

Photophysical Properties of Hematoporphyrin and Dihematoporphyrin Ether (Ester) in Homogeneous and Microheterogeneous Environments

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Excitation wavelength effects on hematoporphyrin (HP) and hematoporphyrin derivative (HPD, Photofrin II) fluorescence spectra, quantum yields and decays were comparatively studied in homogeneous (at different pH and substrate concentrations) and microheterogeneous (Triton X100 micelles and Bovine serum albumin) aqueous environments, in which the porphyrins are expected to be present in different monomer/aggregate equilibria. The observed wavelength dependences of the fluorescence yield and of short lived contributions to the total fluorescence do not correlate to the porphyrin aggregation state obtained from absorption spectra. In micro-heterogeneous environment the influence of local interactions between the porphyrin and hydrophobic sites of the cosolutes on HP and HPD photophysics is demonstrated.

Key words: Hematoporphyrine, Hematoporphyrine derivative, Fluorescence, Fluorescence lifetime, Environmental effects.

Introduction

Photophysical and photochemical properties of hematoporphyrin derivative (HPD, obtained by chemical modifications from hematoporphyrin (HP) [1]) received considerable interest in recent years. Research was mainly stimulated by the increased use of HPD in the photochemotherapy of tumors in man [2]. Understanding of HPD properties is, however, complex, since: i) Its true composition is only partially resolved. The relative amounts of porphyrines covalently linked via ester (ether) bonds (dihematoporphyrin ester (ether), DHE) and the main monomeric components (hematoporphyrin (HP), protoporphyrin (PP), hydroxyethylvinyldeuteroporphyrin (HVD)) vary in different preparations. ii) Water-soluble porphyrins in solution are simultaneously involved in two sets of equilibria: the protolytic and the monomer/dimer/aggregate equilibrium for each ionic species present [3, 4]. All these factors can significantly influence HPD photophysical properties.

HP fluorescence and triplet yields have been shown previously to depend on the medium polarity, which determines its aggregation state [5, 6]. Smith [7] suggested that the concentration dependence of the HP fluorescence yield in solvents with high water content can be explained assuming coexistence of monomers and non-fluorescent as well as fluorescent aggregates. This statement was based on results of Andreoni et al. [5, 6], who observed at least two independent fluorescing species of HP and HPD contributing to the total fluorescence yield: one with slow fluorescence decay (lifetime of 12–16 ns), associated with monomeric HP, and a second faster decaying component (lifetime ~4 ns) associated with some aggregated species. From these data and observation of Potier et al. [4] it follows, however, that fluorescence of HPD should be mainly due to monomeric “impurities” (HP, PP, HVD, etc.) with negligible contribution from DHE. HPD has been shown to aggregate even under conditions where HP is monomeric. These properties of HPD were attributed to the higher hydrophobicity of covalently linked dimers of DHE. As HPD fluorescence is enhanced in binary water/tetrahydrofuran mixtures at high content of organic cosolvent, DHE disaggregation might take place, thus contributing to the total HPD fluorescence [8].

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Concomittant with the decrease of the fluorescence yield, the Soret absorption band (excitation into the second excited porphyrin singlet state) undergoes characteristic shifts, as porphyrines aggregate [7]. Maxima at $\lambda_{\text{max}} = 392.7 \text{ nm}$, 390 nm and 370 nm were assigned to HP monomers, dimers and aggregates, respectively. Shifts of HPD absorption maxima were found to be less pronounced [4]. Besides porphyrin disaggregation at low concentration or high content on organic phase, disaggregation was assumed to occur also in detergents. In such systems, shifts of the Soret absorption band and the fluorescence emission spectra to longer wavelengths were reported [5, 9]. Furthermore, HP and HPD monomerization is also expected in complexes with serum albumin [10–12]. This system is of practical importance, since serum albumin is involved in HP and HPD blood stream transport when HPD is applied as a photosensitizer “in vivo”.

In the present work, a parallel study on HP and HPD photophysical properties (absorption, emission and fluorescence decay) was carried out in aqueous solutions at different concentrations and pH values as well as in the presence of Triton X100 micelles and Bovine serum albumin (BSA). The main purpose was to obtain more information about fluorescence from aggregated species and its participation to the total HP and HPD fluorescence yields and lifetimes. Therefore emission yields and decays were studied, varying excitation wavelengths in the Soret absorption band in different environments.

Materials and Methods

Hematoporphyrin IX diHCl (HP) was purchased from Koch-Light Laboratories (England). HPD (Photofrin II) was supplied from Photomedica Inc. (N.J., USA)*. Both porphyrins were used without further purification. HP (MW 671) was dissolved in a small amount of 0.1 N NaOH , and the appropriate pH and concentration was then adjusted by 0.1 N HCl and 0.1 M phosphate buffer. The stock solution of HPD (MW was taken to be 1200) in physiological saline (as supplied) was dissolved in water or phosphate buffer to obtain the desired concentration. Triton X100 (Merck, FRG) was used in a concentration higher than the critical micellar point. Bovine serum albumin

(BSA, research grade) was supplied from Serva (FRG). The BSA/porphyrin ratio used in the experiments was 2:1 (mol%) to ensure porphyrin binding to the protein moiety.

Absorption spectra were registered on a Hitachi 150-20 model spectrometer (quartz cells of 1 or 0.5 cm optical depth). The emission spectra monitored at 620 nm (bandpass $\geq 15 \text{ nm}$; 22°C) were obtained under different excitation wavelengths using the spectrofluorimeter (a photomultiplier Hamamatsu, R 955) described previously [13, 14]. Time resolved fluorescence measurements were performed as described elsewhere (PMT PM 2233 B) [13, 14].

Results and Discussion

1. Absorption and Fluorescence Spectra of HP and HPD

Absorption spectra of HP in different environment are presented in Figure 1A. In alkaline solution (pH 12) the absorption spectrum (Fig. 1A, spectrum 1) shows an almost symmetrical Soret band, without the blue shifted shoulder characteristic for aggregated porphyrins. In this case only HP monomers are expected to be present in the solution. This band corresponding to the monomer is also observed in the buffered solutions (pH 7.4, spectrum 2) at almost the same wavelength. Additionally, the band for aggregated HP occurs at about 375 nm . In Triton X100 this latter band is only very weak, and the total maximum is red shifted to about 400 nm (Fig. 1A, spectrum 4). This spectral change is usually interpreted as disaggregation by the detergent [9], but should also reflect specific interactions with the hydrophobic interior of the micelle. The protonation state and the ionic strength could, however, also play a certain role in these absorption band shifts.

HPD absorption spectra obtained under different experimental conditions are shown in Figure 1B. Even at the lowest concentrations, the HPD absorption peak near 374 nm is characteristic for the non-monomeric form. It might be expected that several different species contribute to the broad HPD Soret band. Obviously, the covalently bound porphyrine moieties in DHE can easily form – independent of concentration – intramolecular dimers which would, besides intermolecular dimers and/or aggregates of DHE, HP, PP, etc., contribute to the absorption in this region. Two different mutual orientations of DHE tetrapyrrolic rings can be postulated, the unfolded

* The authors acknowledge the receipt of sample material.

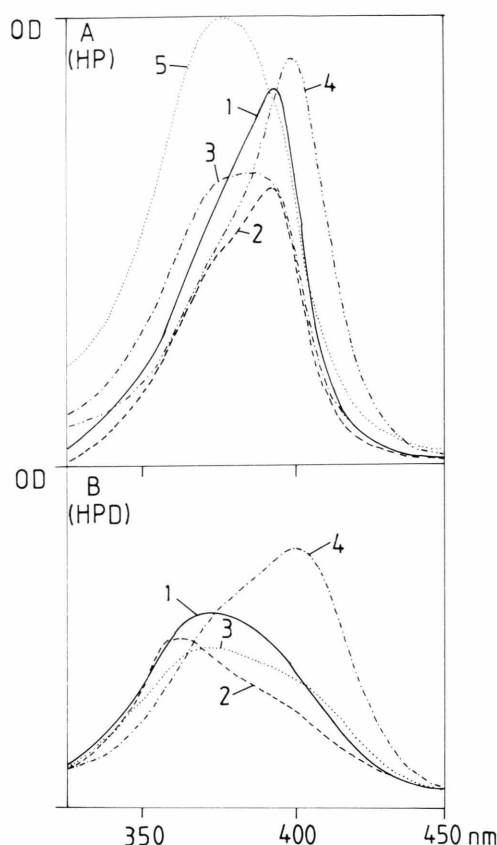


Fig. 1. (A) Absorption spectra of HP solutions (Soret band, relative units): 3×10^{-6} M (pH 12.0) (1); 3×10^{-6} M (pH 7.4) (2); 1.5×10^{-5} M (pH 7.4) (3); 1.5×10^{-5} M in the presence of 2% Triton X 100 (pH 7.4) (4); and 5.0×10^{-5} M (pH 7.4) (5). (B) Absorption spectra of HPD (Photofrin II): 3×10^{-6} M (pH 12.0) (1); 3×10^{-6} M (pH 7.4) (2); 3×10^{-6} M in the presence of 6×10^{-6} M BSA (3) and 3×10^{-6} M in the presence of 2% Triton X 100 [4].

conformation, which is expected to have absorption like HP monomers, and another stacked conformation similar to intermolecular dimers of HP. The absorption properties of the latter conformation should dominate the Soret band observed in polar environment.

In Triton X 100 the absorption maximum is red shifted compared to aqueous solutions (Fig. 1 B, spectrum 4), near to the position of the absorption band of HP under the same conditions (Fig. 1 A, spectrum 4). The half band width is, however, considerably larger. These absorption properties of HP and HPD trapped in Triton X 100 micelles may reflect a superposition of: i) unfolding of DHE dimers in the presence of the

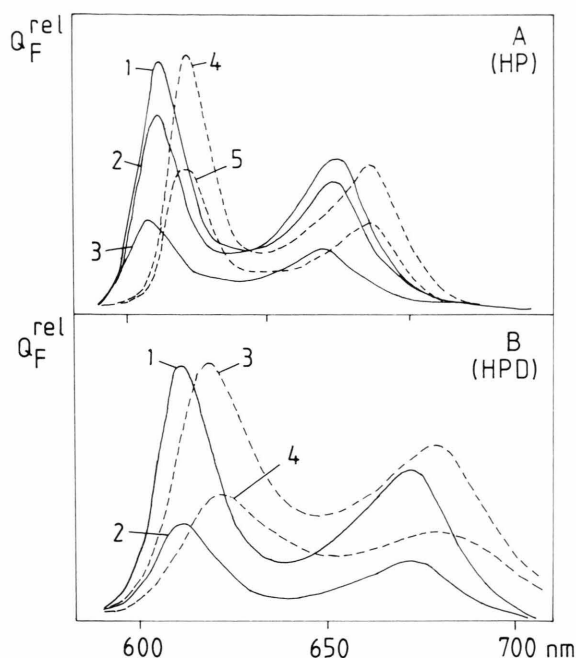


Fig. 2. Corrected fluorescence spectra: (A) 3×10^{-6} M HP in 0.1 M phosphate buffer, pH 7.4 (solid lines) for different excitation wavelengths; $\lambda_{\text{exc}} = 400$ nm (1); $\lambda_{\text{exc}} = 375$ nm (2); $\lambda_{\text{exc}} = 355$ nm (3) and in Triton X 100 (dashed lines) for $\lambda_{\text{exc}} = 400$ nm (4) and 355 nm (5). – (B) 3×10^{-6} M HPD: in phosphate buffer, pH 7.4 (solid lines 1, 2) and as a HPD: BSA complex, pH 7.4 (dashed lines 3, 4), $\lambda_{\text{exc}} = 400$ nm for spectra 1 and 3 and $\lambda_{\text{exc}} = 365$ nm (2, 4).

detergent; ii) porphyrin disaggregation; and iii) influence of the hydrophobicity of the environment. HPD bound to BSA does, however, not show a typical porphyrin monomeric pattern (Fig. 1 B, spectrum 3). This fact was already pointed out by Grossweiner and Goyal [11].

The fluorescence spectra of HP and HPD are shown in Figure 2. Spectral parameters obtained at different conditions are given in Table 1. As can be seen, a significant red shift of the HP emission maxima occurs in solutions containing Triton X 100. No shifts and variations in the band shape were, however, observed on varying the pH or excitation wavelength, especially comparing spectra after excitation near the monomer absorption band or near the maximum of the aggregated species. However, the relative quantum yield varies considerably with excitation wavelength within the Soret band, as excitation at shorter wavelengths leads to an efficient emission quantum yield decrease. This reflects probably an inner filter

Table 1. Fluorescence spectral parameters of HP and HPD in different aqueous solutions.

Concentration (μM)	Solution	λ_{exc} (nm)	$\lambda_{\text{em}}^{\text{max}}$ (nm)	$\frac{Q_{\text{F}}(\lambda_{\text{exc}})}{Q_{\text{F}}(400 \text{ nm})}$
HP	3 pH 12.0	410	610	—
		400	610	1.0
		365	610	0.8
	50 PB, pH 7.4	400	612	1.0
		375	612	0.85
		355	610	0.44
	50 2% Triton X100, PB, pH 7.4	400	623	1.0
		355	622	0.61
	3 pH 12	396	610	—
		400	611	1.0
HPD	3 PB, pH 7.4	365	611	0.39
		410	623	1.0
		400	621	1.0
	3 2% Triton X100, PB, pH 7.4	390	622	0.97
		375	624	0.91
		365	622	0.82
		355	623	0.78
	3 6 μM BSA, PB, pH 7.4	400	620	1.0
		365	622	0.55

effect arising from light absorption by the aggregated species. This observation is consistent with the larger fluorescence decrease as the substrate concentration, and consequently the relative importance of aggregates rises. Nevertheless, there is no quantitative correlation between aggregation and the variation of absolute Q_{F} (2 μM HP: $Q_{\text{F}} = 0.32$, 50 μM HP: $Q_{\text{F}} = 0.13$ [5, 6]) as well as the relative Q_{F} decrease when aggregated species are predominantly excited. For 50 μM HP the concentration of the monomeric form should be negligible, although still remarkable fluorescence is obtained.

HPD emission spectra and fluorescence parameters show a similar trend (Fig. 2B and Table 1). HPD bound to Triton micelles and BSA shows a significant emission red shift, comparable to that observed for HP. HPD quantum yields are, however, smaller than the Q_{F} -values for HP. For example, the quantum yields of HPD ($\lambda_{\text{exc}} = 396 \text{ nm}$) relative to HP ($\lambda_{\text{exc}} = 410 \text{ nm}$) at pH 12 are: $Q_{\text{F}}^{\text{HPD}}(396)/Q_{\text{F}}^{\text{HP}}(410) = 0.54$. This is consistent with recent literature data and reflects the higher aggregation state of HPD [8]. Self-quenching of fluorescence in aggregated porphyrin is assigned to increased singlet-ground state internal conversion as Q_{F} likewise decreases [7]. The decrease of the HPD relative quantum yields observed at

shorter excitation wavelengths reflects the fact that the light is partially absorbed by non-fluorescent aggregates. Like HP there is strong evidence that not only monomeric porphyrin moieties but also some aggregated species, e.g. intramolecular DHE dimers, contribute to the observed fluorescence. This stems from the fact that, based on the absorption spectra, HPD seems nearly totally aggregated, whereas only moderate fluorescence quenching is observed. This might show that the reduction of Q_{F} is no quantitative measure for the aggregation state. Nevertheless, the question remains from what species this additional fluorescence originates.

Fluorescence spectra of HPD in heterogeneous environment, i.e. Triton X 100 and complexed with BSA show significant redshift of the spectrum as was also found for HP. Q_{F} decreases likewise with λ_{exc} , in BSA complexes more strongly than in Triton solutions. This parallels the more efficient disaggregation observed in the micellar environment by absorption measurements. These spectral shifts indicate specific interactions in both systems.

2. Time Resolved Fluorescence of HP and HPD in Different Environment

The fluorescence decay of HP was measured for various concentrations in different environments (pH, presence of Triton X 100 or BSA) for excitation at different wavelengths within the Soret band. The obtained lifetime data for HP are compiled in Table 2. In alkaline aqueous solution (pH 12) a monoexponential decay was observed. In this case HP is present in pure monomeric form and the obtained decay of 5.5 ns should thus be identical to the decay rate of free base HP in polar environment.

For HP in neutral aqueous solution a biexponential decay was obtained (with one exception), the long component generally near to 15.5 ns. The short one near to 3 ns gives generally a low contribution ($\pm 10\%$) to the decay. The weight of the fast component is dependent on the excitation wavelength and is not correlated directly to HP concentration, i.e. the amount of aggregates, as at $3 \times 10^{-6} \text{ M}$ HP exists widely in dimeric forms, whereas above $4 \times 10^{-6} \text{ M}$ substrate higher aggregates predominate [3].

These results are somewhat in contrast to decay data published previously [5, 6]. Herein the amplitude of the short lived component increases in parallel with

Table 2. Fluorescence decay time constants (τ_1 and τ_2), relative initial decay amplitudes (A_1 and A_2) and statistical fitting deviations (χ^2) for different porphyrin concentrations, excitation wavelengths and environments.

Concentration (μM)	Solution	λ_{exc} (nm)	τ_1 (ns)	A_1 (%)	τ_2 (ns)	A_2 (%)	χ^2
HP	3	Water, pH 12	410	15.5 ± 0.02	100	—	0.99
		PB, pH 7.4	403	15.4 ± 0.2	90	2.2 ± 0.2	1.03
	3		373	15.1 ± 0.04	99	2.8 ± 0.8	0.99
						1	
	15	PB, pH 7.4	404	15.85	98	2.24	1.05
			378	15.66	100	—	0.98
	50	PB, pH 7.4	403	15.9 ± 0.08	94	2.7 ± 0.24	1.06
			356	16.4	94	2.8	0.95
	50	2% Triton X 100, PB, pH 7.4	403	18.2 ± 0.10	94	5.4 ± 0.3	1.12
			365	17.5 ± 0.10	95	3.2 ± 0.2	
HPD	3	Water, pH 12	404	17.25	100	—	1.2
		2% Triton X 100, PB, pH 7.4	403	16.6 ± 0.5	88	4.6 ± 0.4	1.00
			390	16.0 ± 0.5	92	3.8 ± 0.3	1.00
	3	6 μM BSA PB, pH 7.4	355	16.0 ± 0.5	89	3.2 ± 0.5	1.10
			404	15.82	100	—	0.98
			373	15.60	100	—	1.00
			373	15.5 ± 0.1	98	1.0 ± 0.6	1.11
			356	15.5	94	0.6	1.15

the HP concentration. Discrepances might arise from the different technique used. Nevertheless, also by these authors no correlation of the amplitudes with monomer and/or dimer concentration was observed. In this respect, it should further be pointed out that besides the monomer/dimer/aggregate equilibrium also protolytic equilibria exist in aqueous solutions, and furthermore impurities by other monomeric porphyrines varying in different preparations could also contribute to the decay.

In heterogeneous environment (adding Triton X 100 or BSA) HP is located either in the interior of the micelle or complexed with the albumin. Both cases lead to effective monomerization of HP, which is clearly demonstrated by absorption. In both cases the lifetimes lengthens, but in micellar environment the decay is still double exponential. This is not the case for HP/BSA-complex, where a monoexponential decay was obtained. The short component could also result from specific interactions with the hydrophobic interior of the micelle.

Fluorescence lifetimes of HPD were measured in aqueous solution of pH 12 and as HPD/BSA complexes, as well as in Triton X 100 micelles (pH 7.4). The obtained data are presented in Table 2. HPD decay in alkaline solutions can be described by a superposition of two exponential functions. The lifetime values ob-

tained are somewhat higher than those for HP in aqueous solution. The relative amplitudes show generally a higher contribution of the short lived component. On interpreting HPD fluorescence lifetime data it must be pointed out that besides HPD and intramolecular dimers of HPD also intermolecularly bound dimers and aggregates are important. Furthermore, monomeric porphyrine impurities could also contribute.

The fluorescence decay of HPD bound to BSA was found to be biexponential too. The obtained lifetimes at 373 nm excitation are smaller than those for HPD in aqueous solution, and at 355 nm the short lived component is even smaller, close to that found previously [5]. The absorption spectrum of the HPD/BSA complex indicates clearly the nonuniform distribution of differently aggregated species in this case. Probably the bound dimers exists in both, the intramolecular dimeric as well as the monomeric form.

Differently to BSA complexes, HPD in micellar solutions show a clear monoexponential decay with a lifetime similar to that obtained in aqueous solutions. This strictly monoexponential decay correlates with efficient disaggregation in this environment. Although there is no clear cut correlation between the monomer/dimer/aggregates ratio observed in absorption spectroscopy and the contribution of a short lived fluorescence com-

ponent, the latter seems to be caused by some aggregated species. Also wavelength effects on Q_F show clearly that fluorescent aggregates might exist. Also DHE should contribute to the fluorescence of HPD.

Conclusion

The data obtained can be summarized as follows:

a) Absorption spectra show the importance of HP and DHE aggregation in neutral aqueous solutions and the influence of concentration and environment. Furthermore, there are specific interactions in the heterogeneous solutions.

b) The fluorescence spectral shape and position does not vary in aqueous solutions, neither with pH, concentration, or excitation wavelength. However, in both, BSA or Triton containing environment strong bathochromic shifts represent local interactions. These data show that primarily only monomeric species should contribute to the fluorescence spectrum.

c) The fluorescence quantum yield is largest for excitation near the monomeric band maximum but decreases as aggregates are predominantly excited. The decrease is, however, smaller than it is to be expected

from the fractional absorption by monomers/dimers/aggregates, as at 5×10^{-5} M HP the monomeric fraction should be minimal. This can be compared to the overall decrease of the fluorescence yield, which is at 5×10^{-5} M HP just half that at 2×10^{-6} M.

d) The fluorescence decay is doubly exponential in most cases where aggregation occurs. Nevertheless there is no clear cut correlation between aggregation and the relative contribution of the short lived fluorescent component. Double exponential decay in heterogeneous media could result from specific complexation with hydrophobic sites of BSA or Triton micelles.

e) Differences between HP and HPD result from the ability of DHE to form stacked intramolecular dimers independent of concentration. The dimeric form should associate more easily than monomers to form higher aggregates.

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