Supramolecular Assembly of Porphyrins and Monoclonal Antibodies

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The interaction of porphyrins and metalloporphyrins with the monoclonal antibody for *meso*-tetrakis(carboxyphenyl)porphine (TCPP) was studied by UV−vis, emission, and circular dichroism (CD) spectroscopy. The UV−vis spectra of TCPP and TCPP-M(Cu, Zn) in the presence of the antibody showed shifts of the Soret bands to a wavelength about 10 nm longer and hypochromicity. The UV−vis and emission spectra of TCPP showed isosbestic points and isoemisive points, respectively, in the presence of various concentrations of the antibody. The spectra of TCPP-Zn showed an isosbestic point in the presence of less than an equimolar amount of the antibody and further increase in the absorption at higher concentrations of antibody, suggesting the existence of a binding mode other than one-to-one binding. The CD spectra of TCPP-M(Cu, Zn) in the presence of the antibody showed strong induced Cotton effects on the Soret band of TCPP-M. The CD spectra of TCPP-Zn with the antibody showed sharp and strong splitting of the Soret band. The CD spectra of TCPP-Cu with the antibody showed a plus-to-minus switch according to the concentration of the antibody. These results suggest the existence of a higher order of association. The interactions between the antibody and some other porphyrin derivatives have been studied by spectroscopic methods. The binding modes are discussed.

Introduction

Recently, the organizations of porphyrins on DNA and polypeptides have attracted much attention because it may be possible to construct supramolecular complexes with conductive, magnetic, and catalytic properties by arrangement of porphyrins into various domains.[1] Such complexes will require the assemblies of metalloporphyrins in definite orientations. For example, in bacterial photosynthetic systems, reaction center porphyrins form dimers in a protein domain in the membrane.[2] The incorporation of porphyrins into certain protein domains in cytochrome *d* oxidase has been demonstrated.[3] More recently, self-assembly of porphyrins on nucleic acids and polypeptides has also been observed.[4,5] One of the best methods to incorporate porphyrins into protein domains is to make monoclonal antibodies for porphyrins. Here we found that porphyrins are incorporated in the combining site of the antibody, which was elicited against tetrakis(4-carboxyphenyl)-porphyrin, to form supramolecular assemblies.

Previously, we reported preparation of monoclonal antibodies against *meso*-tetrakis(4-carboxyphenyl)porphine (TCPP) and found that these antibodies bind TCPP strongly.[6] We have already reported that the CD spectra of TCPP in the presence of one of the antibodies showed induced Cotton effects.[7] Later, Keinan et al. reported that the antibodies against [*meso*-tetrakis-(4-(carboxyvinyl)phenyl)porphinato]tin(IV) dihydroxide bind porphyrins to give induced Cotton effects and suggested the formation of only one-to-one complexes.[8] In this study we found that one of the antibodies binds not only TCPP but also TCPP-M(Cu, Zn, Fe) and that it forms not only one-to-one complexes but also higher order complexes with TCPP-M. This is the first demonstration that the antibodies show a higher order of association. The structures of porphyrins used in this study are shown in Chart 1.

Antibodies have also attracted much attention as catalysts,[9] and the antibodies for other porphyrins have been prepared.[10]

Results and Discussion

UV−Vis Spectra. Figure 1 shows the UV−vis absorption spectra of TCPP-Zn(a) and TCPP-Cu(b) in the presence of monoclonal antibody 03−1. The spectra show shifts of the Soret bands of TCPP-M(Cu, Zn) to a wavelength about 10 nm longer on addition of the antibody (03−1), indicating that the metalloporphyrins are strongly bound to the antibody. The absorption spectra of TCPP in the presence of the antibody show an isosbestic point at 418 nm. Although TCPP-Zn in the presence of various concentrations of the antibody showed an isosbestic point at 427 nm until the molar ratio reached one-to-one, the spectra showed further increase in the absorption at higher concentrations of the antibody. These results indicate the existence of a higher order of association. Although the spectra of TCPP-Cu showed an isosbestic point at higher concentrations of the antibody at 413 nm, they did not show isosbestic points at lower concentrations of the antibody, indicating that the equilibrium is not a simple one-to-one binding and suggesting the existence of another type of binding. When Fab fractions were used instead of the antibody, results similar to those for the antibody were obtained, indicating that these spectroscopic changes are due to intermolecular interactions rather than intramolecular interactions.

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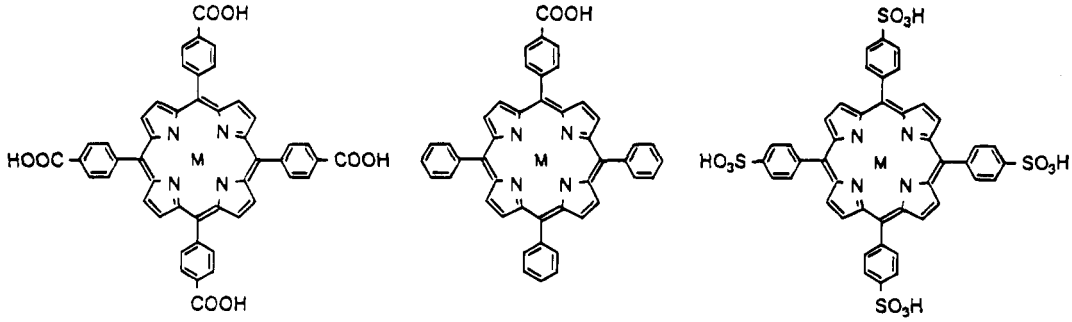
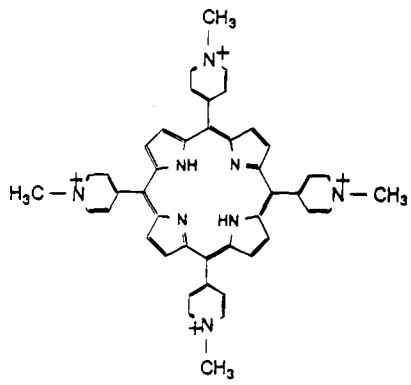
Chart 1



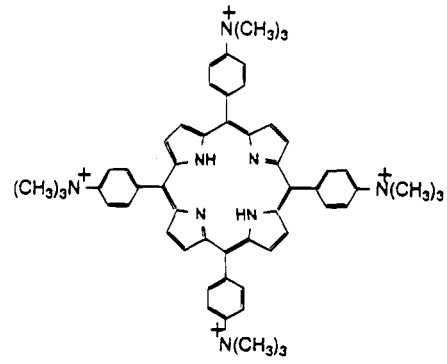
M: 2H TCPP
Zn TCPPZn
Cu TCPPCu
Fe TCPPFe(III)

M: 2H CATPP
Zn CATPPZn

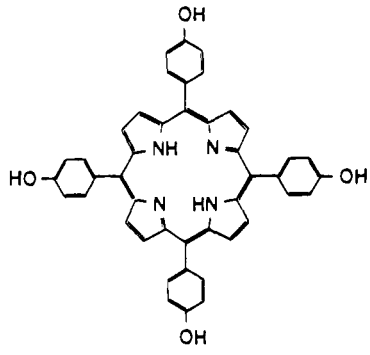
M: 2H TSPP
Zn TSPPZn



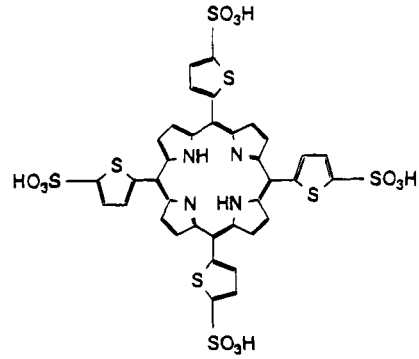
TMPyP



TTMAPP



TOHPP



T(5-ST)P

The binding of antibody 03-1 by TCPP and its metal complexes has been studied by fluorecence quenching of the antibody with the porphyrins. Table 1 shows the dissociation constants of the complexes of the antibody 03-1 with TCPP and its metal complexes assuming one-to-one complex formation. Antibody 03-1 has been found to bind TCPP, TCPP-Cu, and TCPP-Zn strongly with the dissociation constant of 10^{-7} M, but it did not bind TCPP-Co efficiently.

Emission Spectra. Figure 2 shows the emission spectra of TCPP-Zn at the fixed concentration (2.0×10^{-7} M) in the presence of various amounts of the antibody. The spectra show increase in the fluorescence bands and the absence of isoemissive points, although the spectra of TCPP in the presence of the antibody show three clear isoemissive points. The maxima of the emission of 0−0 and 0−1 bands also show shifts to a longer wavelength. These results indicate that TCPP-Zn is fixed in the antibody combining site firmly and the species in solution are not only one-to-one complexes.

Scatchard plots for the complex formation between antibody 03−1 and TCPP or its metal complexes show that the number of binding sites is 0.5; that is, two binding sites of the antibody bind a single porphyrin molecule in this concentration range (Figure 3).

Circular Dichroism Spectra. Figure 4 shows the circular dichroism (CD) spectra of TCPP-M(Cu, Zn) in the presence of the antibody. Both spectra show very strong induced Cotton effects ($\Delta\epsilon$ = (+)249 dm^3 mol^{-1} cm for TCPP-Cu, (+)317 dm^3 mol^{-1}cm, and (−)256 dm^3 mol^{-1} cm for TCPP-Zn). The CD spectrum of TCPP-Cu in the presence of the antibody shows behavior similar to that of TCPP in the presence of the antibody.

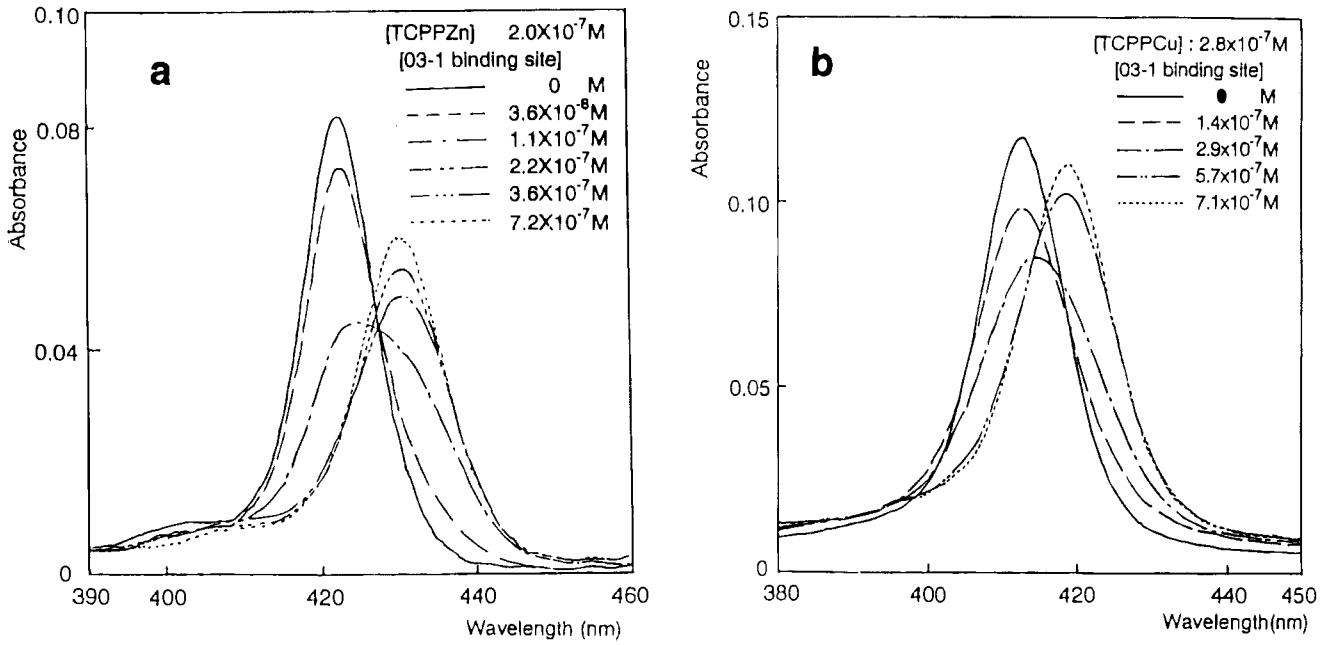


Figure 1. Absorption spectra of TCPP-Zn (2.0×10^{-7} M) (a) and TCPP-Cu (2.0×10^{-7} M) (b) in the absence and presence of antibody 03-1.

Table 1. Dissociation Constants for Antibody 03-1 and Porphyrins[a]

	dissociation constant/M		
	TCPP	TCPP-Cu	TCPP-Zn
03-1	5.0×10^{-7}	1.0×10^{-7}	5.0×10^{-7}

[a] Estimated by fluorescence spectroscopy (excitation wavelength 280 nm) assuming 1:1 complex formation.

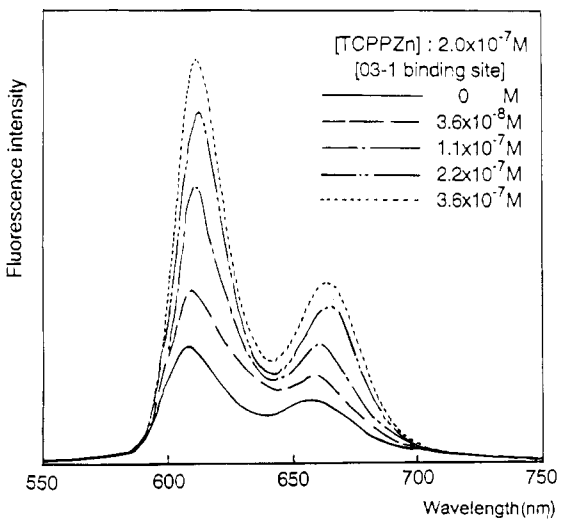


Figure 2. Emission spectra of TCPP-Zn (2.0×10^{-7} M) in the absence and presence of antibody 03-1. The excitation wavelength was 422.4 nm.

The plus Cotton effects were observed at lower concentrations of the antibody until the molar ratio reached one-to-one. Then the plus Cotton effects decreased sharply with increase in the antibody concentration. The plots show a maximum at approximately one-to-one. When the molar ratio exceeds one-to-one, the positive ICD decreases sharply and finally negative ICDs appear. These results clearly indicate the existence of at least two kinds of species in the complexes according to the concentration of the antibody. On the other hand, the spectrum of TCPP-Zn in the presence of the antibody shows sharp splitting at the Soret bands with long wavelength negative and short wavelength positive bands. These bands can be assigned

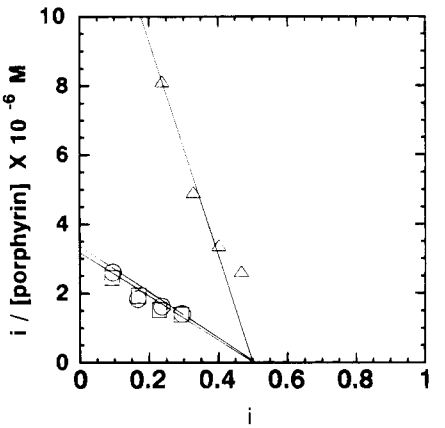


Figure 3. Scatchard plots for the complex formation of antibody 03-1 with TCPP (○), TCPP-Cu (△), and TCPP-Zn (□).

to an exciton coupling of the band. This result can be interpreted as indicating that two TCPP-Zn molecules are close together in the combining site of the antibody. In aqueous solution, CD spectra of the antibody from 200 to 300 nm showed no change on addition of TCPP. These results indicate that no large changes in the ordered structure of the antibody occurred on addition of TCPP.

Figure 5 shows the intensities of the maxima and minima of the induced CD on TCPP-Zn as a function of the concentrations of the antibody. The plus Cotton effects and minus Cotton effects increased and decreased sharply with increase in the concentration of the antibody. The saturations were observed in both cases at a molar ratio of TCPP-Zn to antibody of 1:1. Then the intensity of the Cotton effects decreased with increase in concentration of the antibody over a molar ratio of one-to-one. These results indicate that there is another binding mode at molar ratios of over one-to-one. When Fab fractions were used instead of the antibody, results similar to those for the antibody again were obtained, indicating that these spectroscopic changes are due to intermolecular interactions rather than intramolecular interactions.

Figure 6 shows the effects of NaCl on the absorption and CD spectra of TCPP in the presence of the antibody. Addition of salt to the solution of TCPP in the presence of excess antibody

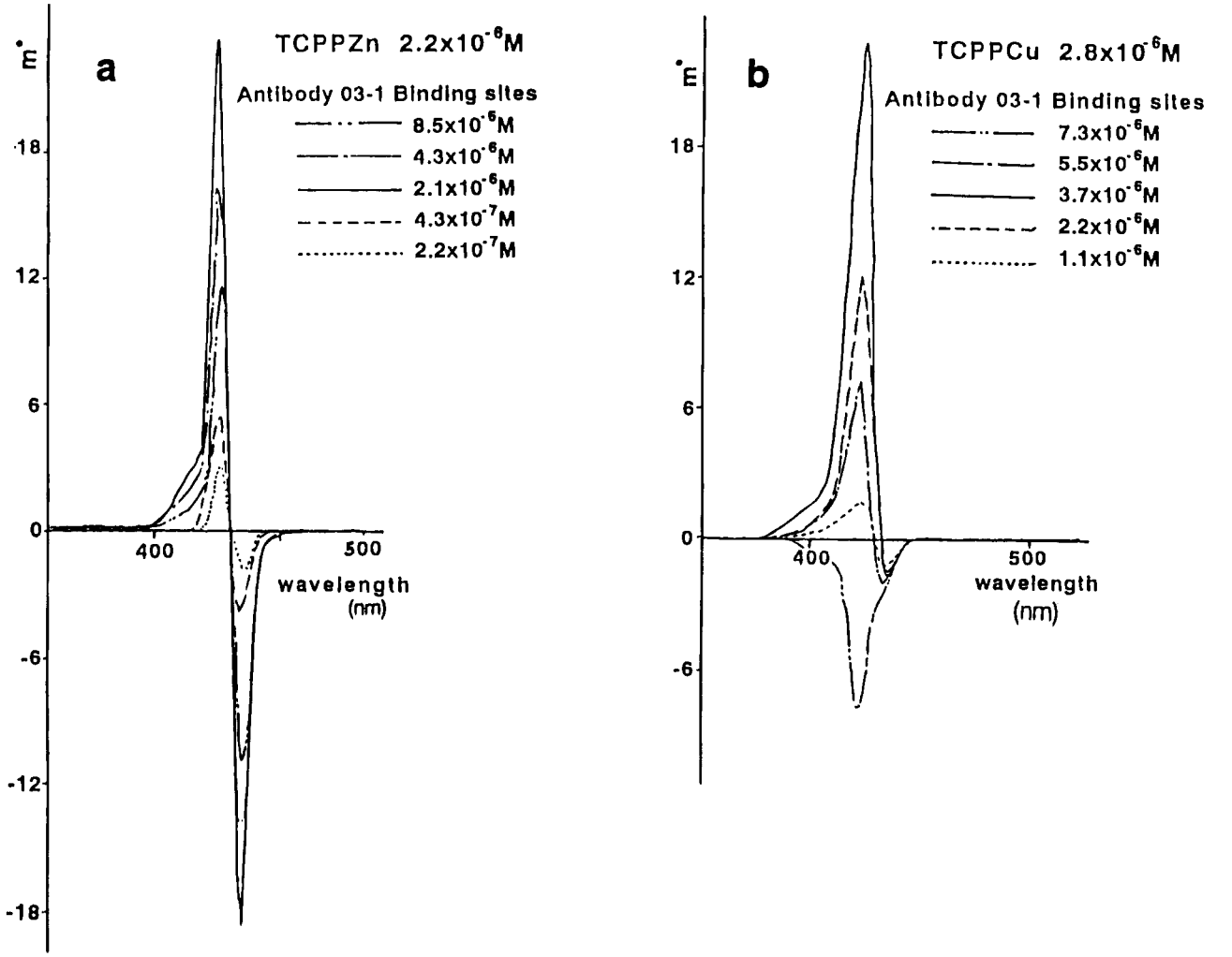


Figure 4. Circular dichroism spectra of TCPP-Zn (a) and TCPP-Cu (b) in the presence of antibody 03-1.

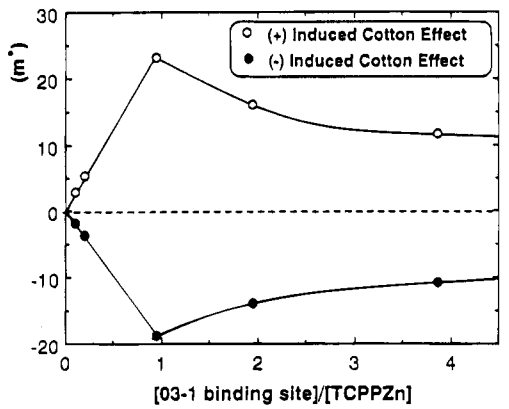


Figure 5. Intensities of the maxima and minima of the induced Cotton effects observed from the circular dichroism spectra of TCPP-Zn in the presence of antibody 03-1 as a function of the ratio of the concentration of the antibody-combining site to that of TCPP-Zn.

results in increase in the Cotton effects. This result indicates that the coagulation of antibody molecules collapses on addition of the salt. In other words, electrostatic interactions play an important role in stabilizing the supramolecular assemblies.

Binding Modes. Figure 7 shows a proposed mechanism for the complexation of TCPP and TCPP-M(Cu, Zn) with antibody 03-1. In each case, a one-to-one complex is first formed between porphyrin and the antibody. Then, another antibody binds TCPP or TCPP-Cu to form a two-to-one complex (two antibodies and one porphyrin). In the case of TCPP-Zn, two one-to-one complexes associate to form a two-to-two complex between porphyrin and TCPP-Zn.

Specificity. Table 2 shows the changes in the UV−vis and CD spectra of various porphyrins on addition of antibody 03-1. A cationic porphyrin, *meso*-tetrakis(*N*-methylpyridinium-4-yl)porphine (TMPyP), did not show any change in the CD spectrum or UV−vis spectrum on addition of the antibody. Although slight hypochromism was observed with *meso*-tetrakis(4-(trimethylamino)phenyl)porphine (TTMAPP), no Cotton effects were observed in the presence of the antibody. A neutral porphyrin, *meso*-tetrakis(4-hydroxyphenyl)porphine (TOHPP) did not show any changes in the spectra on addition of the antibody, indicating that it did not interact with the antibody. *meso*-Tetrakis(5-sulfothienyl)porphine (T(5-ST)P), which is an anionic porphyrin but with thiophene rings in place of the phenyl rings of TCPP, did not show any effects on the CD spectra and little effects on the UV−vis spectra. These results indicate that antibody 03-1 recognizes porphyrins with high specificity.

The absorption spectra of *meso*-tetrakis(4-sulfonatophenyl)-porphine (TSPP) in the presence of various concentrations of the antibody showed a clear isosbestic point at 418 nm, indicating equilibrium between the free state and complexed state (Figure 8). In the emission spectra of TSPP in the presence of various concentrations of the antibody, three isoemisive points were seen at 645, 670, and 710 nm with a long wavelength shift. The CD spectra of TSPP in the presence of the antibody showed that minus Cotton effects appeared at 414 and

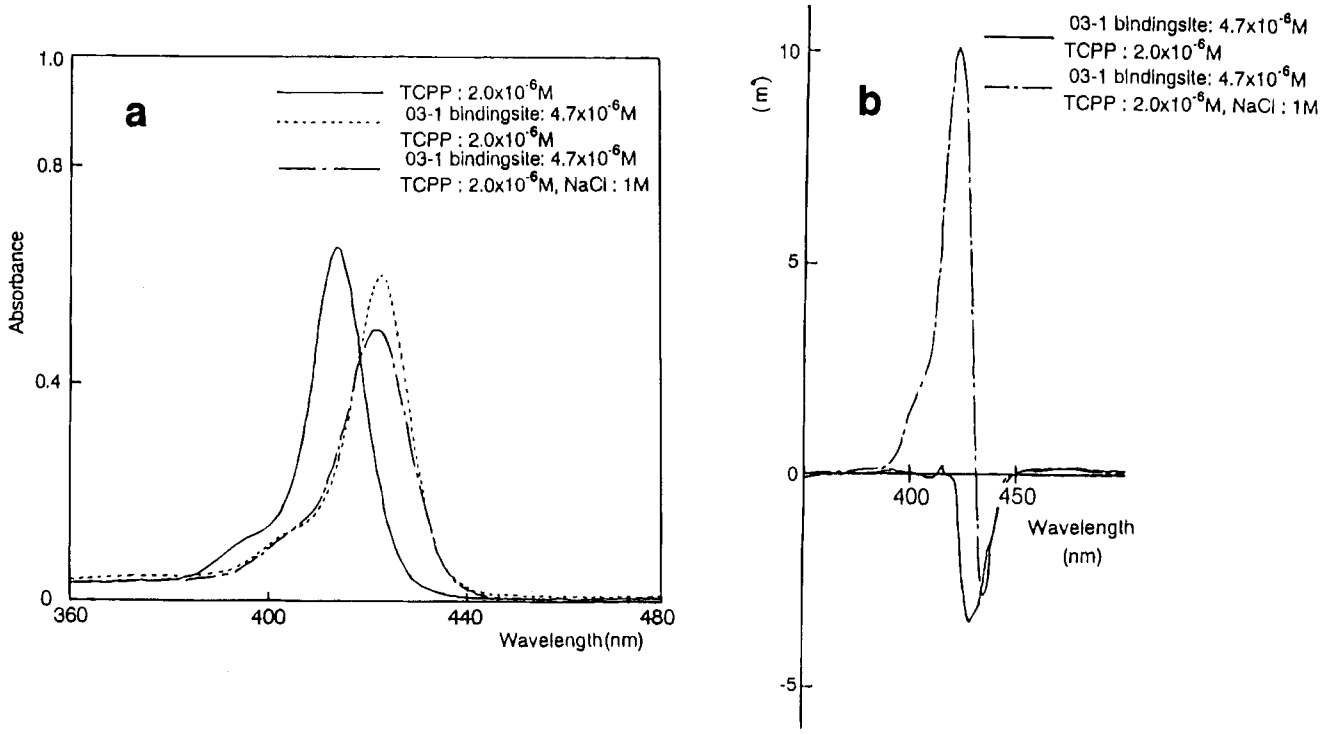


Figure 6. Effects of NaCl on the absorption spectra (a) and circular dichroism spectra (b) in the presence of antibody 03-1.

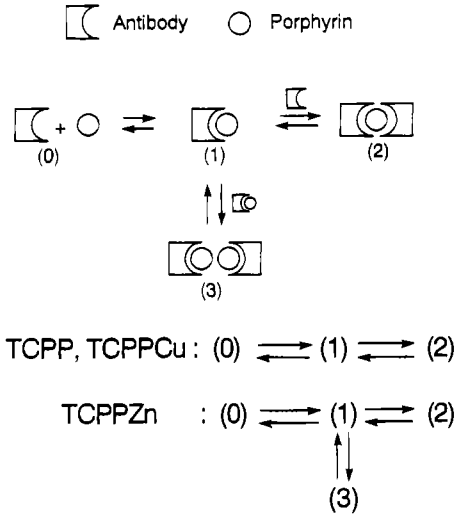


Figure 7. Proposed mechanism for complex formations of TCPP, TCPP-Zn, and TCPP-Cu with antibody 03-1.

432 nm at low concentrations of the antibody, splitting Cotton effects at an equimolar concentration, and a minus Cotton effect at higher concentrations of the antibody. These results indicate a one-to-one-complex at low concentrations of the antibody and a two-to-one (Ab:porphyrin) complex at higher concentrations.

The absorption spectra of TSPP-Zn with the antibody were similar to those of TCPP-Zn with the antibody. The CD spectra of TSPP-Zn in the presence of the antibody showed splittings similar to those of TCPP-Zn with the antibody, but the intensity was one-tenth of the latter. Thus antibody 03-1 is able to differentiate TCPP-Zn and TSPP-Zn in terms of the intensity of induction of Cotton effects.

TCPP-Fe(III), which has chlorine as an axial ligand, was bound to the antibody, resulting in a monotonous red shift of the Soret band and plus Cotton effects, indicating one-to-one binding.

When added at 200 molar excess to a solution of TCPP (2.1×10^{-6} M) and antibody (03-1) (5.3×10^{-6} M), benzoic acid, *p*-carboxybenzaldehyde, and *p*-toluenesulfonic acid, which represent parts of the TCPP and TSPP, did not replace TCPP and TSPP in the binding site of the antibody. Thus the antibody binds to most parts of TCPP and TSPP molecules.

As mentioned above, antibody 03-1 gives not only a 1:1 complex but also other higher orders of aggregation. CATPP, which has only one carboxy group and is more hydrophobic than TCPP, was used as a guest molecule. The Soret band of CATPP decreased by about half on addition of antibody 03-1 and shifted to a longer wavelength. In the presence of antibody 03-1, the CD spectra of CATPP were different from those of TCPP and similar to those of TCPPZn. However, the order of the Cotton effects was different, showing a longer wavelength positive and short wavelength positive band. These CD spectra suggest that CATPP also forms a dimer in the combining site of the antibody.

These spectroscopic data observed with an intact antibody were almost identical to those with a Fab fragment obtained by cleaving the antibody with papain. This fact indicates that cooperativity is not an intramolecular process but an intermolecular interaction.

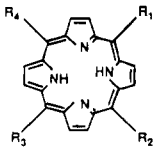
After our paper describing circular dichroism studies on the interactions between TCPP and a monoclonal antibody had appeared,[7] Keinan et al. reported that the UV and CD spectra of porphyrin in the presence of an antibody for [*meso*-tetrakis-(4-(carboxyvinyl)phenyl)porphinato]tin(IV) dihydroxide follow Beer's law, exhibit isosbestic behavior, and show no significant change in the general shape of the Cotton effect as a function of the antibody:porphyrin ratio.[8] This indicates that a simple one-to-one hapten−antibody complex was formed.

We conclude from this study that the monoclonal antibody for TCPP shows a high specificity for porphyrins and forms not only a one-to-one complex but higher orders of association to give supramolecular complexes.

Experimental Section

Materials. Porphyrins were prepared in the usual way starting from pyrrole and aldehydes. *meso*-Tetrakis(4-carboxyphenyl)porphine (TCPP) was prepared by the reaction of *p*-carboxybenzaldehyde and pyrrole.

Table 2



Name	Substrate	U.V.-Vis spectra	CD spectra
CATPP[a]	R1: —COOH R2,R3,R4:	red shift 5 nm hypochromism	(+) 452nm (-) 435nm
CATPPZn[a]	R1: —COOH R2,R3,R4: Zn Complex	red shift 10 nm hyperchromism	(-) 446nm (+) 436nm
TSPPZn	R1,R2,R3,R4: —SO_3H Zn Complex	red shift 7 nm hypochromism and hyperchromism	(-) 437nm (+) 426nm
T(5-ST)P	R1,R2,R3,R4: —SO_3H	red shift 2 nm hypochromism	X[b]
TMPyP	R1,R2,R3,R4: —N^+—CH_3	X	X
TTMAPP	R1,R2,R3,R4: —$N^+(CH_3)_3$	red shift 1 nm hypochromism	X
TOHPP[a]	R1,R2,R3,R4: —OH	X	X

a) Water insoluble porphyrin

b) X means no change between in the presence and absence of Antibody 03-1.

5-(4-Carboxyphenyl)-10,15,20-triphenylporphine (CATPP) was prepared from *p*-carboxybenzaldehyde, benzaldehyde, and pyrrole. *meso*-Tetrakis(4-hydroxyphenyl)porphine (TOHPP) was prepared from *p*-hydroxybenzaldehyde and pyrrole. Porphyrins were metalated by heating them with metal chloride or metal acetate in dimethylformamide (DMF).

***meso*-Tetrakis(4-carboxyphenyl)porphine (TCPP).** *meso*-Tetrakis(4-carboxyphenyl)porphine (TCPP) was prepared by the reaction of *p*-carboxybenzaldehyde and pyrrole according to the method of Longo *et al.*[12] ^{1}H NMR (DMSO-d_6): δ 8.85 (s, 8H), 8.39 (d, J = 8 Hz, 8H), 8.33 (d, J = 8 Hz, 8H), −2.92 (s, 2H). Anal. Calcd for $C_{48}H_{30}N_4O_8 \cdot 1.8H_2O$: C, 70.03; H, 4.11; N, 6.81. Found: C, 70.12; H, 3.92; N, 6.74.

5-(4-Carboxyphenyl)-10,15,20-triphenylporphine (CATPP). A suspension of *p*-carboxybenzaldehyde (15.0 g; 0.10 mol) and benzaldehyde (30.6 mL; 0.30 mol) in propionic acid (1 L) was heated and refluxed. To the mixture was added dropwise in 20 min 28 mL (0.40 mol) of pyrrole, and the mixture was refluxed. Propionic acid was removed by evaporation, and the residue was passed through a silica gel column ($CHCl_3$:acetone:acetic acid = 8:2;0.1). The solvent was evaporated, and the product was purified by recrystallization from $CHCl_3$−MeOH; purple granules. Anal. Calcd for $C_{45}H_{30}N_4O_2 \cdot 0.34H_2O$: C, 81.29; H, 4.56; N, 8.43. Found: C, 81.29; H, 4.65; N, 8.17.

***meso*-Tetrakis(4-hydroxyphenyl)porphine (TOHPP).** Propionic acid (300 mL) was added to *p*-hydroxybenzaldehyde (15.0 g; 1.23 × 10^{-4} mol), and the mixture was refluxed. The pyrrole (8.5 mL; 0.123 mol) was added dropwise in 15 min. Then the mixture was refluxed and allowed to stand overnight. The product was filtered off, purified by column chromatography on silica gel, and recrystallized from MeOH; purple granules. ^{1}H NMR (DMSO-d_6): δ 9.92 (s, 4H), 8.87 (s, 8H), 8.00 (d, J = 8 Hz, 8H), 7.21 (d, J = 8 Hz, 8H). Anal. Calcd for $C_{44}H_{30}N_4O_4 \cdot 2.3H_2O$: C, 73.38; H, 4.84; N, 7.78. Found: C, 73.29; H, 4.85; N, 7.75.

[*meso*-Tetrakis(4-carboxyphenyl)porphyrinato]copper(II) (TCPP-Cu). A solution of $CuCl_2 \cdot 2H_2O$ (310 mg; 1.82 mmol) in DMF was added to a solution of TCPP (260 mg; 0.33 mmol) in 70 mL of DMF. The mixture was heated to 80 °C for 1 h. The reaction was followed by monitoring the absorption spectrum. DMF was removed by distillation, and the residue was recrystallized from DMF−acetone; orange needles. Anal. Calcd for $C_{48}H_{28}N_4O_8Cu \cdot 0.6H_2O$: C, 66.80 H, 3.41; N, 6.49. Found: C, 66.88; H, 3.45; N, 6.32.

[*meso*-Tetrakis(4-carboxyphenyl)porphyrinato]zinc (TCPP-Zn). TCPP-Zn was prepared from TCPP by a method similar to that for TCPP-Cu. ^{1}H NMR(DMSO-d_6): δ 8.80 (s, 8H), 8.37 (d, J = 8 Hz, 8H), 8.30 (d, J = 8 Hz, 8H).

[5-(4-Carboxyphenyl)-10,15,20-triphenylporphyrinato]zinc (CATPP-Zn). CATPP (186 mg; 2.83 × 10^{-4} mol), dissolved in 30 mL of DMF, was mixed with a solution of ZnCl (30 mg; 2.43 × 10^{-3} mol) in DMF (2 mL). The reaction mixture was heated to 100−110 °C with stirring for 2 h, and the reaction was followed by monitoring the absorption spectrum. DMF was removed by reduced distillation, and the residue was recrystallized from $CHCl_3$−MeOH; purple crystals. Anal. Calcd for $C_{45}H_{28}N_4O_2Zn \cdot 1.47H_2O \cdot 0.29CHCl_3$: C, 69.49; H, 4.02; N, 7.16. Found: C, 69.49; H, 4.02; N, 7.34.

[*meso*-Tetrakis(4-sulfonatophenyl)porphinato]zinc (TSPP-Zn). TSPP-Zn was prepared according to the method of Kalyanasundaram.[13] Crude TSPP-Zn was purified by passage through a Sephadex LH-20 column with water as eluent. ^{1}H NMR (DMSO-d_6): δ 8.78 (s, 8H), 8.12 (d, J = 8 Hz, 8H), 8.00 (d, J = 8 Hz, 8H).

meso-Tetrakis(4-sulfonatophenyl)porphine (TSPP) and *meso*-tetrakis-(*N*-methylpyrid-4-yl)porphine (TMPyP) were obtained from Tokyo Kasei Inc. *meso*-Tetrakis(4-(*N*-trimethylamino)phenyl)porphine (TTMAPP) and *meso*-tetrakis(5-sulfothienyl)porphine (T(5-ST)P) were purchased from Dojin Chemicals Inc.

Preparation of Monoclonal Antibodies. Preparation of Protein Conjugates. TCPP (6 mg) and carbonyldiimidazole (CDI) (1.2 mg) were dissolved in 2 mL of dimethylformamide (DMF), and the solution was stirred at 0 °C for 2 h. The mixture was then added dropwise to 6 mL of PBB buffer (pH 9.0) containing 5 mg of KLH (keyhole-limpet hemocyanin) (Sigma) with stirring at 0 °C overnight. The concentration of protein was determined by the BCA method (Pierce). The concentration of TCPP was determined from the absorbance at the Soret band (450 nm). The number of TCPP on the protein was calculated from the molar ratio of TCPP to KLH. The product was purified by column chromatography on Sephadex G100 with PBB buffer as eluent.

Immunization. Balb/c mice (8-week-old) were immunized with the KLH−TCPP conjugate emulsified in complete Freund's adjuvant twice with an interval of 1 month between injections and finally an intravenous injection of the conjugate alone 1 month later.

Monoclonal Antibody Production. Three days after the last injection of conjugate, the spleen was removed and spleen cells were isolated and fused with myeloma cells using poly(ethylene glycol) (MW = 1000, Boehringer, Mannheim).[14] They were then plated in twenty 96-well plates in wells containing 150 μL of hypoxanthines, aminopterin, thymidine (HAT), and CelGrosser-H (Sumitomo Chemical). After 2 weeks, the antibodies produced by wells containing macroscopic colonies were assayed by ELISA for TCPP binding. Colonies were cloned, screened, and cultured. Cells were injected into pristane-primed Balb/c mice to generate ascitic fluid. IgG was purified from the ascitic fluid by ammonium sulfate precipitation and anion exchange chromatography. Antibodies were further purified on a protein A column (Amersham Ampure™ PA kit). Monoclonal antibodies were obtained by dialysis against PBB buffer (pH 8.0). The purity of antibodies was checked by SDS−PAGE electrophoresis.

Production of the Fab Fragment. Antibody (25 mg) in 10 mL of PBB buffer (pH 8) was mixed with 0.1 M L-cysteine solution (1.11mL) and then 25 mL of papain solution (Sigma). The mixture was incubated

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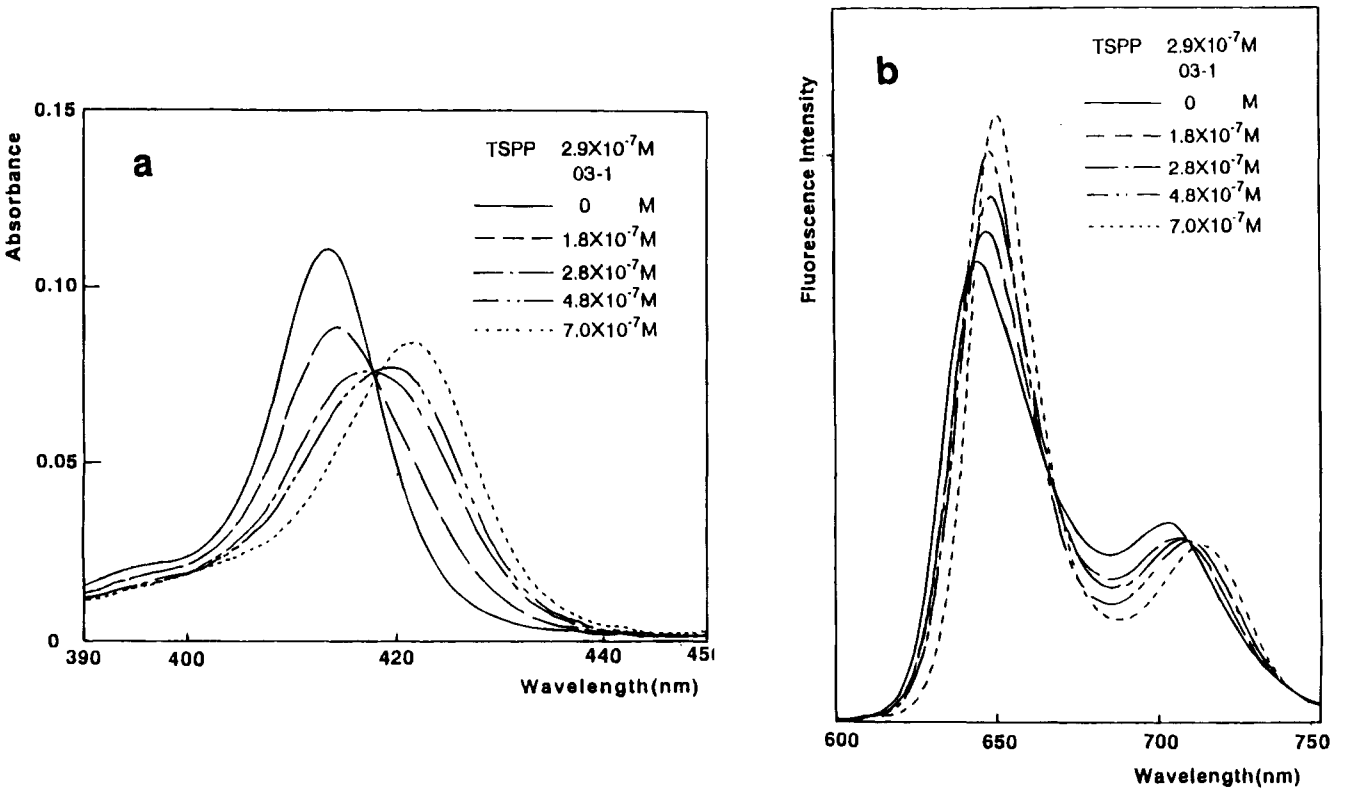

Figure 8. Absorption spectra (a) and emission spectra (b) of TSPP in the absence and presence of various concentrations of antibody 03-1. The excitation wavelength was 417.7 nm.

at 37 °C for 4 h. The reaction was terminated by the addition of iodoacetamide to a final concentration of 30 mM. The reaction mixture was passed through a prepacked protein A column (Pierce immunopure kit). The Fab eluted (16.3 mg) was passed through a Sephadex G100 column. The purity was checked by SDS–PAGE.

Measurements. Measurements were carried out in phosphate borate buffer (PBB) (pH 8.0). The concentration of the antibody was expressed as the concentration of binding sites; the antibody has twobinding sites, so the concentration of binding sites was twice that of the antibody.

UV–vis spectra were recorded on a Shimadzu UV-2100 UV–visible spectrophotometer. CD spectra were recorded on a JASCO J-40A spectrophotometer with a JASCO ORD/CD data processor using a 10 mm quartz cell. Proton NMR spectra were taken at 270 MHz in $CDCl_3$ on a JEOL JNM GSX-270 NMR spectrometer. Chemical shifts were determined with reference to TMS. Emission spectra were recorded on a Shimadzu RF-502A spectrofluorophotometer. The excitation wavelength was 280 nm.

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