Light-Induced Acidification by the Antiviral Agent Hypericin

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Keywords
antivirus agent, hypericin, acidification, antiviral activity, electron transport, fluorescence, light

Disciplines
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Light-Induced Acidification by the Antiviral Agent Hypericin

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Abstract: The naturally occurring polycyclic quinone hypericin possesses light-induced antiviral activity against the human immunodeficiency virus (HIV) and other closely related enveloped lentiviruses such as equine infectious anemia virus (EIAV). We have previously argued that hypericin undergoes a fast proton transfer reaction in its singlet state (J. Phys. Chem. 1994, 98, 5784). We have also presented evidence that the light-induced antiviral activity of hypericin does not depend upon the formation of singlet oxygen (Bioorg. Med. Chem. Lett. 1994, 4, 1339). It is demonstrated here that steady-state illumination of a solution containing hypericin effects a pH drop. When hypericin and an indicator dye, 3-hexadecanoyl-7-hydroxycoumarin, are both imbedded in vesicles, hypericin transfers a proton to the indicator within a time commensurate to its triplet lifetime. 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.

Introduction

Hypericin (Figure 1) possesses light-induced antiviral activity against the human immunodeficiency virus (HIV) and other closely related enveloped lentiviruses such as equine infectious anemia virus (EIAV). Hypericin has a large triplet yield (0.70 in ethanol) and is capable of generating significant quantities of singlet oxygen. It has up till now been assumed that the virucidal activity of hypericin is a result of its production of singlet oxygen. We, however, have recently reported that oxygen is not required for antiviral activity. On the other hand, solutions of the chromophore of the photoreceptor of the protozoan ciliate S. coeruleus, which is very similar both structurally and spectrally to hypericin, produce a pH decrease upon optical excitation. We have argued that hypericin undergoes excited-state proton transfer in its singlet state and that, consequently, it possesses labile protons. We have suggested that the virucidal activity of hypericin may be related to its ability to acidify its environment upon optical excitation; we have proposed chemical methods of illuminating hypericin for antiviral therapies. Given the potentially great importance of photogenerated protons from hypericin as an antiviral or antitumor therapy, the present work was performed in order to determine the nature (singlet or triplet) of the source of the photogenerated proton. These studies were undertaken largely in phospholipid vesicles suspended in aqueous medium in order both to circumvent the insolubility of hypericin in water as well as to provide a simplified model of the viral membrane, within which hypericin is thought to partition.

Experimental Section

In order to observe and measure the deprotonation of hypericin with, for example, a pH indicator dye, it is necessary that the proton donor and the acceptor be in close enough proximity so that the proton transfer event can be efficiently observed. Since hypericin is insoluble in water from pH 2 to 11, a system that takes all of these factors into account is provided by optically clear phosphatidylcholine vesicles, such as dipalmitoylphosphatidylcholine (DPPC), suspended in aqueous buffer. Hypericin is soluble in the vesicle bilayer; although hypericin is hydrophobic, a portion of the hypericin population may be reasonably assumed to orient so that the ejected protons are available to the bulk solvent. The vesicles have been prepared by the method described by Huang and are expected to have an outer diameter of ~250-300 Å and an inner diameter of ~120 Å. Huang reports a maximum bilayer dimension of 73 Å, of which ~30 Å corresponds to the hydrocarbon region where hypericin is assumed to be located. The X-ray structure of hypericin indicates that it has a long axis of 10.5 Å and a short axis of 9.6 Å. Consequently, single hypericin molecules are not capable of spanning the bilayer.

The pH indicator dyes used here are incorporated either in the aqueous interior of the vesicle or in the hydrocarbon portion of the lipid bilayer. Two indicators were used to probe the deprotonation of hypericin. BCECF (2,7′-bis(2-carboxyethyl)-5-(and 6)-carboxyfluorescein) was obtained from Molecular Probes and used exclusively in the steady-state experiments. The second indicator, used for the time-resolved absorption experiments, was the lipophilic pH indicator 3-hexadecanoyl-7-hydroxycoumarin (Molecular Probes). All procedures discussed below were carried out under subdued lighting. All solutions were stored in the dark and were usually purged with argon.

(13) Huang, C. Biochemistry 1969, 8, 344.

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The specific indicator used in the preparation depended on whether the vesicles were destined for steady-state fluorescence or transient absorption measurements. DPPC (Sigma) was dissolved in 95% ethanol and then evaporated to dryness on a Rotovap. Two milliliters of a 0.12 M NaCl/0.03 M NaN₃ solution were 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 interior of the vesicle was removed by passing the vesicle system over a size exclusion column.

Steady-state fluorescence excitation spectra were obtained on a SPEX Fluoromax. For steady-state pH experiments, hypericin was excited by a 300-W tungsten bulb fitted with 575-nm cut-off filters. Background light with the bulb on was less than 0.3% of the signal. Light available at the cuvette was 8–9 mW. Time-resolved absorption data were obtained with the microsecond flash photolysis system,\(^{(15)}\) generously made available to use by Professor J. H. Espenson and Dr. A. Bakac. 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. Steady-state absorption spectra were recorded on a Shimadzu UV-2101PC.

Results and Discussion

A. Hypericin Produces a Light-Induced pH Drop. Steady-State Measurements. Figure 1 presents the steady-state absorption and fluorescence spectra of hypericin in DPPC vesicles in water at pH 8.4. Hypericin is insoluble in pure water from pH 2 to 11, where it forms aggregates.\(^{(16)}\) The steady-state spectra resemble those of hypericin in DMSO, which indicates that aggregation is not occurring. In DPPC vesicles, the absorption maximum of hypericin is 598 nm and the fluorescence emission maximum is 599 nm. The spectra at pH 5.9 and pH 8.4 are essentially identical.

Figure 2 demonstrates the ability of hypericin to acidify a solution of the indicator dye BCECF and hence its capacity to produce a light-induced pH drop much like the structurally and spectrally analogous stentorin chromophore.\(^{(17)}\) BCECF possesses four to five negative charges at pH values between 6.5 and 7.5, which are responsible for its retention in the aqueous interior of the vesicle. BCECF possesses pH-dependent emission and absorption spectra. Light-induced pH changes were monitored by collecting fluorescence excitation spectra of BCECF at an emission wavelength of 535 nm. The presence of the isobestic point at 439 nm (which corresponds to an isoemissive point, assuming the exact equivalence of the absorption and fluorescence excitation spectra) facilitates accounting for dye degradation and for dye leakage from the vesicles.

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Figure 2. Fluorescence excitation spectra of BCECF (0.52 µM) entrapped in DPPC vesicles. Hypericin is dissolved in the lipid bilayer at a concentration of 23 µM. Solid lines (—) denote the indicator spectrum in the absence of hypericin illumination; dashed lines (— — —), in the presence of hypericin illumination. See Experimental Section. Three sets of experiments are depicted in the figure: (a) initial pH = 8.1; (b) initial pH = 7.5; (c) initial pH = 6.45.

Of the three separate sample preparations, at different initial pH values, a maximum pH change of 0.5 unit is observed. It is important to note that this pH change neither reflects the total number of protons ejected by hypericin nor the macroscopic pH of the solution. Rather, it is a measure of the number of protons detected by the indicator. Here, it is useful to consider the following. In a single small unilamellar vesicle (SUV) there is a 50-fold excess of hypericin to BCECF. Not all of the hypericin, however, is necessarily located in a region where dissociated protons can enter the vesicle interior. Because the ratio of phosphatidylcholine between the inner and the outer vesicle bilayer is roughly 1:1.5, hypericin may partition into the outer portion of the vesicle bilayer, away from the interior. It is also likely that one or both of the following occurs: either the majority of the dissociated protons recombine with the parent hypericin anion within the bilayer and never protonate the indicator or the dissociated protons escape from the parent anion but remain undetected because they are released to the bulk solvent where no indicator is present.

B. A Source of the Photogenerated Proton. Time-Resolved Measurements. In order to determine from which excited electronic state the proton originates, time-resolved measurements were required. The lipophilic indicator, 3-hexadecanoyl-7-hydroxycoumarin, was most suitable for time-resolved absorption measurements (given the available experimental apparatus) because the extinction coefficient of its unprotonated form in the region from 400 to 430 nm is larger than that of hypericin and because its extinction coefficient at the laser excitation wavelength (490 nm) is very small (Figure 3).

The decay of the absorbance due to the hypericin triplet in vesicles at 505 nm is presented in the inset to Figure 1. This decay is biphasic with time constants of 10 and 75 µs. It is reasonable to attribute the biphasic decay to various orientations of hypericin in the vesicle bilayer. (The variation in the lifetimes is to be expected given the distribution of vesicles in a given preparation.) Figure 4a presents the transient at 400 nm of the 3-hexadecanoyl-7-hydroxycoumarin indicator subsequent to excitation of hypericin at 490 nm. The transient is a bleach whose recovery is represented by two time constants: 32 and 170 µs. This signal is interpreted as proton transfer from hypericin to the indicator on a time scale commensurate to that of the lifetime of triplet hypericin. It is reasonable for the proton transfer event to be so rapid. Diffusion of the proton is not expected to rate limiting (assuming the diffusion constant of the proton to be that in water \(9.3 \times 10^{-5} \text{cm}^2/\text{s}\). Protoneation of the anionic form of the indicator decreases its population and consequently reduces its absorbance at 400 nm. The persistent bleach of the indicator at long times can be attributed to the slow reestablishment of equilibrium between the acidic and basic forms of the indicator, as is observed in other systems.

Although hypericin has a ground-state absorption at 400 nm (Figure 1), this signal cannot be attributed to the ground-state of hypericin for the following reasons: 1. At 400 nm the extinction coefficients of the indicator and hypericin are 35 900 and 10 100 M⁻¹ cm⁻¹, respectively; the molar ratio of indicator to hypericin is approximately one to one. 2. The time constants for the bleaching recovery in the presence of the indicator are longer than those observed for hypericin alone in vesicles. 3. Most importantly, at long times the transient at 400 nm for hypericin alone in vesicles yields a net absorption, whereas in the presence of indicator on the same time scale the bleaching has not yet recovered.

Furthermore, the signal cannot arise from the indicator itself since 3-hexadecanoyl-7-hydroxycoumarin exhibits no transient absorption at 400 nm (Figure 4a).

Another confirmation of the trace in Figure 4a to a transfer of a proton from hypericin to the indicator is based on the reasoning that if the long component of the kinetic trace representing a persistent bleach is due to the protonation of the indicator by hypericin, performing the experiment at a pH where the indicator is already completely protonated ought to replace this persistent bleach with a net absorption at long times, which is characteristic of the control experiment using hypericin alone. This result is in fact observed (Figure 4b).

Finally, a goal of this work is to determine if the proton is ejected from the triplet or singlet state. The triplet yield of hypericin in ethanol and in BRIJ 35 micelles is ~0.70. Molecular oxygen efficiently quenches triplet hypericin to form

Figure 4. (a) Transient absorption at 400 nm of 3-hexadecanoyl-7-hydroxycoumarin subsequent to excitation of hypericin at $\lambda_{ex} = 490$ nm. Both the indicator (23.3 $\mu$M) and hypericin (21.0 $\mu$M) are contained in the bilayer of DPPC vesicles at pH 8.4. The decrease in the anionic form of the indicator owing to excited-state protonation by hypericin was monitored by a transient reduction of the induced bleach of the anionic form of the indicator at 400 nm. $\Delta A(t) = -0.012 \exp(-t/32 \mu s) - 0.0050 \exp(-t/170 \mu s)$. The control experiment for hypericin alone in vesicles at pH 8.3 yields a trace that is fit to the form $\Delta A(t) = -0.0062 \exp(-t/15 \mu s) - 0.0067 \exp(-t/55 \mu s) + 0.0080$. A second control experiment using only the indicator alone in vesicles at pH 8.3 yields the trace about zero. This trace demonstrates that in the absence of hypericin no transient absorption is induced in the indicator at 400 nm subsequent to excitation at 490 nm. (b) Induced bleaching and its recovery at 400 nm for 3-hexadecanoyl-7-hydroxycoumarin and hypericin in DPPC vesicles at acidic and basic pH; $\lambda_{ex} = 490$ nm. At pH 5.7, $\Delta A(t) = -0.0030 \exp(-t/9.4 \mu s) - 0.0099 \exp(-t/56 \mu s) + 0.00080$. At pH 8.2, $\Delta A(t) = -0.0045 \exp(-t/26 \mu s) - 0.0065 \exp(-t/130 \mu s)$. (c) Induced bleaching and its recovery at 400 nm for 3-hexadecanoyl-7-hydroxycoumarin and hypericin in DPPC vesicles in oxygenated and deoxygenated solution; $\lambda_{ex} = 490$ nm. The absence of a signal in the oxygenated sample is taken as proof that the triplet state of hypericin is responsible for the protonation event. (Under oxygen levels at which the signal is quenched, hypericin is still fluorescent and the indicator absorption spectrum remains unchanged. Consequently the absence of the signal cannot be a result of quenching the singlet state or of destruction of the indicator.) The transient signal for the deoxygenated sample is described well by $\Delta A(t) = -0.0045 \exp(-t/26 \mu s) - 0.0065 \exp(-t/130 \mu s)$.

Conclusions

Our previous picosecond experiments provide strong evidence for intramolecular proton transfer in the excited singlet state of hypericin and suggest that hypericin is a source of light-induced protons. That hypericin does indeed produce a light-induced pH drop is demonstrated by steady-state experiments (Figure 2). Flash photolysis experiments on the microsecond time scale using molecular oxygen as a triplet quencher indicate that the triplet state of hypericin is a proton donor (Figure 4). No conclusions concerning the role of the first excited singlet state of hypericin as a proton donor can be drawn since the time-resolved measurements discussed above cannot detect rapid protonation and deprotonation equilibria between the donor and the acceptor. It is likely that much will be learned by studying the detailed interactions of hypericin with the viral membrane, which have been crudely mimicked here by vesicles.

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