Roles of Oxygen and Photoinduced Acidification in the Light-Dependent Antiviral Activity of Hypocrellin A†

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ABSTRACT: Hypocrellin A displays photoinduced antiviral activity, in particular against the human immunodeficiency virus (HIV), as does its counterpart, hypericin. Although hypocrellin A, like hypericin, executes an excited-state intramolecular proton transfer, it differs from hypericin in two important ways. Unlike hypericin, hypocrellin A absolutely requires oxygen for its antiviral activity. Also, whereas we have previously demonstrated that hypericin functions as a light-induced proton source, we do not observe that hypocrellin A acidifies its surrounding medium in the presence of light. These results are discussed in the context of the ground- and excited-state photophysics of hypericin and its mechanisms of photoinduced virucidal activity.

Hypocrellin A (Figure 1a) is a naturally occurring perylene quinone found in a parasitic fungus that is common in parts of the People's Republic of China and Sri Lanka (Diwu, 1995; Diwu & Lown, 1990; Diwu et al., 1989). Hypocrellin has been used as a phototherapeutic agent for various skin diseases and tumors and has been taken orally as a folk medicine for several centuries in China (Diwu & Lown, 1990; Diwu et al., 1989). Like the related polycyclic quinone hypericin (Meruelo et al., 1988, 1992; Degar et al., 1992; Lenard et al., 1993; Carpenter & Kraus, 1991), hypocrellin A possesses light-induced toxicity against the human immunodeficiency virus, HIV, and related viruses (Hudson et al., 1994). This common property of hypocrellin A and hypericin has led us to examine in more detail the similarities and differences between these chromophores.

Hypericin has a large triplet yield [0.70 in ethanol (Jardon et al., 1986)] and is capable of generating significant quantities of singlet oxygen (Meruelo et al., 1988, 1992; Degar et al., 1992; Lenard et al., 1993). The virucidal activity of hypericin results, in part, from production of singlet oxygen. We, however, have recently reported that oxygen *is not required* for antiviral activity (Fehr et al., 1994). We have shown that hypericin undergoes excited-state proton transfer in its singlet state (Gai et al., 1993, 1994a,b) and that, consequently, it possesses labile protons. We have hypothesized that the virucidal activity of hypericin may be related to its ability to acidify its environment upon optical excitation (Fehr et al., 1994, 1995; Gai et al., 1993, 1994a,b), and we have proposed chemical methods of illuminating hypericin for antiviral therapies (Carpenter et al., 1994). We have, furthermore, demonstrated that illumination of a solution containing hypericin effects a pH drop. When hypericin and an indicator dye are kept in relatively close proximity by the use of vesicles, hypericin transfers a proton to the indicator within its triplet lifetime (Fehr et al., 1995). Proton transfer to the indicator is not observed when the indicator is protonated or when the system is oxygenated. Since hypericin is known to form triplets and to generate singlet oxygen with high efficiency, this latter result is taken to confirm triplet hypericin as a source, but not necessarily the only source, of protons.

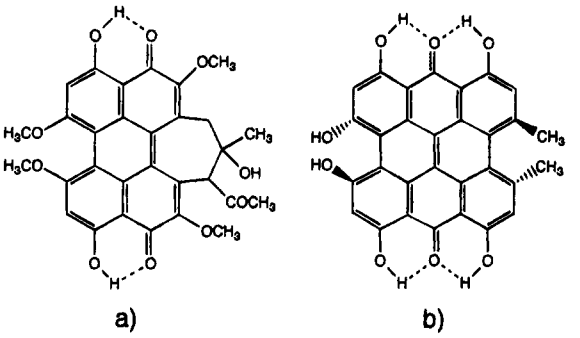

FIGURE 1: Structures of (a) hypocrellin A and (b) hypericin.

Hypocrellin A has a quantum yield for singlet oxygen formation of 0.83 in benzene (Diwu & Lown, 1992). It also possesses structural features that are very similar to those of hypericin: in particular, the hydroxyl groups β to the carbonyl groups. Given this latter feature coupled with our understanding of the photophysical properties of hypericin, as summarized above, we would expect hypocrellin A to exhibit other similarities in its light-induced antiviral activity. In particular, we would expect that, like hypericin, hypocrellin does not require oxygen for its virucidal activity and that it is also capable of intermolecular proton transfer. That we observe neither of these phenomena in hypocrellin suggests an important role for the aromatic skeleton of hypericin. This will have implications for the future design of other light-induced antiviral agents.

MATERIALS AND METHODS

Titration of Infectious Virus. As in our previous work, antiviral assays employ EIAV (equine infectious anemia

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Table 1: Effect of Oyxgen and Serum Concentration of the Light-Dependent Antiviral Activity of Hypericin and Hypocrellin

	hypericin				hypocrellin A			
	0% sera[a]		10% sera[b]		0% sera[a]		10% sera[b]	
	dark	light[c]	dark	light[c]	dark	light[c]	dark	light[c]
aerobic[d]	4.39 ± 0.91[e]	0.50 ± 0.86	4.56 ± 0.50	0.72 ± 1.24	4.74 ± 0.16	0.00 ± 0.00	4.38 ± 0.45	0.88 ± 1.53
hypoxic[f]	4.29 ± 0.84	0.76 ± 1.31	4.53 ± 0.55	0.98 ± 1.70	4.73 ± 0.21	4.19 ± 0.47	4.48 ± 0.61	4.57 ± 0.40

[a] No additional serum besides that which was used to store virus was added (total serum ~1%). [b] Additional serum (fetal calf serum) was added to PBS to give 10% total volume of fetal calf serum. [c] Illumination was effected with a 300-W projector bulb fitted with a cutoff filter blocking wavelengths shorter than 575 nm. [d] The oxygen content of the sample was determined by letting it equilibrate with the atmosphere. [e] Results are expressed as the mean $\log_{10}$ virus titer ± standard deviation. [f] Hypoxic conditions were obtained by passing argon gas over the samples for 45 min before and during illumination.

virus). EIAV is exceptionally well-suited to assay for activity against HIV since it is an enveloped lentivirus structurally, genetically, and antigenically related to HIV (Chiu et al., 1985; Casey et al., 1985; Gonda et al., 1986). All experimental manipulations were performed in subdued light. Cell-free stocks of the MA-1 isolate of equine infectious anemia virus (Carpenter & Kraus, 1991) were diluted 1:10 in phosphate-buffered saline (PBS) containing no or 10% fetal bovine serum. Hypericin (Carl Roth GmbH & Co.) or hypocrellin A (Molecular Probes) was added to a final concentration of 10 μg/mL. Deoxygenation and illumination of samples are described below. A focal immunoassay similar to that previously described (Carpenter & Kraus, 1991) was used for quantifying infectious virus. Results are given for three independant experiments, and are expressed as focus-forming units (ffu) per milliliter of sample.

The analysis of the data involved a comparison between the results collected in the absence and the presence of light *and* in the absence and presence of oxygen (see below). Also, because the results (ffu/mL) involved the comparison of standard deviations of populations that have vastly different means, they were placed on a logarithmic scale. It is well-known that the standard deviation increases with the mean of the sample. Consequently, if one is working with biological samples where populations will vary by orders of magnitude, the appropriate scale on which to base the analysis is not linear but logarithmic (Snedecor & Cochran, 1989).

The effects of light and dark and of aerobic and hypoxic conditions were first evaluated by the application of a randomization test (Snedecor & Cochran, 1989). A second and more involved method of analyzing the data is based on a factorial comparison (Snedecor & Cochran, 1989) that scales the data logarithmically. The factorial comparison permits an evaluation of the combined roles of the absence and presence of light as well as of the absence and presence of oxygen on the virucidal activity. Analyses of these experiments were performed with the standard software package SAS (1988). An analogous factorial analysis indicated that the role of sera on antiviral activity was insignificant. The results are presented in Table 1. They indicate that light is required for virucidal activity in both hypericin and hypocrellin and that oxygen is absolutely required for virucidal activity in hypocrellin but not in hypericin.

Oxygen Assays. Samples were deoxygenated by bubbling argon in light-tight containers and exposed to light for 15 min from a 300-W projector bulb fitted with a cutoff filter blocking wavelengths shorter than 575 nm. The irradiance at the sample was estimated to be 170 W/m^2 (i.e., 8−9 mW) in the spectral range in which hypericin absorbs, 575−600 nm. Hypocrellin/EIAV samples were exposed to identical conditions. Deoxygenation efficiency was evaluated as described previously (Fehr et al., 1994). A dissolved oxygen test kit (Hach, OX-2P) showed dissolved oxygen levels after 1 h of deoxygenating to have fallen from an initial concentration of 5 mg/L (1.56×10^{-4} M) to below the detection limit of 0.2 mg/L (6.25×10^{-6} M). An alternate method of testing for dissolved oxygen is via the bioluminescence of the firefly luciferase/luciferin reaction. Oxygen is necessary in this system for the production of light (McElroy & Deluca, 1985). Light output was measured with a liquid-nitrogen cooled charge-coupled device (CCD) (Princeton Instruments LN/CCD-1152UV) mounted on an HR320 (Instruments SA, Inc.) monochromator with a grating (1200 g/mm) blazed at 5000 Å. A solution of 1.0×10^{-5} M luciferin and 1.6×10^{-8} M luciferase and a solution of 1.0×10^{-4} M ATP were simultaneously deoxygenated in the same apparatus as the EIAV/hypericin and EIAV/hypocrellin experiments. The reaction was initiated by injecting 0.5 mL of the deoxygenated ATP solution into the luciferin/luciferase solution. Three successive 30-s integrations yielded spectra that were superimposable on the background spectra of the CCD. Light could be generated from the reaction system by opening it to the atmosphere. Lack of light generation was taken to indicate that oxygen levels were negligible.

Light-Induced Acidification. Attempts to observe a light-induced acidification in both steady-state and time-resolved measurements were performed as described previously (Fehr et al., 1995). Vesicles were prepared according to the procedure of Huang (1969). The specific indicator used in the preparation depended on whether the vesicles were destined for steady-state fluorescence or transient aborption measurements.

For steady-state fluorescence measurements, 5 mL of dipalmitoylphosphatidylcholine (DPPC) (Sigma) solution (2 mM in 95% ethanol) and a hypocrellin/ethanol solution (1 mg/mL) were mixed and then evaporated to dryness on a rotovap. One milliliter of a 0.12 M NaCl/0.03 M NaN_3 solution, in which 1 mg of 2′,7′-bis(2-carboxyethyl)-5(and 6)-carboxyfluoroscein (BCECF) (Molecular Probes) was dissolved, was added to the dry product; and the solution was heated to 10 °C above the DPPC transition temperature (54−56 °C) until all of the DPPC/hypocrellin/indicator mixture was suspended. NaN_3 was introduced to scavenge oxygen and thus to obviate singlet oxygen production. Vesicles were formed by sonicating the resulting suspension until it was optically clear, using either a Cole Palmer Model 8890 bath sonicator for approximately 1.5 h or a Fisher Sonic Dis-

membrator Model 300 fitted with a microtip for 40 min. BCECF that was not entrapped inside the vesicle was removed by passing the vesicle system over a size-exclusion column (Sepharose 4B).

For time-resolved mesurements, 5 mL of the DPPC solution, a hypocrellin/ethanol solution (1 mg/mL), and a 3-hexadecanoyl-7-hydroxycoumarin (Molecular Probes)/ethanol solution (1 mg/mL) were mixed and then evaporated to dryness on a rotovap. Two milliliters of a 0.12 M NaCl/0.03 M NaN_3 solution was added to the dry product and the solution was heated to 10 °C above the DPPC transition temperature until all the DPPC/hypocrellin/indicator mixture was suspended. Vesicles were prepared as described above. Since, however, all of the indicator is assumed to be partitioned into the bilayer, the system was not passed over a size-exclusion column.

Steady-state fluorescence excitation spectra were obtained on a SPEX Fluoromax. For steady-state pH experiments, hypocrellin A was excited by a 300-W tungsten bulb fitted with 575-nm cutoff filters. Background light with the bulb on was less than 0.3% of the signal. The visible power available at the cuvette was 8−9 mW. Steady-state fluorescence excitation spectra were also corrected by subtracting a blank of the difference in spectra collected with lamp on and lamp off of BCECF alone in vesicles. Time-resolved absorption data were obtained with the microsecond flash photolysis system (Fehr et al., 1995) made available to us by Professor J. H. Espenson. Kinetic traces were the average of 4 shots. The excitation pulse had a duration of ~600 ns and an energy of ~70 mJ at 490 nm. The picosecond kinetic traces displayed in Figure 2 were obtained with the apparatus described elsewhere (Gai et al., 1993, 1994a,b). The transients observed cannot be attributed to another process such as the generation of solvated electrons. The solvated electron absorbs strongly at 600 nm (Bent & Hayon, 1975). The signal observed at 600 nm (Figure 2a) is stimulated emission, consistent with the decay of an excited state. Also, care was taken to ensure that experiments were performed using pump intensities sufficiently low to avoid nonlinear optical phenomena.

RESULTS AND DISCUSSION

Table 1 compares the antiviral activity of hypericin and hypocrellin A under hypoxic and aerobic conditions, as well as under different serum concentrations. The results indicate that, as we observed previously, hypericin possesses significant antiviral activity both in the presence and in the absence of oxygen. In three trials hypocrellin A exhibited minimal, if any, antiviral activity in the absence of oxygen. The differences in efficiency of virucidal activity, which are evident under hypoxic conditions, suggest that hypericin possesses alternate or additional mechanisms of action. The absence of virucidal activity in hypocrellin under hypoxic conditions also provides a further verification of the extent of deoxygenation provided by our experimental protocol.

Table 1 also shows that the serum concentration has negligible effect under either hypoxic or aerobic conditions. The serum concentration was varied to evaluate whether the increased solubility afforded by its increase would positively affect the hypoxic experiment. Since hypericin and hypocrellin A are very hydrophobic, the concentration of the chromophore in the virus membrane is determined by the initial mixing of the phosphate-buffered saline (PBS), the

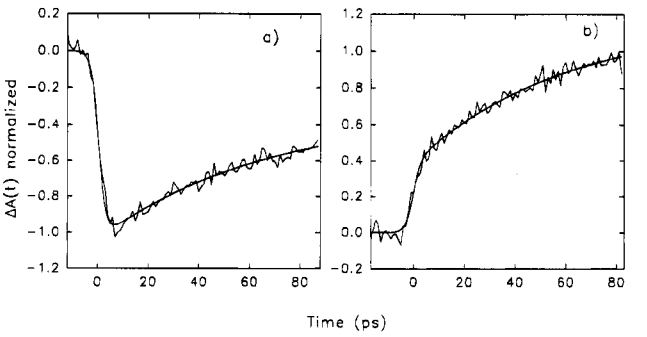

FIGURE 2: Stimulated emission (λ_{probe} = 600 nm) (a) and induced absorption (λ_{probe} = 570 nm) (b) of hypocrellin in ethanol. The pump wavelength is 588 nm. The finite rise time of the induced absorbance equals the decay time of the stimulated emission within experimental error: ~80 ps. An interpretation of these data is that the excited state (monitored by the stimulated emission) is produced instantaneously and executes a proton transfer with an ~80-ps time constant. The rise of the induced absorption can be attributed to the appearance of the excited-state tautomer.

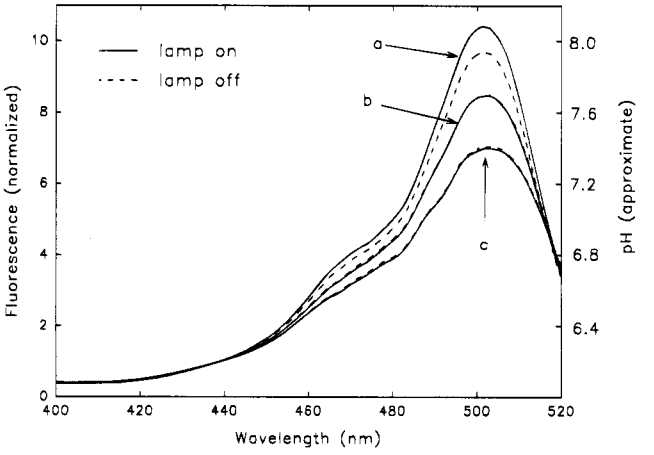

FIGURE 3: Steady-state acidification by hypericin or hypocrellin A of DPPC vesicle interior as probed by the pH indicator BCECF. A decrease in fluorescence of BCECF indicates an increase in the proton concentration. Chromophores were excited with a 300-W tungsten lamp fitted with cutoff filters ($\lambda \geq 575$ nm) to ensure that only they were excited. Fluorescence (as fluorescence excitation) was collected at 535 nm and normalized at the isosbestic point of 439 nm to account for dye degradation and leakage. Data are presented as pairs of curves with solid lines representing the system *without* any excitation source (lamp off) and with dotted lines representing the system *with* an excitation source (lamp on). (a) Photoinduced acidification by hypericin. (b, c) Lack of photoinduced acidification by hypocrellin A.

virus, and the chromophore/dimethyl sulfoxide (DMSO) solution. We hypothesized that, by varying the amount of serum, we might more efficiently incorporate the chromophore into the viral membrane and consequently obtain higher virucidal activity. The results in Table 1 indicate, however, that 0 or 10% additional serum provide comparable results.

Hypericin executes an excited-state intramolecular proton transfer on a time scale of 6−12 ps (Gai et al., 1993, 1994a,b), and Figure 2 indicates that hypocrellin behaves similarly, although the intramolecular proton transfer event is significantly slower. *We have previously reported that hypericin affords photoinduced acidification and that this may play a role in its antiviral activity. Much to our surprise, hypocrellin A does not display similar intermolecular proton transfer behavior under steady-state conditions (Figure 3).* Similarly, we do not observe any evidence

of transient acidification in microsecond experiments (not shown). We are careful in interpreting these results not to conclude that hypocrellin A is incapable of acidifying its surroundings. It is possible that under these experimental conditions one cannot observe such a protonation event. For example, hypocrellin A has slightly different solubility properties from those of hypericin. Hypericin is soluble in some polar protic and aprotic solvents, and it is insoluble in nonpolar solvents. On the other hand, hypocrellin A is soluble in a wider range of polar protic and aprotic solvents as well as in some nonpolar solvents such as cyclohexane and benzene. Consequently, the absence of observed acidification may be a result of an orientation of hypocrellin A in the vesicle that impedes excited-state intramolecular proton transfer to the indicators, as we have placed them.

CONCLUSIONS

Hypericin clearly has multiple modes of light-induced antiviral activity, one of which involves the production of singlet oxygen. Previously we had reported that hypericin is also active under hypoxic conditions and had speculated that this mode of action involved excited-state proton transfer. Optimum pH values are important in the life cycles of many enveloped viruses, including influenza virus (Bullough et al., 1994) and paramyxoviruses (Zhirnov, 1990), and it is possible that hypericin-induced proton transfer disrupts a critical stage during the viral life cycle (Fehr et al., 1994, 1995). Finally, we cannot exclude as an antiviral mechanism the ability of hypericin to perform oxidation−reduction chemistry (Redepenning & Tao, 1993). Given the gross similarities between the structures of hypocrellin A and hypericin, it is surprising that hypocrellin A absolutely requires oxygen for antiviral activity and does not produce observable intramolecular excited-state proton transfer under our experimental conditions. The contrast with hypericin is instructive.

It appears that the more complicated and extended structure of hypericin has a much more important role in its antiviral activity than merely to serve as a substrate for hydroxyl and carbonyl groups. The data suggest that the hypericin structure greatly influences its preferential solubility for the viral membrane and that it may play an important role in its ability to shuttle a proton away from itself. With regard to this latter point, previous steady-state work (Diwu et al., 1989) and preliminary time-resolved work from our laboratory suggest that a large percentage of hypocrellin A is already tautomerized in the ground state. If this is so, it is likely that exposure to light merely regenerates the original, untautomerized form (Figure 1a). Furthermore, the absence of a second hydroxyl group β to the carbonyl group may hinder charge separation that would be required in order to deliver the proton to the solvent, an external pH indicator, or, for example, a capsid protein of the virus (Meruelo et al., 1992; Fehr et al., 1994). The results presented here indicate the utility of studying hypericin analogs in unravelling the origins and the mechanisms of the light-induced antiviral activity of hypericin.

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