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PORPHINATO ZINC COMPLEXES INCORPORATED INTO THE BILAYER MEMBRANE OF LIPID LIPOSOMES

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ABSTRACT

3,8,13,17-Tetramethyl-7,12-divinyl-2,18-bis(18-hydroxyoctadecyl propionate) porphinato zinc (BHPZn) and a model compound, dimethyl ester of protoporphinato zinc (DMPZn), were synthesized and incorporated into the hydrophobic region of bilayer membrane of dipalmitoylphosphatidylcholine (DPPC) liposome. The introduction of long alkyl groups onto the porphyrin ring is effective for restriction of porphyrin aggregation in the bilayer membrane of DPPC liposome. When the molar ratio of DPPC lipid to porphyrin is above 100, the spectrum of BHPZn in the liposome suggests that it is in a typically monomeric state. Quenching of BHPZn fluorescence in the hydrophobic bilayer membrane by hydrophilic quenchers is slow and shows smaller Stern-Volmer constants, while the quenching by hydrophobic quenchers shows much larger Stern-Volmer constants than that of the model compound, DMPZn. These results suggest that the location of the porphyrin ring of BHPZn is fixed at a certain depth in the hydrophobic bilayer membrane of DPPC liposome, and that that of DMPZn is widely distributed in the whole hydrophobic region.

INTRODUCTION

In the approach to mimic the oxygen-transporting function of hemoglobin or myoglobin with modified porphyrin derivatives, one of the efficient methods for protecting the oxygen-binding active center against the μ-dioxo dimer formation and the proton-driven irreversible oxidation is the incorporation of these compounds into the hydrophobic bilayer membrane of phospholipid liposomes [1].

Therefore, studying the incorporation of porphyrins with phospholipids involves finding out 1) whether the porphyrins are trapped or embedded in the liposome particles and 2) what are the dispersion state and the distribution profile of porphyrins in the bilayer membrane of lipid liposomes. Although it has been reported that long alkyl-group-substituted porphyrins have good compatibility with lipids and can be embedded in the bilayer membrane of liposomes [2-5], there are few publications concerning the dispersion state and distribution profile of porphyrins in liposomes [6]. It is well known that porphyrin compounds tend to aggregate in aqueous solution [7]. The relative concentrations of porphyrins in the bilayer membrane of liposomes are much higher than the apparent concentrations. Thus, it is important to clarify the aggregation of porphyrins in the lipid liposomes, especially when the molar ratios of lipid to porphyrins are small. Besides these, the elucidation of the location, i.e., the distribution profile of porphyrins in the bilayer membrane of lipid liposomes, is important for the design of biomimetic models and the functional energy migration path at the molecular level. Porphyrins located in the center of a liposome membrane and near the surface of a liposome particle may have different environments and thus exhibit different properties, such as the reactivity toward water-soluble substrates which are dissolved in the aqueous phase. The compatibility and the orientation effect of substituents of porphyrins affect, in some cases, the distribution profile of porphyrins in the bilayer membrane of liposomes. To elucidate the dispersion state and the distribution profile of porphyrins with different substituents in liposome bilayer membranes will facilitate the design and preparation of functional molecular assemblies, such as oxygen carriers or other biomimetic systems.

We now describe the incorporation of the bis(18-hydroxyoctadecyl)ester of protoporphinato zinc complex and the model compound, dimethyl ester of protoporphinato zinc complex with phospholipid, and the investigation of the dispersion state and the location of these compounds in the bilayer membrane of lipid liposomes. The aggregation or stacking of porphyrins in the bilayer membrane of lipid liposomes, compared to that in aqueous organic

solvents and micelles, are examined. In the presence of various quenchers, the fluorescence spectra of porphyrin-containing liposome suspension and the quenching effect of porphyrin fluorescence by addition of various quenchers are used to elucidate the distribution profile of porphyrins in the bilayer membrane of lipid liposomes.

EXPERIMENTAL

Materials

Dipalmitoylphosphatidylcholine (DPPC) (Sigma), Triton X-100 (Tokyo Kasei Co.), cetyltrimethylammonium chloride (CTAC) (Tokyo Kasei Co.), 2,2,6,6-tetramethyl-4-piperidinol-1-oxide (OH-TEMPO), and 16-nitroxide-stearic acid or 16-DOXY-stearic acid (16-DSA) (Aldrich) were commercial products and used as received. Anthraquinone-2,6-disulfonic acid sodium salt (AQDS) (Tokyo Kasei Co.) was recrystallized from aqueous alcohol solution. 3,8,13,17-Tetramethyl-7,12-divinyl-2,18-bis(18-hydroxyoctadecyl propionate) porphyrin (BHP) and protoporphyrin IX dimethyl ester (DMP) were prepared from protoporphyrin IX disodium, and the preparation and characterization of these compounds will be reported elsewhere.

Complexation Reaction of Porphyrins

Excess zinc acetate was added to the porphyrin solution in chloroform-methanol mixed solvent (2:1), and the mixture was heated under reflux for 6 h. After the reaction the solution was evaporated by rotary evaporator to dryness, chloroform was added to dissolve the product, and the chloroform solution was washed with distilled water to remove the unreacted zinc acetate. The product was purified by silica gel column chromatography (2.5 cm i.d. × 25 cm long) with chloroform-methanol mixed solvent (10:1) as eluent.

Incorporation of Porphinato Zinc Complexes into Micelles

Porphinato zinc and surfactant were dissolved in chloroform to form a homogeneous solution, and then the solvent was removed by vacuum evaporation. After phosphate buffer (pH 7.0) was added, the surfactant-porphinato zinc mixture in the buffer was stirred gently to dissolve porphinato zinc into the formed micelle.

Incorporation of Porphinato Zinc Complexes into Lipid Liposomes

The incorporation of porphinato zinc complex with DPPC was carried out by ultrasonication. The liposome suspension solution obtained was applied to a Sepharose 4B column to confirm the presence of porphinato zinc in the liposome.

Absorption Spectra

UV-Vis spectra were recorded on a Hitachi MPF-2000 spectrophotometer at 25°C. In order to keep the absorbance in a measurable range, quartz cells with path lengths from 1.0 to 10.0 mm were used.

Quenching Experiments

Stock solutions of quenchers, AQDS or OH-TEMPO, were prepared by dissolving these compounds in phosphate buffer of pH 7.0 and stored at 0°C. The quenching of the fluorescence of porphinato zinc complex in liposome was carried out by addition of quencher stock solutions to the liposome suspension solution. The concentration of porphinato zinc complex and the solution ionic strength were kept constant by addition of buffer or NaCl solution. Hydrophobic quencher, 16-DSA, was dissolved in the porphinato zinc-lipid chloroform solution before the ultrasonication treatment and incorporated into the bilayer membrane of lipid liposome together with porphinato zinc complex. The fluorescence intensity was recorded on a Hitachi MP-4 spectrofluorometer equipped with a temperature-control unit. The concentrations of porphinato zinc complexes were kept around 10^{-6} *M*.

RESULTS AND DISCUSSION

1. Incorporation of Porphinato Zinc Complexes in Lipid Liposomes

The incorporation of 3,8,13,17-tetramethyl-7,12-divinyl-2,18-bis(18-hydroxyoctadecyl propionate)porphinato zinc (BHPZn) and the model compound, the dimethyl ester of protoporphinato zinc (DMPZn), into dipalmitoylphosphatidylcholine (DPPC) lipid liposome particles was confirmed by GPC separation, ultracentrifugation, and transmission electron micrograph (TEM). The GPC elution curve for porphinato zinc complex was coincident with that for DPPC lipid. The supernatant of ultracentrifugation of the liposome suspension contained neither porphinato zinc nor DPPC lipid. TEM indicated that porphinato zinc-

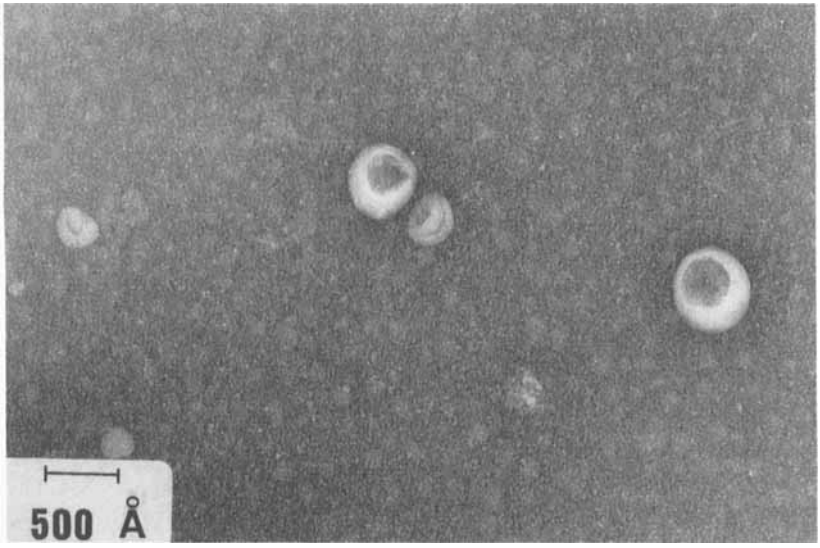

FIG. 1. Transmission electron micrograph of DPPC liposomes containing 1% protoporphinato zinc.

containing liposome was SUV of about 400-500 Å in diameter (Fig. 1). These results showed unambiguously that porphinato zinc complexes were incorporated into the bilayer membrane of lipid liposomes.

2. Dispersion State of Porphinato Zinc Complexes in the Bilayer Membrane of Lipid Liposomes

Figure 2 shows the electronic absorption spectra of porphinato zinc complexes incorporated in the bilayer membrane of DPPC liposome with different molar ratios of DPPC to porphyrin derivatives. When the molar ratio of DPPC to porphinato zinc was low, a shoulder (at about 390 nm) appeared on the Soret band of the porphyrin derivative, and the intensity of the shoulder increased with decreasing molar ratio of DPPC to porphinato zinc. In other words, the intensity of this new band increased with increasing relative concentration of porphinato zinc. These results suggest that the new shoulder band can be attributed to the aggregated porphyrin derivative. Unlike the results reported in the literature [8, 9]. DMPZn exhibited obvious aggregation in the bilayer membrane of DPPC liposome, especially when the molar

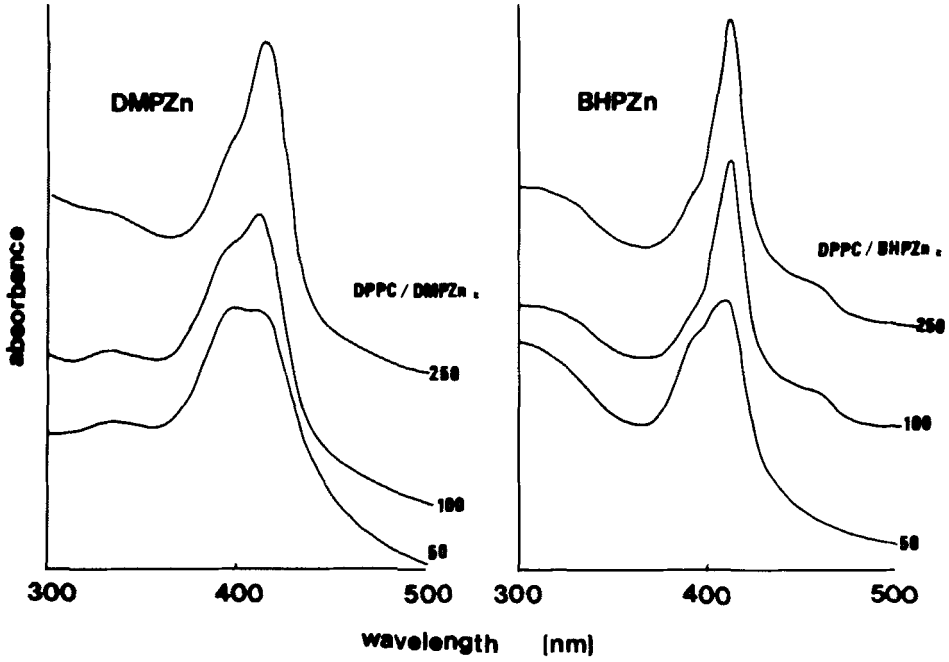

FIG. 2. Absorption spectra of porphinato zinc in DPPC liposome with different molar ratios of DPPC to porphinato zinc. Concentration of porphinato zinc: 10^{-6} *M*.

ratio was low. Against this, the spectra of long-alkyl-group-substituted porphyrin, BHPZn, exhibited typical monomeric characteristics when the molar ratio was above 100, suggesting that the introduction of long alkyl groups on the porphyrin ring suppressed the aggregation of porphyrin compounds.

Spectral changes of porphyrin derivatives in various solvents or micelles were examined for comparison with the aggregation behavior of porphyrins in liposome bilayer. In organic solvents such as chloroform, the spectra of both BHPZn and DMPZn showed them to be typically monomeric in the measurable concentration range. The λ_{max} of the Soret band for porphinato zinc was, of course, a function of the solvent dielectric constant and showed a shift with increasing dielectric constant (Fig. 3). The λ_{max} of protoporphinato zinc complexes in DMSO (dielectric constant 48.9) and in DPPC liposome was about 413 nm, suggesting that the dielectric constant of the environment

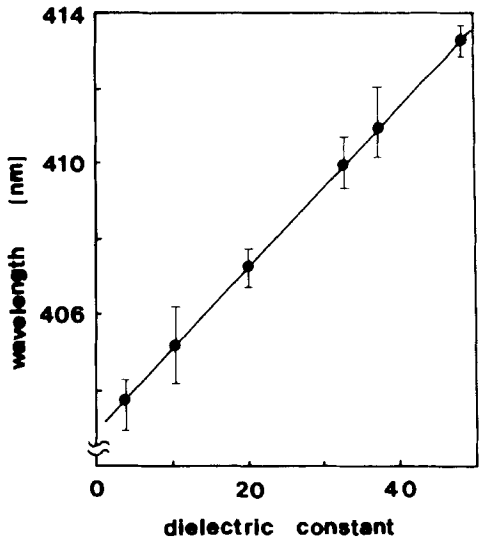

FIG. 3. Spectral maximum of BHPZn as a function of dielectric constant of solvent.

of porphinato zinc complexes in the bilayer membrane of DPPC liposome is around 49. This value is much higher than expected, probably due to the influence of the surface charge of the liposome.

The electronic absorption spectra of BHPZn and DMPZn in 40% acetone aqueous solution are shown in Fig. 4. A shoulder which can be attributed to the aggregated porphyrin appeared beside the Soret band of monomeric porphyrin with increasing DMPZn concentration. This shoulder became dominant at concentrations above 2.5×10^{-5} *M*. The concentration of porphyrin in aqueous organic solvent at which aggregation takes place was much lower than that in liposome membranes, as shown in Fig. 4, i.e., the hydrophobic environment of porphyrins in the bilayer membrane of DPPC liposome restricts the aggregation of porphyrin derivatives.

Micelles are well known to provide hydrophobic environments in aqueous media, and hence are expected to dissolve porphyrins molecularly. The electronic absorption spectra of DMPZn and BHPZn in the ionic micelle of cetyltrimethylammonium chloride (CTAC) are shown in Fig. 5. The concentration of porphyrin aggregation in this micelle was much higher than that in aqueous acetone. A nonionic surfactant, Triton X-100, which can form

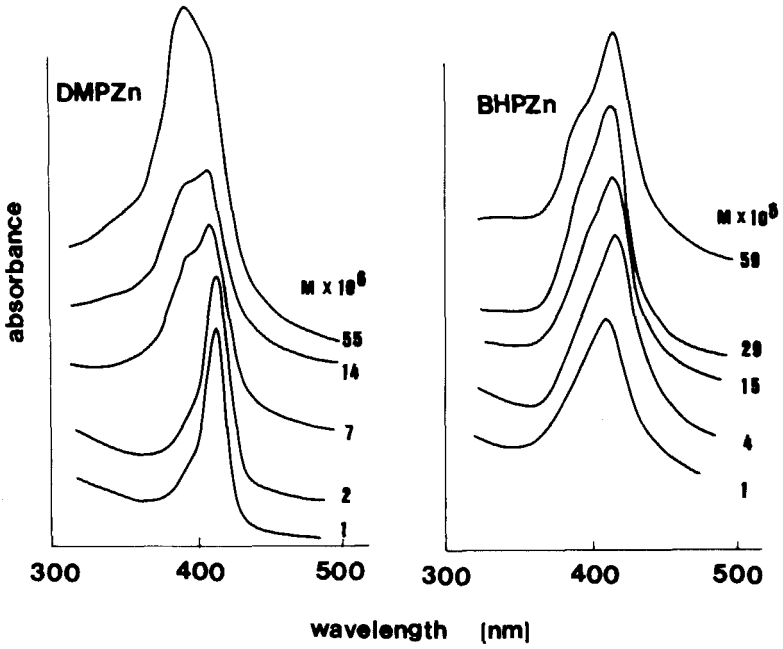

FIG. 4. Absorption spectra of porphinato zinc in 40% acetone-water at different porphinato zinc concentrations.

larger micelle particles, showed the same results, as seen in Fig. 6. Contrary to the observation reported in the literature that protoporphyrin IX in micelles is in the monomeric state [10], our experiments indicate that the dimethyl ester of protoporphinato zinc exhibits obvious aggregation in micelles when the molar ratio of surfactant to porphyrin is lower than 500:1. The introduction of long alkyl substituents onto the porphyrin ring has a marked effect on the inhibition of porphyrin aggregation, similar to the case of DPPC liposome. This is in agreement with the early observation that the larger the side chain, the less aggregation of porphyrin that can be expected [7, 11].

3. Location of Protoporphinato Zinc Complexes in the Bilayer Membrane of Lipid Liposome

Bilayer membranes of DPPC liposome provides a hydrophobic environment for the incorporated porphyrins, and the reactions between the incorporated

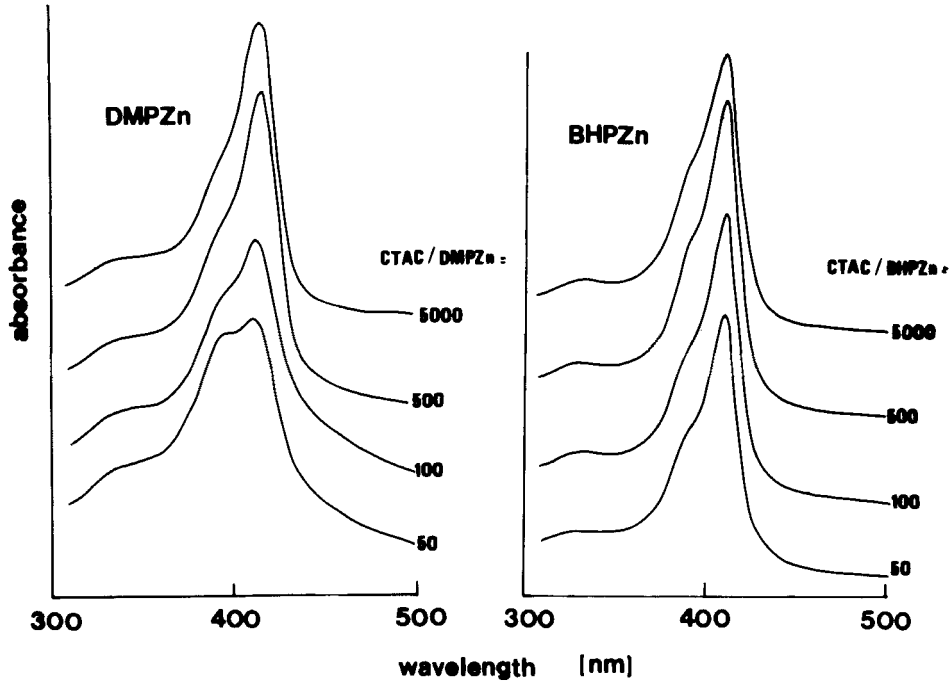

FIG. 5. Absorption spectra of porphinato zinc in micelle of cetyl trimethyl ammonium chloride (CTAC) with different molar ratios of CTAC to porphinato zinc. Concentration of porphinato zinc: 10^{-6} *M*.

porphyrins and water-soluble substrates in aqueous media should be limited. As water-soluble quencher, anthraquinone-2,6-disulfonic acid sodium salt (AQDS) was added to the aqueous phase of a liposome suspension, the fluorescence of both DMPZn and BHPZn in the liposome bilayer was quenched, but the quenching of DMPZn fluorescence was more noticeable than that of BHPZn (Fig. 7). Because the quenching of porphinato zinc fluorescence by AQDS involves electron transfer between the porphyrin ring and the AQDS molecule [12], the electron transfer efficiency, i.e., the quenching efficiency for porphinato zinc fluorescence, depends on the relative position of the redox centers [13]. The higher Stern-Volmer constant (K_{sq}) of DMPZn quenched by AQDS than that of BHPZn (Fig. 7 and Table 1) implies that DMPZn is located, at least partially, near the interface between the liposome and the aqueous phase. As 2,2,6,6-tetramethyl-4-piperidinol-1-oxide

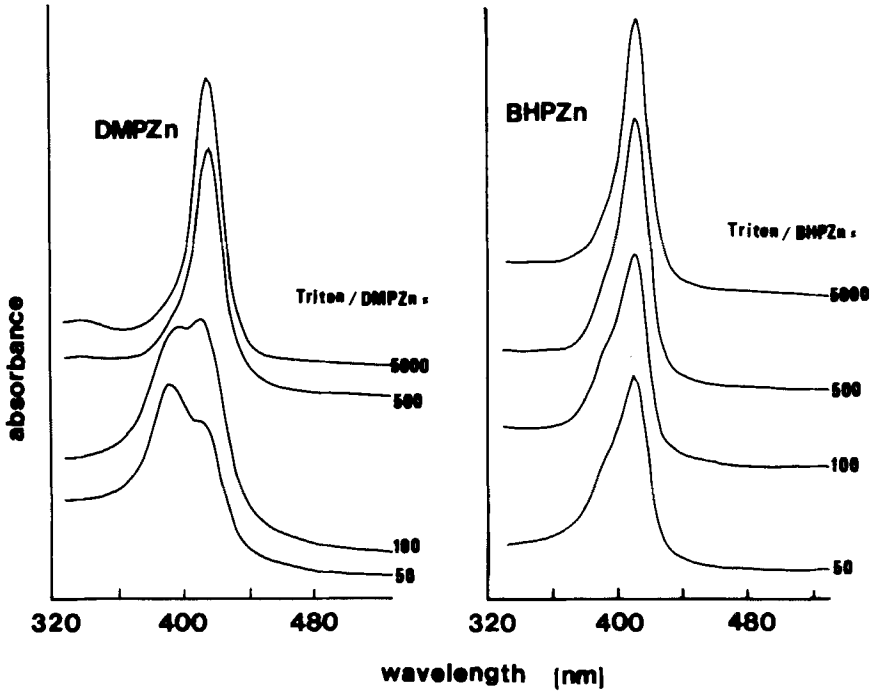

FIG. 6. Absorption spectra of porphinato zinc in micelle of Triton X-100 with different molar ratios of Triton to porphinato zinc. Concentration of porphinato zinc: 10^{-6} *M*.

(OH-TEMPO) was added to the aqueous phase of the liposome suspension, the quenching of the DMPZn fluorescence was also more marked than that of BHPZn (Fig. 8 and Table 1). The quenching of the porphyrin fluorescence by the TEMPO derivative is strongly distance dependent [14]. Thus, the effective quenching of DMPZn fluorescence by OH-TEMPO also suggests that the location of DMPZn in the bilayer membrane of the DPPC liposome was near the surface of the liposome. The hydroxy-terminated long alkyl substituents of BHPZn, which have good compatibility with DPPC molecules, can be packed parallel to the side chains of DPPC in the liposome. As a result, the porphyrin ring of BHPZn can be located in the bilayer membrane, with the depth decided by the length of substituents of BHPZn, and the quenching of BHPZn fluorescence by compounds dissolved in the aqueous phase of the liposome showed smaller Stern-Volmer constants.

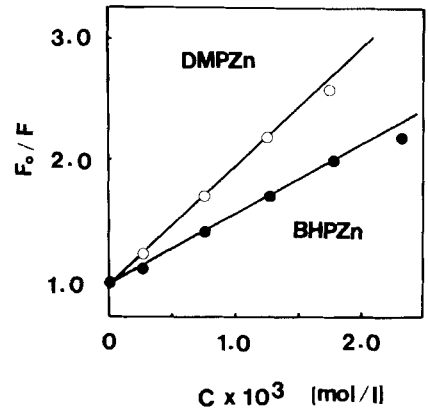

FIG. 7. Stern-Volmer plots for the quenching of fluorescence of porphinato zinc embedded into DPPC liposome by AQDS. Fluorescence of porphinato zinc E_m = 585 nm, E_x = 548 nm. Molar ratio of DPPC to porphinato zinc 500:1. Concentration of porphinato zinc: 10^{-6} *M*.

In the case of 16-DOXY-stearic acid (16-DSA) incorporated into DPPC liposome together with porphyrins, the Stern-Volmer constant of BHPZn was much larger than that of DMPZn (Table 1). Because 16-DSA is a TEMPO derivative of the long alkyl substituent with a carboxyl terminal group and can be incorporated in the bilayer membrane of the liposome with the depth decided by the side chain length [14], the larger Stern-Volmer constant of BHPZn quenched by 16-DSA implies that the porphyrin ring of BHPZn is located near the site of the 16-DSA active center in the hydrophobic region of DPPC liposome. This result also supports the deduction that the porphyrin

TABLE 1. Quenching Parameter for Porphinato Zinc Complexes Embedded in DPPC Liposome by Hydrophilic or Hydrophobic Quenchers

	$K_{sq} \times 10^{-2}, M^{-1}$		
	AQDS	OH-TEMPO	16-DSA
BHPZn	5.6	0.23	11.9
DMPZn	9.6	0.35	1.3

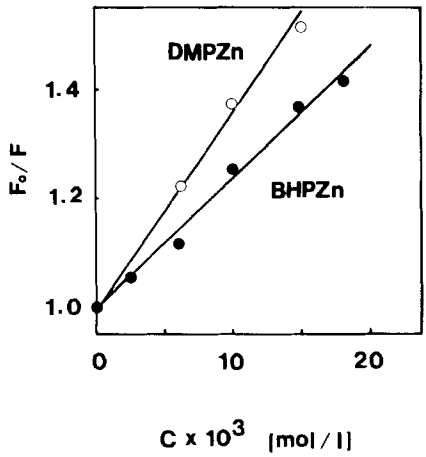

FIG. 8. Stern-Volmer plots for the quenching of fluorescence of porphinato zinc embedded in DPPC liposome by OH-TEMPO. Fluorescence of porphinato zinc E_m = 585 nm, E_x = 548 nm. Molar ratio of DPPC to porphinato zinc 500:1. Concentration of porphinato zinc: 10^{-6} *M*.

ring of BHPZn is incorporated into the bilayer membrane of DPPC liposome to the depth of the side-chain length. Thus, the active center of BHPZn in the hydrophobic region of DPPC liposome can be protected against the attack by water-soluble substrates, as in the case of hemoglobin, in which the active center is surrounded by the hydrophobic environment of the protein matrix. It is expected that this kind of artificial molecular assembly may play a certain role in the mimicking of some functions of biological materials. Further investigations of the properties and applications of porphyrin-lipid assemblies are proceeding in our laboratory.

CONCLUSION

In summary, 3,8,13,17-tetramethyl-7,12-divinyl-2,18-bis(18-hydroxyoctadecyl propionate)porphinato zinc (BHPZn) and the model compound dimethyl ester of protoporphinato zinc (DMPZn) can be incorporated in the hydrophobic bilayer membrane of diplamitoylphosphatidylcholine (DPPC) liposome. The bilayer membrane of DPPC liposome provides the incorporated porphyrins with a hydrophobic environment, and the aggre-

tion of both BHPZn and DMPZn in DPPC liposome is restricted. Introduction of long alkyl substituents on the porphyrin ring not only endows porphyrin with good compatibility with lipid molecules but also inhibits porphyrin aggregation in the liposome bilayer membrane. BHPZn can be dispersed molecularly in DPPC liposome, and the aggregation of BHPZn is less than that of the DMPZn model compound. Due to the orientation effect of hydroxy-terminated long alkyl substituents, the porphyrin ring of BHPZn can be embedded to a certain depth into the hydrophobic region of the DPPC liposome. Thus, the resulting molecular assembly is expected to serve as a model for biomimetic hemoglobinlike oxygen carriers or photoredox systems.

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