Hypericin, Hypocrellin, and Model Compounds: Primary Photoprocesses of Light-Induced Antiviral Agents

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The excited-state photophysics of the light-induced antiviral agents hypericin and hypocrellin are compared with those of the hexa- and tetramethoxy analogues of hypericin. The results are consistent with the interpretation of the primary photoprocess in hypericin and hypocrellin as that of excited-state intramolecular proton or atom transfer.

Introduction

Hypericin and hypocrellin are naturally occurring pigments[1,2] that are remarkable because of their light-induced antiviral activity[3] against enveloped lentiviruses such as the human immunodeficiency virus.[4,5] Although the requirement of light for this activity in hypericin and hypocrellin is absolute, these two molecules possess very different modes of reactivity.[6] Consequently, we have undertaken the task[6−15] of unraveling the excited-state primary photophysical processes of hypericin and hypocrellin.

It has been our thesis from the very first that a significant nonradiative process in hypericin and its analogues is intramolecular proton (or atom) transfer.[7−9] The argument for such a process is the following. The hypericin analogue lacking labile protons, mesonaphthobianthrone (Figure 1g), is significantly fluorescent and has optical spectra that resemble those of hypericin only when its carbonyl groups are protonated. *In hypericin, the fluorescent state grows in on a time scale of several picoseconds, as measured by the rise time of stimulated emission.* Therefore, the combined observations of the requirement of protonated carbonyls for strong hypericin-like fluorescence and the rise time of fluorescence in hypericin were taken as evidence for intramolecular excited-state proton transfer in hypericin. The possible role of labile protons in the light-induced antiviral activity of hypericin and its analogues is discussed in detail elsewhere.[6,10,11,13] In this article, we shall frequently refer to the process in question as a proton transfer, but it should be kept in mind that not enough data are available to determine whether a proton or a hydrogen atom is being transferred.

Two potential arguments against intramolecular excited-state proton transfer in hypericin are the lack of an isotope effect for the process in question[8,9] and the observation of near mirror-image symmetry between its absorption and emission spectra (Figure 2a). The first of these points is discussed in detail in the companion article. As for the mirror-image symmetry, this is typically taken as a signature of negligible structural changes between the absorbing and the emitting species. Intramolecular excited-state proton transfer usually generates a broad, shifted, and structureless emission spectrum. A classic example of such behavior is given by 3-hydroxyflavone.[16]

The issue of the shape of the spectra may be addressed in several ways. It is possible that the structural changes induced by proton transfer do not significantly affect the electronic structure of the tautomeric species (parts a and b of Figure 1) in such a way as to destroy the mirror-image symmetry. High-level quantum chemical calculations will have much to offer in understanding this problem. It is also possible, as we have argued elsewhere,[8,9,15] that the ground state of hypericin is already partially tautomerized and that this ground-state heterogeneity yields the observed mirror-image symmetry between absorption and emission spectra. Another strategy is to study the excited-state photophysics of synthetic analogues that are unable to execute excited-state proton or atom transfer. We have already begun such an examination by comparing the photophysics of hypericin with that of its hexamethoxy analogue (Figure 1e).[15] Here, we pursue this line of investigation more thoroughly by examining the tetramethoxy analogue of hypericin (Figure 1f), which can be considered a methoxy hybrid of hypericin and hypocrellin.

Experimental Section

A. Synthesis of the Tetramethoxy Analogue.[28] *Di(3,5-dimethoxy-2-methoxycarbonylphenyl)acetylene.* The compound was obtained as a white solid from the palladium-catalyzed reaction of methyl 2-ethynyl-3,5-dimethoxybenzoate and methyl 2-(trifluoromethanesulfonyloxy)-3,5-dimethoxybenzoate[17] (mp 149 °C). ^{1}H NMR ($CDCl_3$): δ 3.82 (s, 12H), 3.92 (s, 6H), 6.48 (d, 2H, J = 0.9 Hz), 6.62 (d, 2H, J = 0.9 Hz).

9,10-Bis[(2′-methoxycarbonyl-3′,5′-dimethoxy)phenyl]phenanthrene. To a 2 dram vial equipped with a stir bar is added Pd$(OAc)_2$ (0.125 mmol), NaOAc (0.50 mmol), LiCl (0.25 mmol), 2-iodobiphenyl (0.25 mmol), di(3,5-dimethoxy-2-methoxycarbonylphenyl)acetylene (0.275 mmol), and 5 mL of DMF. The vial is then flushed with N_2, capped with a screw cap containing a Teflon liner, and placed in an oil bath at 100 °C for 48 h. The vial is then removed from the oil bath, diluted with Et_2O and EtOAc, washed with saturated NH_4Cl and water, dried over $MgSO_4$, and concentrated. This residue was then purified by column chromatography using 4:1 hexanes/EtOAc (49% yield, mp 210−212 °C (toluene)). ^{1}H NMR ($CDCl_3$): δ 3.18 (s, 6H), 3.63 (s, 6H), 3.83 (s, 6H), 6.39 (d, 2H, J = 3.0 Hz), 6.81 (d, 2H, J = 3.0 Hz), 7.50 (dt, 2H, J = 7.8, 0.9 Hz), 7.62 (m, 4H), 8.70 (d, 2H, J = 7.8 Hz). ^{13}C NMR ($CDCl_3$): δ 51.5, 55.6, 55.9, 98.5, 106.9, 117.2, 122.3, 126.3, 126.5, 127.8, 129.8, 130.5, 134.8, 141.0, 157.7, 161.5, 167.9. IR ($CDCl_3$): 3010, 1717, 1103, 1047 cm^{-1}. HRMS for $C_{34}H_{30}O_8$: calcd 566.1941; found 566.1952.

2,4,2′,4′-Tetramethoxynaphthodianthrone ("Tetramethoxyhypericin", Figure 1f). This compound was obtained as an orange-brown solid in 91% yield by treatment of 9,10-bis(2′-

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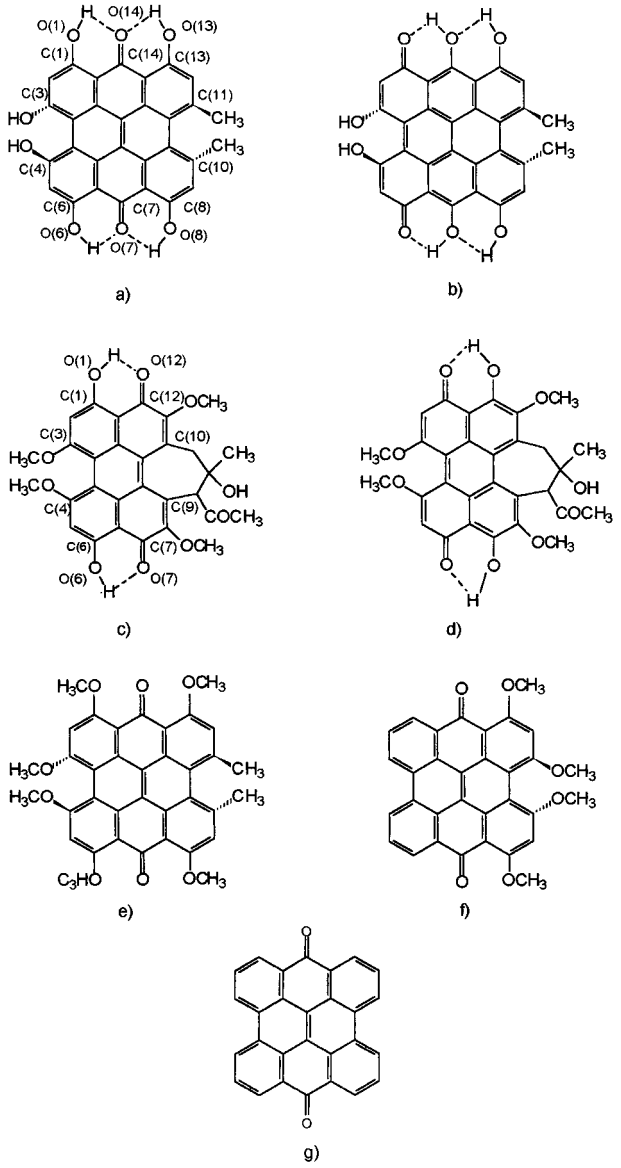

Figure 1. Two-dimensional structures of (a) "normal" form of hypericin, (b) hypericin bitautomer, (c) hypocrellin, (d) hypocrellin tautomer, (e) hexamethoxyhypericin, (f) tetramethoxyhypericin, (g) mesonaphthobianthrone. We have suggested, and our results are consistent with the idea, that hypericin and hypocrellin exist in more than one tautomeric form in the ground state. We have proposed, however, that the hypericin bitautomer (b) is thermodynamically unstable in the ground state. Thus, any excited-state hypericin bitautomer that returns to the ground state will not accumulate, but will rapidly form a lower energy tautomeric form.

methoxycarbonyl-3′,5′-dimethoxyphenyl)phenanthrene with PPA[18] and then concentrated H_2SO_4 and sunlight.[18] ^{1}H NMR ($HCCl_3$): δ 4.18 (s, 6H), 4.26 (s, 6H), 6.94 (s, 2H), 7.90 (t, 2H, $J = 7.8$ Hz), 8.77 (d, 2H, $J = 7.5$ Hz), 8.93 (d, 2H, $J = 8.1$ Hz). The HRMS for $C_{32}H_{20}O_6$ was not obtained because of limitations in the HRMS ionization techniques. Electrospray low-resolution mass spectroscopy found 500.8 ($m + 1$). UV−vis absorption λ_{max} (DMSO): 398, 419, 475, 503 nm. UV−vis fluorescence excitation λ_{max} (DMSO): 396, 418, 474, 502 nm. UV−vis emission λ_{max} (DMSO): 531, 559 nm. The fluorescence excitation spectrum is superimposable on the absorption spectrum. Further characterization was not possible owing to the low solubility of this product.

B. Optical Measurements. Hypericin was used as received from Carl Roth GmbH (distributed by Atomergic Chemetals Corp.). Hypocrellin A (Molecular Probes) was used as received at >98% purity as determined from the supplied TLC and NMR measurements. The hexamethoxy analogue was prepared as described elsewhere.[15] DMSO was dried over 4 Å molecular sieves. Care was taken to prevent sieve dust contamination. All experiments were performed at ambient temperature (22 °C). The time-correlated single-photon counting experiments were performed with an apparatus described elsewhere.[20] The instrument response function is typically about 70 ps. Samples were prepared for pump−probe experiments with an optical density of 0.4−0.7 at the pump wavelength; for time-correlated single-photon counting, samples were diluted by 10-fold or more. The time-resolved fluorescence data provide a powerful assay of the purity of the tetramethoxy analogue. In addition to a 820 ps decay component, there is a small amount (~5%) of a ~2 ns component.

Steady-state fluorescent measurements were made using a Spex Fluoromax with a 4 nm band-pass, and corrections were made for detector response and excitation lamp spectrum. Steady-state absorbance measurements were made using a Perkin-Elmer Lambda 18 double beam UV−vis spectrophotometer with 1 nm band-pass. Fluorescence quantum yield measurements were made relative to hypericin by exciting solutions of each compound at several wavelengths where they had equal optical densities and integrating the emission spectrum over the entire band on a wavenumber scale.

Pump−probe transient absorption measurements were performed with laser systems producing pulses of 150−200 fs

TABLE 1: Fluorescence Parameters in DMSO

compound	Φ_f	τ_f (ps)
hypericin	0.35 ± 0.02	5500 ± 500
hypocrellin	0.014 ± 0.008[a]	1300
hexamethoxyhypericin	0.07 ± 0.01[c]	490[b]
tetramethoxyhypericin	0.11 ± 0.01[c]	820[b]

[a] In all other solvents investigated, the quantum yield was 0.07 ± 0.02. [b] This is the major component of the fluorescence decay and comprised at least 95% of the decay intensity. See Figure 7. [c]) The steady-state fluorescence quantum yields provide upper limits on the true value because they are contaminated by the few percent of long-lived fluorescent impurity in our samples. A more accurate comparison of integrated fluorescence intensity could in principle be given by the ratio of the fluorescence lifetimes in the table, assuming the radiative rate is the same for all the compounds being compared. Such an assumption is probably not warranted given the anomalously low value for the quantum yield of hypocrellin in DMSO as opposed to other solvents.

TABLE 2: Global Fitting Parameters for Tetramethoxyhypericin

probe wavelength[a] (nm)	a_1	τ_1 (ps)	a_2	τ_2 (ps)	a_3	τ_3 (ps)
500[c]	0.23	9.9	0	820	0.77	∞
520[d]	0	9.9	0.35	820	0.65	∞
530[d]	0	9.9	0.31	820	0.69	∞
540[b]	0	9.9	0	820	1.0	∞
550[b]	0	9.9	0	820	1.0	∞
560[b]	0.10	9.9	0	820	0.90	∞
570[b]	0.25	9.9	0	820	0.75	∞
620[b]	0.25	9.9	0	820	0.75	∞
630[b]	0.16	9.9	0	820	0.84	∞

[a] The functional form used to fit the data is indicated by a superscript in the leftmost column, which gives the probe wavelength. The preexponential factors are all normalized and should not be interpreted in terms of absolute optical density changes owing to changes in pump intensities and gain settings on data acquisition hardware. [b] $\Delta A(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + a_3 \exp(-t/\tau_3)$. [c] $\Delta A(t) = a_1[1 - \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2) + a_3 \exp(-t/\tau_3)$. [d] $\Delta A(t) = a_2[1 - \exp(-t/\tau_2) + a_3 \exp(-t/\tau_3)$.

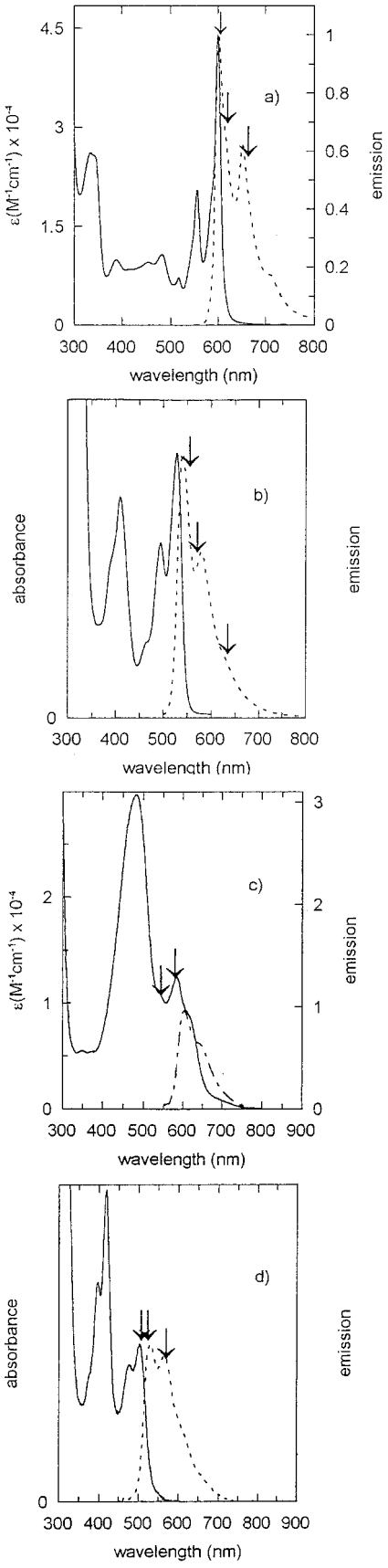

Figure 2. Normalized absorption (−) and emission (- - -) spectra in DMSO of (a) hypericin (λ_{ex} = 550 nm), (b) hexamethoxyhypericin (λ_{ex} = 410 nm), (c) hypocrellin (λ_{ex} = 400 nm), and (d) tetramethoxyhypericin (λ_{ex} = 412 nm). The extinction coefficients for hypericin[8,25] and hypocrellin[27] are taken or estimated from the literature. The arrows indicate the wavelengths at which pump−probe transient absorption experiments were performed.

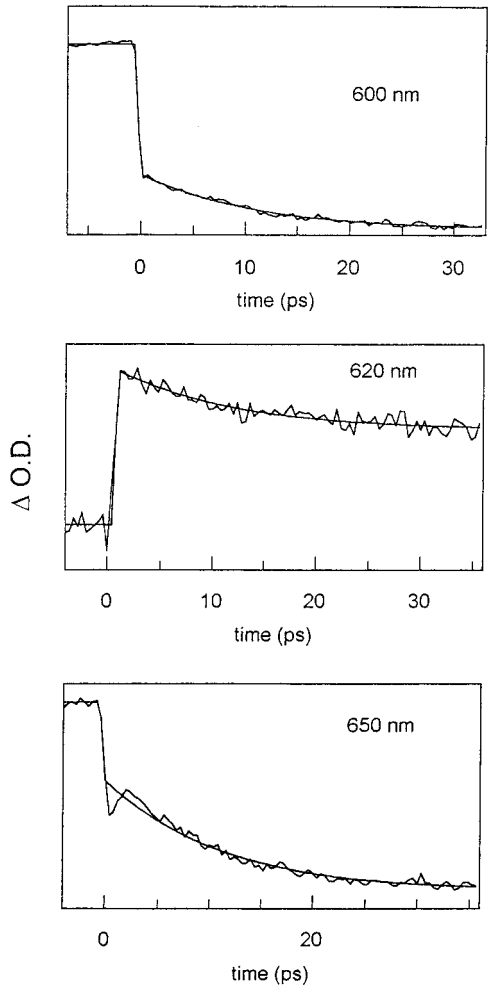

Figure 3. Absorption transients of hypericin in DMSO, λ_{pump} = 415 nm; probe wavelengths are indicated in the panels. The "spike" at zero time that is most apparent in the data in this figure is a result of cross-phase modulation of the pump pulse with the probe pulse.[21] The time constant obtained by globally fitting these and other data is 11.6 ps.[15]

duration at 2 kHz[15] or of 1−3 ps duration at 30 Hz.[14] Sample preparation for the tetra and hexamethoxy analogue samples[15] required bubbling with argon and filling the spinning sample holder in a glovebag of argon to reduce oxygen content. This was done after observing rapid degradation of the hexamethoxy sample in the presence of oxygen. Deoxygenation sufficiently preserved the samples to withstand pump incidence for several hours. Hypericin is stable for hours without deoxygenation and gives the same experimental results in either case.

Kinetic traces acquired at various wavelengths were fit using a global fitting procedure described in detail elsewhere.[15] The time-resolved absorption/stimulated emission data are displayed in Figures 3−6. The results of the global fits, and the functional forms used to obtain them, are summarized in Table 2. The "spike" that appears at zero time in some of the data arises from the more intense pump pulse modulating the phase of the less intense probe pulse.[21]

Results

A. Steady-State Spectra. The steady-state absorption and emission spectra of hypericin, hexamethoxyhypericin, hypocrellin, and tetramethoxyhypericin are presented in Figure 2. The spectra of hypericin differ significantly from those of the other three analogues. The absorbance maximum of hypericin in DMSO arising from the $S_0 \rightarrow S_1$ transition occurs at 598 nm. Hypericin is unlike the other three compounds investigated

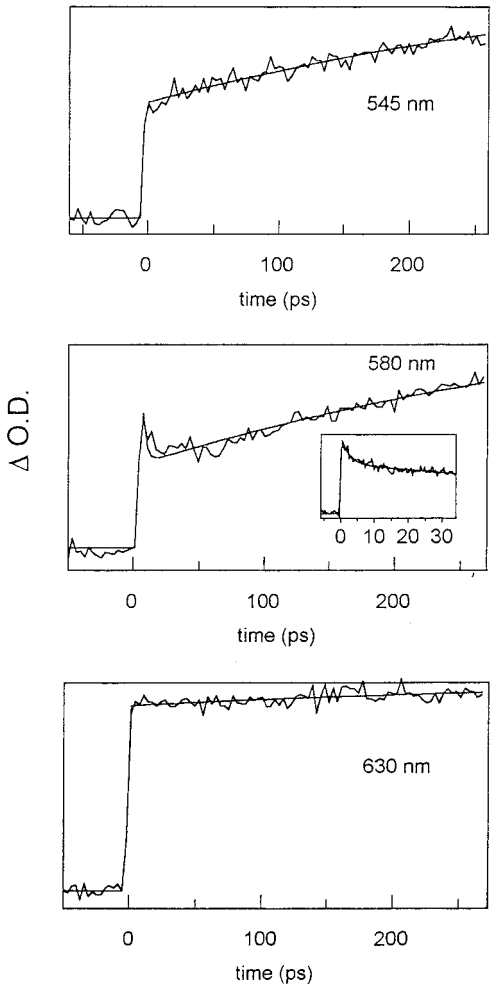

Figure 4. Absorption transients of hexamethoxy hypericin in DMSO, $\lambda_{pump} = 415$ nm; probe wavelengths are indicated in the panels. The full scale is 320 ps. The full scale of the inset is 40 ps. The time constant obtained by globally fitting these and other data is 2.5 ps.[15]

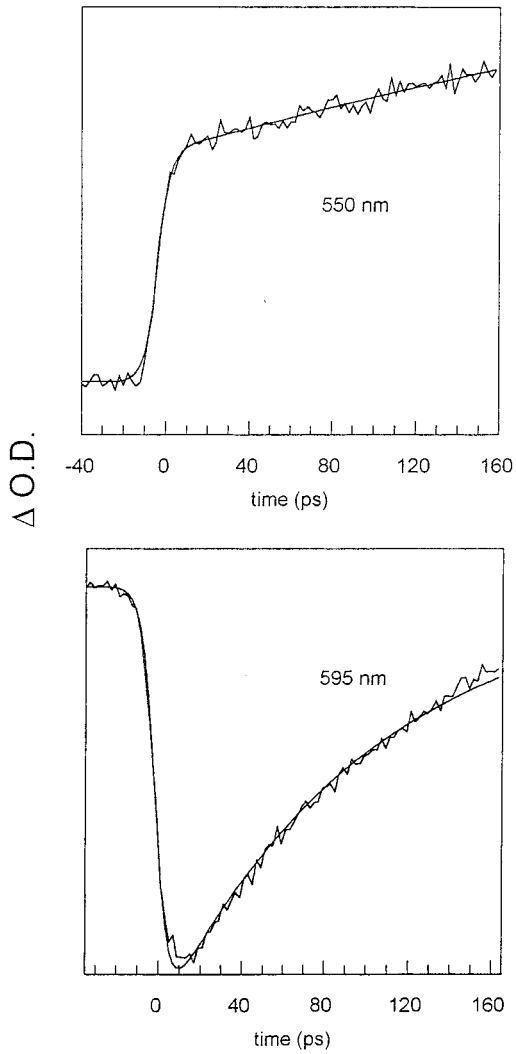

Figure 5. Absorption transients of hypocrellin in DMSO, $\lambda_{pump} = 588$ nm; probe wavelengths are indicated in the panels. The full scale is 200 ps. Comparable results are obtained using a pump wavelength at 415 nm. The time constant obtained by globally fitting these and other data is 90 ps.[26] For $\lambda_{probe} = 550$ nm, $\Delta A(t) = 0.68[1 - \exp(-t/90\ \text{ps})] + 0.32\exp(-t/\infty)$. For $\lambda_{probe} = 595$ nm, $\Delta A(t) = -0.99\exp(-t/90\ \text{ps}) - 0.01\exp(-t/\infty)$.

here in that it lacks an intense transition in the 400−500 nm region, which is most likely due to an $S_0 \rightarrow S_n$ transition. This band is almost as intense as that at 530 nm for the hexamethoxy analogue. In hypocrellin and tetramethoxy hypericin, this band contains the absorbance maximum. These data indicate that methylation of the hydroxyl groups adjacent to the carbonyls (or their removal) provides relative enhancement of the oscillator strength at higher energies. Consequently, these data suggest that interaction of the enol proton with the keto oxygen strongly determines the characteristics of the absorption spectrum. The methoxy analogues are also less fluorescent than their counterparts. This is consistent with our earlier proposals concerning the role of a proton interacting with the keto oxygen for establishing hypericin-like fluorescence.[8,9] Note that in Figure 2 we have normalized the emission spectra so that their maxima are identical with those of the peaks (or the shoulder, in the case of hypocrellin) of the lowest energy absorption features. This is done to indicate the mirror symmetry between the absorption and emission spectra. Table 1 provides a summary of fluorescence data.

B. Excited-State Kinetics. The excited-state kinetics of hypericin, hexamethoxyhypericin, hypocrellin, and tetramethoxyhypericin are presented in Figures 3−6. The probe wavelengths at which these kinetics were interrogated are indicated in the steady-state spectra. Stimulated emission is observed in hypericin but not in the hexamethoxy analogue. The difference in the kinetics of the tetramethoxy analogue and hypocrellin is even more striking. The tetramethoxy analogue exhibits no stimulated emission and possesses a time constant that is 9 times smaller than that observed in hypocrellin.

In all the data presented there is a component that does not decay on the time scale of the experiment or that is characterized by a long-lived time constant that agrees with the fluorescence decay time (Table 1). Consequently, this long-lived component is attributed to absorption by an excited-state singlet or formation of triplet,[23,24] depending on whether the signal is rising or decaying. Figure 7 presents the fluorescence decay of the tetramethoxy analogue in DMSO. Its lifetime can be fit to a time constant of 820 ps, which is consistent with the long-time component extracted from the transient absorption data.

Discussion

We have argued in the Introduction and elsewhere[8,9] that the signature of proton transfer in hypericin is a rise time in stimulated emission. Such a kinetic feature is absent in the hexamethoxy analogue (Figure 1e), which cannot execute such a nonradiative process. This absence consequently confirms the presence of proton transfer in hypericin. A possible objection to this argument is that the description of the traces

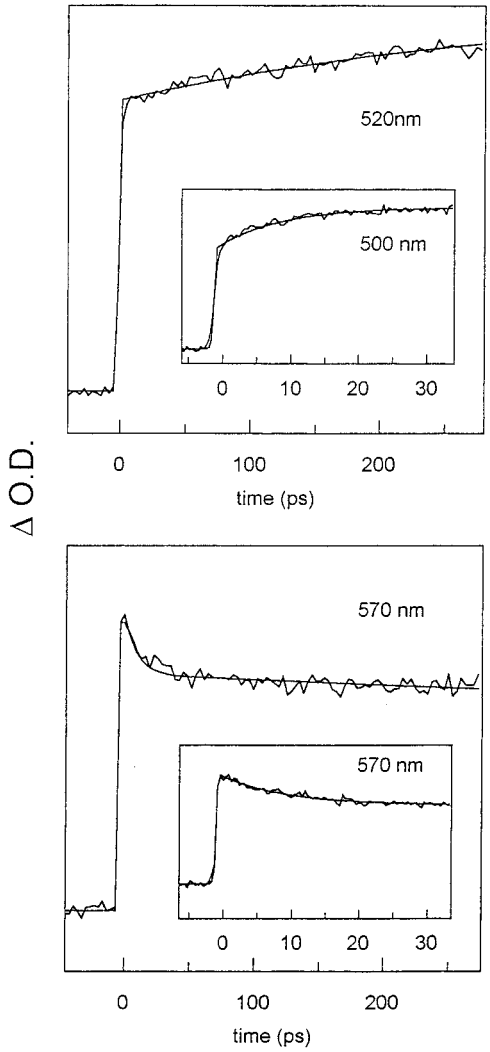

Figure 6. Absorption transients of tetramethoxyhypericin in DMSO, $\lambda_{pump} = 415$ nm; probe wavelengths are indicated in the panels. The full scale is 320 ps. The fast component has a time constant of 9.9 ps. The slowly rising transient apparent in the top panel has a time constant of 820 ps, which matches the fluorescence decay of the analogue in DMSO. The rise in absorbance at 500 nm has the same time constant as the fast decaying component at 570 nm. A summary of absorption data for all the probe wavelengths studied is given in Table 2.

in Figure 3 is not unique. For example, instead of attributing the form of the absorption transients to a rising component in stimulated emission, they may equally well be described by a component of stimulated emission that appears instantaneously and that does not decay on the time scale of the experiment and a component of transient absorption that decays in 11 ps. This latter explanation seems unlikely, since the traces obtained with an excitation wavelength of 588 nm yield much more complicated kinetics that would be difficult to rationalize by this alternative. For excitation at 588 nm, two components of stimulated emission are observed: one rising with a ~10 ps time constant and another that appears instantaneously and that decays in ~1−2 ps.[9]

It is clear that a unique and self-consistent description of the hypericin photophysics requires a range of experiments. We have already indicated the utility of varying the excitation wavelength. Fluorescence upconversion experiments[22] will also provide important complementary information, since only emission is detected. Comparison of a range of analogues also proves to be effective in understanding the origin of the photophysics.

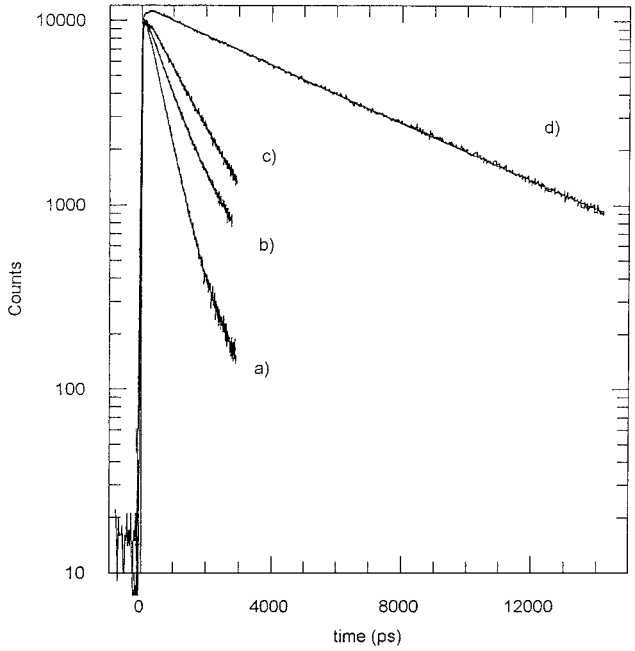

Figure 7. Fluorescence decays obtained from time-correlated single-photon counting at 22 °C. The topmost set of residuals corresponds to hypericin. Curve a is for hexamethoxyhypericin in DMSO: $\lambda_{ex} = 300$ nm, $\lambda_{em} > 530$ nm; $F(t) = 0.97 \exp(-t/490\text{ ps}) + 0.03 \exp(-t/2.1\text{ ns})$, $\chi^2 = 1.15$. The full scale is 3 ns. Curve b is for tetramethoxyhypericin in DMSO: $\lambda_{ex} = 305$ nm, $\lambda_{em} > 530$ nm; $F(t) = 0.95 \exp(-t/820\text{ ps}) + 0.05 \exp(-t/2.0\text{ ns})$, $\chi^2 = 1.15$. The full scale is 3 ns. Curve c is for hypocrellin in DMSO: $\lambda_{ex} = 288$ nm, $\lambda_{em} > 455$ nm; $F(t) = 1.00 \exp(-t/1.3\text{ ns})$, $\chi^2 = 1.05$. The full scale is 3 ns. Curve d is for hypericin in DMSO: $\lambda_{ex} = 305$ nm, $\lambda_{em} > 555$ nm; $F(t) = 1.00 \exp(-t/5.3\text{ ns})$, $\chi^2 = 1.18$. The full scale is 15 ns. To our knowledge, the fluorescence decay of hypericin and its analogues does not depend on excitation wavelength, visible and ultraviolet excitation yielding similar results.[8,25] This result should not be confused with the excitation wavelength dependence of the picosecond transients in hypericin.[15] These latter transients report on primary processes that are not resolvable in the measurement of the longer lived fluorescence decay.

The latter approach is taken in this article, the most striking result of which is the difference in the excited-state kinetics of hypocrellin and the tetramethoxy analogue. The tetramethoxy analogue exhibits no stimulated emission and a time constant that is 9 times smaller than that for hypocrellin (10 ps instead of 90 ps). The absence of a ~100 ps time constant in the transient absorbance of the tetramethoxy analogue is consistent with the assignment of this component to excited-state proton or atom transfer in hypocrellin.

A possible origin of the 2.5 and 10 ps rapid transients in the hexa- and tetramethoxy analogues is dynamic solvation of an excited state (or internal conversion). As the solvent dynamically readjusts itself to the change in the dipole of the solute upon optical excitation, the solute is lowered in energy. This is manifested in the transient absorbance. For example, in both of the methoxy analogues, a decay of the induced absorption at red wavelengths matches a rise in induced absorption at blue wavelengths (Figures 3 and 6). These data are consistent with a picture in which the lowest excited singlet is stabilized relative to a higher-lying state to which it is optically coupled. The dynamic solvation time of DMSO is known to be 3 ps.[22] It may, of course, be fortuitous that the observed transients are of similar magnitude. Ideally, a correlation of decay time in various solvents with the known dynamic solvation times would enable us to make this assignment unambiguously. Unfortunately, DMSO is the only solvent in which we are able to dissolve the analogues in sufficient quantities to perform these experiments and at the same time avoid sample degradation.

Another possibility for the origin of this component is prompt intersystem crossing to form triplets.

Conclusions

The comparison of the transient absorption kinetics of the tetramethoxy analogue with those of hypocrellin (taken in the context of the comparison of those of the hexamethoxy analogue and hypericin) is cogent evidence for the identification of the transients in hypericin and hypocrellin with excited-state proton or atom transfer. In the companion article, further evidence will be provided for the assignment of these processes to proton or atom transfer events. In addition, the order of magnitude difference in the time constant between hypericin and hypocrellin will be discussed in detail.

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