Time-Resolved Fluorescence Studies of Hematoporphyrin in Different Solvent Systems

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Time-resolved fluorescence studies of hematoporphyrin in aqueous solution and in different organic solvents are presented. The observation of two exponential components in the fluorescence decay in aqueous solution reveals the presence of a monomeric and aggregated (probably dimeric) form of the molecule, as confirmed by absorption and fluorescence measurements. The slow component (~ 15 ns in aqueous solution) and the fast one (~ 3.8 ns in aqueous solution) are attributed to monomers and dimers, respectively. Higher aggregated species of hematoporphyrin are also present, which are essentially devoid of fluorescence properties.

Introduction

Hematoporphyrin (HP) is largely used in photodynamic systems for its high photosensitizing activity [1] and for its effectiveness in inducing the regression of different kinds of tumors both *in vitro* [2] and *in vivo* [3]. Recent investigations pointed out that the photosensitizing efficiency of HP is controlled by various parameters, of which the polarity of the surrounding medium [4–6] and the aggregation state of HP [7] play a major role.

The molecular events underlying such effects are largely unknown. Thus, it has been suggested that aggregation appreciably shortens the lifetime of triplet protoporphyrin IX [8], whereas only minor difference in triplet lifetime have been detected between aggregated and monomeric HP (Spikes and Jori, unpublished results). Therefore, it appears of interest to perform detailed photophysical investigations about the influence of the above mentioned factors on the properties of the electronically excited states of HP.

In this paper, results of time-resolved fluorescence spectroscopy of the excited singlet state S_1 of HP are reported. The dependence of the fluorescence decay time on the solvent is studied. An aggregated form, whose relative amount increases with the HP concentration, is shown to exist in aqueous solution and its excited-state lifetime turns out to be shorther than that of the monomeric molecule.

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Materials and Methods

Chemicals

Hematoporphyrin, free base, was a product of Sigma Chemical Co., and it was characterized as described elsewhere [9]. Our sample, when dissolved in 1 M H_2SO_4, gave an absorbance value $E_{1\%} = 7.19 \times 10^3$ in agreement with literature values [10]. Sodium dodecylsulphate (SDS) and cetyltrimethylammonium bromide (CTAB) were obtained from Aldrich Chemical Co., Triton X-100 (alkylphenylpolyethylene glycol) was obtained from Merck. The organic solvents used were spectroscopic grade samples obtained from Merck. All other chemicals were commercially available reagent-grade products.

Preparations of solutions

All samples used for the fluorescence measurements were prepared by suitable dilution of a stock HP solution (1 mM) in either phosphate-buffered aqueous solution at pH 7.4 or absolute methanol and ethanol. Typically, a 2 μM HP solution in 10% aqueous methanol was obtained by adding 20 μl of either aqueous or methanolic stock solution to 10 ml of 10% methanol; control experiments showed that the fluorescence results were independent of the

nature of the original stock solution. The aqueous dispersion of HP-containing surfactants were prepared by adding suitable aliquots of a 1 mM HP solution in 0.1 M Tris at pH 10 to 10 ml of the same solvent containing 20 mg of surfactant per ml.

Unless otherwise stated, all solutions were air-equilibrated. Anaerobic samples were prepared by thorough bubbling with prepurified nitrogen.

Spectroscopic studies

Absorption spectra were monitored by a Perkin-Elmer 576 spectrophotometer using matched quartz cuvettes of 0.1 cm or 1 cm optical path. Difference absorption spectra were recorded at room temperature; the baseline was obtained by reading equimolar HP solutions in CTAB-containing systems in both the sample and reference beams.

Fluorescence emission spectra were recorded at 20° ± 1 °C by a Perkin-Elmer MPF 4 spectrophotofluorimeter. The emission was observed at 90° to the incident beam using 7 nm- and 8 nm-slits for the excitation and emission light, respectively. All measurements were obtained with 405 nm-excitation; when necessary, the solutions of HP were placed in quartz capillary tubes of 0.1 cm optical path to minimize optical artifacts. The emission was registered at wavelengths above 580 nm. Fluorescence quantum yields of HP in different media were estimated by using a value of 0.15 for the emission quantum yield of HP in acetic acid as a standard [1]. The experimental data were corrected for inner filter effects [11].

Time-resolved fluorescence measurements

A nitrogen-pumped dye laser of special design [12], was used as excitation source for the time-resolved fluorescence measurements. The dye laser provides pulses of 150 ps duration (FWHM, full width at half-maximum) with peak power of ~ 50 kW at a repetition rate of up to 100 Hz. Different dye solutions (α-NPO, [2-(1-Naphthyl)-5 phenyloxazole] 10^{-3} M in ethanol, Coumarin 500 (Exciton Chemical Co.) 2×10^{-3} M in ethanol and Fluorescein Disodium Salt (Eastman number 735) 3×10^{-3} M in ethanol were used as lasing media to generate the wavelengths 405, 494, and 544 nm, respectively. The laser beam was passed through the sample cell, consisting of a quartz cuvette with windows as the Brewster angle. The fluorescence was observed at 90 °C through either a Jarrel Ash mod. 82–410 spectrograph with 250 μm slits or Corion interference filters of 5 nm bandwidth or Kodak-Wratten cut-off filters. The fluorescence signal was detected by a Varian 154 M photomultiplier (400 ps FWHM response) and averaged over many repetitions of the excitation pulse by a home-made digital signal averager with ~ 100 ps resolution [13]. The averaged data were then transferred to a Tektronix 4051 Graphic System for processing and plotting.

Results

Time-resolved fluorescence of HP in aqueous solution

The fluorescence decay of HP was measured at several porphyrin concentrations (0.1–100 μM) in aqueous solutions buffered at pH 7.4. The fluorescence was excited at three different wavelengths in the visible region of the absorption spectrum of the porphyrin, namely 405, 494 and 544 nm, and observed at 610 and 675 nm, *i.e.* in each of the two emission peaks of HP, or through a cut-off filter ($\lambda > 570$ nm). A typical decay observed through the cut-off filter for 2 μM HP upon excitation with 405 nm light is shown in Fig. 1. Statistical analysis of the experimental curves by the least-square method yielded exponential components with time-constants 3.94 and 15.40 ns and relative initial amplitudes 6.5% and 93.5%, respectively (Table I). The data collected after 405 nm – excitation were found to be independent of the observation wavelength. The 494 and 544 nm excitations gave results similar to those obtained at 405 nm.

Upon increasing the HP concentration, the relative amplitude of the fast component was found to be enhanced. Table I shows the relative amplitudes and the corresponding decay constants of the two exponential components at different HP concentrations after excitation at 405 nm. It must be noted that no appreciable changes of the shape of the cw emission spectrum have been observed as a function of both HP concentration and excitation wavelength.

Fluorescence lifetimes in different solvent systems

For 2 μM HP in water-organic solvent mixtures, excited at 405 nm, only one exponential component was observed in the fluorescence decay. Changing

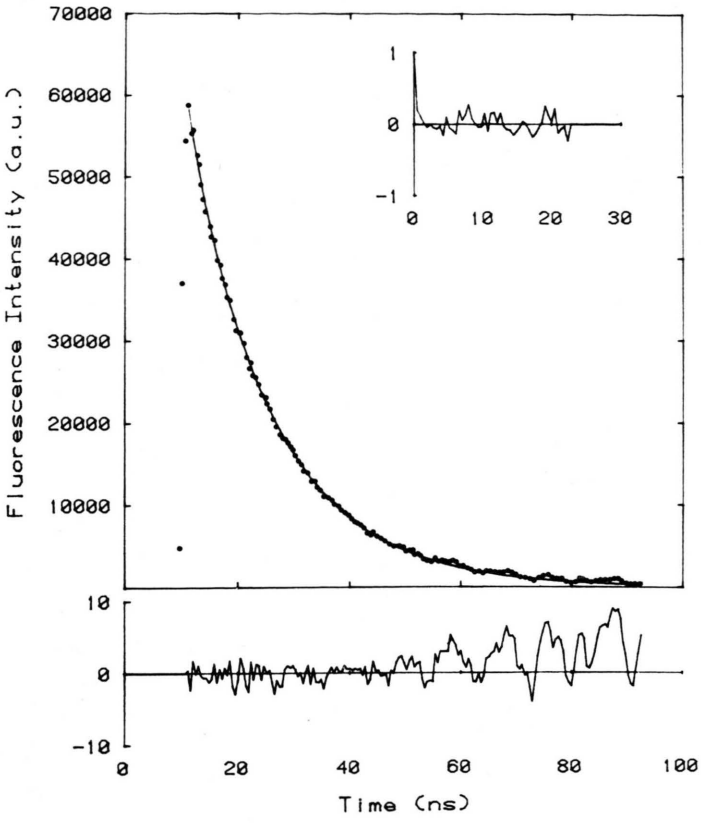

Fig. 1. Experimental fluorescence decay curve of 2 μM HP in buffer pH 7.4 (dots) and two-exponential component fit ($\tau_1 = 15.40$ ns, $\tau_2 = 3.94$ ns) (unbroken line). The weighted residuals (bottom) and their autocorrelation function (top) are shown to evidence the quality of the fit.

the medium by using different solvent mixtures had slight effect on the fluorescence decay time, as shown in Table II. This behaviour was tested with solvents of dielectric constants both lower (methanol and ethanol) and higher (formamide) than that of water. In particular, a decrease of the fluorescence lifetime was observed in absolute methanol and ethanol solutions as compared with water-organic solvent mixtures. Such a decrease was no longer detected if the solutions were deaerated prior to measurements: *e.g.*, the fluorescence lifetime of 2 μM HP in deaerated absolute ethanol was found to be 17.8 ns. Thus, quenching by oxygen, which has a greater solubility in ethanol and methanol, is responsible for this decrease.

Table I. Fluorescence decay time constants (τ_1 and τ_2) and relative initial amplitudes (A_1 and A_2) for different HP concentrations in phosphate buffer at pH 7.4. $\lambda_{exc} = 405$ nm, λ_{obs} above 570 nm.

HP concentration [μM]	τ_1 [ns]	A_1 [%]	τ_2 [ns]	A_2 [%]
2	15.40	93.5	3.94	6.5
10	15.44	89.4	3.65	10.6
25	15.04	89.0	3.54	11.0
50	15.33	78.8	3.64	21.2
75	14.95	78.5	3.80	21.5
100	15.52	70.3	4.07	29.7

For 100 μM HP, the fluorescence decay could be fitted by a single exponential component only in absolute organic solvents. The fast component could be detected (Table II) when a significant amount of water was present in the solvent mixtures.

It is to be remarked that HP, which exists as a stacked aggregated in predominantly aqueous media, is converted to a monomeric species by addition of organic solvents; for 83 μM HP the deaggregation process is virtually complete in 30% methanol or formamide [7].

Fluorescence lifetime and absorption spectra in aqueous micellar dispersions

Hp is known to be incorporated in a monomeric form into SDS, CTAB and Triton X-100 micelles

Table II. Fluorescence decay time constants (τ_1 and τ_2) and relative initial amplitudes (A_1 and A_2) for different HP concentrations in various air-equilibrated solvent mixtures. λ_{exc} = 405 nm, λ_{obs} above 570 nm.

HP concentration [μM]	Organic solvent	Percentage of organic solvent	τ_1 [ns]	A_1 [%]	τ_2 [ns]	A_2 [%]
2	methanol	10	16.24	100	–	–
2	methanol	30	16.79	100	–	–
2	methanol	90	14.28	100	–	–
2	methanol	100	12.33	100	–	–
2	ethanol	100	12.95	100	–	–
2	formamide	10	16.39	100	–	–
2	formamide	90	17.14	100	–	–
100	methanol	10	15.73	83.0	4.18	17.0
100	methanol	30	16.66	88.4	4.06	11.6
100	methanol	90	14.28	91.2	4.06	8.8
100	methanol	100	11.95	100	–	–
100	ethanol	100	12.61	100	–	–
100	formamide	20	16.47	85.3	3.80	14.7
100	formamide	90	16.41	100	–	–

[6]. In one set of experiments the surfactant concentrations were such that about 5% of the micelles contained one HP molecule. Also in these systems, excitation of 2 μM HP with 405 nm-light resulted in a single exponential decay of the fluorescence; the measured time-constants (Table III) were slightly different for each micellar type, possibly owing to the influence of the polar head-group at the micellar surface on the π-electron cloud of the micelle-solubilized HP [14]. In another set of experiments, the HP concentration was increased up to 50 μM in SDS dispersions. No change in the fluorescence decay was observed (Table III).

The incorporation into a micelle induces significant alterations in the HP absorption spectrum [6]. Such changes can be evidenced by difference absorption spectroscopy. Thus, when 2 μM HP in 0.1 M Tris aqueous solution was read against equimolar CTAB-solubilized HP, hypochromicity in the Soret absorption region was observed, with a pronounced negative maximum laying at 408 nm (Fig. 2). A closely similar difference spectrum was obtained by readings SDS-solubilized versus equimolar CTAB-solubilized HP (Fig. 2), although the extent of spectral changes was appreciably smaller. No difference spectrum was detected by comparing Triton-solubilized versus CTAB-solubilized HP.

Table III. Fluorescence decay time constant of HP at different concentrations in various micellar systems. λ_{exc} =405 nm, λ_{obs} above 570 nm.

HP concentration [μM]	Micellar system	τ [ns]
2	CTAB	13.09
2	TRITON	18.46
2	SDS	16.61
10	SDS	16.81
25	SDS	16.78
50	SDS	17.02

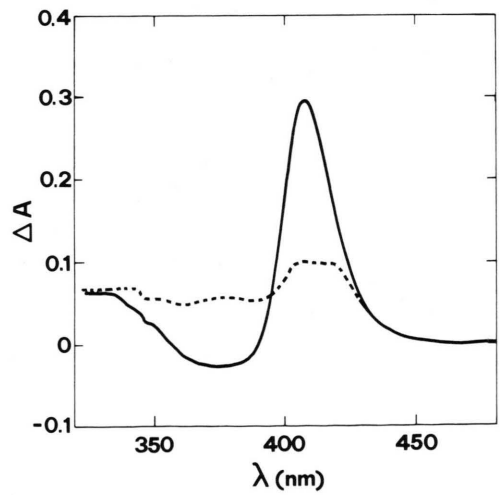

Fig. 2. Difference absorption spectra (ΔA) obtained by reading a 2 μM HP solution in 0.1 M Tris pH 10 (unbroken line) or in 20 mg/ml SDS aqueous dispersion pH 10 (broken line) against equimolar HP in an aqueous dispersion of 20 mg/ml CTAB. Optical pathlength: 1 cm.

Fluorescence quantum yield studies

The fluorescence emission maxima and quantum yield of HP at different concentrations in aqueous solution, in absolute methanol and in aqueous dispersion of SDS micelles are shown in Table IV. HP concentrations higher than 50 μM in SDS-containing media were not tested, owing to the possibility of non-uniform distribution of HP inside the micelles. Clearly, the quantum yield of HP strongly decreased in the 2–50 μM concentration range in phosphate-buffered aqueous solutions. Moreover, a decrease of the quantum yield was observed upon excitation in a spectral region where aggregated species predominantly absorb (*e.g.* 360 nm). On the contrary, no significant change of the quantum yield was observed over the same concentration range in aqueous SDS dispersion and up to 100 μM HP in absolute methanol.

The fluorescence intensity of 2 μM HP for a given excitation power in both anhydrous and 90% methanol solutions was found to decrease monotonically, if the temperature was gradually raised from 5 to 35 °C (Fig. 3). On the contrary, in aqueous solution buffered at pH 7.4, the emission intensity of HP was enhanced by a gradual increase in the temperature (Fig. 3). Only at temperature higher than about 35 °C, a flattening and a subsequent decrease of the plot was observed.

The behaviour of HP in aqueous solutions is clearly abnormal, since increasing the temperature is expected to reduce the emission quantum yield of a fluorescent species as a consequence of the enhanced probability of non-radiative decay from the excited singlet state [15].

Table IV. Fluorescence properties of HP in different solvents (λ_{exc} = 405 nm).

Medium	HP concentration [μM]	Emission λ_{max} [nm]	Emission quantum yield[a]
Phosphate buffer pH = 7.4	2	613, 674	0.32
	50		0.13
	100		0.11
100% Methanol	2	625, 689	0.27
	50		0.26
	100		0.26
SDS 20 mg/ml	2	620, 684	0.43
	50		0.41

[a] Estimated by using Q.Y. = 0.15 as a reference standard for HP in acetic acid solution [1].

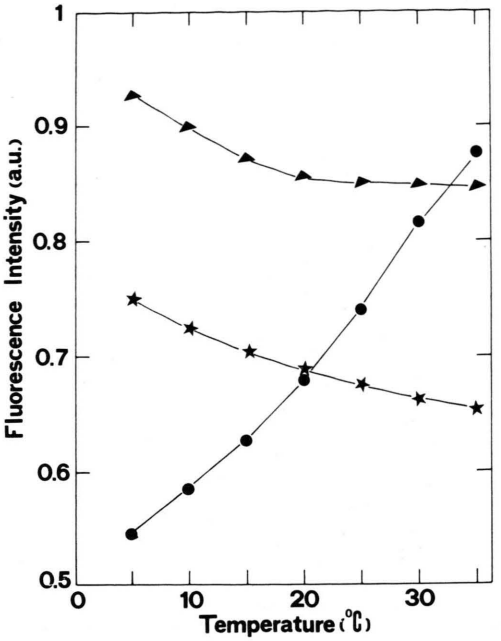

Fig. 3. The effect of temperature on the HP fluorescence emission yield in absolute methanol (▲–▲), 90% methanol (★–★), and 0.05 M phosphate buffer at pH 7.4 (●–●). The spectra were excited at 440 nm, using 7 nm and 8 nm spectral bandwiths for the excitation and emission light, respectively. Each sample was kept at the desired temperature for at least 10 min prior to measurement in order to ensure the attainment of thermal equilibrium.

Discussion

The two exponential components observed in the fluorescence decay of HP in aqueous solution for a wide range of porphyrin concentrations can be ascribed to the presence of a monomeric form, which is responsible for the slow component, and of an aggragated (probably dimeric) form, which is responsible for the fast one. The attribution of the slow component to the monomeric form is based on the fact that it is observed under all our experimental condition: in particular, it is the only component of the fluorescence decay up to 50 μM HP concentration (Table III) in the micellar systems, where HP is known to exist exclusively in the monomeric form [14]. Moreover, in phosphate-buffered aqueous solution, the relative amplitude of the fast component of the fluorescence decay increases upon increasing HP concentration (see Table I). Some discrepancies are found upon comparing the decrease of HP emission quantum yield as a function

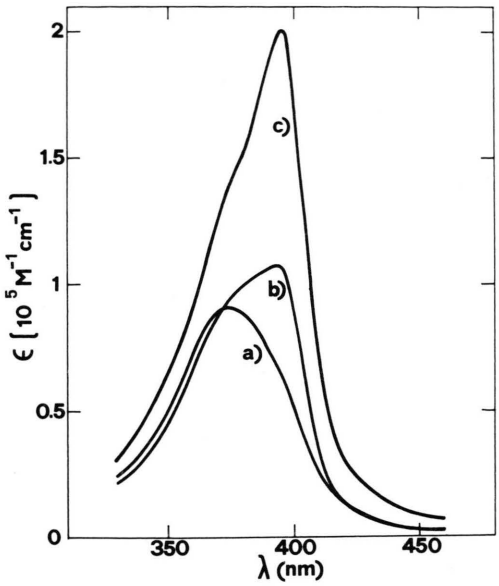

Fig. 4. Molar extinction coefficient (ε) of HP in phosphate buffer pH 7.4 at different concentrations: a) 100 μM; b) 2 μM; c) 0.1 μM.

of concentration in aqueous solution, as obtained by time-resolved* (Table I) and stady-state (Table IV) fluorescence measurements. Therefore, the assumptions underlying the calculation of the quantum yield from the data in Table I may not be valid. In particular, we were unable to fit the absorption spectral measurements at different HP concentrations in aqueous solution to an equilibrium between only two species. As one can see from the HP absorption spectra in Fig. 4, larger amounts of aggregated species are formed upon increasing the porphyrin concentration in agreement with the findings of other authors [16–17]. Likely, HP exists as a mixture of aggregates having different degrees of complexity, whose fluorescence emission is totally quenched. On these bases, we tentatively assign the observed fast decay to dimeric HP.

The amount of aggregates at a given HP concentration is reduced by the presence of organic solvents. This holds for solvents of both higher and lower polarity as compared with water. For example, the addition of 10% formamide or methanol to aqueous solutions of 2 μM causes the disappearance of the fast-decaying component (Table II). Even at a HP concentration as high as 100 μM in aqueous solution, the relative amplitude of the fast component is reduced by small percentages of organic solvent. In anhydrous methanol, no fast-decaying species is detected even for 100 μM HP; in this solvent system, the emission quantum yield is identical for 2 μM and 100 μM HP in agreement with the presence only of a monomeric species. Our interpretation is supported by the essentially identical values of the emission quantum yield for 2 μM HP in SDS aqueous dispersions (Table IV). The influence of organic solvents is also evidenced by the temperature-dependence of the HP fluorescence yield in methanol and aqueous solutions. The abnormal behaviour observed in aqueous solution in the low-temperature range (Fig. 3) should reflect the conversion of aggregates to monomers that requires some activation energy to break porphyrin-porphyrin hydrophobic interactions and, possibly, hydrogen bonds. The corresponding fluorescence enhancement overcomes the normal decrease of the fluorescence yield with increasing temperature which is observed, for example, in methanol. In conclusion, the formation of HP aggregates appears to be due to a specific unfavourable interaction of the porphyrin aromatic system with water, and to be not a consequence of a negative interaction with media of low (alcohols) or high (formamide) polarity.

* The fluorescence quantum yield (Q.Y.) is calculated from the data in Table I as Q.Y. $\propto (A_1 \tau_1 + A_2 \tau_2)$, assuming that all decaying molecules can be represented by either τ_1 or τ_2 decay constant and have the same radiative lifetime.

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