internal conversion may occur through CT character developing from the supramolecule formed between base-stacked nucleotides, as has been suggested for the special bacteriochlorophyll dimer in bacterial photosynthetic reaction center.[8]

Nucleotide analogue fluorescence quenching due to base stacking has proven to be a useful tool for studying the phenomenon of base flipping.[9] Base flipping, where a DNA base flips out extrahelically, may occur spontaneously or in response to the action of a DNA binding protein.[10] When the base in question is a fluorescent analogue, the loss of base stacking upon flipping leads to a substantial recovery of the fluorescence quantum yield of the probe base. We have used 2AP in this manner to obtain circumstantial evidence of base flipping in the target lesion of DNA photolyase, a DNA binding protein that repairs pyrimidine photodimers.[11]

The search for other fluorescent base analogues has led to the synthesis of a series of pteridines that can act as guanine and adenine analogues.[12] These analogues are similar to 2AP in that they are sensitive to base stacking in both single and double helical DNA. One of these, 6MAP (4-amino-6-methyl-8-(2-deoxy-β-D-ribofuranosyl)-7(8*H*)-pteridone, Scheme 1), is

* Corresponding author. Tel.: (215) 204-2027. Fax: (215) 204-1532. E-mail: robert.stanley@temple.edu.

† Temple University.

‡ National Cancer Institute.

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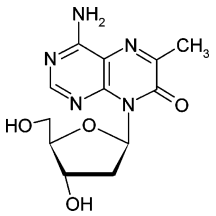

an adenine analogue that makes a minor perturbation on DNA structure but whose fluorescence quantum yield is modulated strongly by base stacking.[12] An attractive property of 6MAP is that its lowest energy absorption band at 330 nm is well removed from the absorption of DNA itself, allowing for selective excitation. We have used 6MAP instead of 2AP and have also observed a fluorescence increase in the photolyase−DNA systems suggestive of base flipping (Yang and Stanley, in preparation). These results point toward the possibility of using these fluorescent base analogues to probe both the structure and the dynamics of this important process using single-molecule microscopy.

A problem facing both microscopies arises when excitation is required in the near-ultraviolet region of the spectrum. This spectral window is useful for many biological cofactors, including DNA analogues and possible photodynamic therapeutics.[13] Because many solution components absorb in this wavelength range as well, there is often unacceptably large background fluorescence (autofluorescence) that obscures the signal of interest. Two-photon excitation (TPE) has been used to reduce this problem.[14] TPE using near-infrared radiation occurs only at the focus of the excitation beam. With suitable optics, this focal volume can be smaller than 1 μm^3, significantly reducing background fluorescence. To our knowledge, there are no reports of measurements of the TPE cross section, δ_{TPE}, for 6MAP. We have used a femtosecond optical parametric amplifier to achieve TPE of 6MAP and have measured its two-photon cross section and corrected emission spectrum. We also present data showing that two-photon excitation of quinine sulfate is suitable for calibrating the spectral responsivity of instruments used for two-photon studies.

Experimental Section

6MAP nucleoside was synthesized as described previously.[12] Fluorescein free base and quinine sulfate dihydrate (99+%) were purchased from Acros and used without further purification. HPLC grade water was used to dissolve the solutes. Tris disodium salt was purchased from Fisher Scientific. All other reagents were of the highest quality obtainable.

6MAP samples 50−240 μM (pH 7.4−8) were prepared by dissolving the 6MAP into 50 mM Tris buffer pH 7.4 and 5% glycerol by sonication. The concentration was determined by UV−vis spectroscopy using an extinction coefficient of $\epsilon_{329} = 8511\ M^{-1}\ cm^{-1}$ (in methanol).[12] Stock solutions of fluorescein in water, pH 13, were characterized spectrophotometrically using an extinction coefficient[15] at 490 nm of 77 898 $M^{-1}\ cm^{-1}$. Fluorescein samples were prepared by dilution to 10−16 μM to use as a standard for measuring the TPE cross section. Quinine sulfate was prepared in 0.1 N H_2SO_4 from 1 to 3 ppb.[16] All measurements were performed at room temperature. All solutions were stored in the dark at −20 °C.

The excitation source for these experiments was a noncollinear optical parametric amplifier (NOPA). The pump for the NOPA was a Ti:sapphire regenerative amplifier (Positive Light Spitfire-LCX) seeded by a mode-locked Ti:Sapphire laser (Kapteyn-Murnane Laboratories L.L.C.), which produced ~120 fs pulses at 788 nm with a repetition rate of 1 kHz. A small part of the fundamental beam was used to produce a white light continuum in sapphire to seed the 2 mm thick type-I BBO crystal for the UV-pumped NOPA.[17,18] This stage was pumped with about 20−30 μJ of second harmonic light generated in a 2 mm type-I BBO crystal using the remaining fundamental. The NOPA is capable of producing ultrashort femtosecond pulses which have a large spectral bandwidth if the angle between seed and pump pulses, α, is set so that phase matching occurs over a broad range of frequencies, about 3°. However, when $\alpha \approx 10°$, phase matching occurs only over a small range of frequencies and highly efficient amplification of narrower band radiation results at the cost of longer pulse widths. For these studies, the NOPA generated pulses from 610 to 700 nm with a pulse width of about 250 fs and a relatively constant spectral bandwidth. The pulse width was determined in one of two ways, either by measuring a cross-correlation trace between 788 nm fundamental and the NOPA pulses in a 2 mm thick type I BBO crystal, or by measuring an auto-correlation trace using two-photon absorption in a SiC photodiode.[19] The spectral width (fwhm) was about 15 nm, indicating that the pulses were not transform limited.

The 690 nm beam was filtered through a near-IR cutoff filter to remove residual fundamental light from the continuum and was passed through a variable neutral density filter to adjust the power. It was focused into the sample cuvette using a fused silica or BK7 plano-convex lens of 6.5 cm focal length. The calculated beam waist for the 6 mm diameter beam was approximately 20 μm at 700 nm. The 2 mm × 10 mm cuvette, made of either fused silica or glass (with a low wavelength cutoff of about 320 nm), was positioned so that excitation was performed along the 10 mm length with emission collected through the 2 mm length. In some cases, this geometry was reversed with excitation through the 2 mm path length. In this case, emission was collected at the front 1−2 mm of the 10 mm dimension to make sure no inner filter effects perturbed the line shape of the emission spectrum. Both the fused silica and the optical glass cuvettes gave similar results, indicating that there was little contribution to the emission spectrum due to harmonic generation at the cuvette−solution interface. Sample solutions were stirred (Spectrocell SYS-D1) to avoid saturation and photobleaching. The excitation pulse energy used was less than 2 μJ as determined with a silicon joulemeter (Molectron J3S-10). Above this value, continuum generation was observed in the cell.

The fluorescence from samples was collected by a 5 cm focal length 1" diameter achromatic lens and then focused into an f/3.9 1/4 m spectrograph (DKSP240-I, CVI Laser Corp.) using either a 10 cm focal length 1" diameter lens or an f/4 UV achromatic lens. A filter (Schott BG 33) was placed in front of the spectrometer to block the 690 nm excitation background. The fluorescence was detected by a cooled (−60 °C) CCD detector (Andor Technology DV420-OE CCI-010). The CCD exposure time was 5−100 s depending on the signal level. All spectra were corrected for background.

The system wavelength responsivity was calibrated using the OPE spectrum from a standard 0.1 N sulfuric acid solution containing quinine sulfate (QS) at a concentration of 1−3 ppb. A 2 mm type I BBO crystal was used to generate 345 nm second harmonic light as an excitation beam for measuring the OPE from QS. As a check, the TPE spectrum of QS was obtained under the same conditions but without the BBO crystal. A comparison of the one- and two-photon-induced fluorescence

(OPIF and TPIF, respectively) spectra of QS is discussed below. The QS spectrum was used along with published absolute emission values to correct the experimental spectra for the responsivity of the optical setup, spectrograph, and detector. A similar calibration was used to correct the one-photon spectra obtained on the conventional fluorimeter, a SPEX Fluoromax-2.

Data Analysis. The two-photon cross section of 6MAP was obtained by comparing its fluorescence intensity with that of a reference fluorophore taken at the same excitation wavelength and laser power. The spectral responsivity of the optical set up must also be taken into account when the emission line shapes of the reference and molecule of interest differ. Therefore, the responsivity of the TPE apparatus was calibrated using the emission from freshly prepared acidic QS solutions as described above. This correction makes a significant difference in the value of δ_{TPE}.

The cross section of 6MAP was obtained relative to fluorescein at 690 nm under identical excitation conditions. The use of the fluorescein as a standard allows for a determination of $\delta_U(\lambda)$ as given the in the equation below:

$$\delta_U(\lambda) = \delta_R(\lambda)\frac{\Phi_R(\lambda)c_R\langle I(t)\rangle_U\langle P(\lambda)\rangle_R^2}{\Phi_U(\lambda)c_U\langle I(t)\rangle_R\langle P(\lambda)\rangle_U^2}$$

where $\delta_R(\lambda)$ and $\delta_U(\lambda)$ are the two-photon cross sections of the reference and unknown compounds (in cm^4 photons^{-1} s^{-1}; 1 Goeppert−Mayer (G.M.) = 10^{-50} cm^4 photons^{-1} s^{-1}), $\Phi_R(\lambda)$ and $\Phi_U(\lambda)$ are the wavelength-dependent fluorescence quantum yields of the reference and unknown, respectively, c_R and c_U are the concentrations of the reference and unknown, respectively, $\langle I(t)\rangle_R$ and $\langle I(t)\rangle_U$ are the integrated fluorescence intensities of the reference and unknown, respectively. The integrated laser powers for the reference and unknown are $\langle P(\lambda)\rangle_R$ and $\langle P(\lambda)\rangle_U$, respectively.

The TPE spectrum of fluorescein has been determined down to 690 nm. We used the value of the two-photon cross section at this wavelength (δ_{TPE} = 16 G.M.) as the standard. More than 10 independent determinations were made at various excitation powers (but always $\langle P(690\text{nm})\rangle_U = \langle P(690\text{nm})\rangle_R$) and concentrations. For wavelengths less than 690 nm, the TPE spectrum for 6MAP was obtained by normalizing the 6MAP fluorescence as a function of wavelength to $\delta_{6MAP}(\lambda = 690\text{ nm})$. The raw emission data below excitation wavelengths less than 690 nm were not corrected for the responsivity of the spectrometer because the emission of 6MAP was used as a reference. However, it was necessary to take into account the laser intensity and the joulemeter spectral responsivity over the excitation wavelength range to correct for the quadratic dependence of the emission intensity with laser power. In addition, the emission integration range was chosen to exclude any residual scattered pump light in the emission spectrum. These corrections are reflected in the calculated cross section.

Results

Two-Photon Emission Spectrum of Quinine Sulfate. Quinine sulfate (QS) is widely used to determine the spectral responsivity of fluorescence instruments. Correction factors for OPIF have been determined from 375 to 675 nm, and it is a primary standard for the determination of the spectral responsivity of emission spectrometers.[16] For the purpose of this study it would be useful, although fortuitous, if the TPIF spectrum of QS coincided with its OPIF spectrum. To our knowledge, the two-photon emission-induced fluorescence spectrum of quinine sulfate has not been reported. A comparison of the OPIF and TPIF spectra is shown in Figure 1. The line shapes of these spectra are the same within the experimental uncertainty. Two-photon excitation of QS was used for all subsequent work to obtain the spectral responsivity calibration curve.

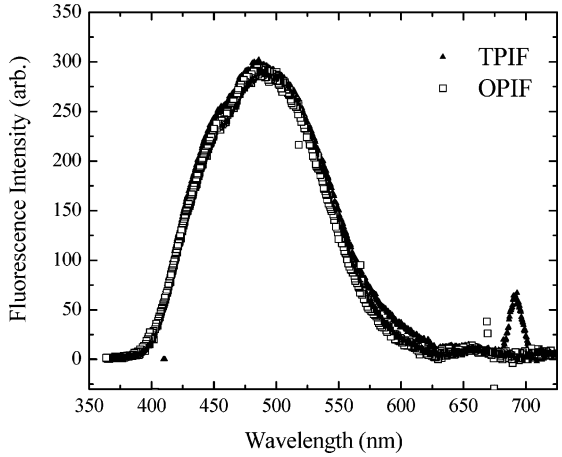

Figure 1. A plot of the one- (□) and two-photon (▲)-induced fluorescence emission of quinine sulfate (1 ppb) in 0.1 N H_2SO_4 collected on the same setup. The one-photon excitation wavelength was 345 nm, and the two-photon excitation wavelength was 690 nm. A small amount of the two-photon excitation can be seen in the TPIF spectrum. These spectra are uncorrected for the spectral responsivity of the instrument.

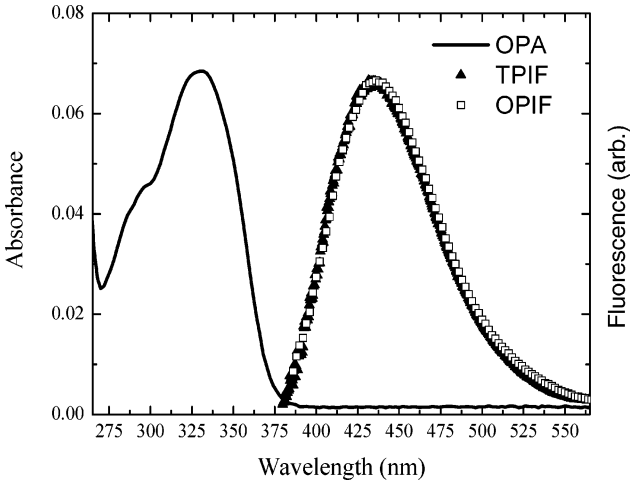

Figure 2. A plot comparing the one- (□) and two-photon (▲)-induced fluorescence emission from 6MAP in buffer at 0.8 and 63 μM, respectively. The one-photon absorption spectrum (−) is shown for reference.

Two-Photon Emission Spectrum of 6MAP: Power and Concentration Dependence. When solutions of 6MAP are excited with focused ultrafast laser pulses from 614 to 700 nm, blue fluorescence can be seen emanating from the focal point in the sample cuvette. For reference, the one-photon absorption spectrum of 6MAP in buffer is shown in Figure 2. The absorption spectrum has a structure with the largest extinction at about 330 nm and a prominent shoulder at 294 nm (the extinction increases to the blue, data not shown).

The two-photon-induced fluorescence spectrum of a 63 μM solution of 6MAP in 50 mM Tris (pH 7.4) with 5% glycerol with excitation at 690 nm is shown in Figure 2. The spectrum has been corrected for the spectral responsivity of the instrument using QS. For comparison, a one-photon-induced fluorescence (OPIF) emission spectrum of 6MAP at 0.8 μM concentration

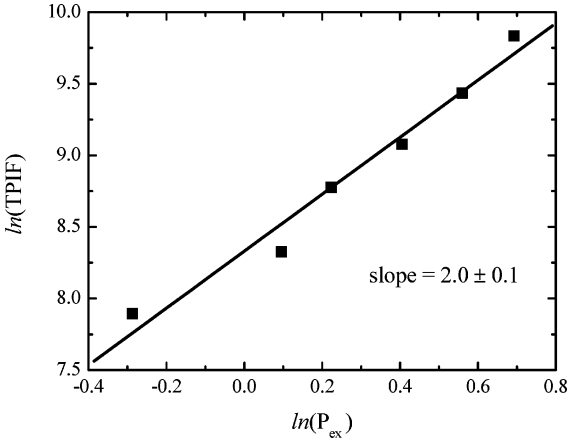

Figure 3. The power dependence of the logarithm of the 6MAP TPIF signal (■) with the logarithm of laser excitation power (P_{ex}). A linear fit returns a slope of 2, indicating that the TPIF signal is due to a two-photon process.

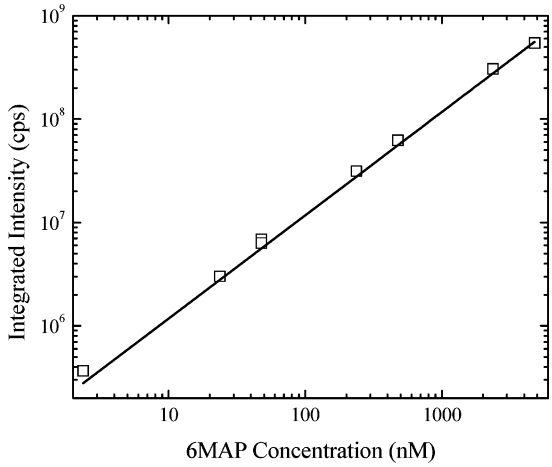

Figure 4. The concentration dependence from 2.4 nM to 4.7 μM of the integrated one-photon-induced fluorescence from 6MAP (□) excited at 345 nm. The solid line is a linear fit to the data ($r^2 = 0.996$).

in the same buffer using 345 nm excitation is also shown, taken on a conventional fluorimeter with the same resolution whose spectral responsivity has been corrected using QS as indicated above. As a control, an OPIF spectrum of 6MAP was obtained on the two-photon setup using 345 nm light generated by frequency doubling the NOPA fundamental in a 2 mm type I BBO crystal and filtering out residual fundamental using a color glass filter. This spectrum was identical to that obtained on the standard one-photon fluorimeter (data not shown).

The OPIF and TPIF fluorescence spectra are characterized by a large Stokes shift of about 100 nm. The emission line shapes, peaked around 436 nm, are identical within the experimental uncertainty. This suggests that the emitting state produced by two-photon excitation is identical to the one-photon state. The lack of vibronic structure suggests that the molecule follows Kasha's rule of fast internal conversion from the state accessed by the strongly allowed vertical transition to a lower energy level well displaced from the initial Franck−Condon allowed region. From the large Stokes shift, it is apparent that the molecule undergoes a large change in nuclear structure before emission.

It was necessary to establish whether the 6MAP emission resulting from red excitation was due to two-photon absorption. To demonstrate this, TPIF spectra were obtained as a function of pump power at $\lambda = 690$ nm. Figure 3 shows the logarithm of the integrated area of the TPIF from 380 to 600 nm plotted against the logarithm of the pump power. A line of slope $= 2.0 \pm 0.1$ was obtained, which is expected for a two-photon process. This result rules out the possibility of either a three-photon excitation from the ground state or a $2 + 1$ photon absorption process and suggests that excited-state absorption is negligible.

To determine whether 6MAP emission is self-quenched, the integrated one-photon-induced fluorescence (OPIF) was measured as a function of concentration over a range of approximately 0.0024−4.77 μM using 345 nm excitation in a standard fluorimeter. The path length for these measurements was 1 cm, giving an optical density of 0.041 for the most concentrated solution. These data are shown as □ symbols in Figure 4. The integrated fluorescence is linear to 4.77 μM, above which the absorbance becomes prohibitively high. Given the high correlation coefficient for the fit ($r^2 = 0.996$), we assume that 6MAP dimerization is not significant at the concentrations used. This is not surprising as similar molecules have low association constants. For example, flavin mononucleotide has an association constant of about 100 M^{-1}.[20] At the concentrations used in this experiment, we expect virtually no dimerization.

Two-Photon Excitation Cross Section of 6MAP. The TPE cross section, δ_{6MAP}, was determined by comparing the corrected integrated emission for 6MAP to fluorescein. This approach has been used previously by others to characterize a variety of two-photon emitters. Because the absorption of 6MAP is blue-shifted as compared to fluorescein, it was not possible to determine the TPE cross section at wavelengths shorter than 690 nm; therefore, the 6MAP cross section was determined at this wavelength and used to calibrate measurements made at shorter wavelengths (see below). Cross-section values were obtained on 11 different preparations of 6MAP and fluorescein. These emission spectra were corrected for the spectral responsivity of the optical setup using quinine sulfate as described above. The TPE cross section at 690 nm is $\delta_{TPE} = 1.9 \pm 0.7$ G.M. This result was invariant over a factor of 10 in laser intensity up to about 2 μJ/pulse. Interestingly, measurements performed from 0.49 to 131 μM in 6MAP showed no concentration dependence of the cross section, despite the quenching obtained in the one-photon experiment.

The TPE spectrum of 6MAP was determined by normalizing the integrated emission from 380 to 600 nm for various excitation wavelengths to that of the integrated emission for 6MAP excited at 690 nm where the cross section was determined to be 2.0 G.M. Two-photon cross sections were determined from 614 to 700 nm (307−350 nm in the one photon spectrum). These data are shown relative to the one-photon absorption spectrum in Figure 5. The maximum two-photon cross section of $\delta_{TPE} = 3.4 \pm 0.1$ G.M. at 659 nm coincides well with the one-photon absorption maximum (330 nm). The line shape of the TPE spectrum appears narrower than the one-photon result, although there is some evidence that the TPE spectrum exhibits similar vibronic structure. This could be verified by increasing the scan range of the OPA, but it is difficult to produce higher wavelength radiation using the NOPA and overlap with the 6MAP emission spectrum occurs at the low wavelength range, making this difficult to confirm. Given that 6MAP has C_s symmetry, there should be no difference between one- and two-photon selection rules. It is possible that the emitting state for TPE exhibits a wavelength-dependent fluorescence quantum yield. This seems unlikely given that the line shapes of the one-photon and two-photon emission spectra are nearly identical.

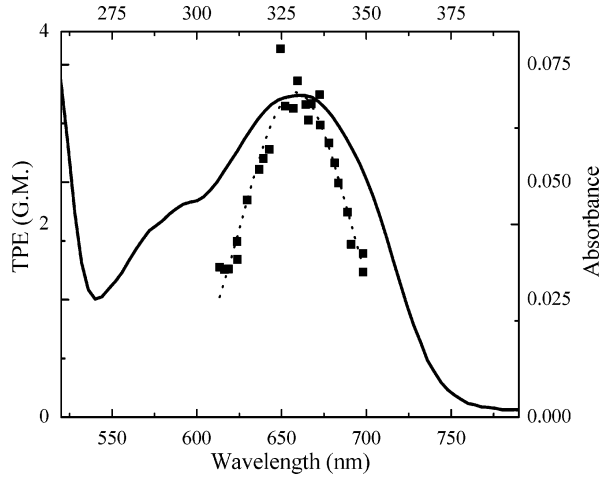

Figure 5. The TPE spectrum of 6MAP (■, 49 μM) from 614 to 700 nm. The bottom axis corresponds to the two-photon wavelength, and the top axis corresponds to the one-photon wavelength. The one-photon absorption spectrum (−) is plotted for reference. The dotted line represents a fit of the data to a Gaussian function whose maximum of 3.4 ± 0.1 G.M. occurs at 659 nm.

It should be pointed out that no correction was made for laser pulse width and this may have introduced some error. However, the spectral width of the NOPA pulse was measured for each excitation wavelength and very little change in the width of the pulse was observed. It is possible that an accurate determination of the pulse width at every wavelength would have produced a smoother TPE spectrum, but the data suggest that any error is small and that the TPE spectrum of 6MAP in Figure 5 is reasonable.

Discussion

6MAP has several attractive qualities as a probe of DNA structure and dynamics. When incorporated into DNA, it undergoes Watson−Crick base pairing with melting points only a few degrees lower than native ds-DNA.[12] Its fluorescent state is well quenched in ds-DNA, and this quenching is a sensitive probe of base stacking in both single- and double-stranded DNA. As compared to 2-aminopurine, its absorption maximum is red shifted about 15 nm (ca. 330 nm) and its emission undergoes a large Stokes shift of about 100 nm as compared to 55 nm for 2-aminopurine. This makes it possible to selectively excite 6MAP in a DNA strand and to separate the resulting fluorescence effectively from the excitation source. The emission overlaps well with the absorption of other biological chromophores (e.g., flavins), opening up the possibility of doing fluorescence energy-transfer experiments for a wide variety of systems. However, because the excitation wavelength is in the near-UV, the utilization of the probe for microscopy is hampered by autofluorescence of other species found in buffers and in cells. Our exploration of the TPE properties of 6MAP shows that this molecule is a potentially powerful probe of nucleic acid structure that could be utilized by two-photon microscopy.

Our results show that there is a moderate two-photon excitation cross section for 6MAP of about 3.4 G.M. at 659 nm. For the purposes of comparison, we contrast the TPE cross of 6MAP at 690 nm with those of other commonly used two-photon probes. At this wavelength, 6MAP has a cross section of about 2 G.M. While this value is small as compared to those obtained for much larger chromophores such as fluorescein (16 G.M.) or rhodamine B (194 G.M.), it is comparable to the cross sections measured for endogenous fluorophores such as flavins (1 G.M.) and nicotiamide adenine dinucleotide (0.1 G.M.), which are commonly used for microscopy.[21] However, several issues remain to be resolved as to whether 6MAP will find wide acceptance as a microscopy probe of nucleic acid structure and dynamics. One issue is its relatively low fluorescence quantum yield, about 0.39 for the monomer.[12] This is smaller as compared to other useful probes. For example, FMN has a quantum yield of about 0.6. Even though other endogenous chromophores have lower TPE cross sections than 6MAP, the overall emission may be larger because it is proportional to the product of the quantum yield and the cross section.

Beyond time-resolved fluorescence studies, there are no data regarding the photophysics of 6MAP, such as whether it has a significant triplet yield or undergoes photobleaching. Recent results for a guanine analogue based on pteridone, 3-MI, suggest that the triplet yield for this class of fluorophore is low but that photobleaching may be significant.[22] Finally, the high degree of quenching in base-stacked DNA may make the probe emission difficult to detect, even though the emission maximum is well matched to the responsivity of highly sensitive photomultiplier detectors. However, this large change in fluorescence as a function of base stacking does have the advantage of making this probe very sensitive to small changes in DNA structure.

Conclusions

We have measured the two-photon excitation spectrum of 6MAP monomer in aqueous buffer. This fluorescent adenosine analogue has a peak cross section of 3.4 G.M. at a wavelength that correlates well with its one-photon absorption maximum. The line shape of the two-photon excitation spectrum is narrower than its one-photon counterpart. A concentration-dependent quenching is observed for the one-photon excited fluorescence quantum yield but is absent in the TPE cross section. The reason for this discrepancy is unknown at this time. We have also shown that the two-photon-induced fluorescence spectrum of quinine sulfate is identical to its one-photon-induced fluorescence spectrum. This observation indicates that the TPIF of QS can be used to correct for the spectral responsivity of two-photon emission instruments in the same manner as for one-photon measurements.

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References and Notes

(1) Pecourt, J.-M. L.; Peon, J.; Kohler, B. *J. Am. Chem. Soc.* **2000**, *122*, 9348.
(2) Pecourt, J.-M. L.; Peon, J.; Kohler, B. *J. Am. Chem. Soc.* **2001**, *123*, 10370.
(3) Ward, D. C.; Reich, E.; Stryer, L. *J. Biol. Chem.* **1969**, *244*, 1228.
(4) Allan, B. W.; Reich, N. O. *Biochemistry* **1996**, *35*, 14757.
(5) Jean, J. M.; Hall, K. B. *J. Phys. Chem. A* **2000**, *104*, 1930.
(6) Fiebig, T.; Wan, C.; Zewail, A. H. *ChemPhysChem* **2002**, *3*, 781.
(7) Larsen, O. F. A.; van Stokkum, I. H. M.; de Weerd, F. L.; Vengris, M.; Aravindakumar, C. T.; van Grondelle, R.; Geacintov, N. E.; van Amerongen, H. *Phys. Chem. Chem. Phys.* **2003**, *6*, 154.
(8) Franzen, S.; Stanley, R. J. *Chem. Phys.* **2002**, *276*, 115.
(9) Roberts, R. J. *Cell* **1995**, *82*, 9.
(10) McCullough, A. K.; Dodson, M. L.; Scharer, O. D.; Lloyd, R. S. *J. Biol. Chem.* **1997**, *272*, 27210.
(11) Christine, K. S.; MacFarlane, A. W., IV; Yang, K.; Stanley, R. J. *J. Biol. Chem.* **2002**, *277*, 38339.
(12) Hawkins, M. E.; Pfleiderer, W.; Jungmann, O.; Balis, F. M. *Anal. Biochem.* **2001**, *298*, 231.

(13) Oh, D. H.; Stanley, R. J.; Lin, M.; Hoeffler, W. K.; Boxer, S. G.; Berns, M. W.; Bauer, E. A. *Photochem. Photobiol.* **1997**, *65*, 91.
(14) Xu, C.; Webb, W. W. *J. Opt. Soc. Am. B* **1996**, *13*, 481.
(15) Diehl, H.; Horchak-Morris, N. *Talanta* **1987**, *34*, 739.
(16) Velapoldi, R. A.; Mielenz, K. D. *NBS Special Publication (United States)* **1980**, *260*−*64*, 122 pp.
(17) Riedle, E.; Beutter, M.; Lochbrunner, S.; Piel, J.; Schenkl, S.; Sporlein, S.; Zinth, W. *Appl. Phys. B* **2000**, *71*, 457.
(18) Cerullo, G.; De Silvestri, S. *Rev. Sci. Instrum.* **2003**, *74*, 1.
(19) Lochbrunner, S.; Huppmann, P.; Riedle, E. *Opt. Commun.* **2000**, *184*, 321.
(20) Gibson, Q. H.; Massey, V.; Atherton, N. M. *Biochem. J.* **1962**, *85*, 369.
(21) Huang, S.; Heikal, A. A.; Webb, W. W. *Biophys. J.* **2002**, *82*, 2811.
(22) Sanabia, J. E.; Goldner, L. S.; Lacaze, P.-A.; Hawkins, M. E. *J. Phys. Chem. B* **2004**, *108*, 15293.