特別寄稿

Probing the Structure of 'Hematoporphyrin Derivative' via
Fluorescence and Absorbance Spectroscopy

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Synopsis

Spectral properties of the tumor-localizing dimer/oligomer fraction of HPD and of several diporphyrin ethers were assessed by fluorescence and absorbance spectroscopy in different solvents and after accumulation by leukemia L1210 cells *in vitro*. Joining 2 porphyrins by an ether linkage caused a blue shift in the Soret bands of the dimers, but not monomers, when the dielectric constant of the solvent was lowered. The presence of ether linkages was associated with enhanced fluorescence at 630–640 nm and decreased fluorescence lifetimes and fluorescence yields. The joining of two mesoporphyrin or protoporphyrin molecules by an ether linkage reduced dye accumulation by L1210 cells, but markedly promoted uptake of the hematoporphyrin analog.

Introduction

The tumor-localizing (dimer/oligomer) fraction of HPD contains hematoporphyrin and HP dehydration products in both ether and ester linkages (Kessel et al, 1987; Dougherty, 1987). Spectroscopic studies on this material have been described (Andreoni & Cubeddu, 1984; Andreoni et al. 1982; Margalit & Cohen, 1983; Bottiroli & Ramponi, 1988; Kessel, 1989). It is clear that the formation of linkages between porphyrin units affects fluorescence and absorbance spectra, fluorescence yields and lifetimes. But the complexity of HPD, and the difficulty of obtaining pure fractions, complicates interpretation of results. For example, the

substantial fluorescence emission at 630–640 nm associated with the dimer/oligomer fraction of HPD (Kessel, 1989) could derive from the presence of vinyl substituents, from ring-ring interactions, or both.

Fluorescence spectra of purified tumor-localizing components from HPD (the dimer/oligomer fraction) were examined. To aid in the interpretation of results, we also examined properties of pure dimers composed of hematoporphyrin, protoporphyrin and mesoporphyrin units joined by ether linkages. These studies have provided new insights with regard to interpretation of spectral data obtained with HPD fractions.

Materials and Methods

The dimer/oligomer fraction of HPD was isolated using an LH-20 column (Kessel, 1989). The porphyrin ethers, provided as tetramethylesters, were prepared as described by Morris and Ward (1988). Porphyrin monomers (free acids and dimethyl esters) were purchased from Porphyrin Products, Logan UT.

* Abbreviations: Bz, benzene; CTAB, cetyl trimethylammonium bromide; DHE, dihematoporphyrin ether; DME, dimesoporphyrin ether; DVE, Divinylether; EtOH, ethanol; FM, formamide; HP, hematoporphyrin; HPD, hematoporphyrin derivative; MP, mesoporphyrin; PDT, photodynamic therapy; PP, protoporphyrin; THF, tetrahydrofuran; Φ_f , fluorescence yield.

All solvents were of HPLC grade or better.

HPLC studies were carried out using an RP-8 column (5×100 mm, 5 μ particle size) in a Waters Z module. The column was eluted with a linear gradient of methanol-THF (0.7 ml/min) applied over 15 min. Absorbance at 400 nm and fluorescence (excitation=400 nm) were monitored.

Absorbance spectra were determined with a Perkin-Elmer dual-beam spectrophotometer. Fluorescence was assessed using an SLM 48000 fluorometer. The excitation wavelength (usually 400 nm) was adjusted to obtain an optimal fluorescence signal. Fluorescence yields were calculated as the ratio of the integrated corrected fluorescence emission (600–725 nm) divided by the optical density at the wavelength of excitation ($OD < 0.1$). The standard used was quinine in 0.1 N H_2SO_4 ($\Phi_f = 0.55$).

Fluorescence lifetimes were determined using the phase-modulation technique with the SLM 48000 instrument; the lifetime standard was POPOP in ethanol (Lakowicz, 1983). The excitation wavelength was 400 nm with emission limited to wavelengths > 610 nm by an interference filter. Both phase and modulation values were used in the lifetime calculations; the χ^2 value for the observed vs. theoretical results was never greater than 3.

To measure the affinity of the porphyrin monomers and ethers (as free acids) for L1210 cells *in vitro*, cells were incubated with 3 μM concentrations of dyes for 15 min at 37° in growth medium (10% horse serum). The cells were then collected by centrifugation, washed with cold isotonic NaCl to remove extracellular dye, and the pellets were homogenized in 10 mM CTAB detergent. An aliquot of the supernatant fluid was mixed with 9 volumes of 10 mM CTAB. The distribution ratio, representing the concentration of dye in cells vs. medium was determined by fluorescence assay.

Results

The fluorescence emission spectrum of the dimer/oligomer fraction isolated from HPD by gel exclusion chromatography is shown in Fig. 1. This material exhibits a greatly enhanced fluorescence at 630–650 nm, compared

Fig. 1. Fluorescence emission of the tumor-localizing (dimer/oligomer) fraction of HPD. Excitation=400 nm, solvent=methanol.

with hematoporphyrin.

Structures of the diporphyrin ethers (as tetramethyl esters) are shown in Fig. 2. There was no evidence of monomer contamination in the ethers used in these studies.

Absorbance spectra of DHE and DVE (as tetramethyl esters) in four different solvents are shown in Fig. 3. Solvents of progressively lower dielectric constant shifted the Soret band toward shorter wavelengths. DME spectra were similar to the DHE results. In contrast, HP, MP or PP dimethyl esters all showed Soret bands at 400 ± 2 nm, regardless of solvent. Moreover, fluorescence excitation optima of both the porphyrin monomers and the ether dimers (as methylesters), in the 4 solvents, were within ± 2 nm of 396 nm, with slight shoulders at 380 nm (not shown). The spectral shifts observed in Fig. 2 were therefore not associated with corresponding changes in excitation spectra.

Fig. 2. Structure of porphyrin ethers. DVE, $R = -CH=CH_2$; DME, $R = -CH_2CH_3$; DHE, $R = -CH(OH)CH_3$.

Fig. 3. Absorption spectra of tetramethyl esters of DHE (top) and DVE (bottom). Solvents=THF (—), CHCl_3 (---), EtOH (....) and formamide (-·-·-).

Fig. 4. Fluorescence emission spectra of dimers and monomers (as methyl esters). Top row: solvent=chloroform; center row, solvent=ethanol; bottom row, solvent=formamide. Left: Solid line=mesoporphyrin, dotted line=DME. Center: solid line=HP, dotted line=DHE. Right: solid line=PP, dotted line=DVE.

Table 1. Fluorescence Properties of Porphyrins

Porphyrin	Solvent	Φ_f	τ_1 (ns)	%	τ_2 (ns)	%
HP	THF	0.09	13.5	100		
DVE	THF	0.017	3.53	51	8.50	49
	EtOH	0.009	2.01	51	8.12	48
	FM	0.005	1.46	63	11.7	37
DME	THF	0.039	2.70	20	8.21	80
	EtOH	0.023	2.13	30	7.52	60
	FM	0.017	1.75	44	8.21	55
DHE	THF	0.089	—	—	8.43	100
	EtOH	0.058	2.26	15	7.49	84
	FM	0.032	3.22	39	10.3	61

All porphyrins were examined as methyl esters. Values shown here are subject to errors of $\pm 10\%$ for lifetimes; $\pm 5\%$ for fluorescence yields. Lifetime values (τ) are shown along with the contribution of each lifetime to total fluorescence.

Abbreviations: HP, hematoporphyrin; DME, dimesoporphyrin ester; DVE, divinylporphyrin ester; THF, tetrahydrofuran; EtOH, ethanol; FM, formamide.

Emission spectra (Fig. 4) of the dimers indicated a substantial promotion of 630–640 nm fluorescence when two porphyrin units were joined by an ether linkage. Data are shown for chloroform and formamide. Spectra obtained with THF (not shown) did not signifi-

cantly differ from the CHCl_3 results. Fluorescence at 630–640 nm was further promoted by the presence of vinyl substituents, i.e., in DVE. We had previously reported such a finding (Kessel, 1969). An anomalous emission spectra obtained with DVE in formamide (see

Fig. 5. HPLC analysis of HP and DHE (as methylesters) using absorbance and fluorescence detection.

Discussion).

Fluorescence lifetime data (Table 1) indicate a substantial decrease in γ associated with the ether structures, regardless of solvent. The decreased lifetimes were associated with lowered fluorescence yields. Expect for DHE dissolved in THF, two distinct lifetime values were always observed. Only one lifetime was seen with the porphyrin monomers (γ and Φ_f data for HP dimethylester are shown in Table 1).

The decreased fluorescence yield of the ethers could be demonstrated (Fig. 5) when both absorbance and fluorescence were monitored during HPLC analysis of a mixture of HP+DHE (as methylesters). Diastereoisomers of both products were also resolved.

Measurements of uptake of the free acid forms of the porphyrins by L1210 cells (Table 2) indicate that ether formation resulted in decreased uptake except in the case of HP. The implications of this finding are discussed below.

Discussion

There is an enhanced fluorescence emission from the dimer/origomer fraction of HPD at

Table 2. Dye Uptake by L1210 Cells

Porphyrin	Monomer	Ether-Dimer
HP	0.01	1.26
MP	3.79	1.06
PP	4.61	1.89

Cells were incubated for 15 min at 37° with 3 μ M levels of porphyrin dimers (free acids) and the resulting distribution ratios (DR) are shown here. In this context, DR = [intracellular dye concentration]/[extracellular dye concentration]. Data represent results of a typical experiment; replicate studies did not differ by more than $\pm 10\%$ of the values shown.

635–650 nm (Fig. 1). In such a mixture, it is difficult to distinguish whether 635 nm fluorescence derives from vinyl substituents or from dimer/origomer formation. The use of the model dimers permits us to assign enhanced fluorescence at 635 nm to at least 2 distinct phenomena: extension of the conjugated system via vinyl groups, and ring-ring interactions which occur in the ether structure even where no vinyl groups are present. These interactions were characterized by a blue shift in the Soret band as the dielectric constant of the solvent was increased, enhanced fluorescence at 630–640 nm and beyond, and reduced fluorescence yields and lifetimes. While absorbance of the dimers in formamide, a solvent with a high dielectric constant, was blue-shifted (Fig. 3), excitation spectra were not. The fluorescence signal from DVE in formamide must therefore derive from a relatively small number of dye molecules which absorb at 400 nm.

In an earlier study (Kessel et al, 1988), we noted no decrease in fluorescence lifetime, nor any alteration in fluorescence emission spectra, when 2 tricarboxylic porphyrins were joined by a bridge of 6 methylene groups. In such a structure, ring-ring interactions are apparently absent, and a side-by-side structure appears to predominate. Molecular models of the ethers examined here indicate 3 possible configurations, with the porphyrin rings parallel (side-by-side), perpendicular, or folded over into a 'clam-shell' configuration. The lifetimes of approx. 2 and 7 ns (Table 1) may represent 'clam-shell' and perpendicular ether

configurations, respectively. Chang et al (1986) had used NMR to show that ring-ring interactions were promoted by solvents of higher dielectric constant, using a relatively hydrophobic diporphyrin. The predominant shorter lifetime and broad 680 nm fluorescence observed with DVE in formamide may derive from extensive ring-ring interactions which occur with a very hydrophobic ether in a solvent of high dielectric constant.

Data summarized in Table 2 indicate that DME and DVE are taken up less efficiently than their monomeric analogs, PP and MP. This result most likely derives from the decreased hydrophobicity of the ethers (four -COOH residues/molecule), as compared with the monomers (two -COOH/molecule). An exception occurs in the case of HP, where ether formation promotes dye accumulation. One interpretation of this result is that DHE molecules assume a configuration, in a biological environment, which results in increased hydrophobicity, e.g., via enhanced ring-ring interactions which mask the effect of hydrophilic substituents on the porphyrin rings. The fluorescence emission spectra shown in Fig. 4 are consistent with the suggestion that DHE and DME have very similar properties in organic solvents. Moreover, the fluorescence emission spectra from cells which have accumulated the ethers (Fig. 7) are consistent with very similar environments for DHE and DME.

The dimeric ethers of hematoporphyrin and its analogs clearly exhibit several unexpected properties which affect dye accumulation in tissues. Additional structure-activity studies may reveal information regarding porphyrin-porphyrin configurations which play a role in the tumor-localization phenomenon.

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