Ground and Excited States of Hematoporphyrin and Its Derivatives

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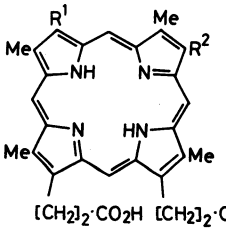
Synopsis. Hematoporphyrin and two derivatives in aqueous solution undergo the molecular association to form each dimer. The association constant of 8(3)-(1-hydroxyethyl)-3(8)-vinyldeuteroporphyrin isomers is much larger than that of hematoporphyrin. No significant difference has been observed in the photophysical properties of their excited singlet and triplet states.

With regard to tumor phototherapy, much attention has been paid to the photochemistry and photophysics of hematoporphyrin (HP) and its derivatives, as reviewed by Kessel.[1] The use of a mixture of chemically treated HP, called "hematoporphyrin derivative (HPD)," has currently been recommended for clinical purposes. It is suggested that the predominant pathway of tumor phototherapy using HPD is attributable to the singlet oxygen generated by energy transfer from excited triplet HPD to ground-state molecular oxygen.[2,3] The superiority of HPD over HP may be understood from the following points of view: The first is high affinity of HPD with tumor cell, and the second is the efficiency for generating cytotoxic molecular singlet oxygen. To verify these questions, we have examined the photophysical properties of three porphyrins which are the main components of HPD,[4] by absorption and emission spectroscopic techniques.

Experimental

Hematoporphyrin (HP, Sigma) was purified by preparative thin layer chromatography. *O,O'*-Diacetylhematoporphyrin (HPdiAc) and the mixture of 8(3)-(1-hydroxyethyl)-3(8)-vinyldeuteroporphyrin isomers (HVD), supplied by Hamari Yakuhin Co. Ltd., were used as received.



HP : $R^1 = R^2 = CH(OH)Me$
HVD : $R^1 = CH{=}CH_2$; $R^2 = CH(OH)Me$
or
$R^1 = CH(OH)Me$; $R^2 = CH{=}CH_2$
HPdiAc: $R^1 = R^2 = CH(OAc)Me$

Spectrograde methanol was used as received. Distilled water used was further deionized. Porphyrins were dissolved in a phosphate buffer (pH 7.2) solution with vigorous stirring. Sample solutions were deaerated by freeze-pump-thaw cycles, when necessary.

Absorption spectra were measured with Hitachi 224 spectrophotometer using quartz cells of 0.1, 0.5, 1.0, 2.0, and 2.5 cm path length. The fluorescence lifetime was determined by a time-correlated single-photon counting system (Ortec) with a D_2 nanosecond light pulser (PRA model 510) and a cut-off filter (Toshiba R-60, ≥600 nm). The transient absorption lifetime was determined by an excimer laser (Lambda Physik EMG-50E, 308 nm) and a monitoring xenon arc lamp system. All the measurements were carried out at room temperature.

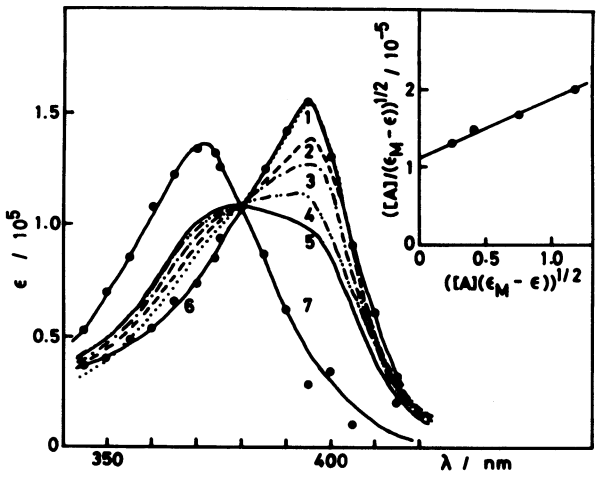


Fig. 1. Soret-band absorption spectra of HP in aqueous buffer solution, pH 7.2; 1, 1.6×10^{-6} M; 2, 3.1×10^{-6} M; 3, 6.3×10^{-6} M; 4, 1.3×10^{-5} M; 5, 2.5×10^{-5} M. 6 and 7 are the calculated absorption spectra of the monomer and the dimer, respectively. Insert: Plot of Eq. 1 for ε's at 394 nm, where ε_M is assumed to be 155000.

Results and Discussion

Ground-State Monomer-Dimer Equilibrium. Figure 1 shows the concentration dependence of absorption spectra (the Soret band) for HP. At low HP concentration the band appears at 394 nm, and a new band appears around 375 nm with increasing concentration, suggesting that the molecular association of HP takes place. To estimate the aggregation number of HP, the absorption intensity at 394 nm was analysed by the Zanker's method, which was successfully applied to the analysis of aggregation of organic dyes.[5,6] The analysis gave the mean aggregation number of two. This fact strongly indicates that the spectral changes in Fig. 1 are attributable to the monomer-dimer equilibrium. Thus, we further analysed the data according to the procedure proposed by Tipping et al.[7] The following equation should hold for the dimerization,

$$\left\{\frac{[A]}{\varepsilon_M-\varepsilon}\right\}^{1/2} = \frac{2}{2\varepsilon_M-\varepsilon_D}\{[A](\varepsilon_M-\varepsilon)\}^{1/2} + \left\{\frac{1}{K_D(2\varepsilon_M-\varepsilon_D)}\right\}^{1/2}. \quad (1)$$

Table 1. Fluorescence Lifetime of HP, HPdiAc, and HVD[a]

	Buffer solution		Methanol solution		k_q[b]
	Deaerated	Aerated	Deaerated	Aerated	
	ns	ns	ns	ns	$10^{10}\,M^{-1}\,s^{-1}$
HP	16.0	15.6	18.1	12.4	1.2
HPdiAc	16.1	15.9	18.4	12.3	1.3
HVD	16.4	15.9	18.5	12.3	1.3

a) Concentrations are 10^{-5} M. b) Quenching rate constants by oxygen, estimated by assuming that oxygen concentration in aerated methanol is 2.12×10^{-3} M.[15]

Here, $[A]$ represents the total concentration of hematoporphyrin in terms of monomer, ε is the observed molar extinction coefficient, ε_M and ε_D are the molar extinction coefficients of the monomer and dimer, respectively, and K_D is the monomer-dimer association constant. The plot of $\{[A]/(\varepsilon_M-\varepsilon)\}^{1/2}$ against $\{[A](\varepsilon_M-\varepsilon)\}^{1/2}$ gives a straight line. The intercept and slope can be used to calculate K_D and ε_D. Insert of Fig. 1 shows the plot of Eq. 1 for HP. The linear relationship in the figure strongly supports the formation of HP dimer in this concentration region, giving the association constant K_D of 0.3×10^5 M^{-1} (M=mol dm^{-3}).

Similar spectral changes were observed for other derivatives and analysed according to the preceding procedure. The K_D's obtained were $0.7\times10^5\,M^{-1}$ (HPdiAc) and 1.8×10^5 M^{-1} (HVD). The association constant of HVD is about six times larger than that of HP.

The aggregation of porphyrins in an aqueous solution was reported by Brown et al.[8] They suggested that dimerization occurs at a very low concentration ($\leqslant4\times10^{-6}$ M) and that the spectral changes in higher concentration region is attributable to "micellarization," i.e., the generation of large aggregates of unknown size. On the other hand, Tipping et al.[7] reported that the absorption-spectral changes of HP caused by the monomer-dimer equilibrium were observed in the concentration range of 0—200×10^{-6} M. The present result agrees with that of the latter authors.

Excited Singlet States. The position of fluorescence spectra of porphyrins in methanol appearing in 600—700 nm region is insensitive to the side-group substitution of porphyrin ring. The fluorescence decays exponentially at a concentration of 10^{-5} M in both methanol and aqueous solution as shown in Table 1. Lifetime values of HP in aerated methanol and aqueous solution agree with those of HP monomer reported by Andreoni et al.[9] The absence of any shorter component due to the dimer in aqueous solution is attributable to wide pulse width of the excitation light source (2—3 ns) in the present study. The quenching rate constants (k_q) of the excited singlet porphyrins by oxygen estimated in methanol are given in Table 1. The rate seems to be diffusion-controlled. No significant difference has been shown in the photophysical properties of the excited singlet porphyrins.

Excited Triplet States. Photophysical properties of the excited triplet porphyrins were studied by laser flash photolysis. In the laser flash photolysis of HP in methanol, a transient absorption band appears in 430—600 nm region, which is assigned to the triplet-triplet absorption of HP with reference to the reported data.[10-12] Since the decay curves of the intense triplet-triplet absorption at 450 nm are strongly influenced by the laser excitation intensity, the lifetime of porphyrins was determined at a low laser intensity where the decay is exponential. The results are shown in Table 2. The lifetime values for three porphyrins are similar to each other. The triplet lifetime of HP was reported to be about 20 μs in acetone and 90% methanol/water mixture.[11] This short lifetime seems to be due to the effect of the triplet-triplet annihilation caused by intense laser excitation used.

Table 2. Triplet Lifetime of HP, HPdiAc, and HVD in Methanol[a]

	Deaerated	Aerated	k_q'[b]
	μs	μs	$10^9\,M^{-1}\,s^{-1}$
HP	77.8	0.30	1.6
HPdiAc	61.8	0.27	1.7
HVD	76.3	0.21	2.2

a) Concentrations are 10^{-5} M. b) Quenching rate constants by oxygen, estimated by assuming that oxygen concentration in aerated methanol is 2.12×10^{-3} M.[15]

In an aerated solution, the lifetime becomes shorter, where the quenching rate constants of the excited triplet porphyrins by oxygen were in the order of 10^9 $M^{-1}\,s^{-1}$. These values are about one order of magnitude smaller than those for the quenching in excited singlet states. Although molecular oxygen quenches both the excited singlet and triplet states efficiently, molecular singlet oxygen is exclusively generated via the excited triplet energy-transfer process as was discussed by Turro.[13] The relative yield of singlet oxygen for these porphyrins is similar to each other.

From the results mentioned above, the monomer-dimer association constant of HVD in the ground-state is larger than those of HP and HPdiAc, while there are no significant difference in the photophysical properties of their excited states. The molecular association (dimer formation) of porphyrins in aqueous solution is relevant to hydrophobicity of the compounds. On the other hand, affinity of porphyrins to tumor cell relates to their hydrophobicity, since photo-induced damage has been reported to occur at the cell membrane which is highly hydrophobic.[14] It is likely, therefore, that the dimer association constant of porphyrins reflects their affinity to tumor cell.

The present results showing similar yields of singlet oxygen for three porphyrins indicate that the affinity of porphyrins to tumor cell is one of determinants for the efficiency of phototherapy.

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