Photophysical Properties of Atto655 Dye in the Presence of Guanosine and Tryptophan in Aqueous Solution

ARTICLE *in* THE JOURNAL OF PHYSICAL CHEMISTRY B · MAY 2011

Impact Factor: 3.3 · DOI: 10.1021/jp200876d · Source: PubMed

4 AUTHORS, INCLUDING:

Anchi Yu
Renmin University of China
46 PUBLICATIONS **539** CITATIONS

SEE PROFILE

THE JOURNAL OF PHYSICAL CHEMISTRY B

ARTICLE

pubs.acs.org/JPCB

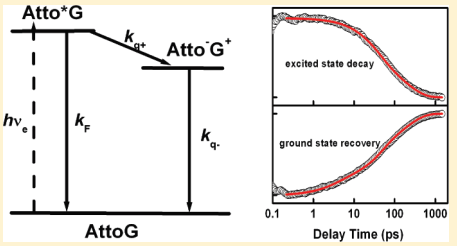
Photophysical Properties of Atto655 Dye in the Presence of Guanosine and Tryptophan in Aqueous Solution

Ruixue Zhu,[†] Xun Li,[‡] Xin Sheng Zhao,*[,‡] and Anchi Yu*[,†]

[†]Department of Chemistry, Renmin University of China, Beijing 100872, China

[‡]Beijing National Laboratory for Molecular Sciences, State Key Laboratory for Structural Chemistry of Unstable and Stable Species, Department of Chemical Biology, College of Chemistry and Molecular Engineering, and Biodynamic Optical Imaging Center, Peking University, Beijing 100871, China

ABSTRACT: Atto655 has been widely used as an excellent probing dye through photoinduced electron transfer (PET) for biochemical processes in oligonucleotides or polypeptides. However, its photophysical properties in the presence of the quenchers guanosine and tryptophan have not been carefully studied. In this work, we investigated the dynamics of PET between Atto655 and the two quenchers in aqueous solution with femtosecond transient absorption experiments. We derived that the charge separation rate is 8.1×10^9 s^{-1} and the charge recombination rate is 7.7×10^{10} s^{-1} for the collision complex between Atto655 and guanosine and that the corresponding values for the collision complex between Atto655 and tryptophan are 4.0×10^{11} and 5.0×10^{12} s^{-1}, respectively. These experimental results are quite consistent with the prediction of Marcus-type theory for electron transfer. The implications of this work for the data analysis of PET-based fluorescence correlation spectroscopy are discussed.



INTRODUCTION

Photoinduced-electron-transfer-based fluorescence correlation spectroscopy (PET-FCS) is a powerful tool for studying the conformational dynamics of biopolymers.[1−16] Because it requires contact formation between the fluorophore and quencher at a van der Waals distance of subnanometer scale,[8] PET is an elegant alternative to conventional fluorescence resonance energy transfer (FRET). To date, many fluorophores such as 2-aminopurine,[5,6,17−19] pyrene,[20−23] coumarin,[24] rhodamine,[7,25] oxazine,[2,4,10,11,26−28] fluorescein,[3,29] and boron-dipyrromethene[12,30] have been reported to exhibit a PET interaction with the nucleobase guanosine and the amino acid tryptophan. Efficient PET often occurs between the first excited singlet state of the fluorophore and the ground state of the quencher.[13,19,24] Two mechanisms, so-called static and dynamic quenching, have been proposed for the deactivation of the excited fluorophore.[6,8,24] However, Zewail et al. pointed out that this distinction depends on the actual time scale of the experimental methods.[18]

Although PET-FCS has been extensively applied, some questions remain as to how to correctly interpret PET-FCS data. For example, Qu et al. employed tetramethylrhodamine (TMR) as an electron acceptor to measure the rates of intramolecular collision in unstructured overhang oligonucleotides using PET-FCS.[16] They found that the equilibrium constant obtained from their PET-FCS data through the standard two-state model is not identical to that from the ensemble static fluorescence measurement. They suggested a three-state model for their PET phenomenon, in which the closed state is composed of a fluorescent state and a nonfluorescent charge-separated state. Recently, we followed the ultrafast PET process between TMR and guanosine in aqueous solution with femtosecond transient absorption spectroscopy. We found that the charge separation and recombination rates in the collision complex of TMR and guanosine are much higher than the rates of forward and reverse intramolecular diffusion determined by the PET-FCS measurements, so that one ought not to interpret the lifetime of the dark state in the FCS experiment as that of the charge-separated state.[31] We also found that, in the case of TMR, the assumption of a completely nonfluorescent dark state would introduce noticeable error in the calculation of the forward and reverse rate constants in the FCS experiment.[31] Both findings are in agreement with the suggestions made by Qu et al.[16] Based on the three-state model provided by Qu et al. and the ultrafast experimental observations, we suggested an equivalent two-state model, in which a dark state with nonzero brightness is composed of a fluorescent state and a nonfluorescent state, to accurately interpret the PET-FCS data.[31]

Atto655 is a member of the oxazine family[32] and is also a good candiate for PET.[8] Its fluorescence can be prominently quenched by guanosine or tryptophan,[8,28,33,34] and it has been widely used in PET-FCS experiments.[32,35−41] Compared with other red absorptive and emissive dyes such as Cy-5 and Alexa Fluor 647, Atto655 has excellent photostability and brightness.[42] However, in the literature, we have not found any ultrafast study on the PET process between Atto655 and the two quenchers guanosine and tryptophan. In this work, we studied the PET kinetics of Atto655 in the presence of guanosine or tryptophan in

Received: January 27, 2011
Revised: March 29, 2011
Published: April 08, 2011

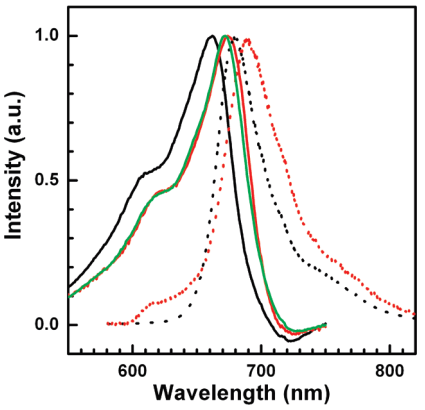


Figure 1. Normalized static absorption (solid line) and fluorescence (dotted line) spectra of Atto655 alone (black line), in 1.00 M guanosine (red line), and in 50.0 mM tryptophan (green line) in 1 × TE buffer.

aqueous solution by implementing femtosecond transient absorption spectroscopy. We recorded the transient absorption spectra of Atto655 in 1.00 M guanosine and 50.0 mM tryptophan. The decay of the first excited electronic state and the recovery of the ground electronic state of Atto655 were investigated, in order to derive the charge separation and recombination rates of the charge-separated state in the collision complexes of Atto655 with the respective quenchers. We also discuss the implications of this work for PET-FCS data analysis and our recommendations.

EXPERIMENTAL SECTION

Materials. Atto655 was purchased from ATTO-TEC GmbH, Siegen, Germany, and used as received. Deoxy-guanosine and tryptophan were purchased from Sigma, St. Louis, MO, and used as received. 1 × TE buffer (pH 8.0) was diluted from 20 × TE (Molecular Probes, Carlsbad, CA). Ultrapure water (18.2 MΩ· cm^{-2}) was obtained through either a PALL or Milli-Q water purification system.

Femtosecond Transient Absorption Measurements. We used an amplified Ti:sapphire laser system (Spitfire, Spectra Physics, Mountain View, CA) that generates about 100-fs laser pulses at 840 nm with a repetition rate of 1 kHz and an average power of around 1.0 W. These fundamental pulses were used to pump an optical parametric amplifier (OPA), as well as to generate the white-light continuum. The OPA pulses were about 100 fs and could be tuned from 450 to 700 nm. The white-light continuum was generated in a spinning fused-silica disk with the 840-nm pump pulse, and its spectrum covered the range of 420−750 nm. The OPA outputs were used as the pump pulses, and the white-light continuum was used as the probe pulse. The timing between the pump and probe pulses was controlled using a motorized translation stage (M-ILS250CC, Newport, Irvine, CA). The pump and probe beams were noncollinearly focused into the sample cell using two achromatic lenses (300-mm focal length for the pump and 100-mm focal length for the probe). At the sample position, the average powers were about 0.5 mW for the pump beam and about 10 μW for the probe beam. The signals were collected by a photomultiplier tube (R955, Hamamatsu, Hamamatsu City, Japan) that was attached to an output port of a monochromator (SP2358, Princeton Instruments, Acton, MA) and sent to a lock-in amplifier (SR850, SRS,

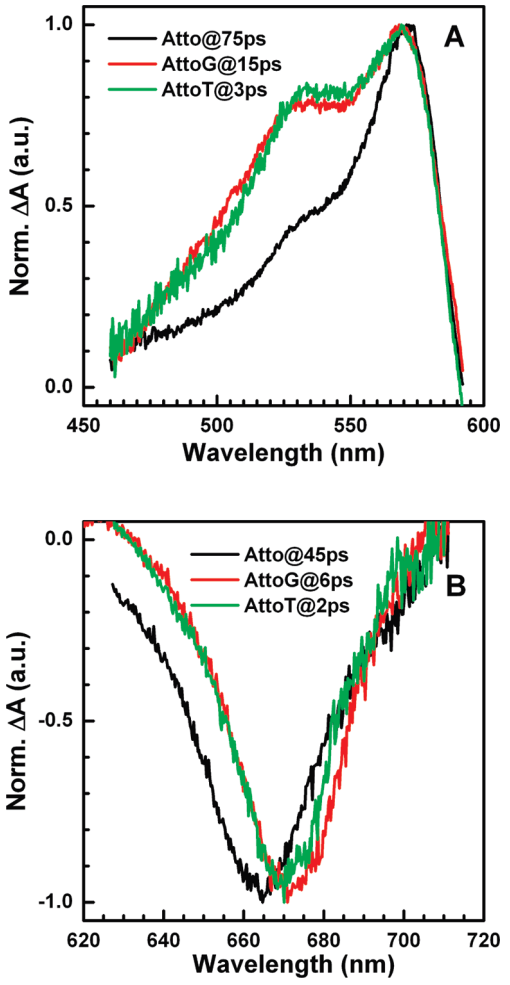


Figure 2. Transient absorption spectra of Atto655 alone (black line), in 1.00 M guanosine (red line), and in 50.0 mM tryptophan (green line) in the wavelength ranges of (A) 450−600 nm (690-nm pump) and (B) 620−720 nm (560-nm pump).

Sunnyvale, CA), where it was synchronized with an optical chopper (75160, Newport, Irvine, CA). The chopped frequency was 160 Hz. The polarization of the pump was set at 54.7° with respect to the polarization of the probe to eliminate the molecular reorientation effect. The time resolution of this apparatus was estimated to be about 150 fs through the cross-correlation between the pump and probe pulses in buffer solution.

To record the transient spectrum at a certain delay time, we used a charge-coupled device (CCD; Spec-10:400B, Princeton Instruments, Acton, MA) as the detector, which was coupled to an axial output port of a monochromator (SP2358, Princeton Instruments, Acton, MA). The spectral window was about 300 nm, with a 300 groove/mm grating. A shutter was used to sequentially block and unblock the pump beam for an equal amount of time (around 0.5 s). During the open and closed cycles, the spectra of the probe (continuum) were collected and averaged, and the net spectrum with the pump on was divided by the net spectrum with the pump blocked. The logarithm of the ratio yielded the absolute magnitude of the pump-induced transient absorption difference spectrum.

Ensemble Static Absorption and Fluorescence Measurements. Static absorption spectra were recorded on a commercial

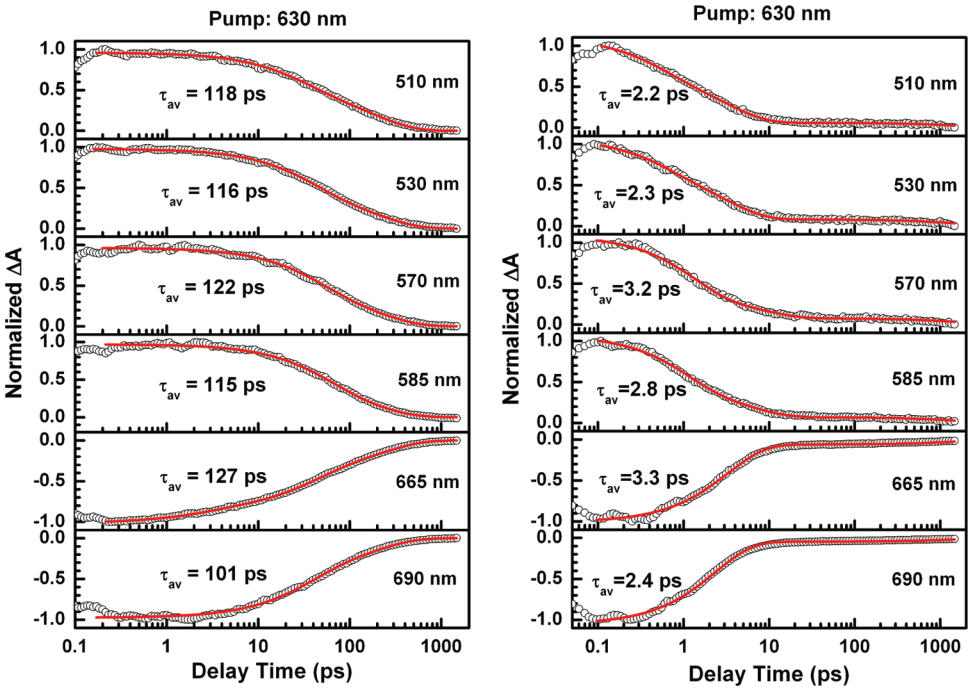


Figure 3. Magic-angle femtosecond pump−probe transients of Atto655 in 1.00 M guanosine (left panel) and in 50.0 mM tryptophan (right panel) in 1 × TE buffer. Pump: 630 nm.

UV−vis spectrometer (U3100, Hitachi, Tokyo, Japan, or Cary 50, Varian, Forest Hill, Victoria, Australia). Static fluorescence spectra were recorded on a LS-55 luminescence spectrometer (Perkin-Elmer, Waltham, MA). To record the weak fluorescence, a CCD detector (Spec10:400B, Princeton Instruments, Acton, MA) and laser excitation were employed.

In all ultrafast measurements, the concentration of Atto655 was adjusted to have an optical density of about 0.1 at its maximum-absorption position in a 1-mm-path-length sample cell. To keep the Atto655 solution fresh, a homemade magnetic stirring bar was placed inside the sample cell and rotated by an external magnet motor. The static absorption spectra of the sample were measured before and after each ultrafast kinetic measurement to check the sample's quality and stability. The data were disregarded when the sample degradation was greater than 5%.

RESULTS

Ensemble Static Absorption and Fluorescence Spectra of Atto655. The evidence for the formation of ground-state complexes between Atto655 and the quencher, guanosine or tryptophan, can be revealed through their ensemble static absorption and fluorescence spectra.[2,28,33,34] Figure 1 displays the ensemble static absorption and fluorescence spectra of Atto655 with and without 1.00 M guanosine and with 50.0 mM tryptophan in aqueous solution. Clearly, the absorption and fluorescence emission maxima of Atto655 in 1.00 M guanosine and 50.0 mM tryptophan are both red-shifted, indicating the formation of ground-state complexes between Atto655 and each of the quenchers, guanosine and tryptophan.

Transient Absorption Spectra of Atto655. To understand the photophysical properties of Atto655 in the presence of guanosine or tryptophan, we recorded their transient absorption spectra. Figure 2 shows the transient absorption spectra at certain delay times after Atto655 had been excited into its first excited electronic state with and without 1.00 M guanosine in aqueous solution. The same experiments were carried out for Atto655 with 50.0 mM tryptophan, and the results are also included in Figure 2. Figure 2A displays the transient absorption spectra in the range of 450−620 nm, and Figure 2B displays the transient absorption spectra in the range of 620−720 nm. We also scanned from 850 to 1150 nm but did not detect any absorptive species in that range. Because the molecular structure of Atto655 is classified and has not been released by the manufacturer, we cannot make a definite assignment of the spectra with respect to the structure. However, because the character of the transient absorption spectra shown in Figure 2A is very similar to that of the first excited electronic state absorption spectra of rhodamine dyes[43] and the transient absorption spectra shown in Figure 2B are identical to the ensemble static absorption spectra shown in Figure 1, we assigned the absorption spectra in the range of 450−620 nm to the transition of the first excited electronic state ($S_1 \rightarrow S_n$) of Atto655 and the absorption spectra in the range of 620−720 nm to the transition of the ground electronic state ($S_0 \rightarrow S_1$) of Atto655. Within the scanning range of our equipment, we did not find a spectrum that could be assigned to the absorption from the charge-separated state.

Kinetic Trace of Atto665 Transient Absorption. To understand the PET process between Atto655 and the quencher, guanosine or tryptophan, we measured the kinetic traces of the Atto655 transient in 1.00 M guanosine or 50.0 mM tryptophan in aqueous solution at different probe and pump wavelengths, as shown in Figures 3 and 4. From these two figures, we determined, first, that the ground-state recovery and excited-state decay times of Atto655 in tryptophan are much shorter than those in guanosine; second, that the ground-state recovery time of Atto655 in guanosine or tryptophan is longer than the

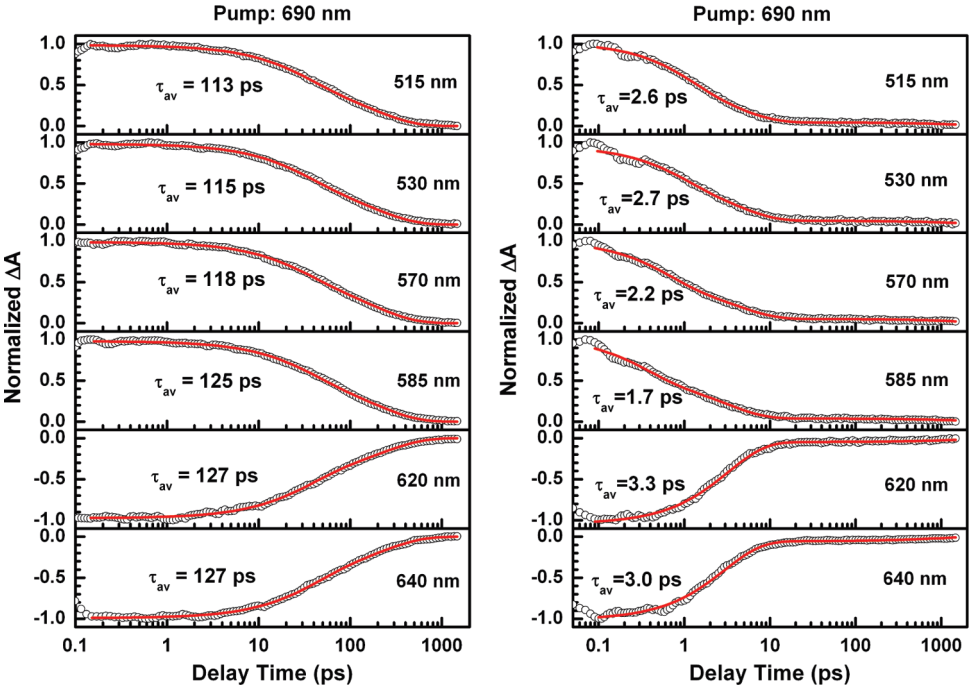


Figure 4. Magic-angle femtosecond pump−probe transients of Atto655 in 1.00 M guanosine (left panel) and in 50.0 mM tryptophan (right panel) in 1 × TE buffer. Pump: 690 nm.

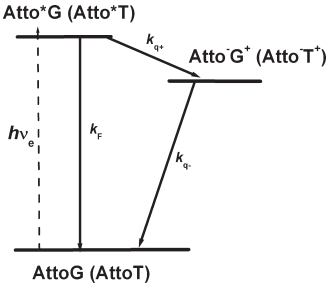


Figure 5. Dynamic model for radiative and nonradiative (PET) pathways of excited Atto655.

respective excited-state decay time; and third, that the ground-state recovery and excited-state decay times did not change with the pump wavelength.

DISCUSSION

Excited-State Decay and Ground-State Recovery of Atto655. In this work, we measured the ultrafast fluorescence quenching kinetics of Atto655 with guanosine or tryptophan in aqueous solution by monitoring their excited-state transition ($S_1 \rightarrow S_n$) and their ground-state transition ($S_0 \rightarrow S_1$). These two measurements provide different information. For the PET scheme displayed in Figure 5, it is easy to derive that monitoring of the $S_1 \rightarrow S_n$ transition provides the excited-state depopulation

$$S_1(t) = S_{1,0} \exp[-(k_F + k_{q+})t] \quad (1)$$

where $S_{1,0}$ is the initial population in the excited state immediately after the pump pulse arrives, k_F is the fluorescence rate without the quencher, and k_{q+} is the charge separation rate. In contrast, monitoring of the $S_0 \rightarrow S_1$ transition provides the ground-state repopulation

$$S_0(t) - S_{0,0} = -\frac{(k_F - k_{q-})S_{1,0}}{(k_F + k_{q+} - k_{q-})} \exp[-(k_F + k_{q+})t] - \frac{k_{q+}S_{1,0}}{(k_F + k_{q+} - k_{q-})} \exp(-k_{q-}t) \quad (2)$$

where $S_{0,0}$ is the initial population in the ground state before the pump pulse arrives and k_{q-} is the charge recombination rate.

Because of the difficulty of preparing a sample of Atto655 completely quenched by guanosine or tryptophan and the solvent solvation effect on the kinetics, we added two terms to eqs 1 and 2 to fit the data of the excited-state decay and ground-state recovery kinetics

$$S_1(t) = a_0 \exp(-t/\tau_0) + a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) \quad (3)$$

$$S_0(t) - S_{0,0} = -a_0 \exp(-t/\tau_0) - a_1 \exp(-t/\tau_1) - a_2\left[\exp(-t/\tau_2) + \frac{\tau_3(\tau_0 - \tau_2)}{\tau_2(\tau_3 - \tau_0)} \exp(-t/\tau_3)\right] \quad (4)$$

where τ_0 is the fluorescence lifetime of Atto655 without the quencher, τ_1 is the solvent solvation time, τ_2 is the fluorescence lifetime of Atto655 with the quencher, τ_3 is the lifetime of the charge-separated state in the collision complex between Atto655 and the quencher, and a_0−a_2 are the amplitudes of the corresponding components. Figure 6 shows a typical global fit (with χ^2 ranging from 1.0 to 1.1) with eqs 3 and 4 for Atto655 in 1.00 M guanosine and 50 mM tryptophan, respectively, and the fitting parameters are summarized in Table 1. With the parameters listed in Table 1, we derived the following values: $k_{q+} = (8.1 \pm 0.6) \times 10^9\ s^{-1}$, $k_{q-} = (7.7 \pm 0.9) \times 10^{10}\ s^{-1}$, and $k_F = (5.6 \pm 0.1) \times 10^8\ s^{-1}$ for Atto655 in 1.00 M guanosine and

dx.doi.org/10.1021/jp200876d |J. Phys. Chem. B 2011, 115, 5001–5007

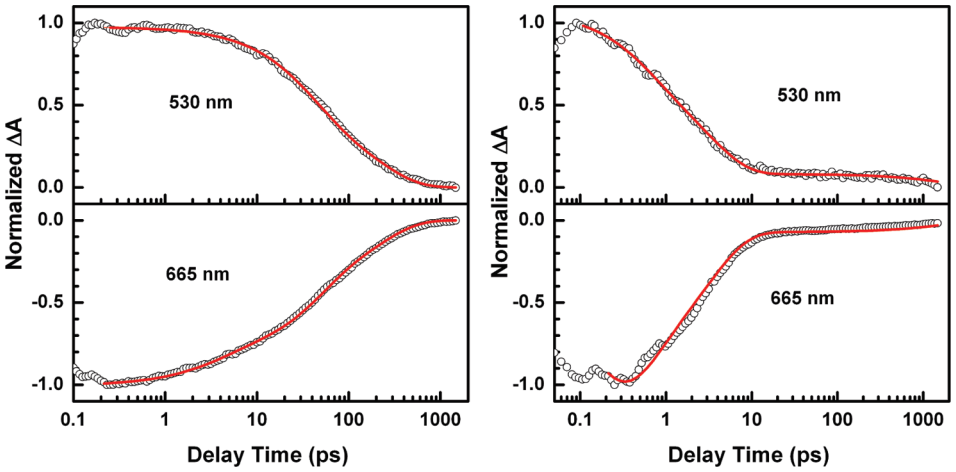


Figure 6. Global fits for the magic-angle femtosecond pump−probe transients of Atto655 in 1.00 M guanosine (left panel) and in 50.0 mM tryptophan (right panel) in 1 × TE buffer. Pump: 630 nm.

Table 1. Excited-State Decay and Ground-State Recovery Parameters for Atto655 in 1.00 M Guanosine (G) and in 50.0 mM Tryptophan (T) in Aqueous Solution

	probe	a_0	τ_0 (ps)	a_1	τ_1[a] (ps)	a_2	τ_2[b] (ps)	τ_3 (ps)
Atto655 + 1.00 M G	530 nm					1.00	116	
	665 nm			0.18	3.7	0.82	116	13
Atto655 + 50.0 mM T	530 nm	0.07	1800			0.93	2.3	
	665 nm	0.05	1800			0.95	2.3	0.2

[a] Fixed to match the reported lifetime for free Atto655 in the literature.[28,34] [b] Because of the nonexponential decay of the excited-state population, τ_2 is an average time.

$k_{q+} = (4.0 \pm 1.0) \times 10^{11}\ s^{-1}$, $k_{q-} = (5.0 \pm 2.0) \times 10^{12}\ s^{-1}$, and $k_F = (5.6 \pm 0.1) \times 10^{8}\ s^{-1}$ for Atto655 in 50.0 mM tryptophan.

In the literature, it has been suggested[44] that, when fluorescence quenching occurs in a collision complex, the relevant states might have different geometries such that the electron-transfer rate could exhibit strong heterogeneity. In our case, the kinetic traces illustrated in Figures 3 and 4 did not show a noticeable wavelength dependence, indicating that the heterogeneity in the electron transfer between Atto655 and the two quenchers is not significant. Therefore, we described our system with single charge separation and recombination rates.

In addition, according to the PET scheme shown in Figure 5, the relative brightness of the collision complex of Atto655 and the quencher compared to that of Atto655 alone is

$$Q = \frac{k_F}{k_F + k_{q+}} = \frac{\tau_2}{\tau_0} \tag{5}$$

With the fitting parameters shown in Table 1, we derived the values $Q = 0.06 \pm 0.01$ for the collision complex of Atto655 and guanosine and $Q = 0.0014 \pm 0.0009$ for the collision complex of Atto655 and tryptophan.

Theoretical Estimation of PET Rate. We measured the PET rates of Atto655 with guanosine or tryptophan and found that the charge recombination rate is higher than the charge separation rate for both systems and that the charge separation and recombination rates between Atto655 and guanosine are much lower than those between Atto655 and tryptophan.

The driving force for charge-transfer processes is known to be determined by the difference in the reversible redox potentials of the donor and acceptor. The energetics of photoinduced charge separation and recombination can be estimated using Weller's equations[8,13,45]

$$\Delta G_{CS} = E_{ox} - E_{red} - E_S + C \tag{6}$$

$$\Delta G_{CR} = E_{red} - E_{ox} \tag{7}$$

where E_{ox} is the first one-electron oxidation potential of the donor; E_{red} is the first one-electron reduction potential of the acceptor; E_S is the energy of the zero−zero transition to the lowest excited singlet state; and C is the solvent-dependent Coulombic interaction energy, which can be neglected in moderately polar environments. Although the reversible potential of Atto655 is not available in the literature, Sauer et al. found that Atto655 exhibited very similar spectroscopic characteristics to the oxazine fluorophore MR121.[8,33] The peak potential for one-electron reduction of MR121 has been determined to be −0.42 V (vs SCE), and the one-electron oxidation potentials of guanosine and tryprophan are ~1.24 V (vs SCE) and ~0.81 V (vs SCE), respectively.[8] From the ensemble static absorption and fluorescence spectra shown in Figure 1, we calculated the zero−zero transition energy of Atto665 in aqueous solution (i.e., E_S) to be 1.85 eV (670 nm). Substituting these values into eqs 6 and 7, we derived values of $\Delta G_{CS} = -0.2$ eV and $\Delta G_{CR} = -1.7$ eV for charge transfer between Atto655 and guanosine and $\Delta G_{CS} = -0.6$ eV and $\Delta G_{CR} = -1.2$ eV for charge transfer beween Atto655 and tryptophan.

The charge-transfer rate can be calculated using the Marcus-type equation[46]

$$k_{et} = \frac{2\pi |J|^2}{\hbar\sqrt{4\pi\lambda kT}} \exp\left[\frac{-(\Delta G + \lambda)^2}{4\lambda kT}\right] \tag{8}$$

where J is the electronic coupling matrix element, λ is the reorganization energy, $\hbar$ is Planck's constant, k is the Boltzmann constant, and T is the temperature. Assuming a typical reorganization energy for aqueous solution of $\lambda = 1.2$ eV[18,24] and an electronic coupling matrix element of $J = 200\ cm^{-1}$ for both the

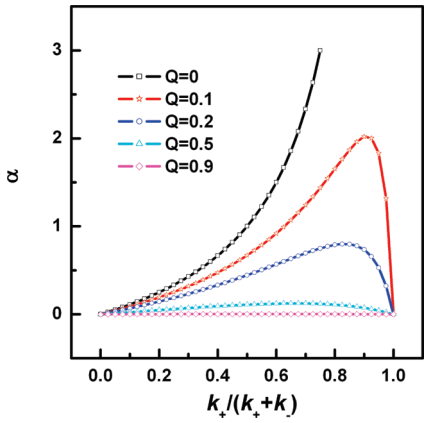


Figure 7. Plot of α against $k_+/(k_+ + k_-)$ for a few chosen Q values. Notice that at the limit of $k_+/(k_+ + k_-) = 1$ all curves of nonzero brightness approach $\alpha = 0$, except for the $Q = 0$ curve, which is divergent.

reduction and oxidation processes, we derived charge separation and recombination rates (k_{CS} and k_{CR}) of 3×10^9 and 1×10^{12} s^{-1}, respectively, for the collision complex between Atto655 and guanosine and 5×10^{11} and 9×10^{12} s^{-1}, respectively, for the collision complex between Atto655 and tryptophan. These estimated values are quite consistent with our femtosecond transient absorption measurements, in which $k_{q+} = (8.1 \pm 0.6) \times 10^9$ s^{-1} and $k_{q-} = (7.7 \pm 0.9) \times 10^{10}$ s^{-1} for Atto655 and guanosine and $k_{q+} = (4.0 \pm 1.0) \times 10^{11}$ s^{-1} and $k_{q-} = (5.0 \pm 2.0) \times 10^{12}$ s^{-1} for Atto655 and tryptophan.

Implications for PET-FCS Data Analysis. Consider a chemical relaxation

$$A \underset{k_-}{\overset{k_+}{\rightleftarrows}} B \tag{9}$$

where A is a fluorescent bright species; B is a dark species; and k_+ and k_- are the forward and reverse relaxation rate constants, respectively. The equilibrium constant is

$$K = \frac{k_+}{k_-} = \frac{C_B}{C_A} \tag{10}$$

where C_A and C_B are the concentrations of species A and B, respectively. In a PET-FCS experiment, the autocorrelation function can be fitted by[31]

$$G(t) = G_D(t)\left[1 + \alpha \exp\left(-\frac{t}{\tau}\right)\right] \tag{11}$$

where $G_D(t)$ is the term that describes the molecular diffusion, with

$$\tau = \frac{1}{k_+ + k_-} \tag{12}$$

$$\alpha = \frac{(1 - Q)^2 K}{(1 + KQ)^2} \tag{13}$$

where Q is the relative brightness of B with respect to A. It is common practice in PET-FCS data analysis to assume $Q = 0$. In Figure 7, plots of α against $k_+/(k_+ + k_-) = K/(1 + K)$ for a few chosen Q values are presented. It is immediately seen that the $Q = 0$ assumption introduces serious error into the relation between α and K when species B has a finite brightness. In particular, when $k_+/(k_+ + k_-) \rightarrow 1$, α goes to the wrong limit of infinity instead of the correct value of zero. Also, as can be seen from Figure 7, with a finite Q value, α will generally correspond to two equilibrium constants, as it should, but the $Q = 0$ curve would predict a one-to-one correspondence between α and K, so that it could provide a completely opposite result regarding the relation between k_+ and k_-. Therefore, even though Q could be tiny, as was seen in this work for the quenching of Atto655 by tryptophan, one still ought to be cautious about whether the finite brightness of the dark species can safely be neglected. Previously, researchers assumed $Q = 0$ because it allows for the derivation of both k_+ and k_- from a single PET-FCS curve, whereas when $Q \neq 0$, independent data for Q and even for K have to be obtained. In ref 31, this issue is discussed thoroughly, and methods to obtain these parameters are proposed. Because of the fatal hidden trouble in the $Q = 0$ assumption, we recommend that Q be determined and used in future PET-FCS studies.

CONCLUSIONS

In summary, we recorded the transient absorption spectra of Atto655 in 1.00 M guanosine and 50.0 mM tryptophan in aqueous solution and investigated the corresponding PET processes using femtosecond transient absorption spectroscopy. We measured the decay rates of the first excited electronic state and the recovery rates of the ground electronic state of Atto655 with the quenchers. In the collision complex of Atto655 and guanosine, the charge separation rate is $k_{q+} = (8.1 \pm 0.6) \times 10^9$ s^{-1}, and the charge recombination rate is $k_{q-} = (7.7 \pm 0.9) \times 10^{10}$ s^{-1}. In the collision complex between Atto655 and tryprophan, the charge separation rate is $k_{q+} = (4.0 \pm 1.0) \times 10^{11}$ s^{-1}, and the charge recombination rate is $k_{q-} = (5.0 \pm 2.0) \times 10^{12}$ s^{-1}. Those values are quite consistent with the prediction of Marcus-type electron-transfer theory. In addition, with these ultrafast measurements, we derived the relative brightness of Atto655 in its complex with the respective quenchers as $Q = 0.06 \pm 0.01$ with guanosine and $Q = 0.0014 \pm 0.0009$ with tryptophan. We argue that neglect of the finite brightness of the dark state in PET-FCS data analysis could induce unacceptable errors or even more serious problems.

AUTHOR INFORMATION

Corresponding Author

*E-mail: a.yu@chem.ruc.edu.cn (A.Y.), zhaoxs@pku.edu.cn (X.S.Z.). Phone: +86-10-6251-4601 (A.Y.), +86-10-6275-1727 (X.S.Z.). Fax: +86-10-6251-6444 (A.Y.), +86-10-6275-1708 (X.S.Z.).

ACKNOWLEDGMENT

This work was supported by the National Natural Science Foundation of China (20603047, 20733001, 20973015), by the 973 Projects (2006CB910300, 2010CB912302), and by the Fundamental Research Funds for the Central Universities and the Research Funds of Renmin University of China (10XNJ008).

REFERENCES

(1) Neuweiler, H.; Sauer, M. *Curr. Pharm. Biotechnol.* **2004**, *5*, 285–298.

(2) Heinlein, T.; Knemeyer, J. P.; Piestert, O.; Sauer, M. *J. Phys. Chem. B* **2003**, *107*, 7957–7964.

(3) Nazarenko, I.; Pires, R.; Lowe, B.; Obaidy, M.; Rashtchian, A. *Nucleic Acids Res.* **2002**, *30*, 2089–2095.

(4) Neuweiler, H.; Schulz, A.; Bohmer, M.; Enderlein, J.; Sauer, M. *J. Am. Chem. Soc.* **2003**, *125*, 5324–5330.
(5) Rachofsky, E. L.; Seibert, E.; Stivers, J. T.; Osman, R.; Ross, J. B. A. *Biochemistry* **2001**, *40*, 957–967.
(6) Rachofsky, E. L.; Osman, R.; Ross, J. B. A. *Biochemistry* **2001**, *40*, 946–956.
(7) Edman, L.; Mets, U.; Rigler, R. *Proc. Natl. Acad. Sci. U.S.A.* **1996**, *93*, 6710–6715.
(8) Doose, S.; Neuweiler, H.; Sauer, M. *ChemPhysChem* **2009**, *10*, 1389–1398.
(9) Kelley, S. O.; Barton, J. K. *Science* **1999**, *283*, 375–381.
(10) Kim, J.; Doose, S.; Neuweiler, H.; Sauer, M. *Nucleic Acids Res.* **2006**, *34*, 2516–2527.
(11) Knemeyer, J. P.; Marme, N.; Sauer, M. *Anal. Chem.* **2000**, *72*, 3717–3724.
(12) Kurata, S.; Kanagawa, T.; Yamada, K.; Torimura, M.; Yokomaku, T.; Kamagata, Y.; Kurane, R. *Nucleic Acids Res.* **2001**, *29*, e34.
(13) Lewis, F. D.; Letsinger, R. L.; Wasielewski, M. R. *Acc. Chem. Res.* **2001**, *34*, 159–170.
(14) Lewis, F. D.; Wu, T. F.; Zhang, Y. F.; Letsinger, R. L.; Greenfield, S. R.; Wasielewski, M. R. *Science* **1997**, *277*, 673–676.
(15) Wang, W. J.; Chen, C. L.; Qian, M. X.; Zhao, X. S. *Sens. Actuators B: Chem.* **2008**, *129*, 211–217.
(16) Qu, P.; Yang, X. X.; Li, X.; Zhou, X. X.; Zhao, X. S. *J. Phys. Chem. B* **2010**, *114*, 8235–8243.
(17) Jean, J. M.; Hall, K. B. *Proc. Natl. Acad. Sci. U.S.A.* **2001**, *98*, 37–41.
(18) Fiebig, T.; Wan, C. Z.; Zewail, A. H. *ChemPhysChem* **2002**, *3*, 781–788.
(19) Wan, C. Z.; Fiebig, T.; Kelley, S. O.; Treadway, C. R.; Barton, J. K.; Zewail, A. H. *Proc. Natl. Acad. Sci. U.S.A.* **1999**, *96*, 6014–6019.
(20) Huber, R.; Fiebig, T.; Wagenknecht, H. A. *Chem. Commun.* **2003**, 1878–1879.
(21) Manoharan, M.; Tivel, K. L.; Zhao, M.; Nafisi, K.; Netzel, T. L. *J. Phys. Chem.* **1995**, *99*, 17461–17472.
(22) Marquez, C.; Pischel, U.; Nau, W. M. *Org. Lett.* **2003**, *5*, 3911–3914.
(23) Wanninger-Weiss, C.; Valis, L.; Wagenknecht, H. A. *Bioorg. Med. Chem.* **2008**, *16*, 100–106.
(24) Seidel, C. A. M.; Schulz, A.; Sauer, M. H. M. *J. Phys. Chem.* **1996**, *100*, 5541–5553.
(25) Eggeling, C.; Fries, J. R.; Brand, L.; Gunther, R.; Seidel, C. A. M. *Proc. Natl. Acad. Sci. U.S.A.* **1998**, *95*, 1556–1561.
(26) Piestert, O.; Barsch, H.; Buschmann, V.; Heinlein, T.; Knemeyer, J. P.; Weston, K. D.; Sauer, M. *Nano Lett.* **2003**, *3*, 979–982.
(27) Neuweiler, H.; Schulz, A.; Vaiana, A. C.; Smith, J. C.; Kaul, S.; Wolfrum, J.; Sauer, M. *Angew. Chem., Int. Ed.* **2002**, *41*, 4769–4773.
(28) Buschmann, V.; Weston, K. D.; Sauer, M. *Bioconjugate Chem.* **2003**, *14*, 195–204.
(29) Nazarenko, I.; Lowe, B.; Darfler, M.; Ikonomi, P.; Schuster, D.; Rashtchian, A. *Nucleic Acids Res.* **2002**, *30*, e37.
(30) McEwen, D. P.; Gee, K. R.; Kang, H. C.; Neubig, R. R. *Anal. Biochem.* **2001**, *291*, 109–117.
(31) Li, X.; Zhu, R.; Yu, A.; Zhao, X. S. *J. Phys. Chem. B*, in press. DOI: http://dx.doi.org/10.1021/jp200455b.
(32) Vogelsang, J.; Cordes, T.; Forthmann, C.; Steinhauer, C.; Tinnefeld, P. *Proc. Natl. Acad. Sci. U.S.A.* **2009**, *106*, 8107–8112.
(33) Doose, S.; Neuweiler, H.; Sauer, M. *ChemPhysChem* **2005**, *6*, 2277–2285.
(34) Marme, N.; Knemeyer, J. P.; Sauer, M.; Wolfrum, J. *Bioconjugate Chem.* **2003**, *14*, 1133–1139.
(35) Vogelsang, J.; Cordes, T.; Tinnefeld, P. *Photochem. Photobiol. Sci.* **2009**, *8*, 486–496.
(36) Miller, A. E.; Fischer, A. J.; Laurence, T.; Hollars, C. W.; Saykally, R. J.; Lagarias, J. C.; Huser, T. *Proc. Natl. Acad. Sci. U.S.A.* **2006**, *103*, 11136–11141.
(37) Sykora, J.; Kaiser, K.; Gregor, I.; Bonigk, W.; Schmalzing, G.; Enderlein, J. *Anal. Chem.* **2007**, *79*, 4040–4049.
(38) Rosenkranz, T.; Katranidis, A.; Atta, D.; Gregor, I.; Enderlein, J.; Grzelakowski, M.; Rigler, P.; Meier, W.; Fitter, J. *ChemBioChem* **2009**, *10*, 702–709.
(39) Dertinger, T.; Pacheco, V.; von der Hocht, I.; Hartmann, R.; Gregor, I.; Enderlein, J. *ChemPhysChem* **2007**, *8*, 433–443.
(40) van de Linde, S.; Kasper, R.; Heilemann, M.; Sauer, M. *Appl. Phys. B: Lasers Opt.* **2008**, *93*, 725–731.
(41) Eggeling, C.; Widengren, J.; Brand, L.; Schaffer, J.; Felekyan, S.; Seidel, C. A. M. *J. Phys. Chem. A* **2006**, *110*, 2979–2995.
(42) *ATTO-TEC Fluorescent Labels and Dyes Catalogue 2009/2010*; ATTO-TEC GmbH: Siegen, Germany, 2009.
(43) Beaumont, P. C.; Johnson, D. G.; Parsons, P. J. *J. Photochem. Photobiol. A: Chem* **1997**, *107*, 175–183.
(44) Vaiana, A. C.; Neuweiler, H.; Schulz, A.; Wolfrum, J.; Sauer, M.; Smith, J. C. *J. Am. Chem. Soc.* **2003**, *125*, 14564–14572.
(45) Weller, A. *Z. Phys. Chem.* **1982**, *133*, 93–98.
(46) Marcus, R. A.; Sutin, N. *Biochim. Biophys. Acta* **1985**, *811*, 265–322.