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Journal of Photochemistry and Photobiology A: Chemistry 154 (2002) 107–116

Journal of Photochemistry and Photobiology A:Chemistry

www.elsevier.com/locate/jphotochem

A comparison of the excited-state processes of nearly symmetrical perylene quinones: hypocrellin A and hypomycin B

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Received 3 April 2002; accepted 28 May 2002

Abstract

The excited-state photophysics of two naturally occurring nearly symmetrical perylene quinones are discussed: hypocrellin A and hypomycin B. Hypocrellin A has a hydroxyl group *peri* to a carbonyl group on either end of its long molecular axis in addition to a hydroxyl group on its seven-membered ring. On the other hand, hypomycin B is unique among this class of known naturally occurring perylene quinones in that it possesses only one hydroxyl group, which is *peri* to a carbonyl group. These quinones are investigated in different nonionic micellar environments. For hypocrellin A and hypomycin B, a micelle concentration 10 times in excess of that used for hypericin in a previous study, i.e. 100 times the critical micelle concentration, must be employed to obviate aggregation. Under such conditions, the pK_a of the *peri* hydroxyl groups of hypocrellin A have been determined to be 8.9. The pK_a of the protonated carbonyl groups could not be measured. A comparable value is estimated for hypomycin B. The differing solubilities and behaviors of hypericin and hypocrellin in micellar environments are briefly discussed in the context of their biological activity. The excited-state processes in hypocrellin A and hypomycin B are compared on a time scale of several hundreds of picoseconds. No deuterium isotope effect is observed for hypomycin B. This result is discussed in the light of the previous assignment of the primary photoprocess in hypocrellin A to hydrogen atom transfer.
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Keywords: Hypocrellin; Hypomycin; Micelles; Fluorescence and absorption spectra; Time correlated single photon counting; Pump–probe absorption spectroscopy

1. Introduction

Hypericin and hypocrellin (Fig. 1) are naturally occurring perylene quinones that have gained great interest recently owing to their light-induced biological activity [1–5]. They display virucidal activity against several types of viruses, including the human immunodeficiency virus (HIV) [6–8], as well as antiproliferative and cytotoxic effects on tumor cells [9–11]. Hypericin is also a potent antidepressant [12,13], exhibits light-dependent inhibition of protein kinase C (PKC) [14], and is reported to possess numerous other types of biological behavior [15–20]. Hypericin, like other anticancer drugs, also induces apoptosis [10,21,22].

Owing to this important biological activity, over the past few years we have been studying the photophysics of hypericin and hypocrellin [23–34]. By means of H/D substitution, investigation of *o*-methylated analogs, and complementary studies using both transient absorption and fluorescence upconversion spectroscopies, we have argued that the major primary photophysical process in hypericin and hypocrellin A in organic solvents is excited-state hydrogen atom transfer. Considerable effort was required to demonstrate this fact owing to the unusual mirror image symmetry between absorption and emission spectra, the lack of an H/D isotope effect on the proton transfer reaction in hypericin, and the occasional consideration of this ultrafast reaction in terms of equilibrium Förster-cycle type calculations [35].

We have suggested that the labile protons resulting from the intramolecular proton transfer reaction may be important for understanding the light-induced biological activity of hypericin and hypocrellin A. Notably, hypericin and hypocrellin A acidify their surroundings upon light absorption [36–38]. The role of photogenerated protons takes on significance in the context of the growing body of literature

Abbreviations: Hep, heptane; AOT, sodium bis(2-ethylhexyl)sulfosuccinate; CMC, critical micelle concentration; DMSO, dimethyl sulfoxide; Brij-35, Brij-35 micelles

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PII: S1010-6030(02)00309-X

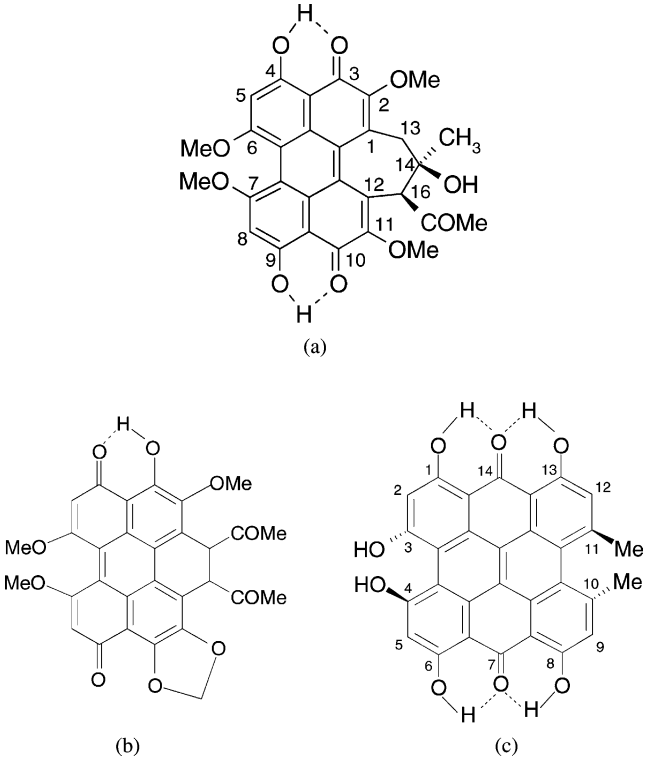


Fig. 1. Structures of (a) hypocrellin A, (b) hypomycin B, and (c) hypericin.

implicating changes in pH with inhibition of virus replication [39], antitumor activity [40,41], and apoptosis (programmed cell death) [42–44]. For example, comparative studies for nine perylenequinones, including hypericin, provide evidence that the quantum yield of singlet oxygen formation is not sufficient to explain the reported antiviral activities of these molecules and that other structural features of perylenequinones are involved [45]. In fact, the quantum yield of singlet oxygen from hypericin is much less than had initially been presumed. Recently, Jardon and coworkers [46,47] have revised their earlier estimation of a singlet oxygen quantum yield of 0.73, essentially equal to the triplet yield, to 0.35 in ethanol and less than 0.02 in water [48]. Based on this result, mechanisms involving only oxygen cannot clearly explain all the activity of hypericin. For example, the ability of photogenerated protons to enhance the activity of activated oxygen species may be of importance.

In this article, we shall be concerned with two topics. First, in the context of the previous body of work referred to above, it is a natural and pressing desire to want to investigate the phenomena as a function of pH. Such a study is, unfortunately, impossible in homogenous aqueous solution owing to the extreme insolubility of these perylene quinones in water at all but the very extremes of pH. Jardon and his coworkers [47,49–53], studying the steady-state spectra and the triplet dynamics of hypericin, were the first to address this problem in a detailed and systematic matter by incorporating hypericin in Brij micelles. Recently we have extended this idea by investigating hypericin in reverse micelles formed by dissolving sodium bis(2-ethylhexyl)sulfosuccinate (AOT) in heptane. Such microemulsions provide an ideal system because their size and properties may be very well controlled. Addition of water to AOT/alkane solutions produces systems that resemble small water pools in bioaggregates [54]. Also the solubilized water is similar to the interfacial water that is present near the biological membranes of protein surfaces [55]. Here we report the behavior of hypocrellin A and hypomycin B [56] in micellar environments, in particular Brij-35 micelles (Brij-35). The behavior of hypocrellin A is significantly different than that of hypericin. Recently, Dumas [57] has performed similar investigations.

Second, given the structure of the perylene quinones of interest here (Fig. 1), with either one (hypocrellin A) or two (hypericin), hydroxyl groups *peri* to a carbonyl on either end of the molecule, one is naturally inclined to inquire how many hydrogen atoms are transferred in the excited-state. And, if more than one is transferred, one must necessarily inquire whether the process is stepwise or concerted. (It is useful to note the difference between a concerted and a synchronous reaction. A concerted reaction takes place in a single kinetic step, with no reaction intermediate, where some of the changes in bonding take place to different extents in different parts of the reaction. A synchronous reaction is one where all the bond-making and bond-breaking processes take place at the same time and proceed at the same extent during the reaction [58,59]. It is a common error to assume that concertedness implies synchrony.) The availability of hypomycin B [56], where there is only one *peri* hydroxyl group, and only one intramolecular hydrogen bond, provides an excellent means to investigate these questions.

2. Materials and methods

Hypocrellin A was purchased from Molecular Probes (Eugene, OR) and was used as received. Hypomycin B was provided by the Shandong researchers and was extracted and prepared as described elsewhere [60]. Sodium bis-(2-ethylhexyl)sulfosuccinate (AOT) purchased from Sigma Chemical Co. (St. Louis, MO), was purified by dissolving in methanol and stirring it overnight in the presence of activated charcoal. Subsequent filtration and removal of methanol by distillation under vacuum, yielded AOT suitable for use. Brij-35 was obtained from Aldrich (Milwaukee, WI) and was recrystallized twice from ethanol before use. Spectrophotometric grade heptane purchased from Aldrich was refluxed over calcium hydride and collected by distillation before use.

2.1. Preparation of sample

An attempt was made to study hypocrellin A in reverse micelles. The solution of AOT in heptane was prepared

in such a way that its concentration was 0.9 M. (The reason for choosing this high reverse micellar concentration is that hypocrellin A has marked solubility in heptane, and hence it was hoped that the higher number of reverse micelles would increase the partitioning of hypocrellin A at the micelle–water interface.) Nevertheless, hypocrellin A was soluble in heptane. Consequently, recourse to Brij micelles was made. An alkaline solution of 2×10^{-2} M, 100 times the critical micellar concentration (CMC), of Brij-35 was prepared by dissolving 480 mg Brij-35 in 20 ml of an aqueous solution of NaOH of pH 13. Solid hypocrellin A was added to a portion of this solution and sonicated for 5 min to obtain a clear solution. In order to obtain solutions of different pH, appropriate volumes of 12 M HCl were added with a microliter syringe. The pH of the resulting solutions were recorded by a manual Fisher-Accumet (915) pH meter. For hypomycin B sample preparation, Brij-35 was dissolved in buffers of different pH (7–13). Solid hypomycin B was added to the solutions in a very small amount followed by sonication so as to ensure complete homogenization of the solution. For hypomycin B, the concentration of Brij-35 solution was also maintained at 100 CMC to avoid complications due to aggregation as much as possible. The concentration of both hypocrellin A and hypomycin B was kept at $\sim10^{-6}$ M except for the transient absorption measurements of hypomycin B, where the concentration was increased to 10^{-5} M to improve the signal-to-noise ratio.

2.2. Steady-state measurements

Steady-state absorption spectra were recorded on a Perkin-Elmer Lambda-18 double-beam UV-Vis spectrophotometer with 1 nm resolution. Steady-state fluorescence spectra were obtained on a SPEX Fluoromax with a 4 nm bandpass and corrected for detector response. The samples were excited at 480 nm. For both fluorescence and absorption measurements, a 1 cm path-length quartz cuvette was used.

2.3. Time-resolved studies

The apparatus for time-correlated single photon counting is described elsewhere [23–25]. Fluorescence decays were collected by exciting at 580 nm and collecting emission >610 nm for a maximum of 10,000 counts in the peak channel. The solutions of hypomycin B in Brij at pH values of 12 and above were not stable enough to carry out lifetime measurements. All measurements were carried out at 298 K.

2.4. Time-resolved pump–probe absorption spectroscopy

The apparatus used for ultrafast kinetic measurements is described in detail elsewhere [61].

3. Results and discussion

3.1. Effects of pH on the absorption and emission spectra of hypocrellin A and hypomycin B

Whereas hypericin is insoluble in heptane and partitions only at the AOT/water interface [62], hypocrellin A exhibits marked solubility in heptane. This solubility in heptane obviates the performance of unambiguous examination of its photophysics in the microemulsion. Consequently, recourse was made to Brij micelles, as in the work of Jardon and coworkers. It was found, however, that whereas hypericin shows no evidence of aggregation at 10 times the CMC (data not shown), i.e. at 2×10^{-3} M Brij, hypocrellin A exhibits aggregation at pH 7. A sensitive signature of aggregation is nonexponential fluorescence decay and the quenching of fluorescence [31] (for comparison of aggregation effects, both hypocrellin A and hypericin were maintained at a concentration of $\sim10^{-6}$ M). A Brij concentration of one hundred times the CMC is required for hypocrellin A to afford an unambiguous single exponential fluorescence lifetime (Fig. 2). A Brij concentration of 2×10^{-2} M was used for all titration studies. At this concentration of Brij, there is one probe molecule for every 250 micelles, thus keeping aggregation to the minimum.

Fig. 3 presents the absorption spectra of hypocrellin A at pH values of 1, 7, and 13. Between pH 13 and 7 there is a distinct change in the spectra, which we attribute to the deprotonation of the *peri* hydroxyl groups. On the other hand,

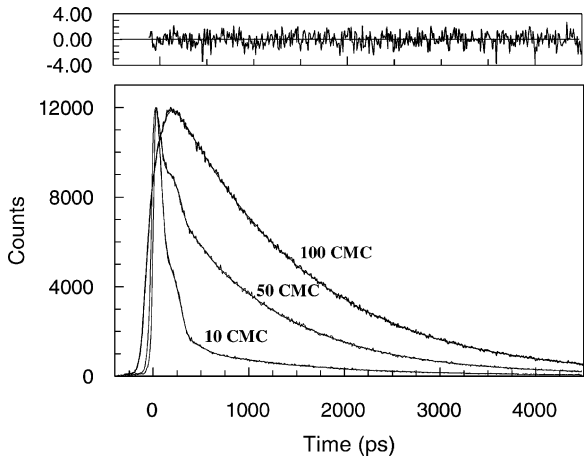


Fig. 2. Fluorescence decays of hypocrellin A in 2×10^{-3} M (10 times CMC), 1×10^{-2} M (50 times CMC) and 2×10^{-2} M (100 times CMC) aqueous Brij-35 solutions at pH 7 (λ_{ex} = 580 nm, λ_{em} > 610 nm). The decay for hypocrellin A in 100 CMC of Brij is fit to a single exponential function with a time constant of 1.3 ns. The decay at 50 CMC is biexponential with time constants of 0.18 and 1.1 ns, with relative amplitudes of 0.55 and 0.45, respectively. The decay at 10 CMC is fit to a triple exponential function, with time constants of 0.15, 0.75 and 2.6 ns, having relative amplitudes of 0.92, 0.05 and 0.03, respectively. The presence of the shorter components in lower concentrations of the micelle indicates the aggregation of hypocrellin A. The residuals shown refer to the fit for the 100 CMC data.

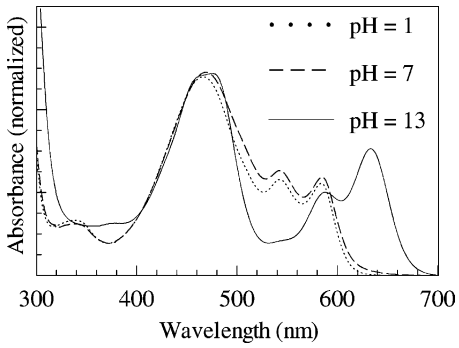


Fig. 3. Normalized absorption spectra of hypocrellin A in 2×10^{-2} M (100 times CMC) aqueous Brij-35 at pH 1, 7, and 13.

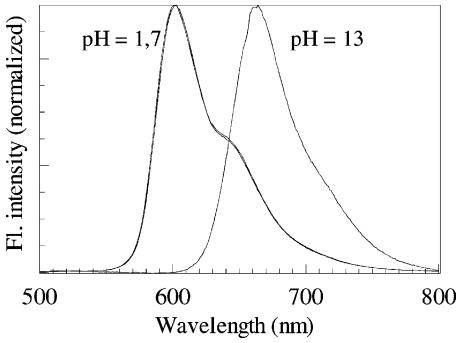
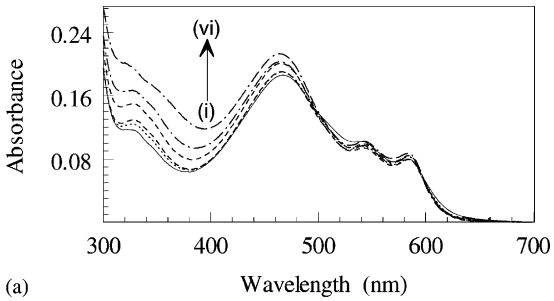
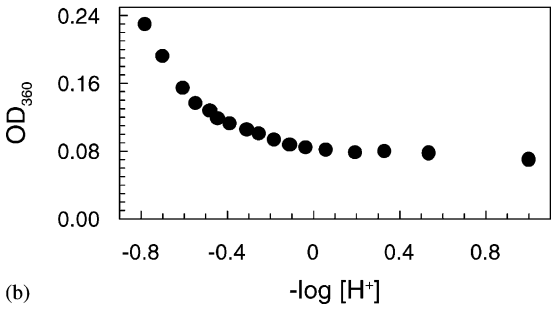


Fig. 5. Normalized fluorescence spectra of hypocrellin A in 2×10^{-2} M (100 times CMC) aqueous Brij-35 at pH 1, 7 and 13 ($\lambda_{ex} = 480$ nm).

the spectra at neutral pH and a pH value of 1 are very similar. We anticipated to see a change in this region, attributed to the protonation of the carbonyl groups because Jardon and coworkers have measured a pK_a of 1 for these groups in hypericin. Consequently, we searched for spectral changes owing to titration of these groups at higher acidity. Fig. 4 represents the absorption changes of hypocrellin A from 0.06 to 3.5 M HCl. Although there are well-behaved spectral changes characterized by an isosbestic point at 596 nm, we are hesitant to interpret them in terms of the titration of the carbonyls. In particular, Fig. 4b gives no evidence of an inflection point over this range of acid concentration. It is possible that at these concentrations we are disrupting the micelles. We suggest that at low acid concentrations where integrity of micelles is maintained, either protonation of the carbonyls is undetectable spectroscopically or that preferential protonation of the –OCH_3 group occurs.



(a)



(b)

Fig. 4. (a) Absorption spectra of hypocrellin A in 2×10^{-2} M (100 times CMC) Brij-35 in (i) 0.06 M, (ii) 0.29 M, (iii) 0.63 M, (iv) 1.5 M, (v) 2 M and (vi) 3.5 M HCl solutions. An isosbestic point is obtained at 596 nm. (b) Plot of absorbance at 360 nm of hypocrellin A in 2×10^{-2} M (100 times CMC) Brij-35 in solutions of high acidity, against $-\log[H^+]$ in the range $0.06\,M \leq [H^+] \leq 3.5\,M$, where $[H^+]$ = concentration of HCl.

Fig. 5 presents the steady-state fluorescence spectra at pH 1, 7, and 13. Again, as in absorption, the spectrum at pH 13 is significantly different from that at 1 and 7. The average fluorescence lifetime of hypocrellin A in Brij micelles is plotted in Fig. 6. The pK_a of the *peri* hydroxyl groups is obtained from the inflection point to be 8.9. The use of lifetimes to perform fluorescence titrations provides a distinct advantage over methods using fluorescence quantum yields or optical densities, which require accurate accounting of concentration changes.

Hypomycin B was investigated under the same conditions as hypocrellin A. Figs. 7 and 8 present the steady-state absorption and emission spectra of hypomycin B in 100 CMC Brij solutions as a function of pH. Above pH 9, the absorption and emission spectra change significantly, revealing the formation of the anionic form of hypomycin B. Fig. 9 presents several examples of the fluorescence decays. A summary of the lifetime behavior as a function of pH is given in Table 1. The fluorescence is well described by a single exponential time constant of ~890 ps below pH values of 8; however, above this pH, we see the appearance of a second,

Table 1
Fluorescence lifetime parameters of hypomycin B in 100 CMC Brij-35 solutions of different pH[a]

pH	τ_1 (ps)	a_1	τ_2 (ps)	a_2
5	890	1.00	–	–
6	890	1.00	–	–
7	890	1.00	–	–
8	910	0.82	200	0.18
9	900	0.75	210	0.25
10	942	0.54	180	0.46
11	920	0.12	190	0.88

[a] All the samples were excited at 580 nm and emission was collected after 610 nm with a cut-off filter. Fluorescence lifetimes were adequately described by two exponentially decaying components: $F(t) = a_1 \exp(-t/\tau_1) + a_2 \exp(-t/\tau_2)$. The absence of values for a_2 and τ_2 indicate that lifetime was adequately described by a single exponential decay. For hypomycin B in Brij micelles in D_2O, the time constant is 900 ps.

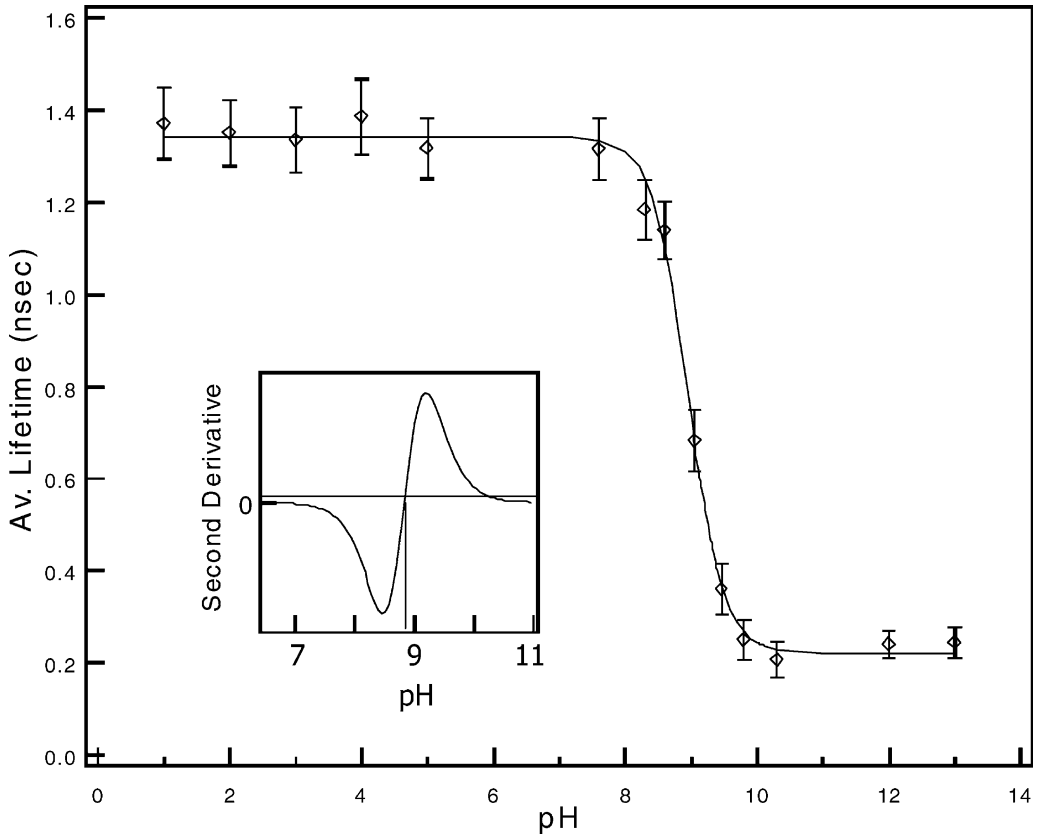


Fig. 6. Plot of the average fluorescence lifetime of hypocrellin A in 2×10^{-2} M (100 times CMC) aqueous Brij-35 solutions against pH. The data were fit to a sigmoid. The inflection point, obtained from the second derivative of the fitted curve, as shown in the inset, yields a pK_a of 8.9. The fluorescence decays were single exponential for all pH values except those in the region of the inflection point (pH ∼ 8–10). At pH values of less than 8, even up to an acid concentration of 3 M, the lifetime remained constant and single exponential.

shorter, component, the amplitude of which increases as shown in Table 1 with increasing pH values. Hence the average lifetime becomes shorter leading to faster decays as shown in Fig. 9. The hypomycin B data reveal a titratable group with a pK_a of approximately 9.7, which is slightly higher than that of hypocrellin A. (We did not have enough material available to construct a detailed titration curve.)

The most significant experiments involving hypomycin B presented here are the picosecond transient absorption measurements in MeOH and MeOD (Fig. 10). Within experimental error, there is no difference in the decaying transient in the two solvents. This result is discussed below.

3.2. Multiple H-atom transfers in the perylene quinones?

Hypomycin B is unique in that it has only one intramolecular hydrogen bond as opposed to two in hypocrellin A and four in hypericin (Fig. 1). Picosecond transient absorption data for hypomycin B fail to reveal any stimulated emission, let alone rise time in stimulated emission (unlike hypocrellin and hypericin), which we have interpreted in terms of excited-state H-atom transfer. Furthermore, a global analysis of the excited-state kinetics at four different probe wavelengths in MeOD and MeOH yields no significant difference in the excited-state kinetics: the time constants are 82 and 75 ps, respectively. Transient absorption data for hypomycin B in other solvents like dimethyl sulfoxide (DMSO) and buffer–DMSO mixtures (data not shown) also failed to reveal any rise time in the stimulated emission. In the context of our previous arguments and criteria for identifying hydrogen atom transfer in hypericin, hypocrellin, and their analogs, one might hastily conclude that hypomycin B does not undergo this process. Compare Figs. 10 and 11. Fig. 11 clearly reveals the presence of a growth in the kinetics for hypocrellin A, but which is not present for the hypomycin B data in Fig. 10. If such a conclusion were made, however, prudence demands that it be only tentative.

If subsequent experiments do indeed demonstrate that excited-state hydrogen atom transfer does not occur in hypomycin B, then one may draw the conclusion that multiple transfers (either concerted or stepwise) must occur in these perylene quinones and that by frustrating the process in one half of the molecule, the process in the other half is impeded. At this point, such reasoning is speculative and contrary to the growing body of evidence provided by theory and experiment. Quantum mechanical calculations indicate that the double hydrogen atom transfer in hypericin [63] and in the perylene quinone nucleus [64] of hypocrellin

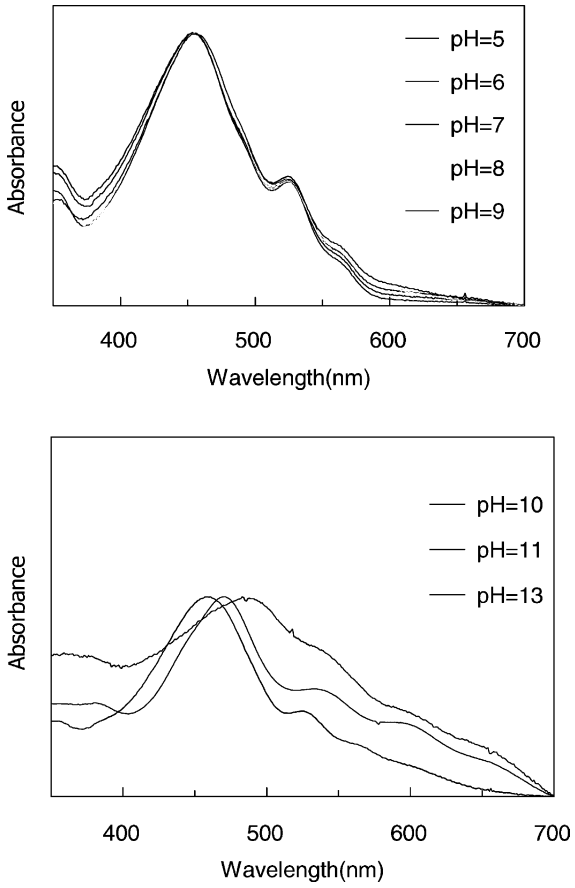


Fig. 7. Absorption spectra of hypomycin B in 100 CMC Brij solutions of pH varying from 5 to 13. All the spectra have been normalized to unity at the peak maximum.

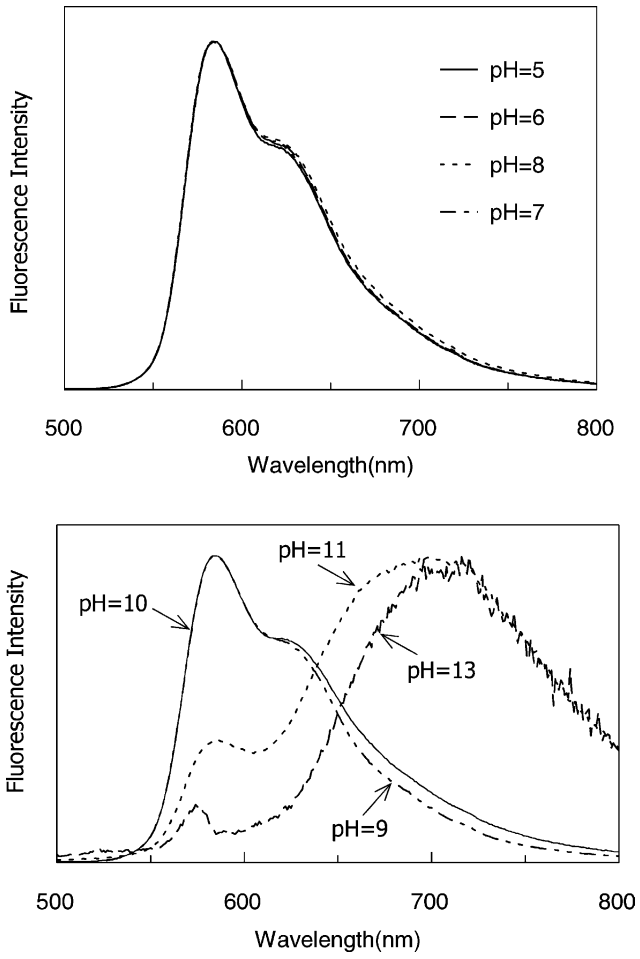


Fig. 8. Emission spectra of hypomycin B in 100 CMC Brij solutions of different pH (λ_{ex} = 480 nm). All spectra have been normalized to an arbitrary value at the peak maximum (beyond pH 9, there is a drastic reduction in fluorescence intensity, indicating that the deprotonated species is far less emissive compared to the normal form of hypomycin B).

is energetically unfavorable compared to the single transfer event. Experiments for hypericin in which the one half of the molecule cannot participate in hydrogen atom transfer owing to protonation of the carbonyl group (or even perhaps complexation with a metal ion) [65] also indicate that the transfer process can still occur.

Given the richness of the photophysics of these perylene quinones and their attendant complexity, it is premature to conclude that hypomycin B does not execute an excited-state hydrogen atom transfer. Certainly, much more work is required to reach a proper judgement. This will include fluorescence upconversion experiments with picosecond resolution in order to interrogate the entire time scale of relevance as well as to monitor only emission, which can simplify the analysis [66]. For example, the failure to detect stimulated emission for hypomycin B may simply be the result of the presence of a strongly absorbing species in the same spectral region. Also, experiments must be performed to determine if hypomycin B, like hypericin [36,37,67] and hypocrellin A [38], acts an excited-state acid. It is clear, however, that hypomycin B is an important system with which to test and refine our current understanding of these naturally occurring perylene quinones. One of the most important questions that the study of hypomycin B might fruitfully address is the elucidation of the reaction coordinate in the H-atom transfer process in the perylene quinones.

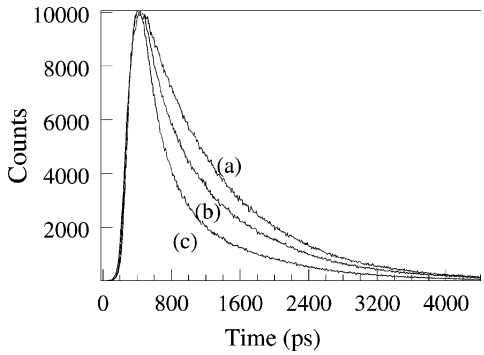


Fig. 9. Comparison of the fluorescence lifetime decays of hypomycin B in 100 CMC Brij-35 solution of varying pH: (a) pH = 9, (b) pH = 10 and (c) pH = 11. For all the samples λ_{ex} = 580 nm. The fitting parameters are reported in Table 1.

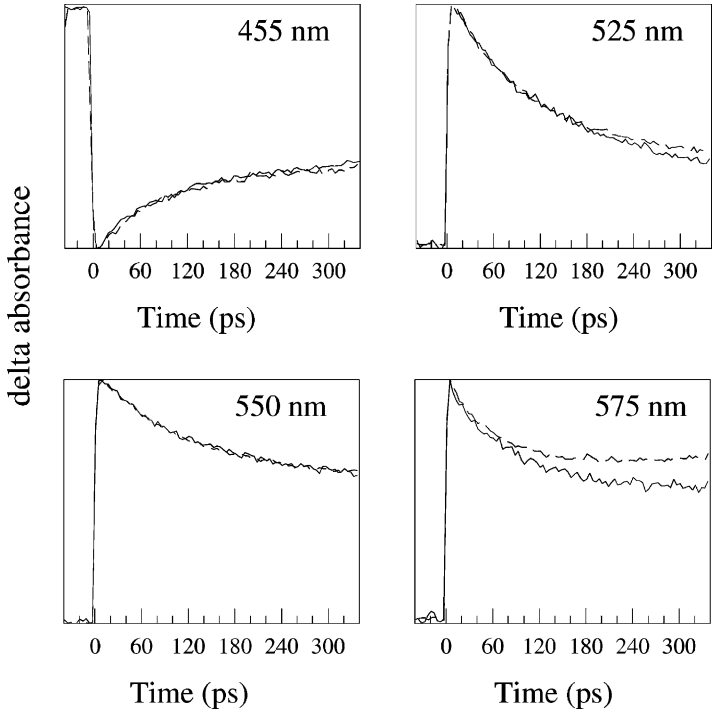


Fig. 10. Comparison of the kinetic traces of hypomycin B in MeOD (solid curves) and in MeOH (broken curves). The probe wavelengths are given in the right hand top corner of each panel. A global fit was carried out to fit the decays of the several wavelengths. In MeOH, the global time constant was 82 ps while that in MeOD was 75 ps ($\lambda_{ex} = 407$ nm).

As we have discussed in depth elsewhere, despite the similarities in the structures of hypericin and hypocrellin, which are centered about the perylene quinone nucleus, their excited-state photophysics exhibit rich and varied behavior. The H-atom transfer is characterized by a wide range of time constants, which in certain cases exhibit deuterium isotope effects and solvent dependence. Of particular interest is that the shortest time constant we have observed for the H-atom transfer is ∼10 ps. This is exceptionally long for such a process, 100 fs being expected when the solute H-atom does not hydrogen bond to the solvent [68]. That the transfer time is so long in the perylene quinones has been attributed to the identification of the reaction coordinate with skeletal motions of the molecule [66,69].

The identification of the reaction coordinate and the question of whether one or more H-atoms are transferred is related. We have previously observed that when hypericin is bound to human serum albumin, it no longer undergoes an excited-state hydrogen atom transfer. Assuming that the binding occurs through the interaction of one of the two carbonyl groups of hypericin and the N_1–H of the single tryptophan residue (W214), which would necessarily impede H-atom transfer on this half of the hypericin molecule, we suggested that the absence of H-atom transfer in the complex was indicative of concerted, double H-atom transfer in the excited-state of hypericin [31]. We suggested that H-atom transfer is completely impeded when hypericin binds to HSA because skeletal motion is coupled to the H-atom transfer [66,69,70]. Fluorescence anisotropy measurements of the HSA/hypericin complex indicate that the hypericin is rigidly bound and that there is no rapid restricted motion of hypericin relative to the protein. By analogy, one might argue that if H-atom transfer does not occur in hypomycin B, it is not because the process requires that two H-atoms be in flight but because the required skeletal motion is restricted by the presence of the O–C–O bond. Although this response is plausible, it is not easy to reconcile it with the observation that hypericin undergoes H-atom transfer in a glass at low temperatures (the energy of activation is 0.05 kcal/mol) [32], where the amplitude of skeletal motion would seem to be less than that in the HSA matrix. These sorts of problems and questions continue to illustrate the need for further elucidation of the reaction coordinate for the H-atom transfer in hypericin and its analogs [69].

To conclude this section, we note that our assignment of excited-state H-atom transfer to the primary photoprocess in hypericin, hypocrellin, and their derivatives has occasioned some objections, to which we refer in the introduction and which we address in detail elsewhere, especially in [66,69]. An additional concern, which has been brought to our attention, that is relevant to address in the light of the previous discussion is the following. As we note above, we have measured the energy of activation for the H-atom transfer in hypericin to be 0.05 kcal/mol (or $\sim 20\,cm^{-1}$). The absence of an isotope effect for the hypericin reaction (and for the ∼10 ps reaction in hypocrellin A) indicates that

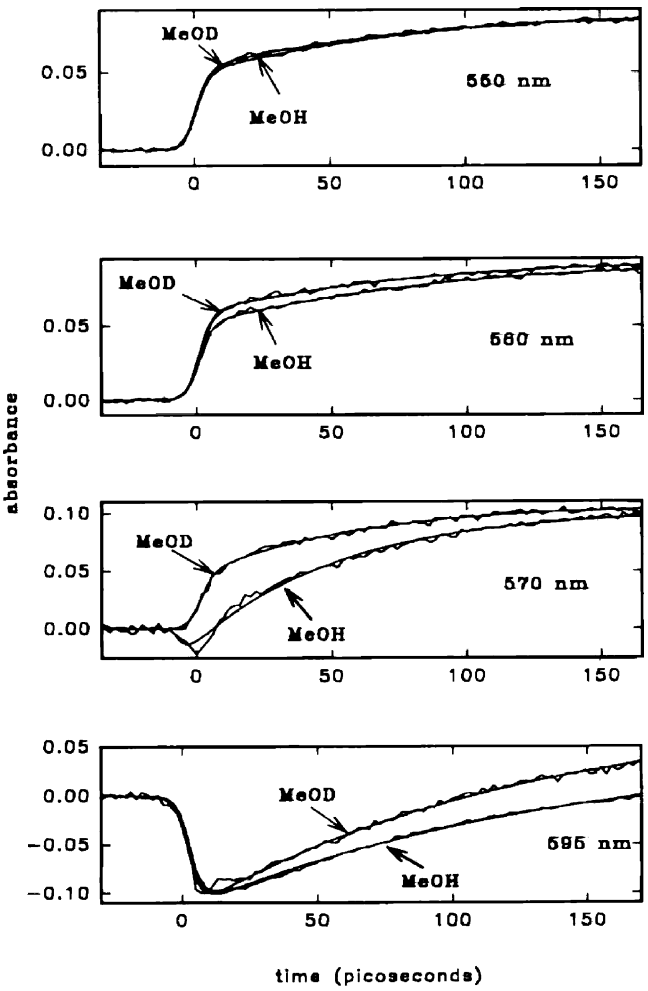


Fig. 11. Kinetic traces of hypocrellin in MeOH and MeOD at four different probe wavelengths. Global fits to the data yield time constants of 67 and 97 ps for MeOH and MeOD, respectively ($\lambda_{ex} = 588$ nm). From reference [27].

the reaction coordinate is not the hydrogen atom coordinate (which theoretical and experimental results suggest is $\sim$1450 cm^{-1} in the hypericin triplet [70]) and consequently must involve skeletal motions invoked above. It has been suggested by an anonymous colleague that if there is a slow hydrogen atom transfer that is not limited by the hydrogen atom coordinate, "then it must be that vibrational excitation of oxygen or ring modes is what limits the rate. This would correspond to a significant vibrational barrier, and hence large activation energy." We disagree with the last statement. Quantum mechanical calculations (see Ref. [63] and http://www.msg.ameslab.gov/Group/Supplementary_Material/Hypericin/) indicate that there are four calculated frequencies (unscaled) below 100 cm^{-1}: 40 cm^{-1}, out-of-plane motion of oxygens and carbons; 48 cm^{-1}, oxygen and carbon displacements; 80 cm^{-1}, mostly OH oxygen motion; 84 cm^{-1}: mostly OH oxygen motion. There is no dearth of low frequency vibrations in large biological molecules as the calculations and many experiments [71–73] suggest; and we believe that such motions may indeed be coupled to the hydrogen atom transfer in these perylene quinone systems.

4. Summary and conclusions

Jardon and coworkers have measured the pK_a's of the bay (3 and 4 positions) and the *peri* hydroxyl groups (1, 6, 8, and 13 positions) of hypericin in Brij-35 and in aqueous liposome media. Their work indicates a pK_a of 7 for the bay hydroxyl, of 11 for the *peri* hydroxyls, and of 1 for the protonated groups [49]. In contrast, hypocrellin A in a similar environment has a pK_a of 8.9 for the *peri* hydroxyls; the pK_a of its protonated carbonyl could not be determined. Recently, using Brij concentrations of 5×10^{-2} M (2.5 times the highest concentration we employ, and 250 times the CMC), Dumas [57] has obtained a similar value of 8.2 for hypocrellin A. Another titratable group is resolved with a pK_a of 11.4. Dumas obtains similar results for hypocrellin B.

Previously we had investigated the light-induced antiviral activity of hypericin and hypocrellin in the presence and absence of oxygen under experimental conditions where the effect of oxygen depletion could be quantified [74]. There was a significant reduction of light-induced activity under hypoxic conditions. Interestingly, antiviral activity of hypocrellin was not observed at the low oxygen levels at which hypericin retained measurable virucidal activity. Based on these results, we proposed that additional pathways, such as the generation of protons from excited-states of hypericin, may enhance the activity of activated oxygen species. The results presented here suggest a reason why this may be the case. There is much evidence indicating that the photophysical and biological properties of hypericin and its analogs are drastically altered when they exist as aggregates. Aggregates do not exhibit excited-state H-atom transfer, have markedly reduced fluorescence quantum yields, shorter fluorescence lifetimes, and reduced virucidal and cytotoxic activity [31]. Under conditions where hypericin exists as the monomer and hypocrellin is still induced to aggregate, such as those modeled in this work, it is not surprising that the biological activity of the latter should be reduced. Similar antiviral and antitumor studies on hypomycin B will provide an interesting comparison with those of hypocrellin A.

Our previous results on hypericin indicate that excited-state H-atom transfer occurs even when one of the carbonyls is prohibited from accepting a hydrogen. The presence of such a transfer is apparent under very acidic conditions in AOT reverse micelles and cannot be excluded upon chelation of Tb^{3+} [65]. There is, thus, no evidence for a concerted hydrogen-atom transfer mechanism in hypericin. In the present study, contrary to our initial expectations, we are not even able to demonstrate that hypomycin B executes an excited-state H-atom transfer; and hence our investigation sheds no light on the general question of how many H-atoms are transferred in the perylene quinones and whether the

transfer is concerted or stepwise. On the other hand, if further investigation does reveal that H-atom transfer does not occur in hypomycin B, the result would have considerable implications for an understanding of the reaction coordinate for the H-atom transfer.

Acknowledgements

This work was partially supported by NSF Grant CHE-9613962 to J.W.P. We thank Professor Pierre Jardon for providing us a copy of the thesis by S. Dumas.

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