Title	Preparation of Functionalized Antibodies
Author(s)	山口, 浩靖
Citation	大阪大学, 1998, 博士論文
Version Type	VoR
URL	https://doi.org/10.11501/3143786
rights	
Note	

Preparation of Functionalized Antibodies

A Doctoral Thesis
by
Hiroyasu Yamaguchi

Submitted to
the Graduate School of Science, Osaka University

February, 1998

Preparation of Functionalized Antibodies

A Doctoral Thesis

by

Hiroyasu Yamaguchi

Submitted to

the Graduate School of Science, Osaka University

February, 1998

Approvals

February, 1998

This thesis is approved as to
style and content by

蒲池幹治

Member-in-chief

中村晃   森島淨太郎
Member   Member

原田明   足立桂一郎
Member   Member

Acknowledgments

Osaka

February, 1998

Hiroyasu Yamaguchi

Contents

Definitions

Adjuvant: A substance which non-specifically enhances the immune response to an antigen.

Affinity: Measure of the binding strength between an antigenic determinant and an antibody combining site.

Antibody: A molecule produced by animals in response to an antigen, which has the particular property of combining specifically with the antigen which induced its formation.

Antigen: A molecule which induces the formation of an antibody.

CDR (Complementarity Determining Regions): The sections of an antibody, responsible for an antigen binding.

Carrier: An immunogenic molecule, or part of a molecule, which is recognized by T-cells in an antibody response.

Clone: A family of cells or organisms having a genetically identical constitution.

Constant Regions: The relatively invariant parts of immunoglobulin heavy* and light** chains.

  * Heavy chain: A polypeptide of ca. 50,000 daltons.

  ** Light chain: A polypeptide of ca. 25,000 daltons.

ELISA (Enzyme-linked Immunosorbent Assay): A group of techniques for measuring the binding affinities of antibodies, in which one of the reagents is coupled to an enzyme generating a colored reaction product.

Fab: The part of an antibody molecule which contains the antigen combining site, consisting of a light chain and part of the heavy chain. It is produced by enzymatic digestion.

Fc: The portion of an antibody which is responsible for binding to antibody receptors on cells.

Framework Segments: Sections of antibody V regions which lie between the hypervariable regions.

Freund's Adjuvant: An emulsion of aqueous antigens in oil. Complete Freund's adjuvant contains killed Mycobacterium tuberculosis.

Hapten: A small molecule which can act as an antigen determinant but which is incapable itself of eliciting an antibody response.

Hinge: The portion of an immunoglobulin heavy chain between the Fc and Fab regions. This allows flexibility within the molecule and allows the two combining sites to operate independently.

Hybridoma: Cell lines created in vitro by fusing two different cell types, usually lymphocytes, one of which is a tumor cell.

Hypervariable Region: The most variable areas of the V domains of immunoglobulin and T cell receptor chains. These regions are clustered at the distal portion of the V domain and contribute to the antigen-binding site.

Immunogen: An antigen which elicits a strong immune response, particularly in the context of protective immunity to a pathogen.

Monoclonal: Derived from a single clone, for example, monoclonal antibodies which are produced by a single clone and are homogeneous.

Myeloma: A lymphoma produced from cells of the B cell lineage.

Polyclonal: A term which describes the products of a number of different cell types.

Protein A: A cell wall component of certain strains of staphylococci, which binds to a site in the Fc region of most IgG isotypes.

Chapter 1

General Introduction

Background

In biological systems, life process is led by the unique behavior of macromolecules such as DNA and proteins. Especially molecular recognition by the macromolecules plays an important role, for example, in substrate specificities of enzyme and antigen-antibody reactions in human life. Selective recognition among macromolecules is achieved through a large number of weak bonding interactions including hydrogen bonds, van der Waals, dipole and/or electrostatic interactions and so on. L. Pauling recognized the similarity between enzymes and antibodies nearly 50 years ago.[1] While antibodies have not been considered to catalyze reactions, Schultz[2] and Lerner[3] found about ten years ago that antibodies might possess the intrinsic catalytic activity through unique sizes and shapes of their binding pockets. We have focused our attention on the special behavior of antibodies, especially monoclonal antibodies because they can recognize a larger and more complex compound with high specificity than that enzymes can. Recently, with the advent of cell technology,[4] it has become possible to prepare individual immunoglobulins which are called "monoclonal antibodies" in large amounts and in homogeneous form. The research groups of Schultz and Lerner isolated monoclonal antibodies using cell technology against the tetrahedral, negatively charged phosphate and the phosphonate transition state analog and they found that these antibodies selectively catalyze the hydrolysis of carbonates and esters, respectively (Figure 1.1).[2,3]

Ester hydrolysis reaction
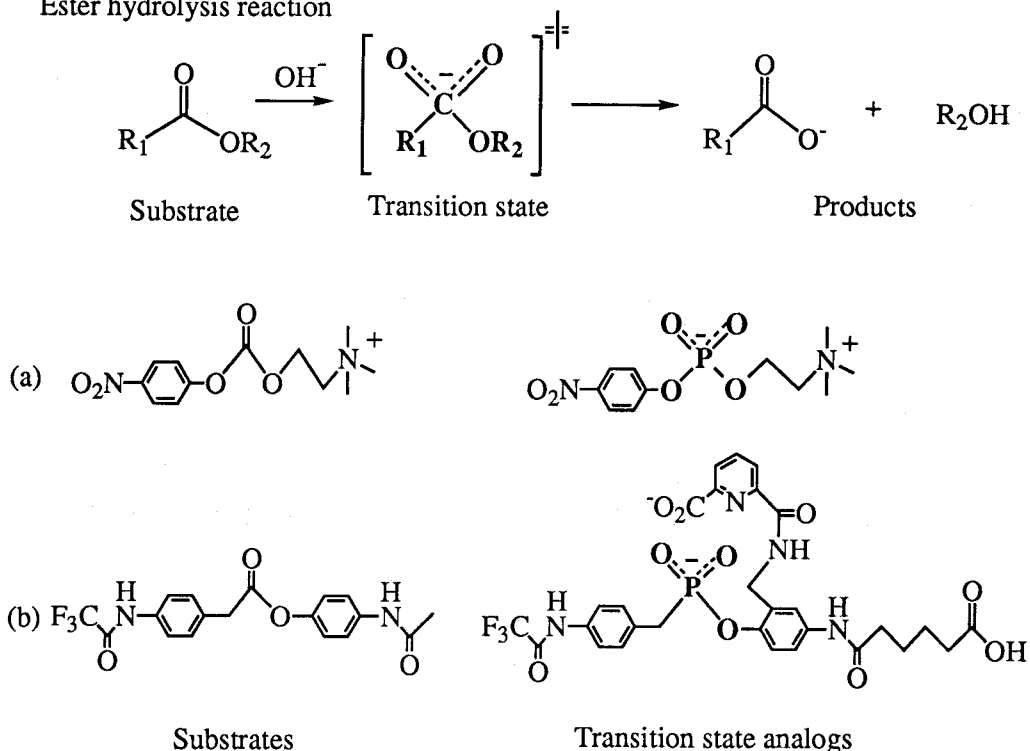
Figure 1.1. Transition state analogs synthesized by Schulz *et al.* (a) and Lerner *et al.* (b) for the catalytic hydrolysis reactions of esters. The isolated monoclonal antibodies specific for transition state analogs (a) and (b) were found to catalyze the hydrolysis of the corresponding substrates (a) and (b).

The development of general strategies for introducing catalytic activity into antibody combining sites should lead to a new class of enzymelike catalysts with tailored specificities. Two general approaches have been proposed on the catalytic behavior of antibodies. The steric and electronic complementarity of an antibody to its corresponding hapten (the ligand against which the antibody is elicited) has been investigated from the following four points: (1) the complementarity to the rate-determining transition state,[2,3,5-65] (2) overcoming the entropy requirements appropriately involved due to orienting reaction partners, (3) cofactor in

5

combining sites,[66-74] or (4) appropriately positioned catalytic amino-acid sequences.[75-79]

The immune system's ability to generate selectively antibodies against virtually any molecule of interest has resulted in the widespread use of antibodies not only as diagnostic agents but also as catalysts in chemical laboratories and industry. They are also used as probes for isolating and unraveling structures of complex biological molecules. Monoclonal antibodies have become more and more important with high possibility as new chemical materials.

Structures of Monoclonal Antibodies. Antibodies are composed of large proteins. Many antibody molecules are produced in the immune system, but these antibodies can be classified into five classes by their structures and amino acid sequences. These five classes are called as Immunoglobulin G (IgG), IgD, IgE, IgA, and IgM. (Figure 1.2). A typical antibody molecule, IgG consists of four polypeptide chains: two identical heavy chains of ca. 50,000 daltons and two identical light chains of ca. 25,000 daltons. They are cross-linked each other by disulfide bonds (Figure 1.3).[80,81] Each type has common characteristic sequences (C in Figure 1.3) and variable sequences characteristic of antibodies (V in Figure 1.3) in five types, respectively. The amino acid sequences are fixed in the regions shown as C, while those in the regions shown as V are unique and highly polymorphic for each immunoglobulin. A number of antibodies have been produced by changing the amino acid sequences of V regions.[82,83]

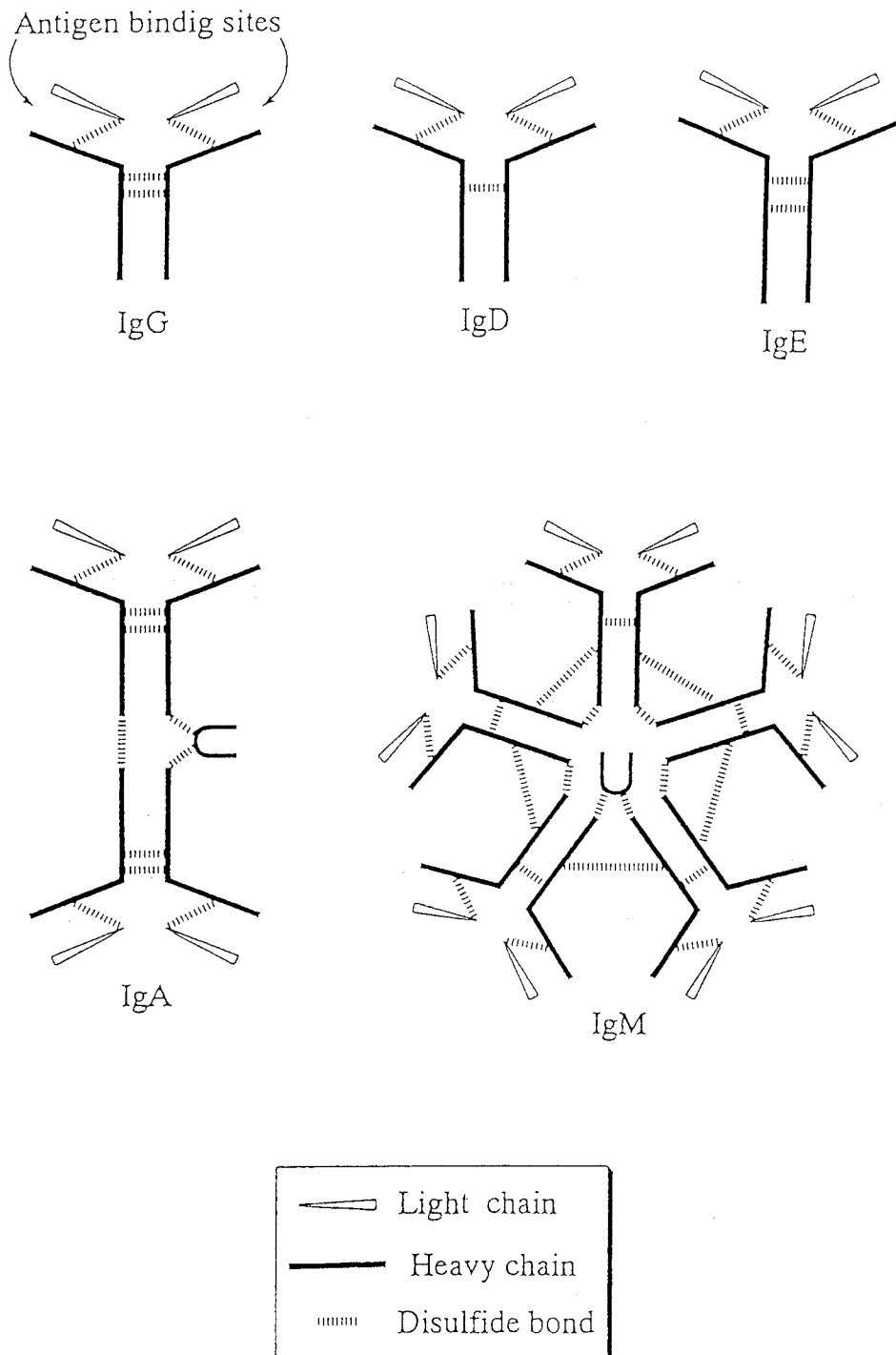**Figure 1.2.** Schematic structures of immunoglobulins.

(a), Immunoglobulin G (IgG); (b), IgD; (c), IgE; (d), IgA and (e), IgM.
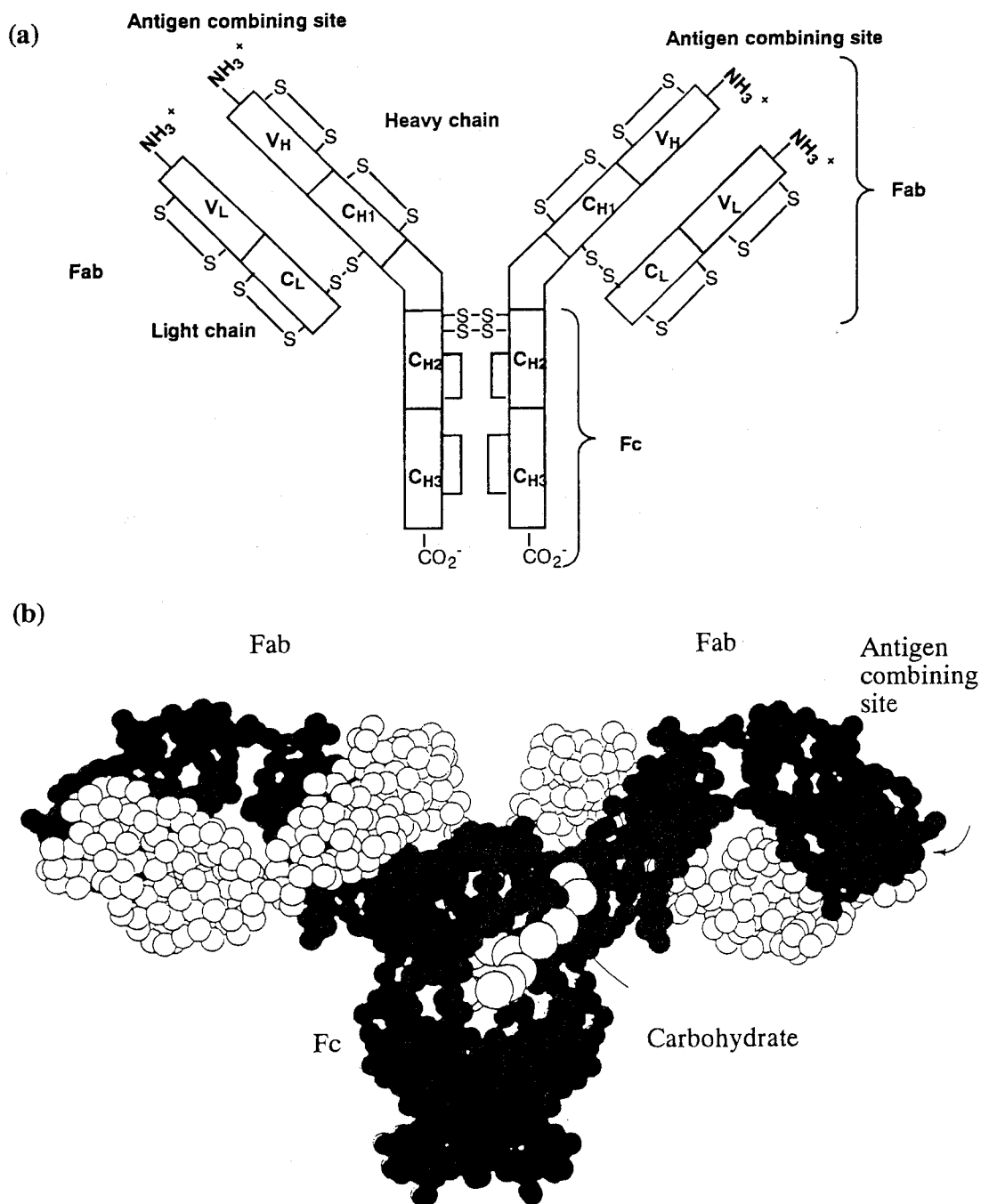In IgA or IgM, U shape structure represents a polypeptide called J chain.

7

(a)

(b)

Figure 1.3. The schematic structure (a) and three-dimensional structure (b) of immunoglobulins (antibodies). Each amino acid residue is represented by a small sphere in (b).

8

Scope of This Research

It is well known that porphyrins play an important role as functional groups in a wide variety of biological systems.[84] In nature, there are a number of functional molecules that have porphyrin derivatives as cofactors in their active sites or their reaction centers (Figure 1.4), for example, oxygen carriers such as hemoglobin and myoglobin, redoxidase such as catalase and peroxidase, electron transfer carriers, cytochrome. It

(a)   (b)
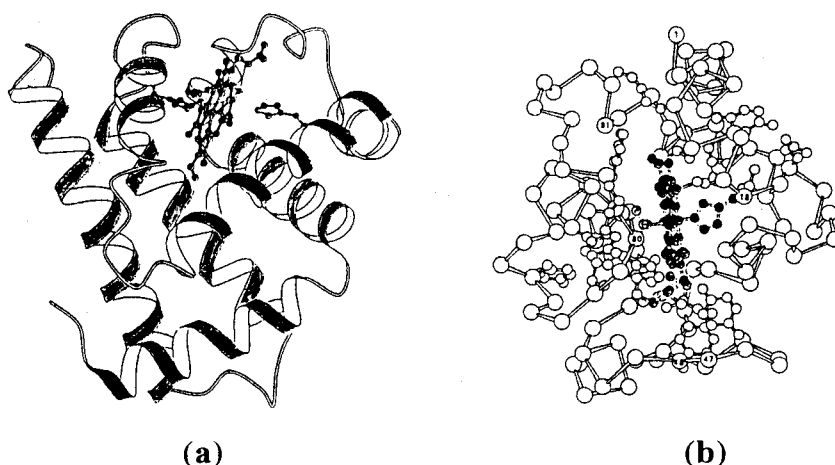
Figure 1.4. Porphyrin-protein complexes in naturally occurring molecules, (a) Cytochrom and (b) Myoglobin.

is well-known that special functions of metalloporphyrins such as oxygen-carrying systems and photosynthetic systems appear in an environment where metalloporphyrin moieties are surrounded by proteins.

In order to realize the functions of porphyrins artificially, there are some attempts to modify heme-protein complexes. Hayashi and Ogoshi[85] have reported a noncovalent electron transfer model that is based on the complexation of methyl viologen and a myoglobin reconstituted with a modified zinc porphyrin (Figure 1.5). Nagamune and Aida[86] have

reported cytochrome b562 reconstructed with mesoporphyrin IX as a metallochelatase mimic
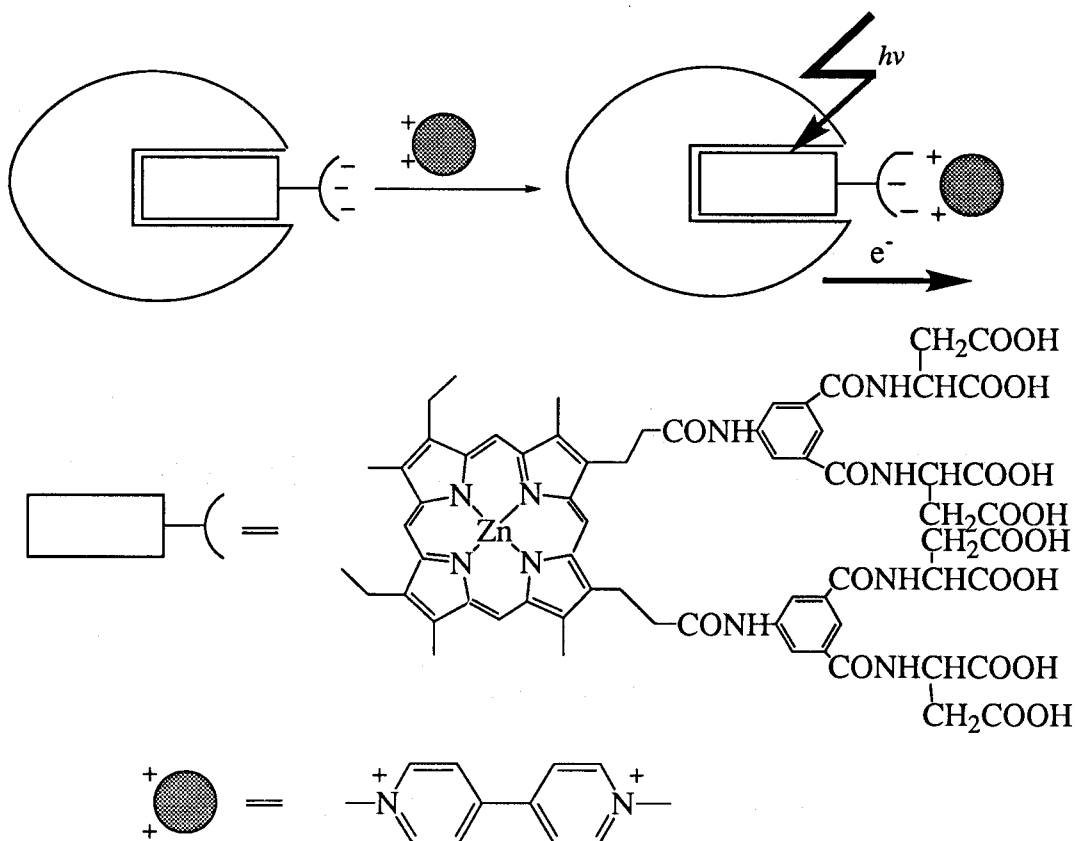
Figure 1.5. Reconstructed porphyrin-protein complex (1). Noncovalent electron transfer model constituted by myoglobin-modified zinc porphyrin-methyl viologen.

(Figure 1.6). Hamachi[87] *et al.* have reported that ruthenium-tris(bipyridine)-appended heme is successfully reconstituted with apo-cytochrome b562 and that the redox state of the active site in the resultant semisynthetic cytochrome b562 can be switched by visible light irradiation (Figure 1.7).
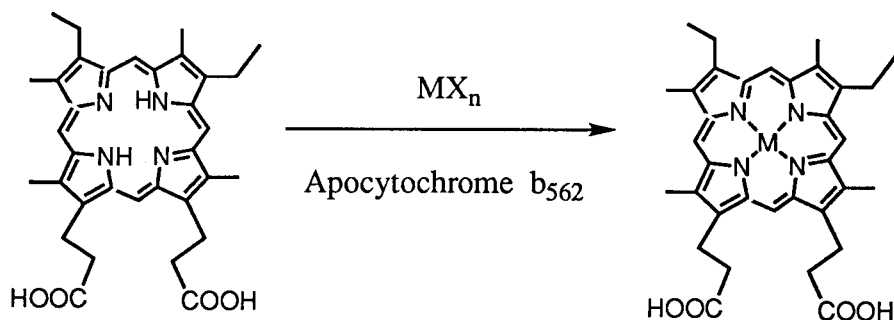
Figure 1.6. Reconstructed porphyrin-protein complex (2). Cytochrome b_{562} reconstructed with mesoporphyrin IX catalyzed a metal chelation reaction.

Apo-cytochrome b_{562} Ru(bpy)$_3$-appended cytochrome b_{562}
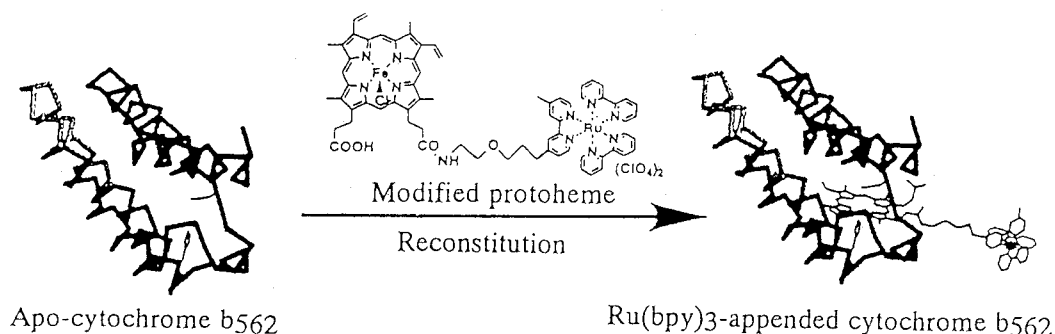
Figure 1.7. Reconstructed porphyrin-protein complex (3). Ru(bpy)$_3$-appended cytochrome b_{562}.

These studies mentioned above are carried out by introduction of porphyrins into naturally occurring protein molecules. The use of naturally occurring proteins might be limited. One of the most convenient incorporation methods of porphyrins into protein matrices is thought to be preparation of monoclonal antibodies for porphyrins. Strategies that allow incorporation of cofactors into antibody combining sites should expand the scope of antibody catalysis.[66-74]

Antibodies as New Functionalized Materials. We can expect to obtain antibodies not only as new materials but also as catalysts by the introduction of porphyrins. We paid much attention to the catalytic functions of the complexes between anti-porphyrin antibodies and

11

porphyrins. Accordingly, monoclonal antibodies for various kinds of porphyrins have been prepared as functionalized antibodies. Anti-porphyrin antibodies obtained bind to some porphyrin derivatives with high specificities. Anti-porphyrin antibodies were used as protein matrices for porphyrin molecules.

Functionalized Antibodies 1. Photochemistry of Porphyrins in the Presence of Antibodies. In chlorophyll, a number of porphyrin molecules and electron acceptors are incorporated in a protein domain, which plays an important role in the regulation of electron flow for the charge separation in the photochemical system. It is important to keep a charge separated to undergo the efficient electron transfer reaction. There are a lot of studies concerned with electron transfer from porphyrins to covalently linked electron acceptors. But there still remain unsolved problems on the electron transfer. In order to solve the problems, it is important to study the non-covalent electron donor-acceptor system. Accordingly, the electron transfer reaction is performed by the anti-porphyrin antibody and porphyrin system. Consequently, it is found that the efficiency of the electron transfer can be controlled by following method: a porphyrin molecule is incorporated into an antibody combining site, and electron acceptors are added to the porphyrin-antibody system. If electron acceptor molecules are placed far from a porphyrin molecule due to its binding to antibodies, long-range electron transfer may be expected.

Functionalized Antibodies 2. Supramolecular Chemistry by the Interaction of Antibodies and Porphyrins. In recent years, much attention has been focused on supramolecular science, science of non-covalent assembly, because of the recognition of the importance of specific non-covalent interactions in biological systems and in chemical processes.[88] By the construction of supramolecules between antibodies

and porphyrins, antibodies against porphyrins can be expected to induce or improve the function that is seen in naturally occurring porphyrin-protein complexes (Figure 1.8).

Supramolecular Chemistry

Receptor
Substrate
Interaction → Supramolecule ──────→ Molecular and Supramolecular Device
  Recognition
  Transformation
  Translocation

Creation of Functionalized Antibody

Antibody
Porphyrin
Molecular Recognition → Porphyrin-Antibody Complex ──→ Catalyst / Photochemical Device
Supramolecule
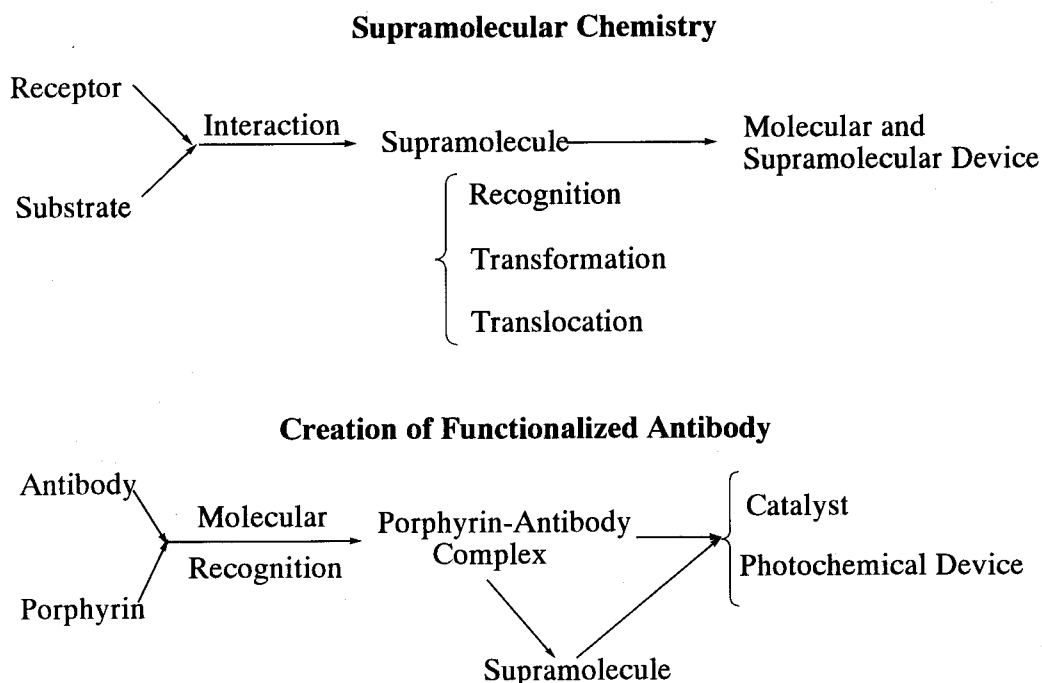
Figure 1.8. Supramolecular chemistry and strategy for the creation of functionalized antibodies.

Functionalized Antibodies 3. Antibody in Polymer Science.
Although the importance of monoclonal antibodies has been recognized, the use of monoclonal antibodies has been limited mainly to diagnoses. But recently, monoclonal antibodies have attracted much attention not only in the field of biology but also in the field of chemistry, because they are homogeneous and have been extensively studied as catalytic antibodies and sensors. In order to make monoclonal antibodies versatile, it is important to make antibody polymers. The study of antibody polymers (covalently linked antibodies) from the point of macromolecular chemistry is indispensable to use antibodies as functional materials. Antibody polymers

may be useful not only for the affinity chromatography but also for the high sensitive immunosorbent assay and a DNA cloning method.

Outline of This Thesis

Figure 1.9 shows the porphyrin molecules used as haptens. In this study, monoclonal antibodies against tetracarboxyphenylporphyrin: **A** and phosphorus(V) porphyrin: **B**, which has two axial ligands have been prepared (Chapter 2).[89] The reason why porphyrin **B** has been used for the preparation of monoclonal antibodies is to obtain the antibody possessing a combining site for both porphyrin and substrate or electron acceptor.
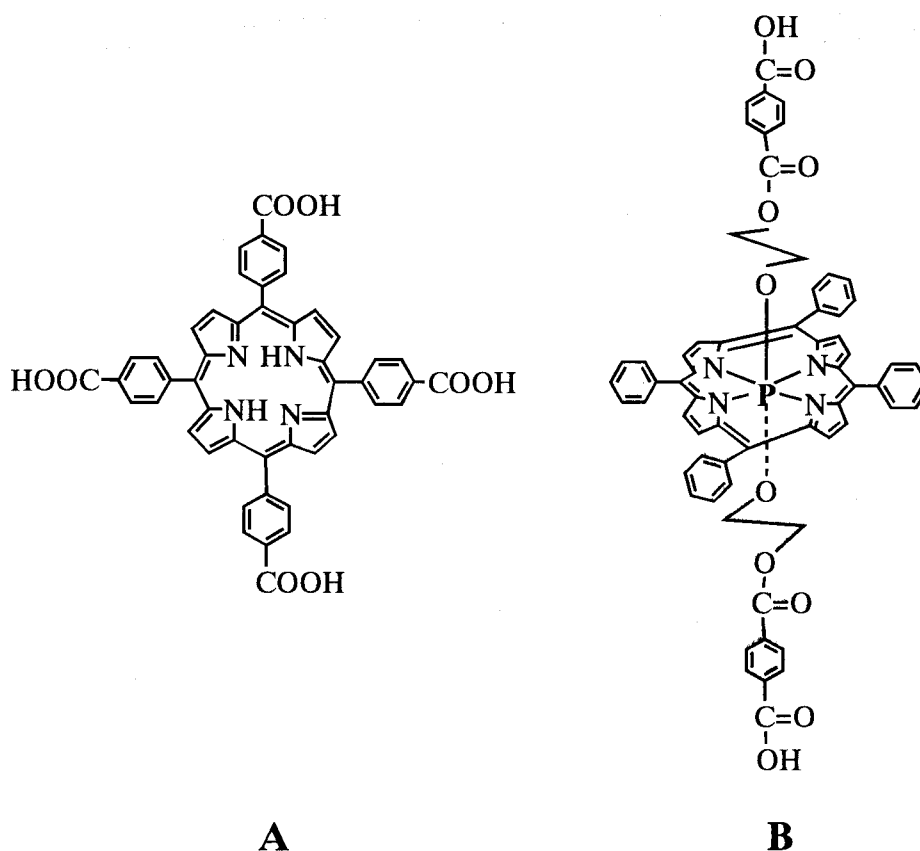

A   B

Figure 1.9. Two porphyrins used as haptens. (A); TCPP and (B) phosphorus(V)TPP.

14

Preparation and characterization of antibodies against porphyrin **A** were described in Chapter 2 and those against porphyrin **B** were described in Chapter 2 and Chapter 5.

The antibodies that raised against **A** were found to have catalytic activity of oxidation of pyrogallol with high selectively(Chapter 3).[90]

It was shown in Chapter 4 and Chapter 5 that the electron transfer reaction from porphyrin molecules to electron acceptors can be controlled by antibodies binding porphyrin molecules in their combining sites (Chapter 4 and Chapter 5).[91,92]

In Chapter 6, the construction of supramolecular structures between antibodies and porphyrins has been studied. Antibody molecules[93] and supramolecules between antibodies and porphyrins have been directly observed with an atomic force microscope (AFM) at room temperature. This supramolecular construction was also supported by biosensors and size exclusion chromatography.

In Chapter 7, an antibody was used as a precursor to obtain a new bio-polymer. Antibody polymer was prepared by coupling antibody with glutaraldehyde.[94]

References

(1) Pauling, L. *Am. Sci.*, **1948**, *36*, 51-58. He submitted the principle that enzymes can reduce the activation energy by binding substrates in the intermediate rather than the ground state.

(2) Pollack, S. J.; Jacobs, J. W.; Schultz, P. G. *Science* **1986**, *234*, 1570-1573.

(3) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1566-1569.

(4) Kohler, G.; Milstein, C. *Nature* **1975**, *256*, 495-497.

(5) Schultz, P. G. *Science*, **1988**,*240*, 426-433.

(6) Lerner, R. A.; Tramontano, A. *Sci. Am.* **1988**, *258*, 58.

(7) Schultz, P. G. *Acc. Chem. Res.* **1989**, *22*, 287-294.

(8) Schultz, P. G. *Angew. Chem., Int. Ed.* **1989**, 28, 1283-1295.

(9) Tramontano, A.; Schloeder, D. *Meth. Enzymol.* **1989**, *178*, 531-548.

(10) Pollack, S. T.; Nakayama, G. R.; Schultz, P. G. *Meth. Enzymol.* **1989**, *178*, 551-568.

(11) Shokat, K. M.; Schultz, P. G. *Annu. Rev. Immunol.* **1990**, *8*, 335-363.

(12) Shokat, K. M.; Schultz, P. G. *Meth. Enzymol.* **1991**, *203*, 327-351.

(13) Lerner, R. A.; Benkovic, S. J.; Schultz, P. G. *Science* **1991**, *252*, 659-667.

(14) Gibbs, R. A.; Benkovic, P. A.; Janda, K. D.; Lerner, L. A.; Benkovic, S. J. *J. Am. Chem. Soc.*, **1992**, *114*, 3528-3534.

(15) Stewart, J.; Liotta, L. J.; Benkovic, S. J. *Acc. Chem. Res.* **1993**, *26*, 396-404.

(16) Schultz, P. G.; Lerner, R. A. *Acc. Chem. Res.* **1993**, *26*, 391-395.

(17) Stewart, J. D.; Benkovic, S. *Chem. Soc. Rev.* **1993**, *22*, 213-219.

(18) Tawfik, D. S.; Eshhar, Z.; Green, B. S. *Molecul. Biotech.* **1994**, *1*, 87-103.

(19) Tramontano, A. *Appl. Biochem. Biotech.* **1994**, *47*, 257-276.

(20) Stewart, J. D.; Benkovic, S. J. *Nature* **1995**, *375*, 388-391.

(21) Schultz, P. G.; Lerner, R. A. *Science* **1995**, *269*, 1835-1842.

(22) Hisen-Wilson, L. C.; Xiang, X. D.; Schultz, P. G. *Acc. Chem. Res.* **1996**, *29*, 164-170.

(23) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Proc. Natl. Acad. Sci. U. S. A.* **1986**, *83*, 6736-6740.

(24) Jacobs, J.; Schultz, P. G.; Sugasawara, R.; Powell, M. *J. Am. Chem. Soc.* **1987**, *109*, 2174-2176.

(25) Tramontano, A.; Ammann, A.; Lerner, R. A. *J. Am. Chem. Soc.* **1988**, *110*, 2282-2286.

(26) Janda, K. D.; Schloeder, S. J.; Benkovic, S. J.; Lerner, R. A. *Science* **1988**, *241*, 1188-1191.

(27) Scanlan, T. S.; Prudent, J. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1991**, *113*, 9397-9398.

(28) Reymond, J. -L.; Jahangiri, G. K.; Stoudt, C.; Lerner, R. A. *J. Am. Chem. Soc.* **1993**, *115*, 3909-3917.

(29) Campbell, D. A.; Gong, B.; Kochersperger, L. M.; Yonkovich, S.; Gallop, M. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 2165-2166.

(30) Zhou, G. W.; Guo, J.; Huang, W.; Fletterick, R. J.; Scanlan, T. S. *Science* **1994**, *265*, 1059-1064.

(31) Suga, H.; Ersoy, O.; Tsumuraya, T.; Lee, J.; Sinskey, A. J.; Masamune, S. *J. Am. Chem. Soc.* **1994**, *116*, 487-494.

(32) Janda, K. D.; Weinhouse, M. I.; Schloeder, D. M.; Lerner, R. A.; Benkovic, S. J. *J. Am. Chem. Soc.* **1990**, *112*, 1274-1275.

(33) Janda, K. D.; Weinhouse, M. I.; Danon, T.; Pacelli, K. A.; Schloeder, D. M. *J. Am. Chem. Soc.* **1991**, *113*, 5427-5434.

(34) Jackson, D. Y.; Jacob, J. W.; Sugasawara, R.; Reich, S. H.; Bartlett, P. A.; Schultz, P. G. *J. Am. Chem. Soc.* **1988**, *110*, 4841-4842.

(35) Hilvert, D.; Nared, K. D. *J. Am. Chem. Soc.* **1988**, *110*, 5593-5594.

(36) Hilvert, D.; Carpenter, S. H.; Nared, K. D.; Auditor, M. -T. M. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85*, 4953-4955.

(37) Janda, K. D.; Schloeder, D.; Benkovic, S. J.; Lerner, R. A. *Science* **1988**, *241*, 1188-1191.

(38) Iverson, B. L.; Lerner, R. A. *Science* **1989**, *243*, 1184-1188.

(39) Blackburn, G. M.; Deng, S. -X. *Biochemical Society Transactions* **1993**, *21*, 1102-1107.

(40) Titmas, R. C.; Angeles, T. S.; Sugasawara, R.; Aman, N.; Darsley, M. J.; Blackburn, G. M.; Martin, M. T. *Appl. Biochem. Biotech.* **1994**, *47*, 277-292.

(41) Martin, M. T.; Angeles, T. S.; Sugasawara, R.; Aman, N. I.; Napper, A. D.; Darsley, M. J.; Sanchez, R. I.; Booth, P.; Titmas, R. C. *J. Am. Chem. Soc.* **1994**, *116*, 6508-6512.

(42) Napper, A. D.; Benkovic, S. J.; Tramontano, A.; Lerner, R. A. *Science*, **1987**, *237*, 1041-1043.

(43) Li, T.; Janda, K. D.; Ashley, J. A.; Lerner, R. A. *Science* **1994**, *264*, 1289-1293.

(44) Hilvert, D.; Hill, K. W.; Nared, K. D.; Auditor, M. -T. M. *J. Am. Chem. Soc.* **1989**, *111*, 9261-9262.

(45) Cochran, A. G.; Sugasawara, R.; Schultz, P. G. *J. Am. Chem. Sci.* **1988**, *110*, 7888-7890.

(46) Balan, A.; Doctor, B. P.; Green, B. S.; Torten, M.; Ziffer, H. *J. Chem. Soc., Chem. Commun.* **1988**, 106-108.

(47) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Nature* **1989**, 338, 269-271.

(48) Shokat, K. M.; Leumann, C. J.; Sugasawara, R.; Schultz, P. G. *Angew. Chem. Int. Ed. Engl.* **1988**, *27*, 1172-1174.

(49) Janjic, N.; Tramontano, A. *J. Am. Chem. Soc.* **1989**, *111*, 9109-9110.

(50) Braisted, A. C.; Schultz, P. G. *J. Am. Chem. Soc.* **1994**, *116*, 2211-2212.

(51) Ulrich, H. D.; Mundorff, E.; Santarsiero, B. D.; Driggers, E. M.; Stevens, R. C.; Schultz, P. G. *Nature* **1997**, *389*, 271-274.

(52) Iverson, B. L.; Cameron, K. E.; Jahangiri, G. K.; Pasternak, D. S. *J. Am. Chem. Soc.* **1990**, *112*, 5320-5323.

(53) Yu, J.; Hsieh, L. C.; Kochersperger, L.; Yonkovich, S.; Stephans, J. C.; Gallop, M. A.; Schultz, P. G. *Angew. Chem. Int. Ed. Engl.* **1994**, *33*, 339-341.

(54) Janda, K. D.; Benkovic, S. J.; Lerner, R. A. *Science* **1989**, *244*, 437-440.

(55) Pollack, S. J.; Hsiun, P.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 5961-5962.

(56) Nakayama, G. R.; Schultz, P. G. *J. Am. Chem. Soc.* **1992**, *114*, 780-781.

(57) Sinha, S. C.; Keinan, E. *J. Am. Chem. Soc.* **1993**, *115*, 4893-4894.

(58) Yli-Kauhaluoma, J. T.; Ashley, J. A.; Lo, C.-H. L.; Coakley, J.; Wirsching, P.; Janda, K. D. *J. Am. Chem. Soc.* **1996**, *118*, 5496-5497.

(59) Uno, T.; Ku, J.; Prudent, R.; Huang, A.; Schultz, P. G. *J. Am. Chem. Soc.* **1996**, *118*, 3811-3817.

(60) Benkovic, S. J.; A. D. Napper, A. D.; Lerner, R. A. *Proc. Natl. Acad. Sci. U. S. A.* **1988**, *85*, 5355-5358.

(61) Janda, K. D.; Lerner, R. A.; Tramontano, A. *J. Am. Chem. Soc.* **1988**, *110*, 4835-4837.

(62) Cook, C. E.; Allen, D. A.; Miller, D. B.; Whisnant, C. C. *J. Am. Chem. Soc.* **1995**, *117*, 7269-7270.

(63) Hilvert, D.; Hill, K. W. *Meth, Enzymol.* **1991**, *203*, 352-369.

(64) Bahr, N.; Guller, R.; Reymond, J.-L.; Lerner, R. A. *J. Am. Chem. Soc.* **1996**, *118*, 3550-3555.

(65) Wiest, O.; Houk, K. N. *J. Am. Chem. Soc.* **1995**, *117*, 11628-11639.

(66) Pollack, S. J.; Nakayama, G. R.; Schultz, P. G. *Science* **1988**, *242*, 1038-1042.

(67) Pollack, S. J.; Schultz, P. G. *J. Am. Chem. Soc.* **1989**, *111*, 1929-1931.

(68) Schwabacher, A. W.; Weinhouse, M. I.; Auditor, M.-T. M.; Lerner, R. A. *J. Am. Chem. Soc.* **1989**, *111*, 2344-2346.

(69) Harada, A.; Okamoto, K.; Kamachi, M.; T. Honda, T.; Miwatani, T. *Chem. Lett.* **1990**, 917-918.

(70) Harada, A.; Okamoto, K. Kamachi, M. *Chem. Lett.* **1991**, 953-956.

(71) Cochran, A. G.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 9414-9415.

(72) Cochran, A. G.; Schultz, P. G. *Science* **1990**, *249*, 781-783.

(73) Keinan, E.; Benory, E.; Sinha, S. C.; Sinha-Bagchi, A.; Eren, D.; Eshhar, Z.; Green, B. S. *Inorg. Chem.* **1992**, *31*, 5433-5438.

(74) Partridge, L. J. *Biochemical Society Transactions* **1993**, *21*, 1096-1098.

(75) Takahashi, M.; Fuller, S. A.; Winston, S. *Method. Enzymol.* **1991**, *203*, 312-327.

(76) Lesley, S. A.; Patten, P. A.; Schultz, P. G. *Proc. Natl. Acad. Sci. U. S. A.* **1993**, *90*, 1160-1165.

(77) Wade, W. S.; Koh, J. S.; Han, N.; Hoekstra, D. M.; Lerner, R. A. *J. Am. Chem. Soc.* **1993**, *115*, 4449-4456.

(78) Jackson, D. Y.; Prudent, J. R.; Baldwin, E. P.; Schultz, P. G. *Proc. Natl. Acad. Sci.* USA **1991**, *88*, 58-62.

(79) Davis, D. R.; Padlan, E. A.; Segal, D. M. *Annu. Rev. Biochem.* **1975**, *44*, 639-667.

(80) Silverton, E. W.; Navia, M. A.; Davies, D. R. *Proc. Natl. Acad. Sci. U. S. A.* **1977**, *74*, 5140-5144.

(81) Davis, D.; Chacko, S. *Acc. Chem. Res.* **1993**, *26*, 421-427. and references in this paper.

(82) Tonegawa, S. *Nature* **1983**, *302*, 575-581.

(83) Dolphin, D. Ed., *The Porphyrins*, Academic Press, New York **1978**.

(84) Hayashi, T.; Takimura, T.; Ogoshi, H. *J. Am. Chem. Soc.* **1995**, *117*, 11606-11607.

(85) Ishida, Y.; Konishi, K; Nagamune, T.; Aida, T. *Abstracts of Symposium on Biofunctional Chemistry* **1997**, *12*, 43-45.

(86) Hamachi, I.; Tanaka, S.; Tsukiji, S.; Shinkai, S.; Shimizu, M.; Nagamune, T. *J. Chem. Soc. Chem. Commun.* **1997**, 1735-1736.

(87) Lehn, J. -M. *Angew. Chem. Int. Ed. Engl.* **1990**, *29*, 1304-1319.

(88) Harada, A.; Shiotsuki, K.; Fukushima, H.; Yamaguchi, H.; Kamachi, M. *Inorg. Chem.* **1995**, *34*, 1070-1076.

(89) Harada, A.; Fukushima, H.; Shiotsuki, K.; Yamaguchi, H.; Oka, F.; Kamachi, M. *Inorg. Chem.* **1997**, *36*, 6099-6102.

(90) Harada, A.; Yamaguchi, H.; Kamachi, M. *J. Inorg. Biochem.* **1997**, *67*, 114.

(91) Yamaguchi, H.; Harada, A.; Kamachi, M. to be submitted.

(92) Harada, A.; Yamaguchi, H.; Kamachi, M. *Chem. Lett.* **1997**, 1141-1142.

(93) Yamaguchi, H.; Harada, A.; Kamachi, M. *Reactive & Functional Polymers* **1997**, in press.

Chapter 2

Preparation of Anti-Porphyrin Antibodies
and Their Binding Properties

Introduction

Unique functions of porphyrins such as oxygen-carriers and photosynthetic pigments appear in an environment where porphyrin moieties are surrounded by protein. We paid attention to the anti-porphyrin antibodies as protein matrices for porphyrin molecules in order to make the best use of functions of porphyrins. In this Chapter, accordingly, preparation of anti-porphyrin antibodies by using the two porphyrin derivatives, (**A**):*meso*-tetrakis-(4-carboxyphenyl)porphine (TCPP) and (**B**): phosphorus(V)tetraphenylporphyrin (P(V)TPP)[1-10] (Figure 2.1) was performed and their binding properties were examined.
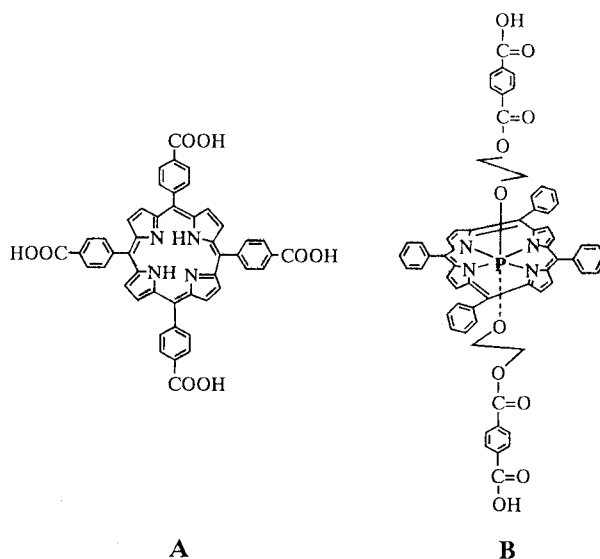

A **B**

Figure 2.1. *meso*-Tetrakis(4-carboxyphenyl)porphyrin (A) and phosphorus(V)tetraphenylporphyrin (B).

Porphyrin **A** was used as a synthetic water-soluble porphyrin. P(V)TPP was used as a hapten to induce substrate (or electron acceptor) binding space into antibody combining sites, because P(V)TPP is able to have two axial ligands. These ligands are bound to the phosphonium atom with covalent bonding at the center of the porphyrin molecule.

In order to prepare monoclonal antibodies, porphyrins coupled to immunogenic carrier proteins such as KLH (keyhole-limpet hemocyanin) or BSA (bovine serum albumin) were used, because amino acids, glycoproteins, etc., and in particular molecules with a molecular weight less than 1,000 Da (for example, porphyrins), can not trigger an immune response by themselves ("haptens"). Binding properties of anti-porphyrin antibodies to porphyrin derivatives were examined by enzyme-linked immunosorbent assay (ELISA), and biosensers (BIAcore), which are well suited to studies of molecular interaction in real time.[11-14]

Experimental Section

Materials
(A)   Preparation of Anti-*meso*-tetra(4-carboxyphenyl)-porphyrin (TCPP) Antibodies

meso-**Tetrakis(4-carboxyphenyl)porphine (TCPP).** *meso*-Tetrakis(4-carboxyphenyl)porphine (TCPP) was prepared by the reaction of *p*-carboxybenzaldehyde and pyrrole in propionic acid.[15] ^{1}H NMR (DMSO-d$_6$): δ 8.85(s, 8H), 8.39(d, 8H), 8.33(d, 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.

Preparation of Protein Conjugates. TCPP (6 mg) and carbonyldiimidazole (CDI) (1.2 mg) were dissolved in 2 mL of DMF, and the solution was stirred at 0 °C for 2 h. The mixture was then added dropwise to 6 mL of phosphate borate buffer (PBB, pH 9.0) containing 5 mg of KLH (keyhole-limpet hemocyanin) with stirring at 0 °C overnight.[16] The concentration of protein was determined by the BCA method (Pierce). The concentration of TCPP was determined from the absorbance at the Soret band. 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 G-100 with PBB as an eluent.

Immunization. Balb/c mice (7-8 week old) were immunized with KLH-TCPP conjugate emulsified in complete Freund's adjuvant twice with an interval of 1 month between injections and finally an injection of the conjugate alone 1 month later.

Monoclonal Antibody Production. Three days after the last injection of conjugate, the spleen was removed from a mouse and spleen cells were isolated and fused with myeloma cells using poly(ethylene glycol) (MW = 1000, Boehringer, Mannheim). They were then plated in twenty 96-well plates in wells containing 150 mL 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. Antibodies were purified on a protein A column (Amersham Ampure[TM] PA kit) from ascites. Monoclonal antibodies were obtained by dialysis against PBB (pH 9.0). The purity of antibodies was checked by SDS-PAGE electrophoresis.

**(B) Preparation of Anti-P(V)tetraphenylporphyrin Antibodies**

(5,10,15,20-Tetraphenylporphinato)dichlorophosphorus (V) Chloride 1.   Porphyrin **1** was synthesized and crystallized according to the reported procedure.[2] Into a 100 mL round bottom flask were placed 1.00g of *meso*-tetrakis-tetraphenylporphyrin (TPP) and 40 mL of pyridine. Approximately 8 mL of POCl3 were gradually added dropwise and the solution was refluxed for 3 days. The pyridine solution was then flash evaporated to yield the crude product. This was then chromatographed on alumina using chloroform and methanol as eluents. Anal. Calcd. for $C_{44}H_{28}N_4PCl \cdot 4H_2O$: C, 64.11; H, 4.35; N, 6.79. Found: C, 64.11; H, 4.35; N, 6.45. λmax / nm (acetonitrile):437, 567, 610. (CHCl3): 436, 526, 565, 609. ^{1}H-NMR (CDCl3, relative to TMS) / δ: 7.8 (m, 12H), 8.0 (m, 8H), 9.1 (d, 8H).

(5,10,15,20-Tetraphenylporphinato)bis(2-hydroxyethoxy) phosphorus(V) Chloride 2.   Porphyrin **2** was synthesized by condensation of the dichloro-P(V)-TPP **1** and ethylene glycol.[10,13] Anal. Calcd. for $C_{48}H_{38}N_4O_4PCl \cdot 2H_2O$: C, 69.37; H, 5.01; N, 6.74. Found: C, 69.37; H, 4.96; N, 6.62.   FAB MS, m/z 765.8 ($C_{48}H_{38}N_4O_4P$, [M]+). The MS showed the expected molecular weight of the axis-coordinated porphyrin derivatives without the counter ion. λmax / nm (CHCl3): 431, 522, 560, 600. ^{1}H-NMR (CDCl3, relative to TMS) / δ: -2.29 (dt, 4H), 0.74 (m, 4H), 7.6-8.1 (m, 20H), 9.01 (d, 8H).

(5,10,15,20-Tetraphenylporphinato)bis(hydrogen-terephthalate-2-ethoxy)phosphorus(V)chloride   3   :   Hapten. Hapten **3** was prepared by the reaction of porphyrin **2** with terephthaloyl chloride. Porphyrin **3** was prepared and characterized by converting into methyl ester **4** (Scheme 2.1).   Methyl ester **4** was

purified by column chromatography on alumina (CHCl3 : MeOH = 9:1).

4: ^{1}H-NMR (CDCl3) / δ: -2.17 (m, 4H), 0.78 (dt, 4H), 1.38 (s, 6H), 7.75-8.01 (m, 28H), 9.06 (s, 8H). λmax / nm: 428, 520, 559, 599. Anal. Calcd. for $C_{66}H_{50}N_4O_{10}PCl$: C, 70.43; H, 4.48; N, 4.98. Found: C, 70.45; H, 4.80; N, 5.11.
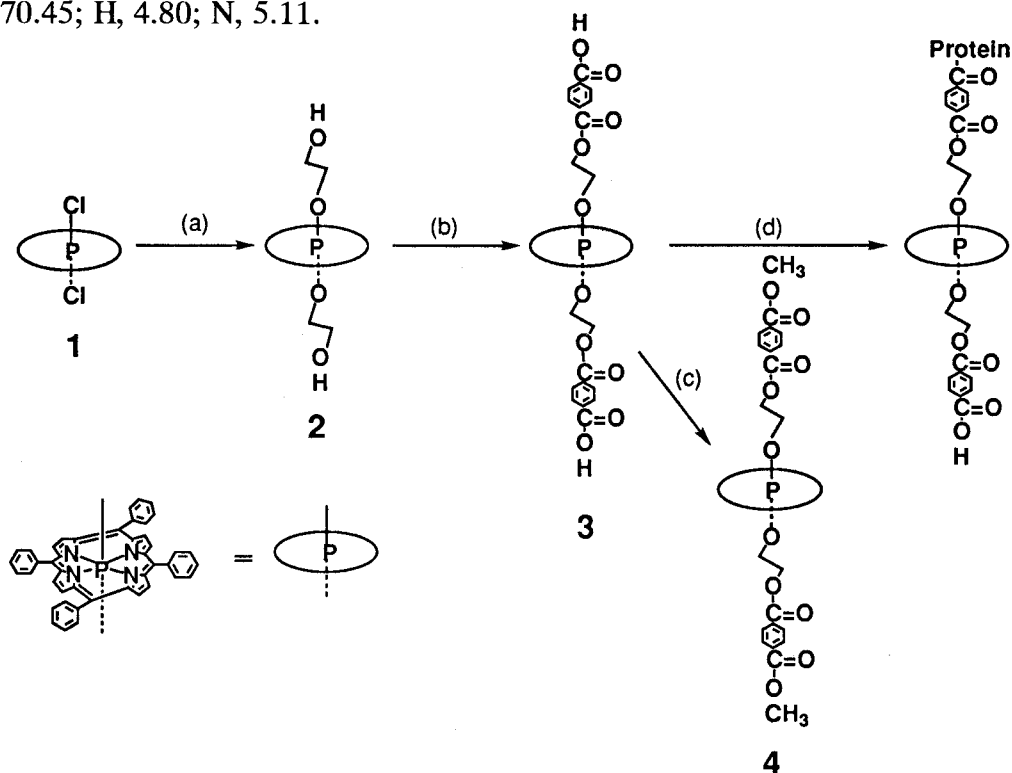
Scheme 2.1. Synthesis of hapten (B); phosphorus(V)porphyrin 3.

Preparation of Antigen and Monoclonal Antibodies. Hapten **3** was covalently attached to the carrier proteins, keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA), using 1-ethyl-3-(3-dimethylaminopropyl)-carbodiimide (EDC) in DMF / phosphate buffer (PB, 0.1 M, pH 7.0). The conjugates were purified by column chromatography on Sephadex G-100 with phosphate borate buffer (PBB, 0.1 M, pH 9.0) as an eluent. Balb/c mice were immunized with the KLH conjugate in complete Freund's adjuvant four times with an interval of 1 week between injections. Serum titer was determined using the BSA

conjugate by an enzyme-linked immunosorbent assay (ELISA). Hybridomas were generated by fusion of spleen cells with SP2/0 myeloma cells by standard protocols. Cells were injected into mice to generate ascitic fluid. Monoclonal antibodies were purified from ascetic fluid on a protein A column. Monoclonal antibodies were obtained by dialysis against 0.1 M PBB (pH 9.0). The purity of the antibody was determined by SDS-PAGE electrophoresis.

[*meso*-Tetrakis(4-carboxyphenyl)porphinato]copper(II) (Cu(II)-TCPP). 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[17]. The mixture was heated to 80 °C for 1 h. The reaction was followed by monitoring the absorption spectrum. DMF was removed, 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)porphinato]manganese (III) chloride (Mn(III)-TCPP). Mn(III)-TCPP complex was prepared by treatment of TCPP with excess $MnCl_2$ in acetic acid and acetic anhydride refluxing for 2 h. After evaporation of the solvents, the residue was recrystallized from methylene chloride[18]. Anal. Calcd for $C_{48}H_{28}N_4O_8MnCl \cdot 5H_2O$: C, 59.69; H, 3.95; N, 5.78. Found: C, 59.73; H, 4.14; N,5.75.

[*meso*-Tetrakis(4-carboxyphenyl)porphinato]iron(III) chloride (Fe(III)-TCPP). Fe(III)-TCPP was prepared by treatment of TCPP with excess $FeCl_3$ in glacial acetic acid with sodium acetate. After the solution was refluxed for 1 h and water added, the resulting crystals were filtered off, washed with water, and recrystallized from 0.5 N NaOH-EtOH [19]. Anal. Calcd for $C_{48}H_{24}N_4O_8FeCl \, Na_4 \cdot 7H_2O$: C, 52.26; H, 3.47; N, 5.08. Found: C, 52.57; H, 3.58; N,4.94.

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) was purchased from Dojin Chemicals Inc.

Measurements

ELISA (Enzyme-linked Immunosorbent Assay). Binding affinities of anti-porphyrin antibodies were determined by ELISA.[19] The antibodies to be assayed are allowed to react with the immobilized antigen in the first step and in the second step the amount of the antibody bound to the antigen is measured using a second antibody labeled with an enzyme. The antigen for immunosorbent assay was prepared by conjugating TCPP to bovine serum albumin (BSA). Alkaline phosphatase conjugated goat anti-mouse immunoglobulin (polyclonal antibody for IgA, IgG, IgM) was used as a second antibody. The absorbance of wavelength at 405 nm was monitored by ImmunoMini NJ-2300 (Inter Med).

Kinetic Measurements. The kinetic parameters of the association and dissociation of antibodies with porphyrins were determined by a BIAcore X system from Pharmacia Biosensor with a sensorchip CM5. Antibody 03-1 was immobilized to the sensor chip by activating carboxymethyl groups on surface matrix by a mixture of *N*-hydroxysuccinimide (NHS) and *N*-ethyl-*N'*-(dimethylaminopropyl)-carbodiimide (EDC). The response signal (RU) of the BIAcore apparatus is proportional to the change in the refractive index at the surface and is generally assumed to be proportional to the mass of substance bound to the chip. By measuring the amount of bound
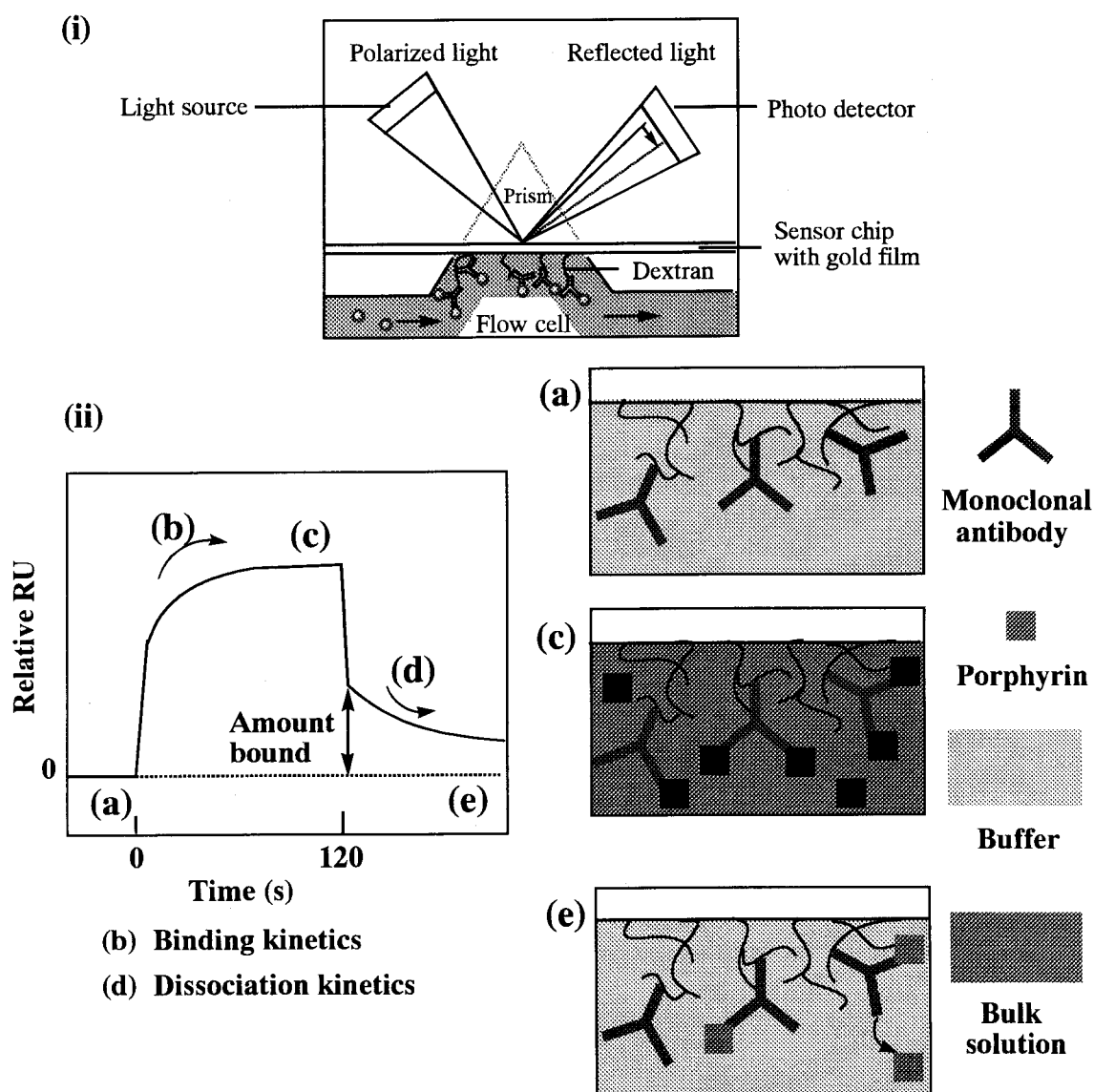
Figure 2.2. (i) The configuration of the surface plasmon resonance detector, sensor chip and integrated fluidic cartridge in the BIAcore system. (ii) The schematic sensorgram, showing association, equilibrium and dissociation phases.

State (a); Baseline signal for the sensor chip immobilized with antibodies.

State (b); Association of the antibody, immobilized to the surface of the sensor chip, with substrates (porphyrins) during sample injection.

State (c); Steady state during sample injection, where the rate of porphyrin binding is balanced by dissociation from the complex.

State (d); Dissociation of porphyrins from the surface during buffer flow.

State (e); The continuous buffer flow.

substrate as a function of time when a solution containing porphyrin passes over the chip surface, the kinetics of association to the immobilized antibody were studied. The dissociation kinetics were monitored by detecting the time-dependence of the mass decrease after the surface was subsequently washed with buffer. Figure 2.2 illustrates the evaluation of kinetic and affinity parameters for a monoclonal antibody. The on and off rates for binary complex formation are described by the equations below:

$$A + B \underset{k_d}{\overset{k_a}{\rightleftharpoons}} AB \tag{1}$$

where A is a porphyrin, B an antibody combining site, AB the complex between the antibody and the porphyrin. k_a and k_d are the association and dissociation rate constants, respectively.

Association rate: $\quad d[AB] / dt = k_a [A] [B]$ $\hfill (2)$

Dissociation rate: $\quad - d[AB] / dt = k_d [AB]$ $\hfill (3)$

If mass transport is much faster than association, the analyte at the surface is maintained at the same concentration as in the bulk phase, and the constants for interaction kinetics. Under these conditions, the rate equation can be written as

$$d[AB] / dt = k_a [A] [B] - k_d [AB] \tag{4}$$

The concentration of unoccupied antibody combining site [B] is the difference between the total amount of antibody combining site on the surface $[B]_0$ and the amount of complex [AB]:

$$[B] = [B]_0 - [AB] \tag{5}$$

Substituting in the rate equation for the formation of AB:

$$d[AB] / dt = k_a [A] ([B]_0 - [AB]) - k_d [AB] \tag{6}$$

If the total amount of antibody combining site $[B]_0$ is expressed in terms of the maximum porphyrin binding capacity of the surface, all

concentration terms can then be expressed as SPR signal (RU), eliminating the need to convert from mass to molar concentration:

$$dR / dt = k_a C (R_{max} - R) - k_d R \qquad (7)$$

where dR/dt is the rate of change of the SPR signal, C the concentration of porphyrins, R_{max} the maximum porphyrin binding capacity of RU, and R the SPR signal at time t.

This equation may be rearranged to give

$$dR / dt = k_a C R_{max} - (k_a C + k_d) R \qquad (8)$$

If R_{max} is known, both k_a and k_d can be determined from a single association sensorgram. In a plot of dR / dt as a function of R, the intercept with the dR / dt axis will give k_a. After the pulse of porphyrin has passed over the sensor chip surface, the surface-bound complex dissociates in a zero-order reaction. Assuming that re-association of released porphyrin is negligible (it means that released porphyrin is effectively washed out by the buffer flow):

$$dR / dt = - k_d R \qquad (9)$$

Separating variables and integrating gives

$$R_t = R_0 \exp [-k_d (t-t_0)] \qquad (10)$$

where R_t is the response at time t and R_0 is the response at the arbitrary starting time t_0.

From equation 10

$$dR / dt = - k_d R_0 \exp [-k_d(t-t_0)] \qquad (11)$$

or $\qquad \ln (dR / dt) = \ln (-k_d R_0) - k_d (t-t_0) \qquad (12)$

Plotting $\ln (dR / dt)$ against $t-t_0$ thus gives a straight line with slope $- k_d$. Both equilibrium and rate constants can be measured by biomolecular interaction analysis (BIA).

Results and Discussion

Although a number of monoclonal antibodies were obtained by using two porphyrins as haptens independently: four monoclonal antibodies were selected against (A) TCPP and five monoclonal antibodies were selected for (B) P(V)TPP, respectively, from the monoclonal antibodies.

Four monoclonal antibodies raised against TCPP were found to belong in immunoglobulin G (IgG) by a second antibody specific to the respective class of immunoglobulin G. Two of Anti-TCPP antibodies, named 03-1 and 13-1, have been further characterized and used for further experiments such as affinity determination.

Five monoclonal antibodies specific for hapten were obtained. One of these antibodies, 74D7A was found to belong to IgG, and was used for further experiments.

Affinity Determination

The binding affinities of the antibodies to various porphyrins were investigated by ELISA (enzyme-linked immunosorbent assay) and biosensor that antibody molecules were immobilized to the sensor chips.

ELISA. The binding of TCPP by the monoclonal antibodies was studied by enzyme-linked immunosorbent assay (ELISA). All four antibodies bound TCPP strongly. Klotz equation[20] is given as follow:

$$A_0 / (A_0 - A) = 1 + K_d / a_0 \qquad (13)$$

where A_0 and A are absorbances at 405 nm of ELISA in the absence and presence of porphyrin, respectively. K_d is the dissociation constant and a_0 the total concentration of porphyrin.

$$K_d = [\text{Antibody}] [\text{Porphyrin}] / [\text{Antibody-porphyrin complex}] \qquad (14)$$

From the slope of the Klotz plot, the dissociation constants of antibodies to the various porphyrins were determined. The dissociation constants were found to be approximately 1.0×10^{-6} to 7.0×10^{-7} M for TCPP (Table 2.1). The antibodies also strongly combined metal-TCPP complexes, such as that of Zn, with similar dissociation constants to those for unmetallyzed TCPP.

Table 2.1. Dissociation constants of porphyrins and monoclonal antibodies.[a]

Monoclonal antibody	Dissociation constants (M)			
	03-1	10-2	12-1	13-1
TCPP	1.0×10^{-6}	2.5×10^{-7}	7.0×10^{-7}	1.4×10^{-6}
Zn-TCPP	1.1×10^{-6}	2.0×10^{-7}	7.0×10^{-7}	1.2×10^{-6}

a) Estimated by ELISA.

Biosensor Technique. Antibody 03-1 was immobilized on the surface of the sensor chip. Figure 2.3 shows the changes in the signal with exposure time of a surface with immobilized antibody 03-1 to a solution of TCPP (a), TSPP (b), TMPyP (c), and TTMAPP (d). In each sensorgram, an upper curve shows a solution of porphyrin at a concentration of 8.0×10^{-5} M, and a lower one shows that of 4.0×10^{-5} M. When a solution containing TCPP passes over the chip surface, the amount of bound TCPP was measured as a function of time. From sensorgrams for anionic porphyrins (TCPP and TSPP), kinetic parameters were determined, respectively (Table 2.2). On the other hand, the amount of bound cationic porphyrins (TMPyP and TTMAPP) were zero. The difference of sensorgrams between anionic and cationic porphyrins were clearly observed in Figure 2.3. These results indicate
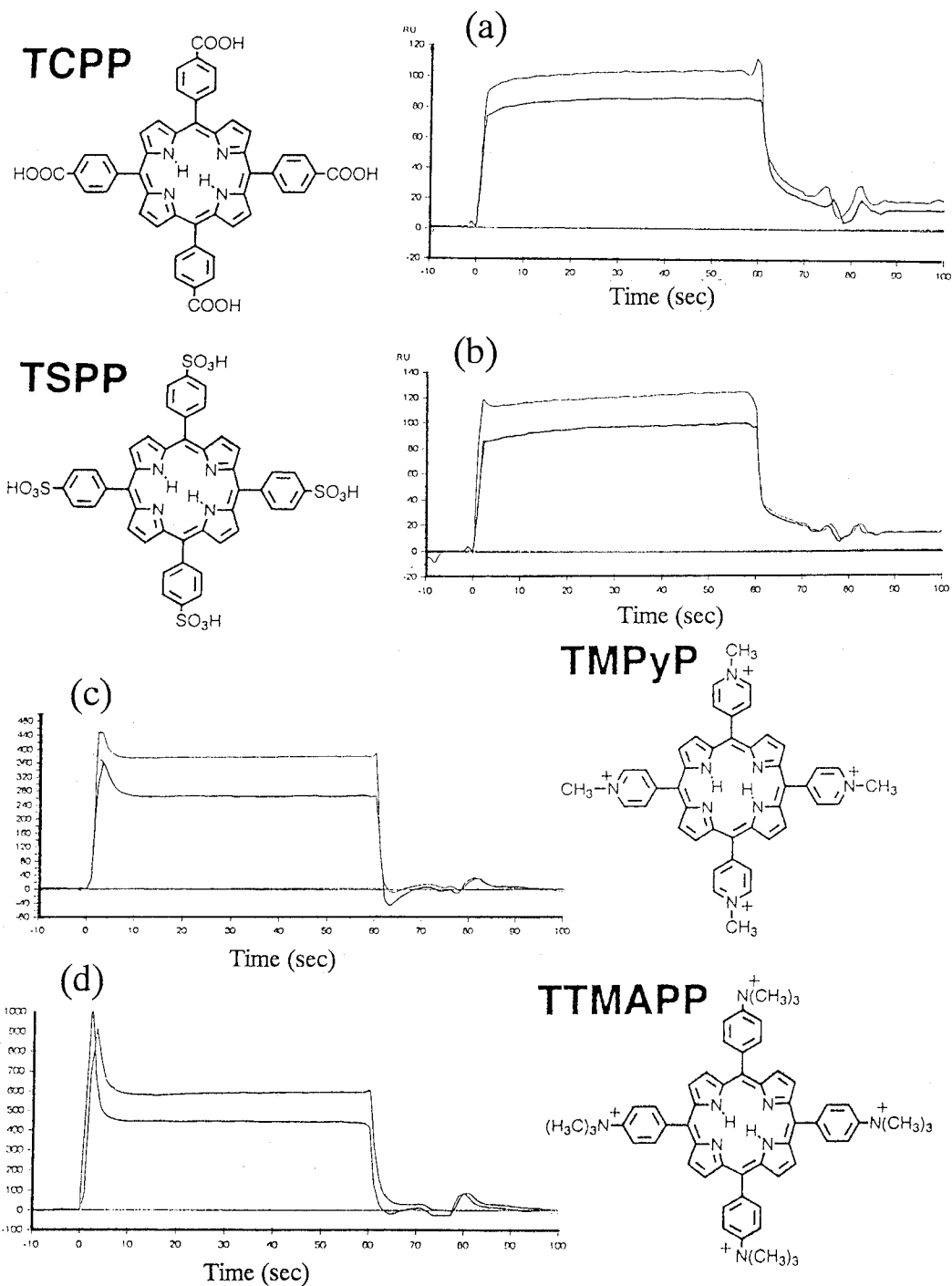
TCPP

TSPP

TMPyP

TTMAPP

(a)

(b)

(c)

(d)

Time (sec)

Time (sec)

Time (sec)

Time (sec)

Figure 2.3. The changes in resonance signals over time during exposure of the surface with immobilized antibody 03-1 to solutions of porphyrins. Specific binding of anionic porphyrins to antibody 03-1 (a); TCPP, (b); TSPP. Non-specific binding of cationic porphyrins to antibody 03-1 (c); TMPyP, (d); TTMAPP. [Porphyrin]; 8.0×10^{-5} M (upper) and 4.0×10^{-5} M (bottom).
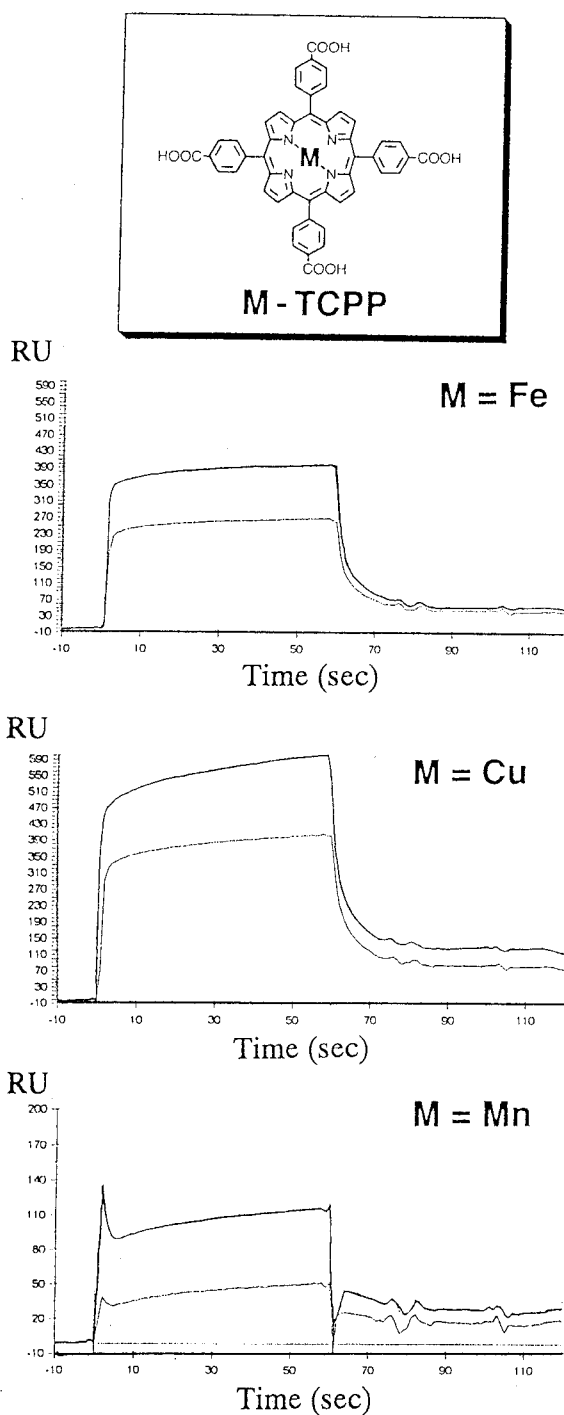
34

Figure 2.4. Sensorgrams showing binding of antibody 03-1 to and dissociation from three different metalloporphyrins. [M-TCPP (M = Cu, Fe(III), and Mn(III))]; 1.0×10^{-4} M (upper) and 2.0×10^{-5} M (bottom).

35

that monoclonal antibody 03-1 has a high specificity for anionic porphyrins.

Various metalloporphyrins (M-TCPP) were tested under the same conditions. Figure 2.4 shows sensorgrams for Cu-TCPP; (a), Fe(III)-TCPP; (b), and Mn(III)-TCPP; (c). Each sensorgram of M-TCPP was similar to that of TCPP. Table 2.2 shows kinetic parameters of antibody 03-1 with metalloporphyrins determined with BIAcore.

Table 2.2. Association and dissociation kinetic parameters between antibody 03-1 and various porphyrins.

Porphyrin	k_a (1/Ms)	k_d (1/s)	K_A (1/M)	K_D (M)
TCPP	1.22×10^4	9.59×10^{-3}	1.28×10^6	7.84×10^{-7}
TSPP	1.29×10^4	7.86×10^{-3}	1.65×10^6	6.07×10^{-7}
Cu-TCPP	1.20×10^4	5.28×10^{-3}	2.27×10^6	4.40×10^{-7}
Fe(III)-TCPP	1.37×10^4	1.11×10^{-2}	1.26×10^6	8.11×10^{-7}
Mn(III)-TCPP	1.82×10^3	5.88×10^{-3}	3.10×10^5	3.22×10^{-6}

Conclusion

One of the monoclonal antibodies 03-1 raised against TCPP shows a high specificity for porphyrins with a dissociation constant on the order of 10^{-7} M. Especially antibody 03-1 recognizes the difference of charge in porphyrin molecules and is sensitive to the metal in the center of porphyrin ring.

References

(1) Sayer, P.; Gouterman, M.; Connell, C. R. *J. Am. Chem. Soc.* **1977**, *99*, 1082-1087.

(2) Carrano, C. J.; Tsutsui, M. *J. Coord. Chem.* **1977**, 7, 79-83.

(3) Gouterman, M.; Sayer, P.; Shankland, E.; Smith, J. P. *Inorg. Chem.* **1981**, *22*, 87-92.

(4) Marrese, C. A.; Carrano, C. J. *Inorg. Chem.* **1983**, *22*, 1858-1862.

(5) Mangani, S.; Meyer, E. F.; Cullen, D. L.; Tsutsui, M.; Carrano, C. J. *Inorg. Chem.* **1983**, *22*, 400-404.

(6) Barbour, T.; Belcher, W. J.; Brothers, P. J.; Rickard, C. E. F.; Ware, D. C. *Inorg. Chem.* **1992**, *31*, 746-754.

(7) Segawa, H.; Kunimoto, K.; Nakamoto, A.; Shimidzu, T. *J. Chem. Soc., Perkin Trans. 1* **1992**, 939-940.

(8) Segawa, H.; Kunimoto, K.; Susumu, K.; Taniguchi, M.; Shimidzu, T. *J. Am. Chem. Soc.* **1994**,*116*, 11193-11194.

(9) Segawa, H.; Nakamoto, A.; Shimidzu, T. *J. Chem. Soc., Chem. Commun.* **1992**, 1066-1067.

(10) Anita, T.; Maiya, B. G. *Inorg. Chem.* **1996**, *35*, 4829-4836.

(11) Cullen, D. C.; Brown, R. G. W.; Lowe, C. R. *Biosensors* **1987**, 211-225.

(12) Lofas, S.; Johnsson, B. *J. Chem. Soc., Chem. Commun.* **1990**, 1526-1528.

(13) Jonsson, U.; Fagerstam, L.; Ivarsson, B.; Johnsson, B.; Karlsson, R.; Lundh, K.; Lofas, S.; Persson, B.; Roos, H.; Ronnberg, I.; Sjolander, S.; Stenberg, E.; Stahlberg, R.; Urbaniczky, C.; Ostlin, H.; Malmqvist, M. *Biotechniques* **1991**, *11*, 620-627.

(14) Malmqvist, M. *Nature* **1993**, *361*, 186-187.

(15) Longo, F. R.; Finarelli, M. M.; Kim, J. B. *J. Heterocycl. Chem.* **1969**, *6*, 927-931.

(16) Harada, A.; Fukushima, H.; Shiotsuki, K.; Kamachi, M. *Supramolecular Chem.* **1993**, *2*, 153-155.

(17) Harada, A.; Shiotsuki, K.; Fukushima, H.; Yamaguchi, H.; Kamachi, M. *Inorg. Chem.* **1995**, *34*, 1070-1076.

(18) Harada, A.; Fukushima, H.; Shiotsuki, K.; Yamaguchi, H.; Oka, F.; Kamachi, M. *Inorg. Chem.* **1997**, *36*, 6099-6102.

(19) Engvall, *Meth. Enzymol.* **1980**, *70*, 419-425.

(20) Friguet, B.; Chaffotte, A. F.; Ohaniance, L. D.; Goldberg, M. E. *J. Immun. Method* **1985**, *77*, 305-312.

Chapter 3

Specific Peroxidase Activity
by Antibody-Metalloporphyrin Complex

Introduction

Recently, much attention has been focused on catalytic antibodies.[1-3] Most catalytic antibodies have been prepared using "transition state analog compounds" as haptens. However, since antibodies are proteins composed of about 20 different amino acids, reactions promoted only by antibodies are limited. Accordingly, development of the catalytic antibody has been directed to metal complexes and organometallic compounds in which catalytic behavior is expected.

Recently, much attention has been focused on monoclonal antibodies for metal complexes. One of the most important families of metal complexes is the metalloporphyrins which function as oxygen carriers, redox enzymes, and photosynthetic mediators. Synthetic porphyrins and metalloporphyrins should function well when they are suitably incorporated into protein domains. One of the most convenient methods to incorporate porphyrins and metalloporphyrins into protein matrices is by preparation of monoclonal antibodies for porphyrins.

Various monoclonal antibodies against *meso*-tetrakis(4-carboxyphenyl) porphyrin (TCPP)[4] have been prepared and these monoclonal antibodies have been found to form complexes with porphyrins and metalloporphyrins specifically.[5-7] If the environment

of the porphyrin complex can be artificially manipulated through the concept of the second sphere coordination,[8] more effective catalytic antibody-metal complex might be devoted. In this Chapter, the catalytic activity of the antibody-metalloporphyrin complex has been investigated. The complexes between antibody 03-1 and metalloporphyrins have been found to have selective catalytic activity on the oxidation of pyrogallol and this activity was compared with the catalytic activity of horseradish peroxidase.

Experimental Section

Materials

The Mn(III)-TCPP complex was prepared by treatment of TCPP with excess $MnCl_2$ in acetic acid and acetic anhydride refluxing for 2 h. After evaporation of the solvents, the residue was recrystallized from methylene chloride. Fe(III)-TCPP was prepared by treatment of TCPP with excess $FeCl_3$ in glacial acetic acid with sodium acetate. After the solution was refluxed for 1 h and water added, the resulting crystals were filtered off, washed with water, and recrystallized from 0.5 N NaOH-EtOH. Monoclonal antibodies for TCPP were prepared as described previously. Fab fractions of antibody 03-1 were obtained by digestion of the antibody with papain (Wako Chemical Ltd.) as described below.

Preparation of Fab Fragment

Antibody (25 mg) in 10 mL of PBB buffer (pH 8) was mixed with 0.1 M L-cysteine solution (1.11 mL) and then 25 mL of papain solution (Sigma). The mixture was incubated at 37 °C for 4 h. The reaction

was terminated by the addition of iodoacetamide at a final concentration of 30 mM. The reaction mixture was passed through a prepacked Protein A column (Pierce immunopure kit). The Fab (antigen binding fragment, 50 kDa) eluted was passed through a Sephadex G-100 column. The purity was checked by SDS-PAGE.

Measurements

(1) Spectroscopy

UV-Vis spectra were recorded on a Shimadzu UV-2100 UV-visible spectrophotometer. Circular dichroism spectra were recorded on a JASCO J-40A spectrophotometer with a JASCO ORD/CD data processor using a 10 mm quartz cell. Emission spectra were recorded with a Shimadzu RF-502A spectrofluorophotometer using an excitation wavelength of 280 nm.

(2) Kinetic Study

Kinetic study was performed under the following conditions. The Fab fraction of antibody 03-1 and TCPP-metal complex was dissolved in Tris buffer (pH 8.0) or PBB (0.1M, pH 9.0), and incubated for 2 days. Hydrogen peroxide was then added (5.0 mM), followed by a substrate (e.g., pyrogallol). The starting time was set at this point. The reactions were followed by measuring the change of absorbance at 420 nm. Reaction mixtures contained 5 mM hydrogen peroxide, 0.5 mM Fe(III) or Mn(III)-TCPP , 4 % (v/v) dimethyl sulfoxide (DMSO), and 90 mM Tris acetate (pH 8.0).

Results and Discussion

Bindings of Metalloporphyrins to Monoclonal Antibodies

The binding of metalloporphyrins to monoclonal antibodies was investigated by enzyme-linked immunosorbent assay (ELISA). One (03-1) of four antibodies against TCPP strongly bound not only to TCPP but also to Mn(III)-TCPP and Fe(III)-TCPP (Figure 3.1),
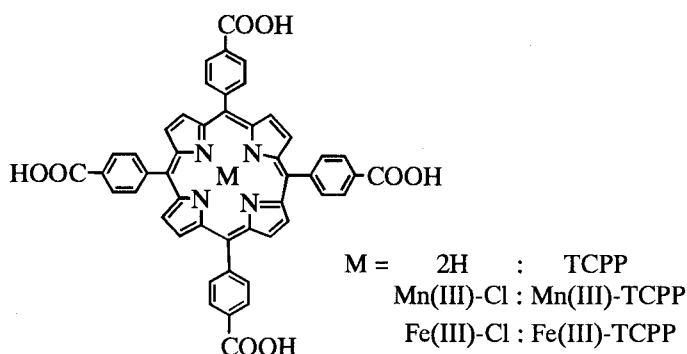

$$M = \quad 2H \quad : \quad TCPP$$
$$Mn(III)\text{-}Cl : Mn(III)\text{-}TCPP$$
$$Fe(III)\text{-}Cl : Fe(III)\text{-}TCPP$$

Figure 3.1. *meso*-Tetrakis(4-carboxyphenyl)porphyrin (TCPP) and its metalloderivatives (Mn(III)-TCPP and Fe(III)-TCPP).

whereas the other three antibodies against TCPP did not form complexes with Mn(III)-TCPP and Fe(III)-TCPP. Antibody 03-1 bound strongly to TCPP with a dissociation constant of 7.8×10^{-7} M and to Mn-TCPP and Fe-TCPP with dissociation constants of 3.2×10^{-6} M and 8.1×10^{-7} M, respectively (Chapter 2, Table 2-2). Under the conditions used for catalytic reactions, most of the metal complexes were incorporated into the combining site of the antibody. Figure 3.2 shows the UV-Vis and circular dichroism spectra of Fe(III)-TCPP in the presence of antibody 03-1. The UV-Vis spectra showed some hyperchromism in the region of the Soret band, indicating that metalloporphyrin is placed in the low-polar environment. The circular dichroism spectra showed induced Cotton effects on Fe(III)-TCPP,

indicating that metalloporphyrin is incorporated into the chiral environment of the combining site of the antibody.
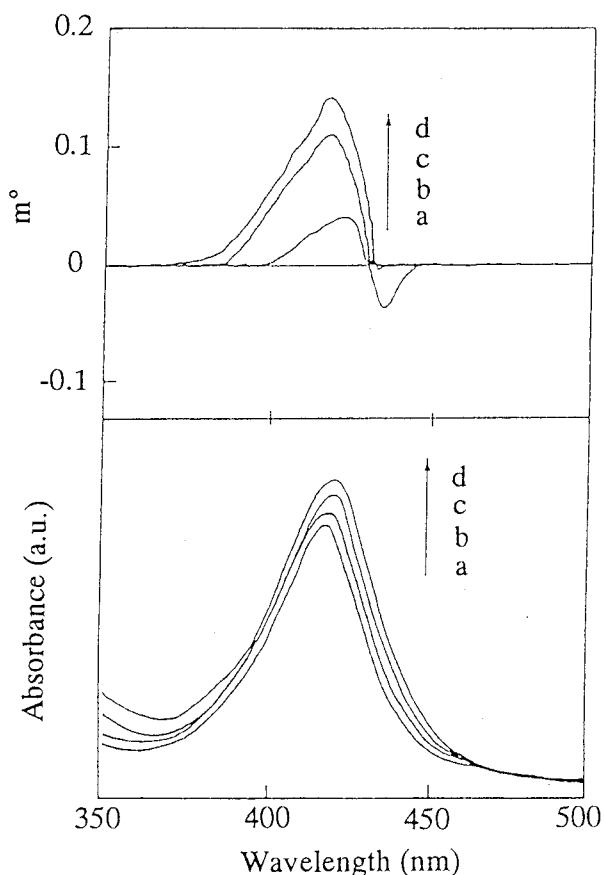

Figure 3.2. Absorption spectra and circular dichroism spectra of Fe(III)-TCPP in the presence of antibody 03-1 (Fab) in phosphate borate buffer (PBB) at pH 9.0. [Fe(III)-TCPP] = 1.8 μM; [antibody 03-1 (Fab)] = (a) 0, (b) 0.49, (c) 1.5, and (d) 2.9 μM.

Catalytic Effects on Peroxidation by Antibody-Metallo-porphyrin (M-TCPP) Complexes

The catalytic effects of complexes Mn(III)-TCPP and Fe(III)-TCPP with antibody 03-1 were investigated on the oxidations of various substrates; hydroquinone, catechol, resorcinol, pyrogallol, guaiacol,

2,2'-azinobis(3-ethyl-benzothiazoline-6-sulfonic acid) 2-ammonate (ABTS), and *o*-dianisidine. For understanding of the catalytic effects, Fab fragments (antigen binding fragments, 50 kDa) are used instead of whole antibody 03-1 (IgG $_{2b}$) because Fab is more soluble in aqueous buffer than whole antibody. Results showed that the complexes of M-TCPP with antibody 03-1 had specific catalytic effects only on pyrogallol. This result is in contrast to the oxidations of various substrates by mesoporphyrin-iron complexes with antibody against *N*-methylmesoporphyrin obtained by Cochran and Schultz,[9,10] who found that their complexes catalyzed not only the oxidation of pyrogallol but also those of other substrates. The difference in the specificities on the catalytic oxidation between our antibody and that of Schultz might be due to a difference in the size of antibody combining sites.

Figure 3.3 shows the time dependencies of oxidation of pyrogallol by metal-TCPP complexes in the presence and absence of antibody 03-1. The antibody 03-1-M-TCPP complexes had higher catalytic effects than M-TCPP alone, indicating that they have a catalytic effect on the oxidation. Further addition of the substrates caused a further catalytic reaction, indicating that the catalysts were still active. Fe(III)-TCPP was more active than Mn(III)-TCPP in the presence of antibody 03-1. The turnover number was 200 with the antibody-Mn(III)-TCPP complex and 300 with the antibody-Fe(III)-TCPP complex. M-TCPP and the Fc fraction (which does not bind antigen), which has no binding site for porphyrin, had no effect on the reaction.
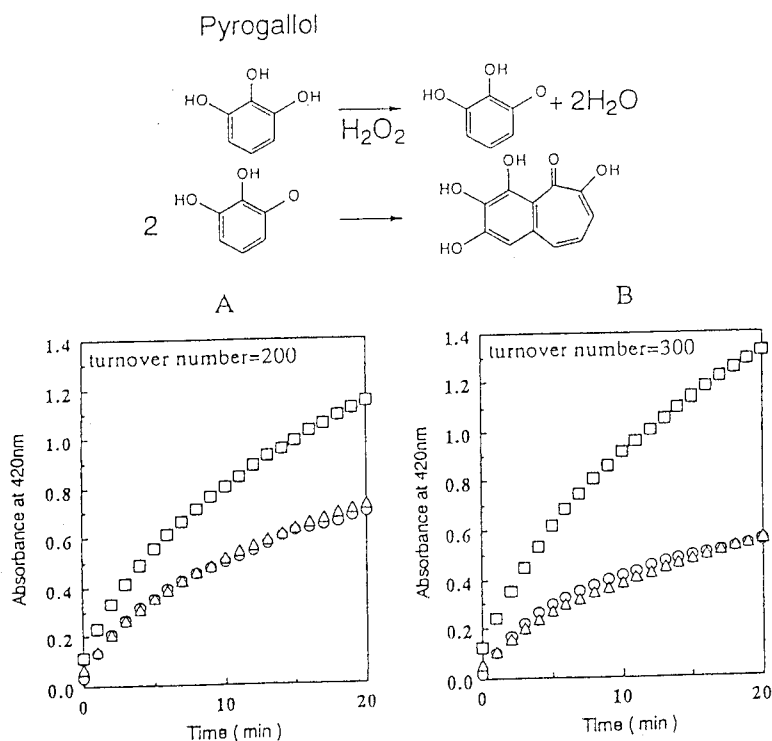
Figure 3.3. Peroxidation of pyrogallol in the presence of Fab-porphyrin complex (□), Fc-porphyrin complex (○), and porphyrin only (△). (A) Mn-TCPP and (B) Fe-TCPP. Reaction mixtures contained 5 mM H_2O_2, 1.2 mM pyrogallol, 0.5 µM Mn-TCPP or Fe-TCPP, 0.8 µM Fab or Fc, 90 mM Tris-acetate buffer, and 4 % (v/v) DMSO.

Figure 3.4 shows a Lineweaver-Burk plot on the oxidation of pyrogallol by the antibody 03-1-Fe(III)-TCPP complex. From the plot, the Michaelis constant K_m and the catalytic constant k_{cat} values were calculated. The K_m value in the presence of antibody 03-1-Fe(III)-TCPP complex is 4.0 mM. The k_{cat} is 50 min^{-1}, which is higher than that in the absence of antibody (8.7 min^{-1}).
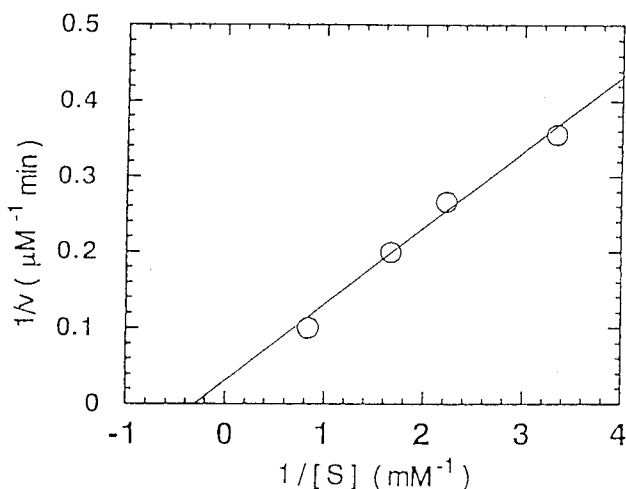
Figure 3.4. Lineweaver-Burk plot for the oxidation of pyrogallol as a function of [pyrogallol] for the Fe-TCPP-antibody 03-1 complex. Whole antibody was used for kinetic analysis. Initial rates of oxidation were determined by measuring the absorbance increase at 420 nm. Reaction mixtures contained 5 mM H_2O_2, 0.5 µM Fe-TCPP, 0.4 µM antibody ([Fab] = 0.8 µM), 90 mM Tris-acetate buffer, and 4 % (v/v) DMSO.

Figure 3.5 shows the effects of NaCl on the catalytic effect of the antibody-metalloporphyrin complexes. The effect increased with an increase in the concentration of salt, indicating that electrostatic interactions between M-TCPP and antibody combining site are important in the formation of the complex. NaCl at 60 mM was the most effective on the reactions, higher concentrations of NaCl possibly having a salting-out effect, causing dissociation of the antibody-metalloporphyrin complex. This kind of dissociation was observed by comparison of the absorption spectra of M-TCPP complexes in the presence with that in the absence of NaCl.
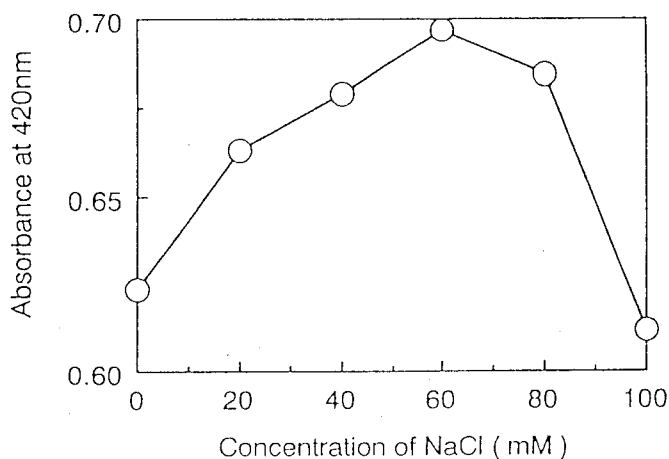
Figure 3.5. Effect of salt concentrations on the oxidation of pyrogallol by the antibody 03-1 (Fab)-Mn-TCPP.

Figure 3.6 shows the effect on the reaction of the molar ratio of antibody combining site to the M-TCPP complex. The plot shows a maximum at a molar ratio of 1:1, indicating that the 1:1 complex was the most effective. These results indicate that excess antibody inhibited
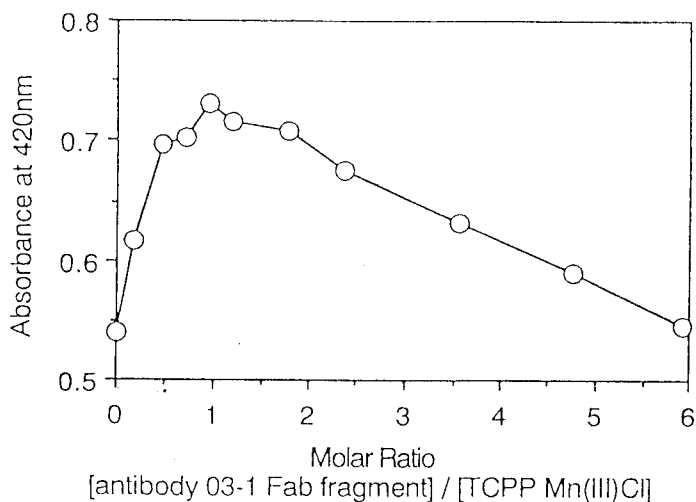

Figure 3.6. Effects of the molar ratio of the Fab fragment of antibody 03-1-Mn-TCPP on oxidation of pyrogallol.

47

the reactions. This suggests the formation of a 2:1 complex (two combining site per porphyrin), as indicated by studies on the binding properties of antibody 03-1 (Chapter 4 and Chapter 6).

In Tris buffer (at pH 8.3), the reactions proceeded rather fast without metalloporphyrins, making comparison of the rates in the presence and in the absence of antibody difficult. So, phosphate borate buffer (PBB, pH 9.0) was used for these oxidation reactions.

If the oxidation reactions proceed in the same way as those with natural enzymes, such as peroxidases and catalase, an imidazole group in the histidine residue should play an important role in an axial position on metals in the porphyrins. So, the effects of amines on the reaction were studied. Addition of various amines, such as imidazole and pyridine, did not have marked effects on the oxidation reactions by the antibody-porphyrin complexes. These results suggest that an imidazole group in the antibody serves as a coordinating ligand on metals in porphyrins. Preliminary results on the sequence analyses showed that there are a few histidine residues around the combining site of the antibody, which is shown in the Chapter 4 (Figure 4.12). There is a possibility that one of the histidine residue is involved in the association with a M-TCPP complex.

Comparison of Catalytic Activities of Antibody-Metalloporphyrin Complexes with Those of Peroxidase

Figure 3.7 shows the changes of absorbance with time curve in the oxidation of pyrogallol with naturally occurring horseradish peroxidase in the presence of Fe(III)-TCPP or the antibody 03-1 (Fab)-Fe(III)-TCPP complex. The results indicated that activity of the antibody-Fe(III)-TCPP complex was high under these conditions, although the metalloporphyrins show some effects on the reaction. The antibody-

48

porphyrin complexes are stable enough to show catalytic activity in the presence of an excess amount of H_2O_2. Horseradish peroxidase showed some loss of activity under the same conditions.
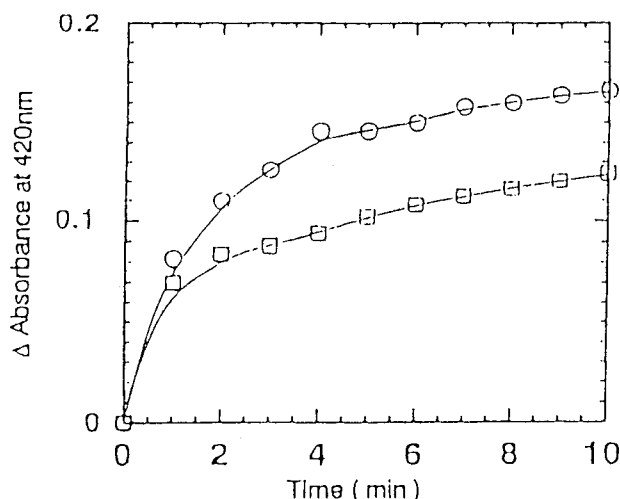

Figure 3.7. Absorbance versus time curve for the oxidation of pyrogallol in the presence of horseradish peroxidase and Fe-TCPP ($\bigcirc$) and that in the presence of antibody 03-1 (Fab)-Fe-TCPP complex ($\square$). Reaction mixtures contained 800 mM H_2O_2, 1.2 mM pyrogallol, 0.5 μM Fe-TCPP, 0.8 μM horseradish peroxidase or Fab, PBB (pH 9.0), and 4 % (v/v) DMSO.

Figure 3.8 shows a Lineweaver-Burk plot in the oxidation of pyrogallol in the presence of horseradish peroxidase. From the plot, the K_m and k_{cat} values for horseradish peroxidase were estimated to be 4.6 mM and 2.5 x 10^3 min^{-1}, respectively. The specificity constant k_{cat}/K_m value was 9.1 x 10^3 M^{-1} s^{-1} being 50 times that of the antibody-Fe(III)-TCPP complex. The antibody-Fe(III)-TCPP was highly reactive. The antibody-Fe(III)-TCPP complex was also highly selective, catalyzing the oxidation of pyrogallol specifically.
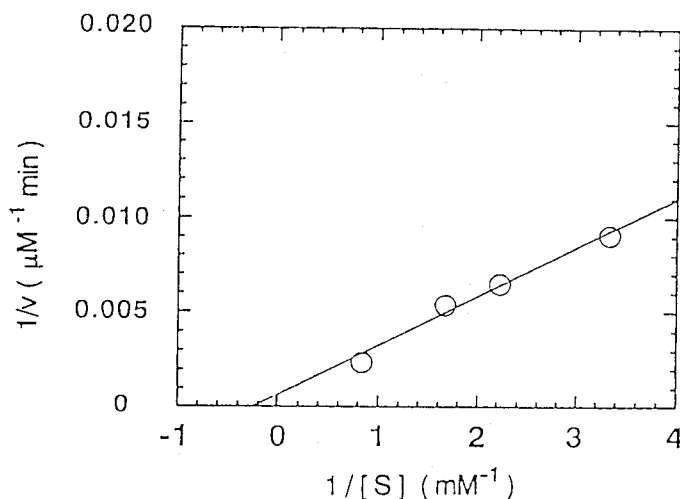
Figure 3.8. Lineweaver-Burk plot for the oxidation of pyrogallol in the presence of peroxidase. Reaction conditions were the same as those given in Figure 3.4.

Naturally occurring catalyst horseradish peroxidase catalyzes the oxidations of various substrates, not only pyrogallol but also hydroquinone, catechol, resorcinol, guaiacol, ABTS, and o-dianisidine. The reactions promoted by peroxidases are nonspecific. In contrast, the antibody 03-1-M-TCPP complex was selective for pyrogallol. High susceptibility of pyrogallol to oxidation was probably because pyrogallol has the lowest redox potential of the substrates tested, and antibody-metalloporphyrin complex selected such a compound as a substrate. The dissociation constant of the complex between antibody 03-1 and pyrogallol was determined to be 10^{-3} M. The antibody has the ability to bind pyrogallol, and metalloporphyrins selected such a compound as a substrate.

Catalytic Effects of Heavy and Light Chains

The effects of the heavy chains (longer peptide chains, 50 kDa) and light chains (shorter peptide chains, 25 kDa) of antibody 03-1 in the presence of M-TCPP complexes were investigated on the oxidation of pyrogallol. For consideration on the heavy and light chain effect on the reaction, the heavy chains and light chains of antibody 03-1 were separated by the reduction of the disulfide bonds with cysteine. Figure 3.9 shows the time-absorbance curve for the oxidation of pyrogallol in the presence of Mn(III)-TCPP and heavy chain of antibody 03-1. Heavy chain-M-TCPP complexes show effects comparable to those of the Fab and M-TCPP complexes. The light chain had lower reactivity, comparable to that of M-TCPP complex alone. These results indicate that heavy chain plays an important role in catalytic effects on the oxidation of pyrogallol.
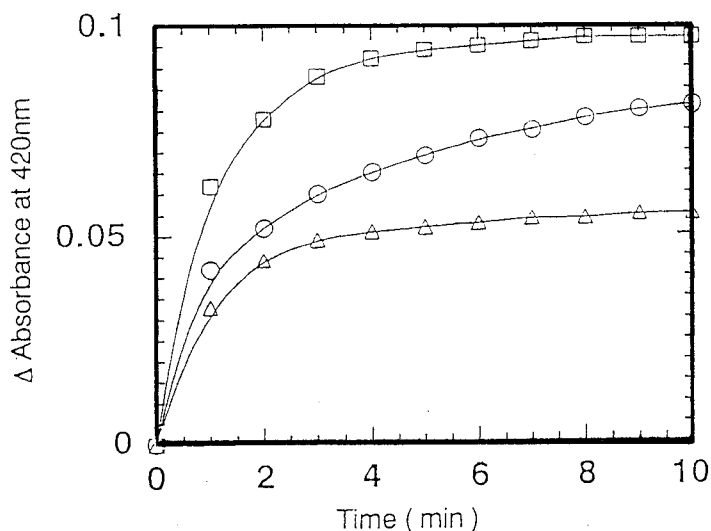

Figure 3.9. Absorbance versus time curve for the oxidation of pyrogallol in the presence of Fab-porphyrin (□), heavy chains-porphyrin (○), and light chains-porphyrin (△). Reaction mixtures contained 800 mM H_2O_2, 3.3 mM pyrogallol, 0.8 μM Mn-TCPP, 1.2 μM Fab, heavy chains, or light chains, 90 mM Tris-acetate buffer, and 4 % (v/v) DMSO.

Conclusion

The complexes of antibody 03-1 with Mn(III)-TCPP and Fe(III)-TCPP were found to catalyze oxidation of pyrogallol selectively. Studies on the effect of the molar ratio of the antibody to metalloporphyrin on the catalytic activity showed that a 1:1 complex was the most effective for the reaction. The antibody-metalloporphyrin complexes are stable enough to show catalytic activity in the presence of an excess amount of H_2O_2.

References

(1) Schultz, P. G.; Lerner, R. A.; Benkovic, S. J. *Chem. Eng. News* **1990**, *68*, 26.

(2) Lerner, R. A.; Benkovic, S. J.; Schultz, P. G. *Science* **1991**, *252*, 659-667.

(3) Jencks, W. P., Ed. Catalytic Antibodies; Ciba Foundation Symposium 159; John Wiley & Sons, **1993**.

(4) Harada, A.; Okamoto, K.; Kamachi, M.; Honda, T.; Miwatani, T. *Chem. Lett.* **1990**, 919-920.

(5) Harada, A.; Okamoto, K.; Kamachi, M. Chem. Lett. 1991, 953-956.

(6) Harada, A.; Shiotsuki, K.; Fukushima, H.; Kamachi, M. *Supramolecules* **1993**, *2*, 153-156.

(7) Harada, A.; Shiotsuki, K.; Fukushima, H.; Yamaguchi, H.; Kamachi, M. *Inorg. Chem.* **1995**, *34*, 1070-1076.

(8) Colquhoun, H. M.; Stoddart, J. F.; Williams, D. J. *Angew. Chem. Int. Ed. Engl.* **1986**, *25*, 487-492.

(9) Cochran, A. G.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 9414-9415.

(10) Cochran, A. G.; Schultz, P. G. *Science* **1990**, *249*, 781-783.

Chapter 4

Photochemistry of Porphyrins
in the presence of Antibodies

Introduction

Much effort has been directed toward mimicking electron transfer in the natural photosynthetic systems.[1-4] Electron transfer in covalently linked donor-acceptor systems has been extensively studied.[5-9] Recently, much attention has been focused on the design of noncovalently assembled donor-acceptor arrays,[10-13] because the chromophores of the photosynthetic reaction center are not covalently linked via spacer groups. Electron transfer carriers, cytochrome, and photosynthetic chlorophyll have porphyrins which are suitably incorporated into protein domains and these functions are regulated by the protein molecules. In order to incorporate artificial porphyrins into protein domains,[14-16] it seems most appropriate to use monoclonal antibodies for porphyrins. In Chapter 2, monoclonal antibodies for meso-tetrakis(4-carboxyphenyl)porphyrin (TCPP) were prepared and their binding affinities to porphyrins were studied.[17-20] In this Chapter, spectroscopic studies on the interactions between antibodies (03-1 and 13-1) and porphyrins are described. Electron transfer from TCPP zinc complex to methyl viologen (MV^{2+}) in the presence of antibody 03-1 is compared with that in the presence of another antibody 13-1 (Scheme 4.1).
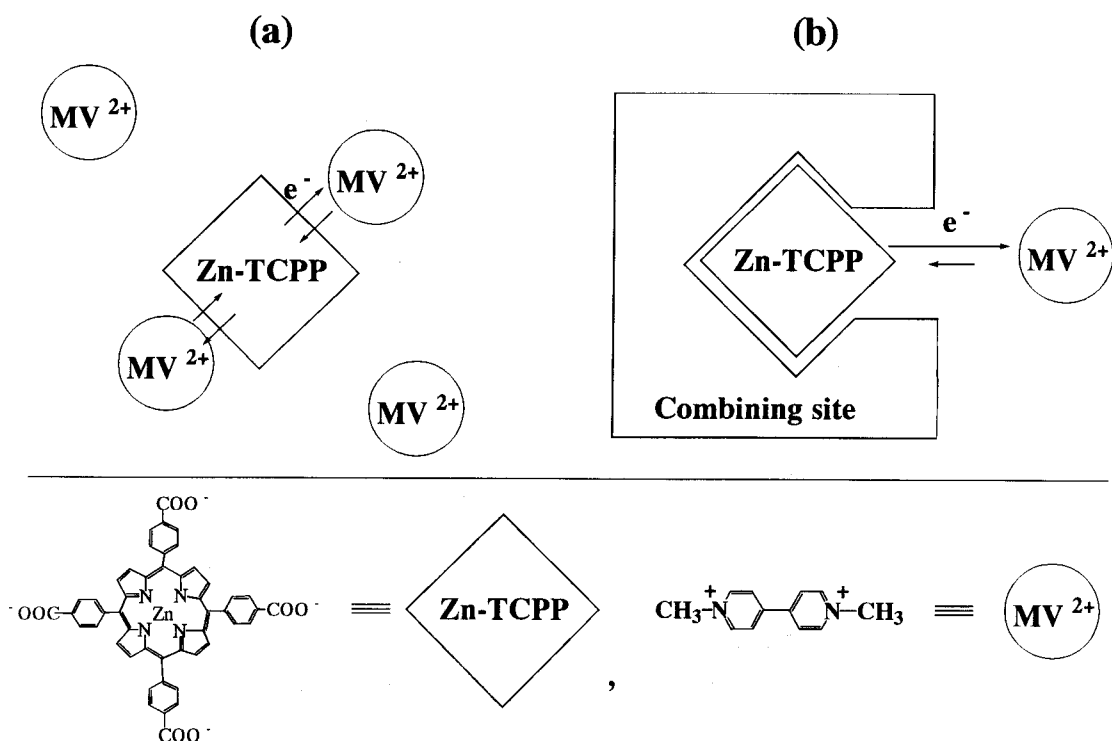
Scheme 4.1. Photoinduced electron transfer from Zn-TCPP to methyl viologen. (a) Zn-TCPP and (b) Zn-TCPP-antibody complex.

Experimental Section

Materials

Monoclonal antibody (03-1) against *meso*-tetrakis(carboxyphenyl) porphine (TCPP) was prepared according to the previous reports.[17] It was purified by affinity chromatography using protein A (Amersham Ampure™ PA kit). The purity of antibody 03-1 was checked by SDS-PAGE electrophoresis. Antibody solutions were stored and treated in phosphate borate buffer with various ionic strength (10 mM and 100 mM) and pH (5.7 and 9.0). Methyl viologen was obtained from Tokyo Kasei Co. Ltd.

Measurements

UV-Vis spectra were recorded on a Shimadzu UV-2100 UV-visible spectrophotometer. Circular dichroism spectra were recorded on a JASCO J-40A spectrophotometer with a JASCO ORD/CD data processor using a 10 mm quartz cell. Emission spectra were recorded on a Shimadzu RF-502A spectrofluorophotometer with an ND-10 filter. Fluorescence lifetimes were measured on a Horiba NAES-550 time-correlated single-photon counting equipment. The error values are less than 0.1 nsec.

Analyses

Scatchard Plot. Dissociation constants of antibodies were determined by the Scatchard plot as follow:[21-23]

$$i \,/\, [\text{porphyrin}] = n \,/\, K_d - i \,/\, K_d \qquad (1)$$

where i is the fraction of antibody combining sites that are occupied by porphyrins, n the number of porphyrins per antibody combining site, and [porphyrin] the concentration of free porphyrin. i is determined by the measurements of the fluorescence quenching of antibodies on addition of porphyrins.

Stern-Volmer Plot. Rate constants of electron transfer were determined by the Stern-Volmer equation given by

$$I_0 \,/\, I = 1 + K_{SV} \,[Q] \qquad (2)$$

where I_0 and I are fluorescence intensities in the absence and presence of the quencher, respectively. K_{SV} is the Stern-Volmer constant and [Q] the concentration of the quencher.

Results and Discussion

Photochemical Behavior of TCPP and Zn-TCPP in the presence of Antibodies

UV-Vis Spectra. Antibodies 03-1 and 13-1 raised against TCPP bound TCPP zinc complex [Zn-TCPP] strongly with a dissociation constants of 10^{-7} M (Table 2.1). The absorption spectra of TCPP in the presence of antibody 03-1 showed that the Soret band shifted to a longer wavelength by about 10 nm (Figure 4.1(a)). However, in the TCPP-antibody 13-1 system, no such changes in the absorption spectra were observed (Figure 4.1(b)). Similar results were obtained in Zn-TCPP-antibody complexes (Figure 4.2). The shifts of the Soret band of Zn-TCPP to a longer wavelength by about 10 nm on addition of the antibody 03-1 indicate that the Zn-TCPP is strongly bound to the antibody. The absorption spectra of TCPP in the presence of antibody 03-1 showed an isosbestic point at 418 nm. Although Zn-TCPP 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 association.

The binding of antibody 03-1 and 13-1 to TCPP and metal complexes has been also estimated from the Scatchard plot. Table 2.1 shows the dissociation constants of the complexes of the antibodies with TCPP and Zn-TCPP complex assuming a one-to-one complex formation.
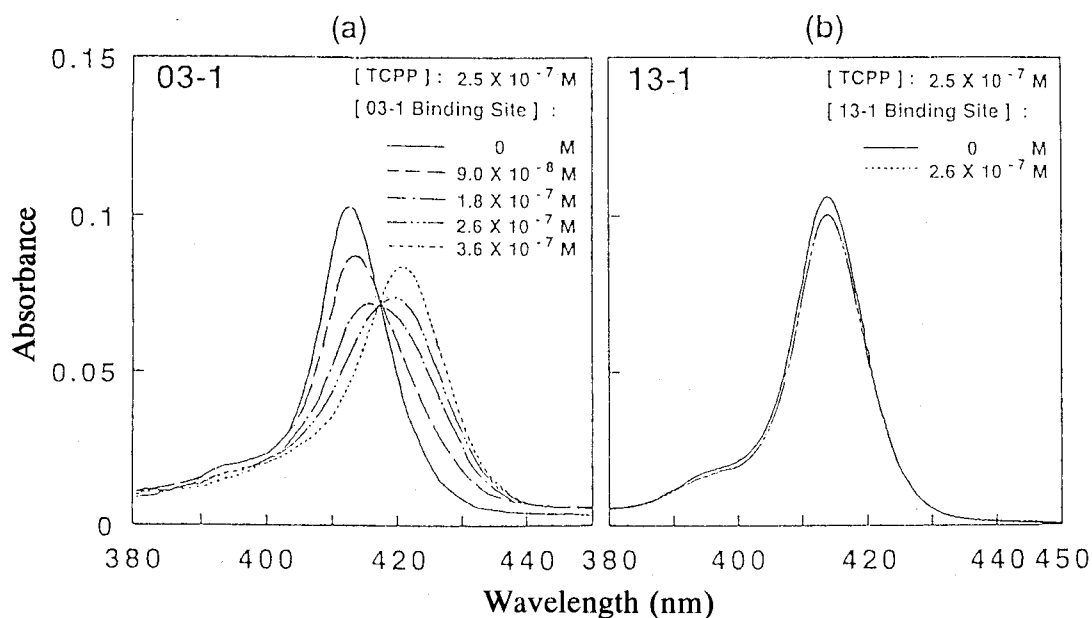
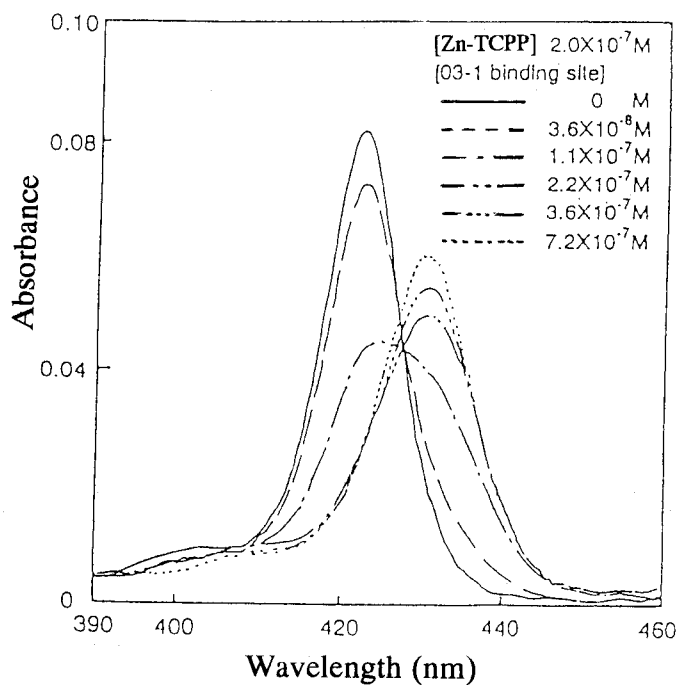**Figure 4.1.** UV. Vis spectra of TCPP in the presence of anti-TCPP antibody 03-1; (a) and Antibody 13-1 (b).

Figure 4.2. UV. Vis spectra of Zn-TCPP in the presence of antibody 03-1.

Emission Spectra. Figure 4.3 shows emission spectra of TCPP at a fixed concentration (2.5×10^{-7} M) in the presence of various amounts of the antibody. The spectra showed three clear isoemissive points in the presence of antibody 03-1 (Figure 4.3(a)). In the Zn-TCPP-antibody 03-1 system, the spectra showed an increase in the fluorescence bands and the absence of isoemissive points. The maxima of the emission of the 0-0 and 0-1 bands showed red-shifts. These results indicate that Zn-TCPP is fixed in the antibody combining site firmly and the species in solution are not only one-to-one complexes (Figure 4.4). Scatchard plots for the complex formation between antibody 03-1 and TCPP or Zn-TCPP 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 4.5). Such changes in the absorption spectra were not observed in the TCPP-antibody 13-1 system (Figure 4.3(b)).
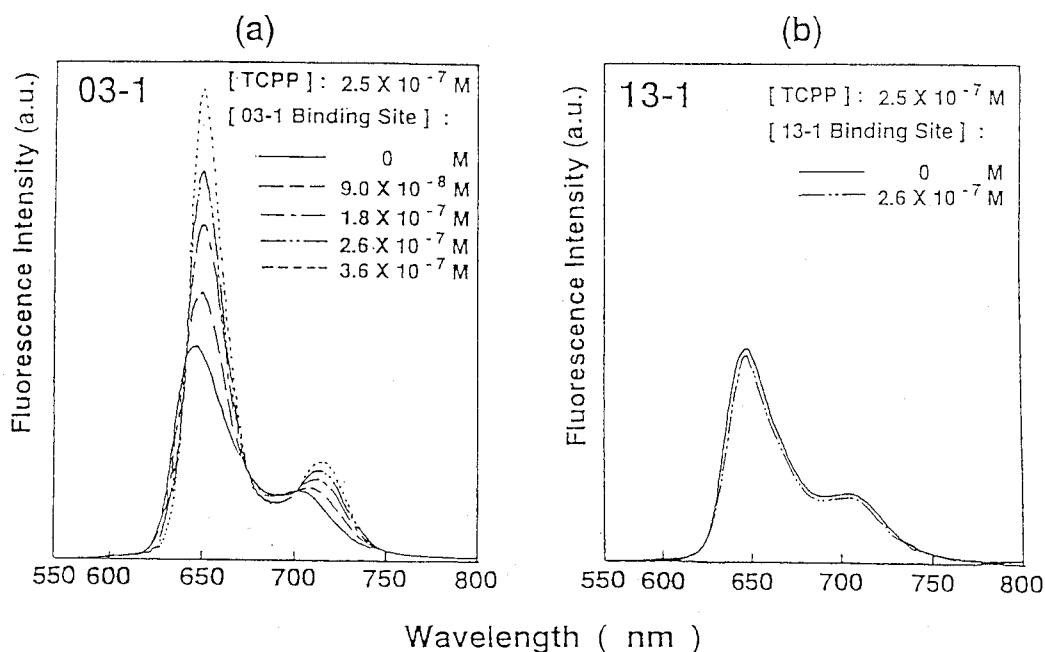

Figure 4.3. Fluorescence spectra of TCPP in the presence of antibody 03-1; (a) and antibody 13-1; (b).
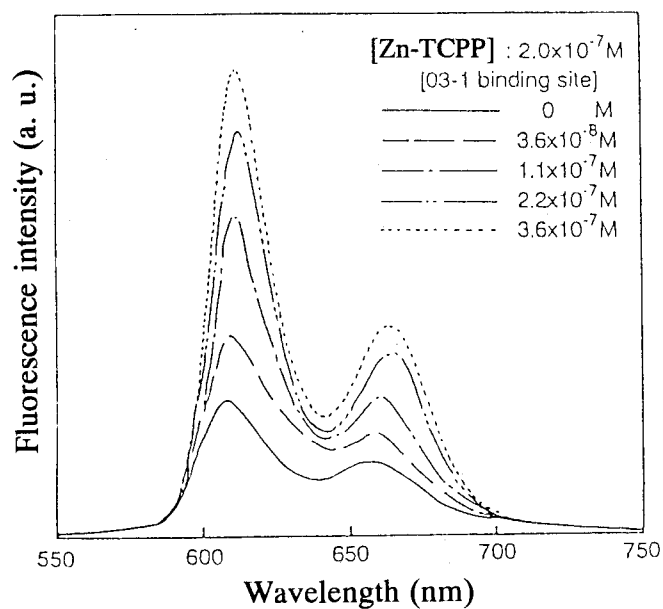
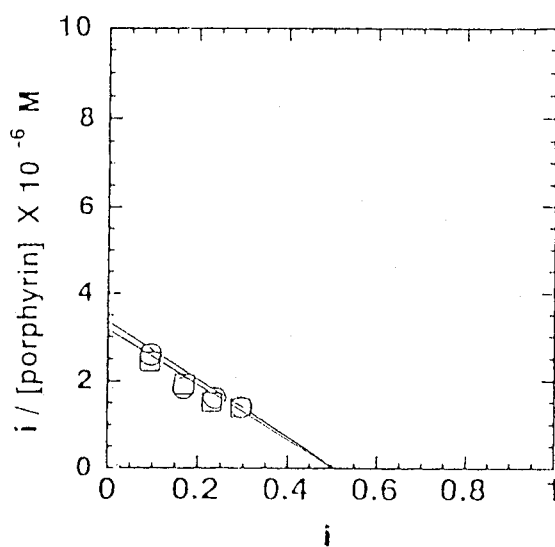**Figure 4.4.** Fluorescence spectra of Zn-TCPP in the presence of antibody 03-1.

Figure 4.5. Scatchard plots for the complex formation of antibody 03-1 with TCPP (○) and Zn-TCPP (□).

60

Circular Dichroism Spectra. Figures 4.6 and 4.7 show circular dichroism (CD) spectra for TCPP and Zn-TCPP, respectively in the presence of antibody 03-1. Both spectra show very strong induced Cotton effects. In the TCPP-antibody 03-1 system, the positive Cotton effects were observed at low concentrations of the antibody until the molar ratio reached one-to-one. Then the positive Cotton effects decreased with increase in the antibody concentration. When the molar ratio exceeds one-to-one, the positive induced CD decreased and finally negative induced CDs appeared. 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 Zn-TCPP in the presence of antibody 03-1 showed sharp splitting at the Soret bands where negative and positive bands were obtained in the longer and shorter wavelength sides, respectively.
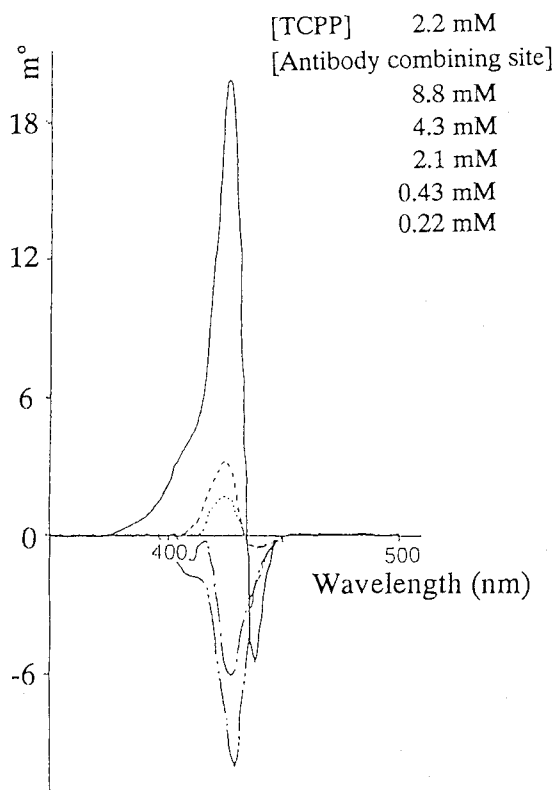

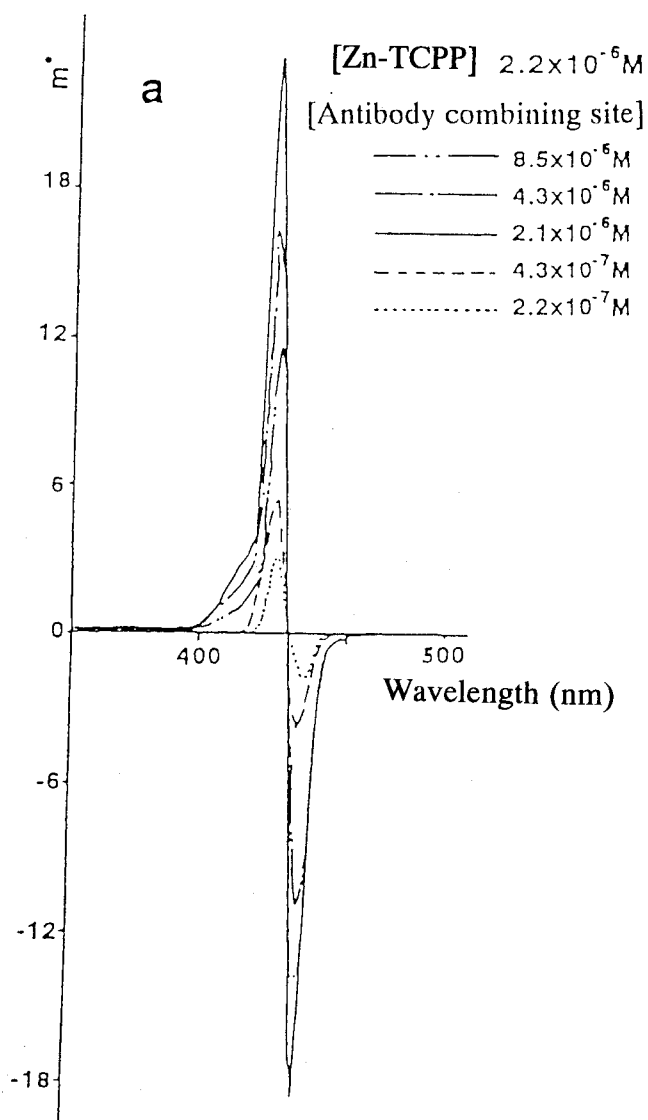Figure 4.6. Circular dichroism spectra of TCPP in the presence of antibody 03-1.

Figure 4.7. Circular dichroism spectra of Zn-TCPP in the presence of antibody 03-1.

These bands can be assigned to an exciton coupling of the band.[24] This result can be interpreted as indicating that two Zn-TCPP molecules are close together in the combining site of the antibody.

It is suggested that a one-to-one complex is first formed between antibody and porphyrin. Then, another antibody binds TCPP (or Zn-TCPP) to form a two-to-one complex, which is composed of two antibodies and one porphyrin.

Similar experiments were performed in one of the other antibodies (13-1). Although antibody 13-1 bound to TCPP and Zn-TCPP strongly, it gave no Cotton effects in the Soret bands of TCPP and Zn-TCPP.

Lifetimes of Excited States for TCPP. Figure 4.8 shows fluorescence decay profiles for TCPP and TCPP bound to the antibody combining site. All measurements were carried out in 0.1 M phosphate borate buffer (PBB, pH 9.0). The decay for the antibody 03-1-TCPP complex was slower than that for TCPP alone. The lifetimes of the excited states of TCPP are elongated on addition of antibody 03-1 (Table 4.1). The lifetime of the singlet excited state of TCPP was the longest in the presence of antibody 03-1 at two-to-one molar ratio (two antibody combining sites and one porphyrin). A similar elongation of lifetime was observed in the triplet excited states of TCPP bound to antibody 03-1.

Table 4.1. Singlet excited state lifetimes (τ_S) and triplet excited state lifetimes (τ_T) of TCPP in the presence and absence of antibodies.

	No antibodies	Antibody 03-1	Antibody 13-1
τ_S (nsec)	8.97	10.2 (1:1)[a]	8.97 (1:1)[a]
		12.1 (1:2)[a]	8.97 (1:2)[a]
		12.1 (1:3)[a]	8.97 (1:3)[a]
τ_T (msec)	0.34	2.7	0.26

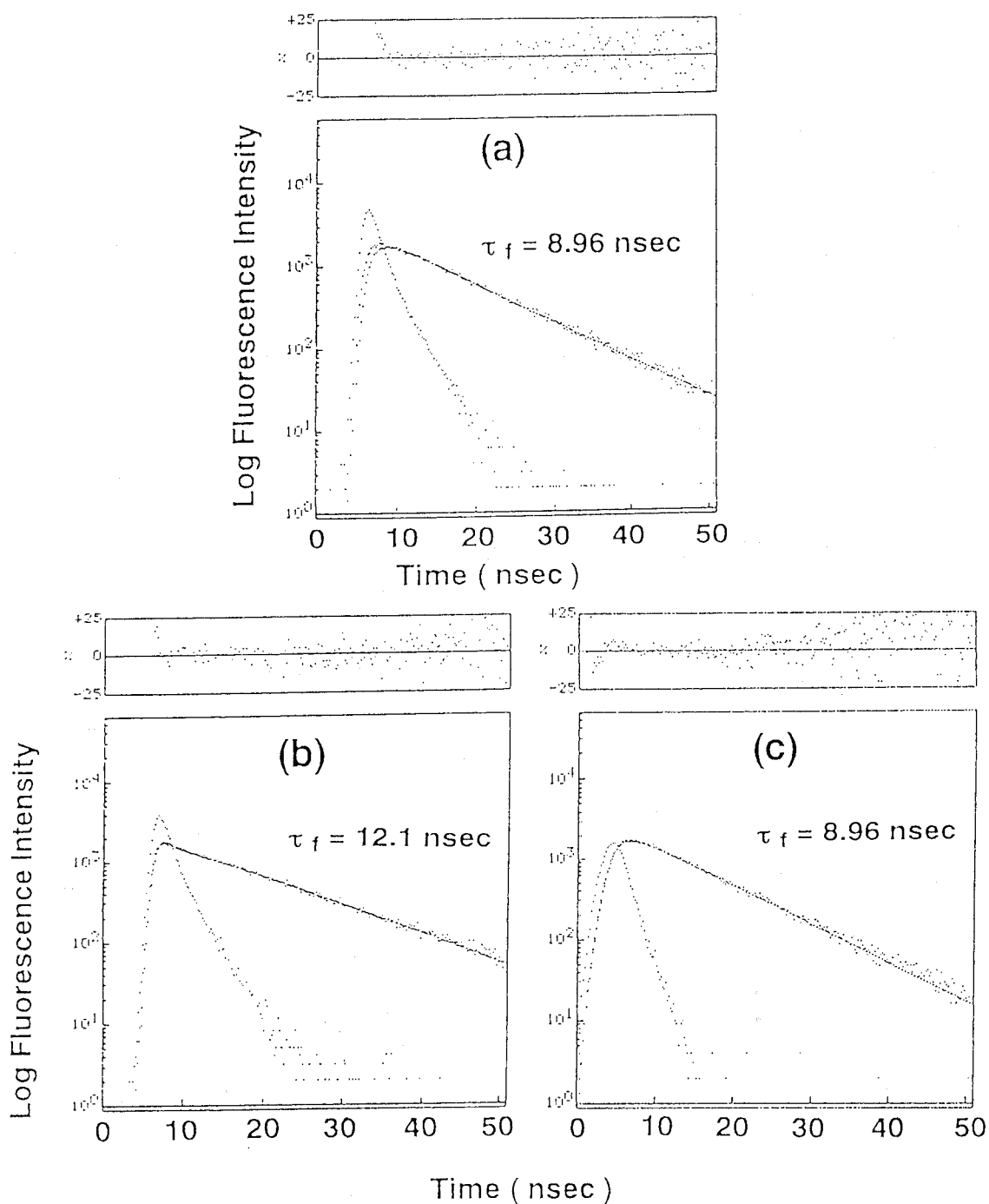a) [TCPP] : [Antibody combining site].

Figure 4.8. Fluorescence decay profiles for TCPP (a), TCPP in the presence of antibody 03-1 (b), and antibody 13-1 (c).

Although most of the catalytic antibodies have been limited to ground state reactions, this antibody 03-1 raised against TCPP was found to stabilize excited states of porphyrins.

Photoinduced Electron Transfer Reactions from Zn-Porphyrin to Methyl Viologen in the presence of Antibodies

UV-Vis Spectra. Figure 4.9 shows spectral changes upon titration of Zn-TCPP with methyl viologen (MV^{2+}) in the absence and in the presence of antibodies (03-1 and 13-1). When MV^{2+} was added to an aqueous solution of Zn-TCPP, no changes were observed for the Soret band of Zn-TCPP in the presence of antibody 03-1, as shown in Figure 4.9(b). This result indicates that there are no interactions between MV^{2+} and Zn-TCPP incorporated in the combining site of the antibody in the ground state.

Emission Spectra. Upon excitation at the Soret band of Zn-TCPP, Zn-TCPP emitted fluorescence peaking at 610 and 660 nm. The fluorescence of Zn-TCPP was quenched by MV^{2+}. The emission spectra of Zn-TCPP in the presence of antibody 03-1 showed that the addition of MV^{2+} resulted in a decrease in the emission intensity, although there are no ground state interactions. It is in contrast with the fact that Zn-TCPP was statically quenched in the ground state by MV^{2+}. Figure 4.10 shows Stern-Volmer plots for quenching of the emission from the Soret band of Zn-TCPP-MV^{2+} in the absence and presence of antibodies. The plots for the Zn-TCPP-MV^{2+} system showed efficient fluorescence quenching. In contrast, the fluorescence
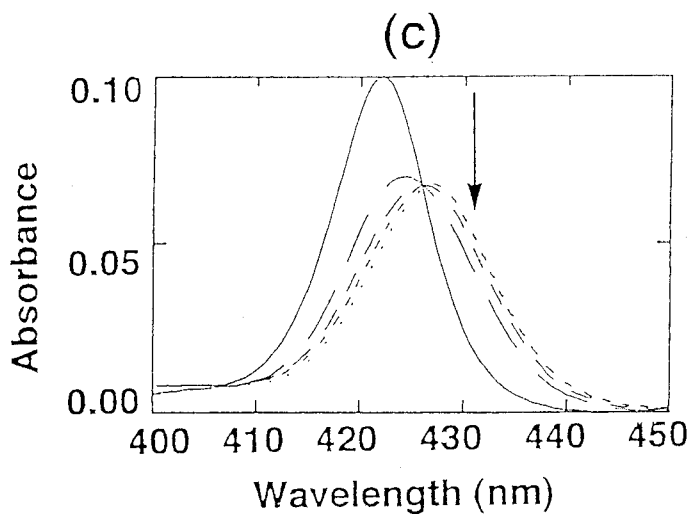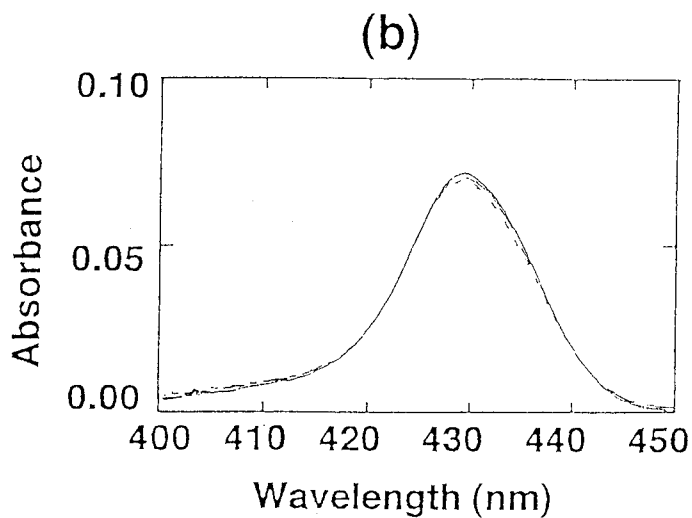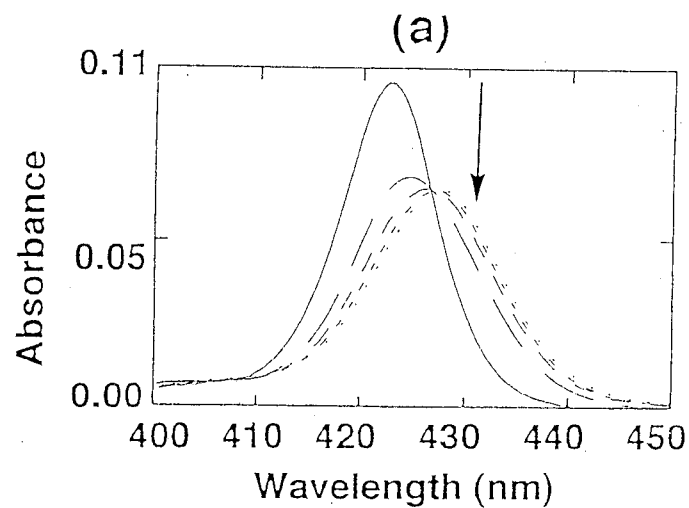
Figure 4.9. Spectral changes upon titration of (a) Zn-TCPP, (b) Zn-TCPP-antibody 03-1 and (c) Zn-TCPP-antibody 13-1 with methyl viologen (MV^{2+}). [MV^{2+}]; 0, 0.30, 0.60, 0.89, 1.20 mM, [Zn-TCPP]; 0.25 µM, and [antibody combining site]; 0.5 µM.

quenching was saturated at $[MV^{2+}] > 500$ mM in the presence of antibody 03-1, where the ratio I_0/I was almost constant at 2.0. Considering the result in Figure 4.10, this saturation behavior indicates the electron transfer from Zn-TCPP in the antibody to MV^{2+} outside the antibody.

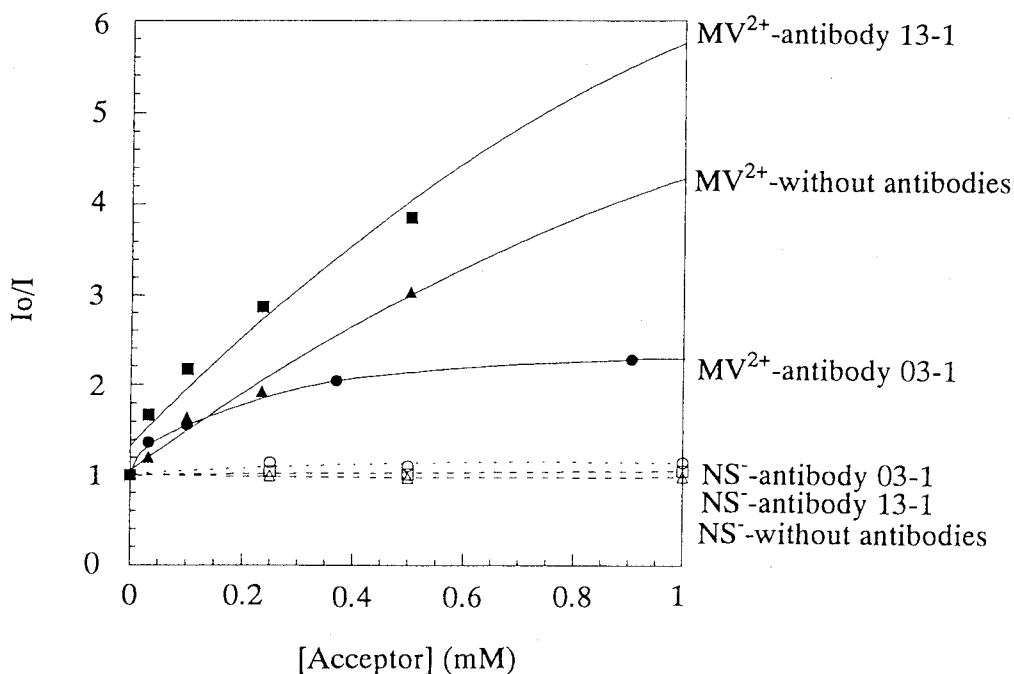**Figure 4.10.** Stern-Volmer plots for the quenching of the emission from the Soret band of Zn-TCPP by methyl viologen (MV^{2+}) or Naphthalenesulfonate (NS^-).
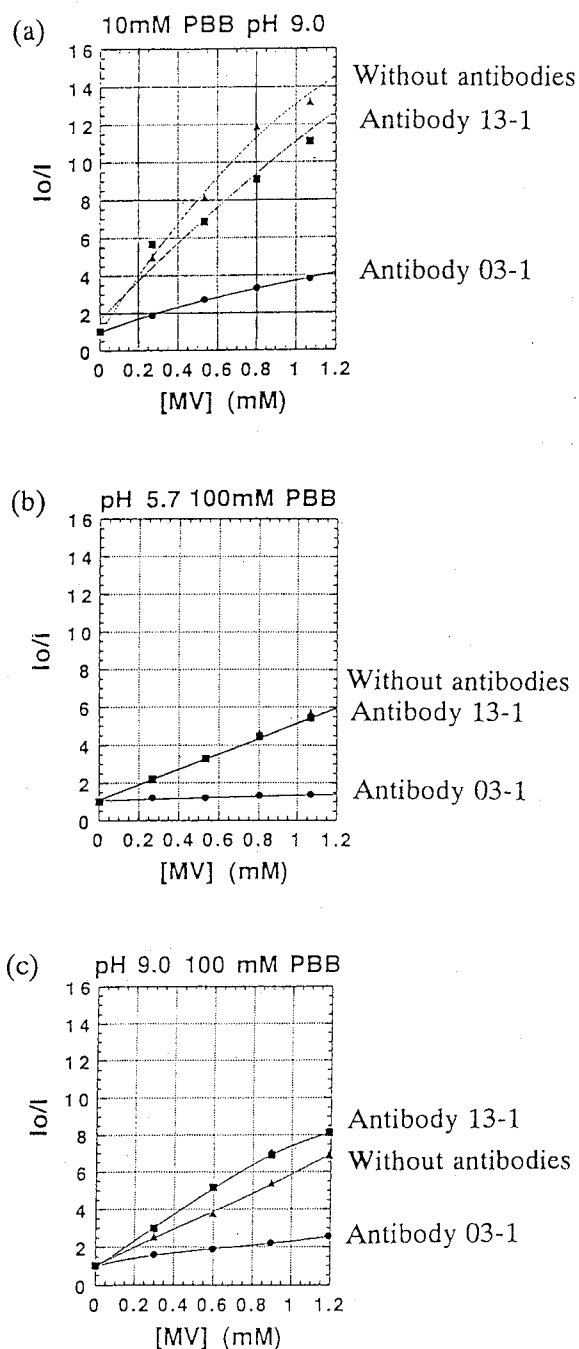
Figure 4.11. Stern-Volmer plots for Zn-TCPP and Zn-TCPP-antibody complexes quenched by methyl viologen. Ionic strength and pH dependency. In 10 mM phosphate borate buffer (PBB) (pH 9.0) (a), 100 mM PBB (pH 5.7) (b), and 100 mM PBB (pH 9.0) (c).

Figure 4.11 reveals that the efficiency of the quenching increases with decreasing ionic strength of the solution. At low ionic strength (pH 9.0, 10 mM PBB), the quenching efficiency is obviously higher than that at high ionic strength. Figure 4.11 also shows that the efficiency of quenching increases with increasing pH of the solution in the presence of antibodies. It is found that the complex formation of antibody 13-1 and Zn-TCPP is effective in the quenching of Zinc porphyrin fluorescence.

Although two monoclonal antibodies, 03-1 and 13-1, have the same affinity to Zn-TCPP, the photochemical behavior of Zn-TCPP on addition of MV^{2+} in the presence of antibody 13-1 was similar to that of Zn-TCPP in the absence of antibodies. On the other hand, in the case of antibody 03-1, the electron transfer from Zn-TCPP to MV^{2+} was controlled by the complex formation between antibody 03-1 and Zn-TCPP with two-to-one (Scheme 4.2).

Lifetimes. The fluorescence decay of Zn-TCPP-antibody was fitted to a monoexponential function with a lifetime of 1.8 nsec, whereas the addition of MV^{2+} brought about a biexponential decay curve with lifetimes $\tau_L = 2.5$ nsec (72%) and $\tau_S = 0.5$ nsec (28%) at $[MV^{2+}] = 5.0 \times 10^{-3}$ M (Table 4.2). The longer- and shorter-lived components of the decay curve are assignable to Zn-TCPP incorporated in an antibody and Zn-TCPP-antibody-MV^{2+} complex, respectively. Taking into account the absence of donor-acceptor complexation at the ground state together with the saturation behavior in the fluorescent quenching, the rate constant (k_{et}) for the electron transfer from Zn-TCPP in the antibody combining site to MV^{2+} was estimated to be 1.6 $\times 10^9$ s^{-1}, using the equation $k_{et} = (\tau_{[MV^{2+}]})^{-1} - (\tau_{[none]})^{-1}$.

Antibody 03-1
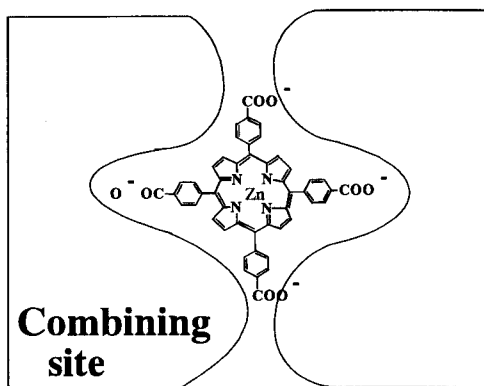
Antibody 13-1
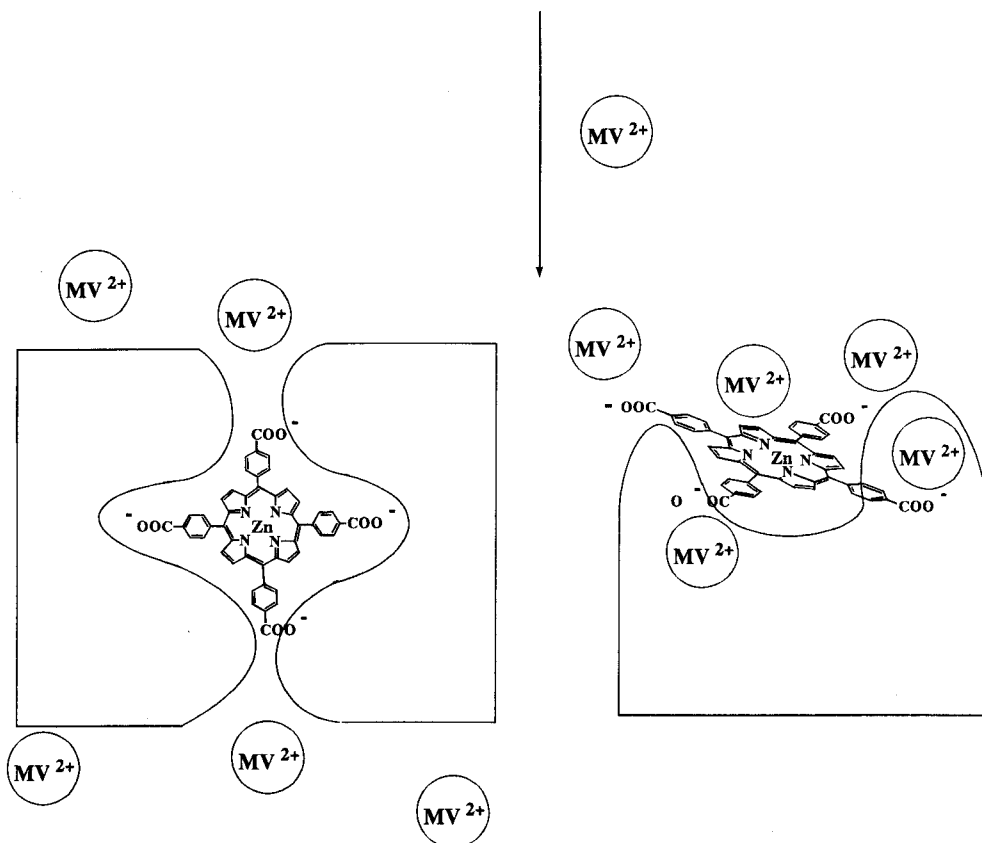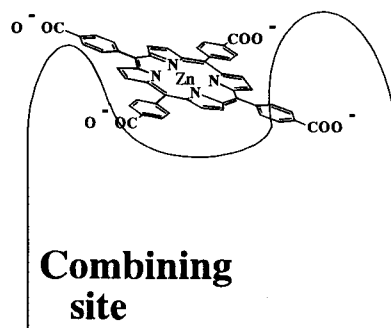
Scheme 4.2. Schematic structures of Zn-TCPP-antibody complexes and the possible positions of methyl viologen molecules approaching to the complexes.

70

Table 4.2. Singlet excited state lifetimes (τ) of TCPP-Zn in the presence of anti-TCPP monoclonal antibodies and methyl viologen [MV^{2+}].

Sample	[MV^{2+}] (mM)	τ_L (nsec)	$A_1(\%)$	τ_S (nsec)	$A_2(\%)$
TCPP-Zn	0.0	1.8	100		
TCPP-Zn	5.0	2.6	50	0.5	50
TCPP-Zn-Ab.03-1	0.0	2.5	100		
TCPP-Zn-Ab.03-1	5.0	2.5	72	0.5	28
TCPP-Zn-Ab.13-1	0.0	1.7	100		
TCPP-Zn-Ab.13-1	5.0	2.3	41	0.4	59

First Order Sequences of Antibody 03-1 and 13-1. Figure 4.12 shows amino acid sequences of the variable region of antibody 03-1 and antibody 13-1. Among the regions shown as CDR which are responsible for the binding of porphyrins, there are three amino acids bearing acidic groups in their side chain in antibody 13-1, compared with two amino acids in antibody 03-1. The difference of the number of the acidic amino acid is considered to be one of the reason why the complex formation of antibody 13-1 and Zn-TCPP is effective in the quenching of Zn-TCPP fluorescence by the addition of methyl viologen.

(a)

Amino Acid Sequences of Heavy (H) Chain Variable Region from the Anti-Porphyrin Antibodies 03-1 and 13-1

```
           1            10               20           30               40
03-1 H    N V Q L V E S G G G   L V Q P G G S R K L   S C A A S G F T F S   S F G M H W V R Q A
13-1 H    E V Q L L E S G G G   L V R P G N S L K L   S C L T S G F T F S   N Y R M H W V R Q P
                                                                            ————————
                                                                             CDR1

                        50               60           70               80
03-1 H    P E K G L E W V A Y   I S - - G G S R K L   Y Y A D T V K G R F   T I S R D N P K N T
13-1 H    P G K R L E W I A V   I T V K S D N Y G A   K Y A E S V R G R F   T I S R D D S K S S
                               ————————————————————
                                      CDR2

                        90               100          110
03-1 H    L F L Q M T S L R S   E D T A M Y Y C A R   - S W L L P F D Y W   G Q G T T L T V S
13-1 H    V Y L Q M N R L R E   E D T A T Y Y C C R   T P W V Y A M D C W   G Q G T S V I V S
                                                     ————————————————————
                                                            CDR3
```

(b)

Amino Acid Sequences of Light (L) Chain Variable Region from the Anti-Porphyrin Antibodies 03-1 and 13-1

```
           1            10               20           30               40
03-1 L    D I V M T Q S P S S   L A M S V G Q K V T   M S C K S S Q S L L   N S R N Q K N Y L A
13-1 L    D I V L T Q S P A S   L A V S L G Q R A T   I S C R A S K S - -   V S A S G Y I Y M H
                                                     ————————————————
                                                            CDR1

                        50               60           70               80
03-1 L    W Y Q Q K P G Q S P   K L L V Y F A S T R   E S G V P D R F I G   S G S G T D F T L T
13-1 L    W Y Q Q K P G Q P P   K L L I S L A T N L   E S G V P A R F S G   S G S G T D F T L N
                               ————————————————————
                                      CDR2

                        90               100          110
03-1 L    I S T V Q A E D L A   D Y F C Q Q H Y S T   P Y T F G G G T K L   E I K R A
13-1 L    I H P V E E E D V A   T Y Y C Q H S R E L   P L T F G A G T K L   E L K R A
                               ————————————————
                                      CDR3
```

Figure 4.12. Amino acid sequences of heavy (H) chain variable region from the monoclonal antibodies 03-1 and 13-1 (a) and those of light (L) chain (b).

Conclusion

The monoclonal antibody 03-1 raised against TCPP shows a high specificity for porphyrins and forms not only one-to-one complex but also two-to-one complex. The photoinduced electron transfer from incorporated Zn-TCPP to MV^{2+} in an antibody-Zn-TCPP noncovalent assembly system suggested that the electron transfer reactions of Zn-TCPP can be controlled by antibodies.

References

(1) Deisenhofer, J.; Epp, O.; Miki, K.; Huber, R.; Michel, H. *J. Mol. Biol.* 1984, 180, 385-398.

(2) Deisenhofer, J.; Michel, H. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 829-847.

(3) Gust, D.; Moore, T. A. *Science* 1989, 244, 35-41.

(4) Huber, R. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 848-869.

(5) Wasielewski, M. R. *Chem. Rev.* 1992, 92, 435.

(6) Giraudeau, A.; Ruhlmann, L.; Kahef, L. EI, Gross, M. *J. Am. Chem. Soc.* **1996**, *118*, 2969-2979.

(7) Osuka, A.; Maruo, S.; Mataga, N.; Taniguchi, S.; Okada, T.; Yamazaki, I.; Nishimura, Y.; Ohno, T.; Nozaki, K. *J. Am. Chem. Soc.* **1996**, *118*, 155-168.

(8) Osuka, A.; Maruyama, K.; Mataga, N.; Asahi, T.; Yamazaki, I.; Tamai, N. *J. Am. Chem. Soc.* **1996**, *118*, 155-168.

(9) Sessler, J. L.; Wang, B.; Harriman, A. *J. Am. Chem. Soc.* **1993**, *115*, 10418-10420.

(10) Sessler, J. L.; Wang, B.; Harriman, A. *J. Am. Chem. Soc.* **1995**, *117*, 704-714.

(11) Berman, A.; Izraeli, E. S.; Levanon, H.; Wang, B.; Sessler, J. L. *J. Am. Chem. Soc.* **1995**, *117*, 8252-8257.

(12) Brun, A. M.; Harriman, A.; Heitz, V.; Sauvage, J. P. *J. Am. Chem. Soc.* **1991**, *113*, 704-714.

(13) Brun, A. M.; Atherton, S. J.; Harriman, A.; Heitz, V.; Sauvage, J. P. *J. Am. Chem. Soc.* **1992**, *114*, 4632-4639.

(14) Sadamoto, R.; Tomioka, N.; Aida, T. *J. Am. Chem. Soc.* **1996**, *118*, 3978-3979.

(15) Hayashi, T.; Takimura, T.; Ogoshi, H. *J. Am. Chem. Soc.* **1995**, *117,* 11606-11607.

(16) Shreder, K.; Karriman, A.; Iverson, B. L. *J. Am. Chem. Soc.* **1995**, *117,* 2673-2674.

(17) Harada, A.; Okamoto, K.; Kamachi, M.; T. Honda, T.; Miwatani, T. *Chem. Lett.* **1990**, 917-918.

(18) Harada, A.; Okamoto, K. Kamachi, M. *Chem. Lett.* **1991**, 953-956.

(19) Harada, A.; Okamoto, K.; Fukushima, H.; Shiotsuki, K.; Kamachi, M. *Supramolecular Chem.* **1993**, *2*, 153-156.

(20) Harada, A.; Shiotsuki, K.; Fukushima, H.; Yamaguchi, H.; Kamachi, M. *Inorg. Chem.* **1995**, *34*, 1070-1076.

(21) Scatchard, G. *Ann. N. Y. Acad. Sci.* **1949**, *51.* 660-672.

(22) Rosenthal, H. E. *Anal. Biochem.* **1967**, *20.* 525-532.

(23) Weder, H. G.; Schildknecht, J.; Lutz, R. A.; Kesselring, P. *Eur. J. Biochem.* **1974**, *42*, 475-481.

(24) Matile, S.; Beroba, N.; Nakanishi, K.; Fleischhauer, J.; Woody, R. W. *J. Am. Chem. Soc.* **1996**, *118,* 5198-5206.

Chapter 5

Specific Interaction between Phosphorus(V)tetraphenylporphyrin-Antibody Complex and Electron Acceptor

Introduction

Recently, antibodies have attracted much attention not only as hosts for molecular recognition but also as catalysts (catalytic antibodies). Most of these catalytic antibodies have been produced by immunizing mice with transition-state analog compounds.[1,2] Another approach is to use a cofactor as an active site.[3-9] The catalytic function, in general, is achieved by the cooperation of proteins and co-factors, such as coenzymes or metal ions. However, it is rather complicated to prepare catalytic antibodies with the cofactors, because this kind of catalytic antibody should be induced by a single immune process to have the combining sites for both the cofactors and the substrates.

Previously, monoclonal antibodies against *meso*-tetrakis(4-carboxyphenyl)porphine (TCPP)[7] have been prepared in our laboratory. It has been found that one of these antibodies 03-1 binds not only TCPP but also Metal-TCPP complexes (Metal = Cu, Zn, Fe).[8,9] To develop antibodies in the field of more effective catalytic system, a new porphyrin hapten is designed in order to induce antibodies for a porphyrin with higher substrate specificity. To obtain an antibody possessing a combining site for both a porphyrin and a substrate, phosphorus(V) porphyrin was chosen as a hapten (Scheme 5.1), because

phosphorus(V)porphyrin with a central six-coordinated phosphorus is a unique nonmetal porphyrin and an unusual hypervalent compound.[10-15] Water-stable, axial dialkoxy hypervalent phosphorus(V)tetraphenyl porphyrins was synthesized by efficient substitution of axial chloride ligands of dichlorophosphorus(V)tetraphenylporphyrin. In this Chapter, the characterization of monoclonal antibodies against the porphyrin with axial ligands, (5,10,15,20-tetraphenylporphinato) bis(hydrogen-terephthalate-2-ethoxy)phosphorus(V)chloride, is described. In addition, selective electron transfer in a combining site of monoclonal antibody is discussed.
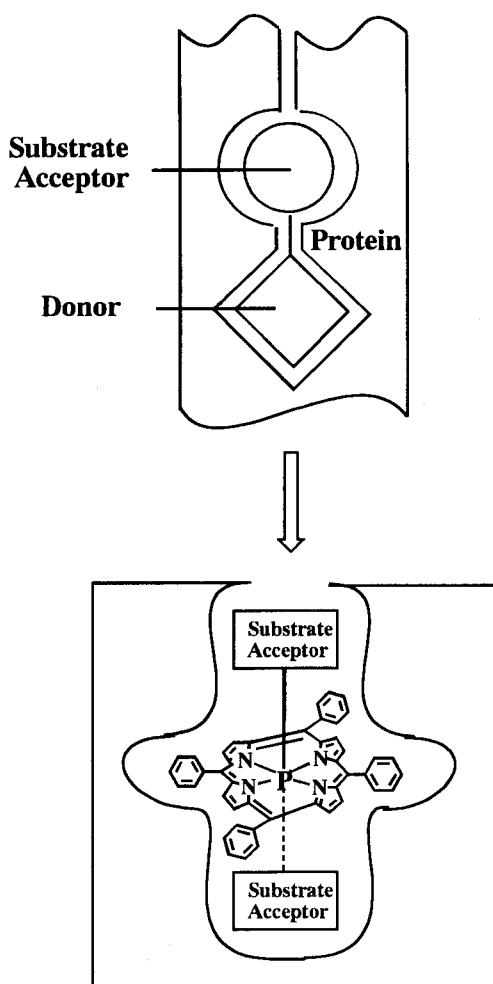
Scheme 5.1. Anti-phosphorus(V)porphyrin antibody.

76

Materials

Preparation of Antigen and Monoclonal Antibodies.
Monoclonal antibodies for (5,10,15,20-tetraphenylporphinato)
bis(hydrogen-terephthalate-2-ethoxy)phosphorus(V)chloride were
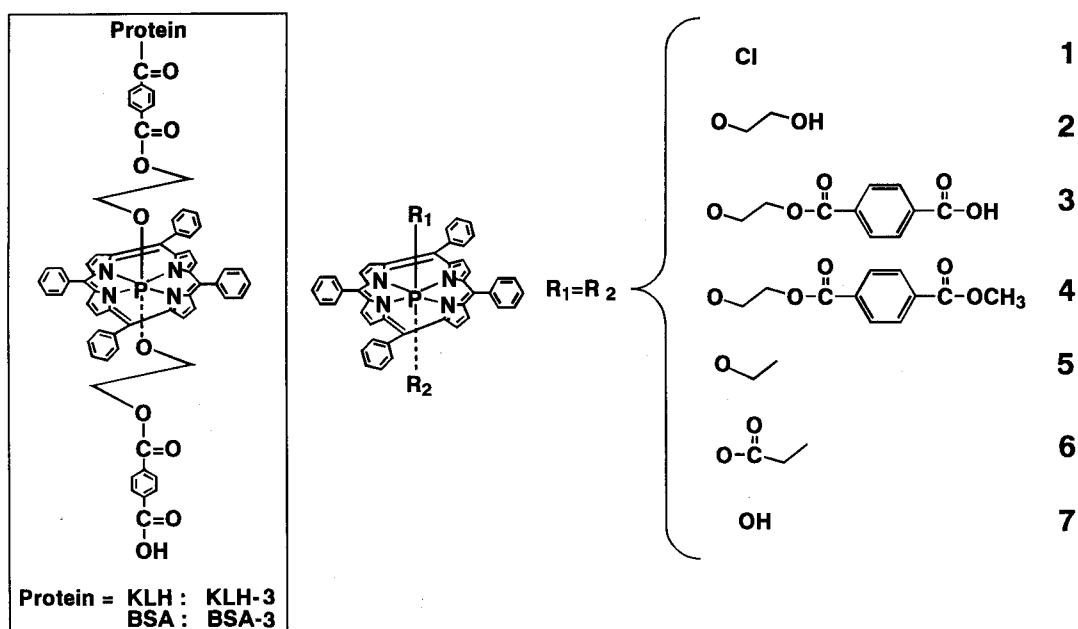prepared as described in Chapter 2.

Figure 5.1. Phosphorus(V)porphyrin derivatives.

Phosphorus(V) Porphyrin Derivatives 5,6,7(Figure 5.1).
The dichlorophosphorus(V)TPP chloride **1** was dissolved in ethanol,
propionic acid and water, respectively, and the solutions were refluxed
2 h. Solvents were evaporated at the end of the reaction and the

products were purified using a column of alumina (chloroform/ MeOH eluent) and dried *in vacua*.[11-15]

Diethoxyphosphorus(V)tetraphenylporphyrin Chloride [P(V)TPP(OEt)$_2$] Cl 5. Anal. Calcd. for $C_{48}H_{38}N_4O_2PCl$: C,74.94; H,4.98; N,7.28. Found: C, 74.65; H, 4.86; N, 7.47. ^{1}H NMR (CDCl$_3$) δ: 9.07 (d, 8H), 7.7-8.1 (m, 20H), -1.73(dt, 6H), -2.34(d, 4H). λ_{max}(MeOH) / nm : 427.0, 558.0, 599.5. MS (FAB), m/z 733.3 ($C_{48}H_{38}N_4O_2P$, [M$^+$]).

Tetraphenylporphine Phosphorus(V) Dipropionate [P(V)TPP(OOCEt)$_2$] Cl 6. Anal. Calcd. for $C_{50}H_{38}N_4O_2PCl$: C,72.77; H,4.64; N,6.79. Found: C, 72.91; H, 4.65; N, 7.07. ^{1}H NMR (CDCl$_3$) δ: 9.07 (d, 8H), 7.7-8.1 (m, 20H), -1.73 (dt, 6H), -2.34 (d, 4H), -1.73 (dt, 6H). . λ_{max}(CHCl$_3$) / nm : 428.0, 556.0. MS (FAB), m/z 789.2 ($C_{50}H_{38}N_4O_4P$, [M$^+$]).

Dihydroxyphosphorus(V)tetraphenylporphyrin Chloride [P(V)TPP(OH)$_2$]Cl 7. Anal. Calcd. for $C_{44}H_{30}N_4O_2PCl$: C, 74.10; H, 4.24; N, 7.86. Found: C, 74.94; H, 4.67; N, 8.42. MS (FAB), m/z 677 ($C_{44}H_{30}N_4O_2P$, [M$^+$]). λ_{max} (H$_2$O/CH$_3$CN = 1:1) / nm : 423.5 (ε=3.1 x 10^5). λ_{max} (CHCl$_3$) / nm : 427.0, 553.5. ^{1}H NMR (CDCl$_3$) δ: 8.82 (m, 8H), 8.10(m, 12H), 7.76(m, 8H). ^{13}C NMR (CDCl$_3$) δ: 116.1 (C$_{meso}$), 127.3 (C$_{3'}$, C$_{5'}$), 128.3 (C$_{4'}$), 131.3 (C$_\beta$), 133.6 (C$_{2'}$, C$_{6'}$), 138.7 (C$_{1'}$), 139.6 (C$_\alpha$).

***meso*-Tetrakis(4-carboxyphenyl)porphine (TCPP).** *meso*-Tetrakis(4-carboxyphenyl)porphine (TCPP) was prepared as described in reference 8.

Measurements

Antibodies were treated in 0.1 M PBB (pH 9.0). The concentration of the antibody was expressed at the concentration of

antigen binding sites. UV-Vis spectra were recorded on a Shimadzu UV-2100 UV-visible spectrophotometer. Proton NMR spectra were taken at 270 MHz in $CDCl_3$ on a JEOL JNM EX-270 NMR spectrometer. Chemical shifts were determined with reference to TMS. Emission spectra were recorded on a Shimadzu RF-502A spectrofluorophotometer.

Determination of Dissociation Constants

Dissociation constants of the complexes between antibodies and various phosphorus(V)-porphyrins, tetracarboxyphenylporphyrin, benzoic acid and terephthalic acid derivatives were determined by fluorescence spectroscopy and enzyme-linked immunosorbent assay (ELISA). The fluorescence measurements were carried out in 0.1 M PBB (pH 9.0). Phosphorus(V) porphyrin derivatives **2** - **7** (Figure 5.1) were dissolved in buffer/acetonitrile or buffer/MeOH (95 : 1 (v/v)). Antibodies were added to the porphyrin solutions at a fixed concentration (2.5×10^{-6} M). Dissociation constants between one of the anti-phosphorus(V)porphyrin antibodies 74D7A and various substrates were determined by enzyme-linked immunosorbent assay. The antibody 74D7A solution (1.6×10^{-8} M) and substrate solutions (9.8×10^{-3} M to 1.1×10^{-4} M) were prepared. The solutions (60 μL) of various concentrations of the substrate were added to the antibody solution 60 μL. Mixtures of antibodies and substrates were allowed to stand for 1 day at room temperature, and then 100 μL of the mixed solutions was transported to the ELISA plates pre-coated with BSA-conjugated phosphorus(V)porphyrin and BSA blocking buffer. Absorbance at 405 nm (the resulting hydrolysis product by alkalinephosphatase of second antibody, *p*-nitrophenolate) was

monitored to determine the amount of the antibody that bound phosphorus(V) porphyrin. The dissociation constants were obtained by Klotz plots.[16]

Results and Discussion

Specificity of Antibody 74D7A

Binding abilities of antibody 74D7A, which was obtained by immunization of (5,10,15,20-tetraphenylporphinato)bis(hydrogen-terephthalate-2-ethoxy)phosphorus(V)chloride, were measured by enzyme linked immunosorbent assay (ELISA). One of these antibodies, 74D7A binds antigen strongly with a dissociation constant Kd = 2.2 x 10^{-7} M (Table 5.1).

Table 5.1. Dissociation constants of antibody 74D7A and phosphorus(V)porphyrins.

Porphyrin	**BSA-3**	**2**	**7**
Kd (M)	2.2 x 10^{-7}	7.5 x 10^{-7}	2.1 x 10^{-6}

BSA-3

80
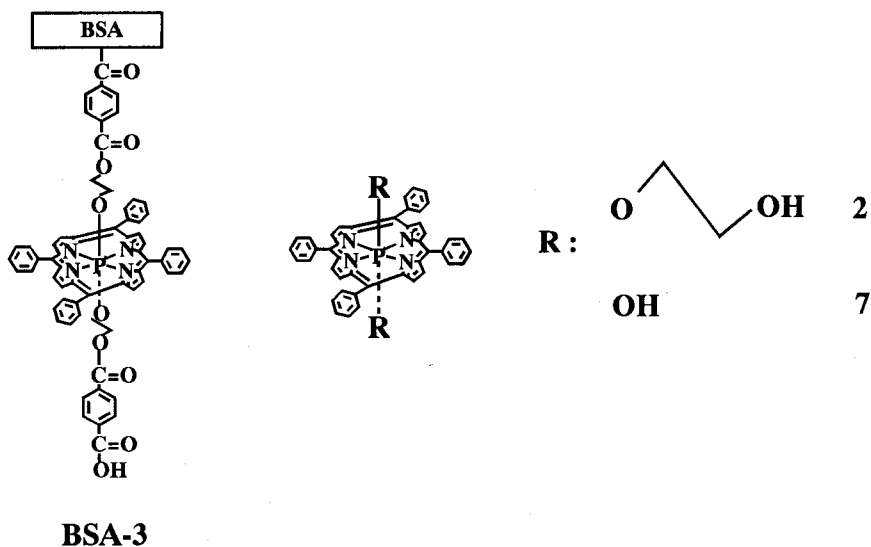
It was difficult to determine the binding constant of the antibody with phosphorus porphyrin derivative **3** in water or in buffer solutions because hapten **3** is insoluble in water and buffer. But it is able to dissolve these porphyrins which are covalently attached to proteins in water. Figure 5.2 shows the UV-Vis absorption spectra of phosphorus(V) porphyrin conjugated BSA (BSA-**3**) in the presence of the monoclonal antibody 74D7A. A hypochromism was observed on the addition of the antibody. When the equimolar antibody was added to the porphyrin solution, absorbance of the porphyrin decreased to 70%. But the absorption maxima of the Q bands of the mixture of BSA-**3** and the antibodies were nearly equal to those of BSA-**3**. In the fluorescence spectra of BSA-**3** in the presence of antibody 74D7A, the intensity of BSA-**3** decreased with an increase in the concentration of the antibody. Unique substrate specificity of antibody 74D7A was observed in the recognition for the porphyrin molecules and in that for the molecules used as axial ligands of phosphorus porphyrin. The specificities of antibody 74D7A are described as follows.
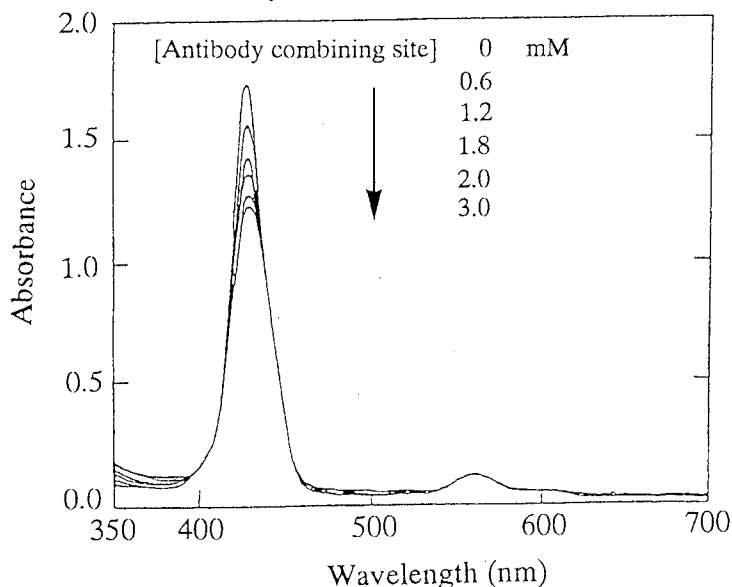

Figure 5.2. Absorption spectra of BSA-3 in the absence and presence of antibody 74D7A. The concentration of porphyrin 3 was fixed at 2.5×10^{-6} M.

Binding of Porphyrin Derivatives

Figure 5.3 shows the fluorescence spectra of the antibody in the presence of porphyrins **5** and **6**. The fluorescence intensity of the antibody decreased with an increase in the concentrations of **5**. On the other hand, the fluorescence intensity of the antibody did not change on addition of **6**. These results indicate that porphyrin **5** was incorporated in the combining site of antibody 74D7A and that the micro-environments around the tryptophan residues in the antibody changed.
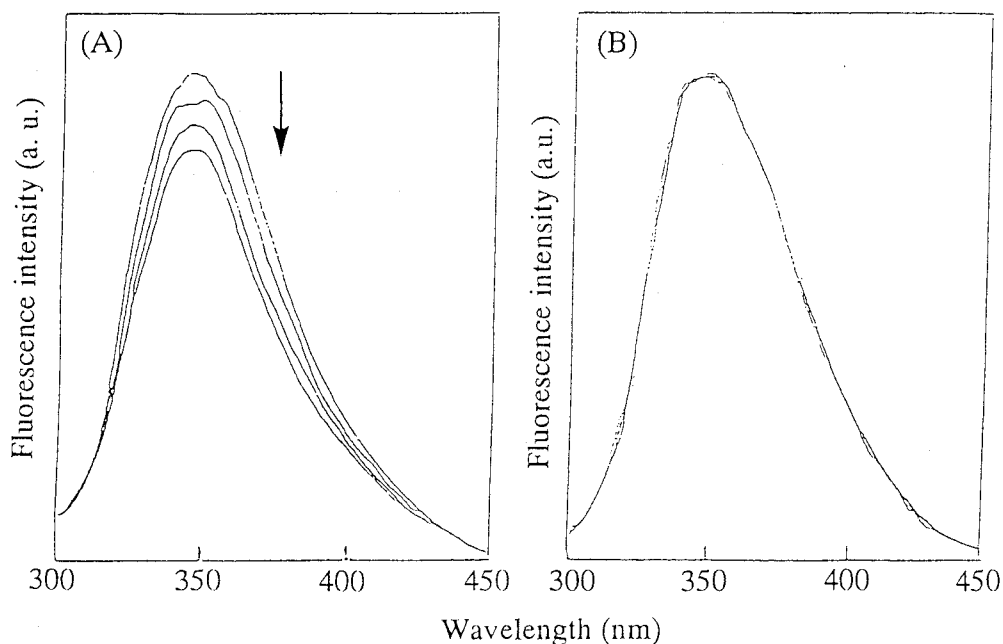
Figure 5.3. Fluorescence spectra of the phosphorus(V)porphyrin derivatives in the absence and presence of antibody 74D7A: (A); **5**, (B); **6** in PBB / MeOH = 95/5 (excitation wavelength : 280 nm).

The antibody was found to be unable to bind tetracarboxyphenyl porphyrin by the study of UV-Vis spectroscopy and ELISA. This result indicates that the antibody recognizes the structures of the tetraphenylporphyrin along with axial ligands simultaneously (Figure 5.4).
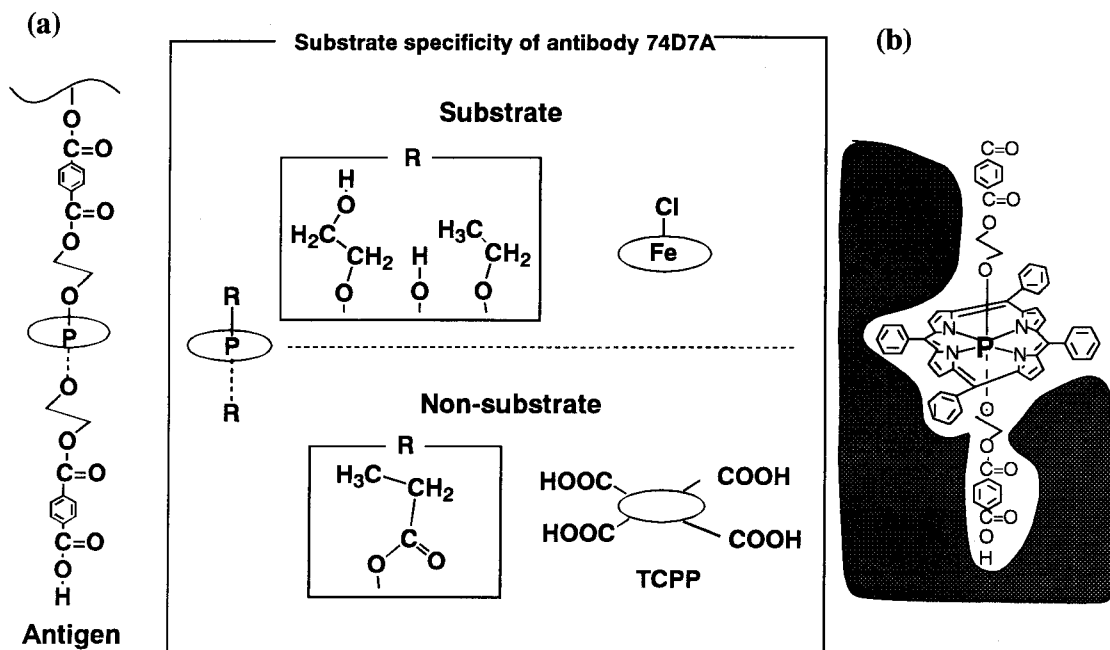
Figure 5.4. Substrate specificity of antibody 74D7A (a) and a schematic representation of the antibody combining site (b).

Table 5.1 shows the dissociation constants for antibody 74D7A and phosphorus(V)-porphyrin derivatives. The antibody shows different dissociation constants for these porphyrins. These different values of the dissociation constants are probably due to the different affinity of the antibody for the axial parts of the phosphorus(V)porphyrin.

Recognition of Axial Ligands

The antibody 74D7A was found to recognize not only a porphyrin ring but also terephthalic acid. Figure 5.5 shows Klotz plots for benzoic acid and phthalic acid derivatives. The dissociation constants of the complexes of antibody with terephthalic acid and benzoic acid were

extremely different. Although the dissociation constant of the antibody with benzoic acid is 1.1×10^{-2} M, that of the antibody with terephthalic acid is 1.2×10^{-5} M. The affinity of the antibody for terephthalic acid is c.a. 1000 times higher than that for benzoic acid. The antibody shows an excellent specificity for the molecules of axial ligands. Table 5.2 shows the dissociation constants of the complexes between antibody 74D7A and various substrates. Comparison of the dissociation constants among isomers of terephthalic acid makes it clear that the specificity of antibody 74D7A is based on the recognition of the molecular structure.

Table 5.2. Dissociation constants between antibody 74D7A and benzoic acid or phthalic acid derivatives.

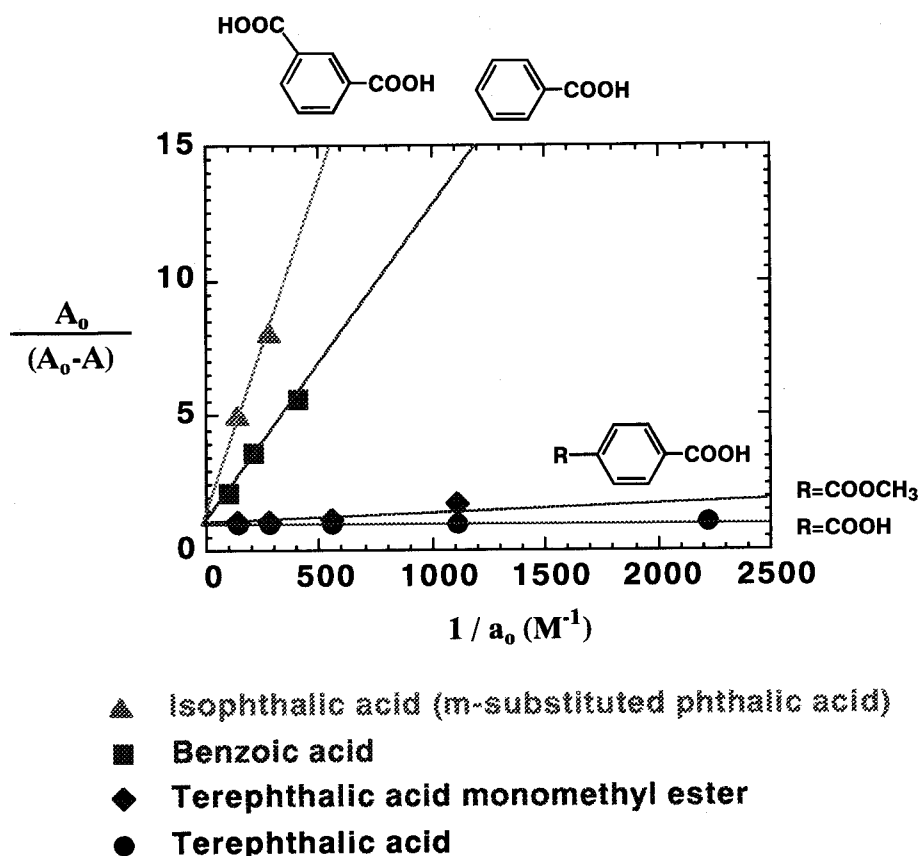| Substrate		Dissociation constant Kd (M)
HOOC–⬡		1.1×10^{-2}
HOOC–⬡COOH	o -	$> 10^{-1}$
	m -	3.0×10^{-2}
	p -	1.2×10^{-5}
HOOC–⬡–COOCH$_3$		4.5×10^{-5}

Figure 5.5. Klotz plots for benzoic acid and phthalic acid derivatives.
Klotz equation : $A_0/(A_0-A) = 1 + K_d/a_0$
where A_0 is the absorbance at 405 nm of ELISA in the absence of substrate (benzoic acid or terephthalic acid), A the absorbance at 405 nm of ELISA in the presence of substrate and a_0 the total concentration of substrate.

Control of the Electron Donor-Acceptor Interactions Using Antibodies (Scheme 5.2). Terephthalic acid and its derivatives act as an electron acceptor[17] for porphyrins. Fixation of terephthalic acid to the axial position of porphyrin induces changes in photochemical properties of phosphorus(V)porphyrin. For example, the lifetime of the singlet excited state of antigen (KLH-**3** conjugate) is shorter than porphyrin **2** (antigen : 0.4 nsec , **2** : 4.4 nsec in PBB-CH3CN). When the complex is formed between porphyrin **2** and antibody 74D7A, it is expected that the antibody may offer the space

where acceptor molecules come in. In the presence of antibody 74D7A (8.0×10^{-7} M), the fluorescence quenching of porphyrin **2** was observed on addition of terephthalic acid. The quenching was not observed in the absence of the antibody under the same conditions (the concentration of porphyrin **2** (8.0×10^{-7} M) and terephthalic acid (1.6×10^{-6} M)). In the presence of antibody 74D7A, the degree of the quenching of porphyrin **2** observed in the terephthalic acid system was ten-fold larger than that of the isophthalic acid system (Table 5.3). These results show that porphyrin (donor)-acceptor interactions occurred in the antigen combining site.
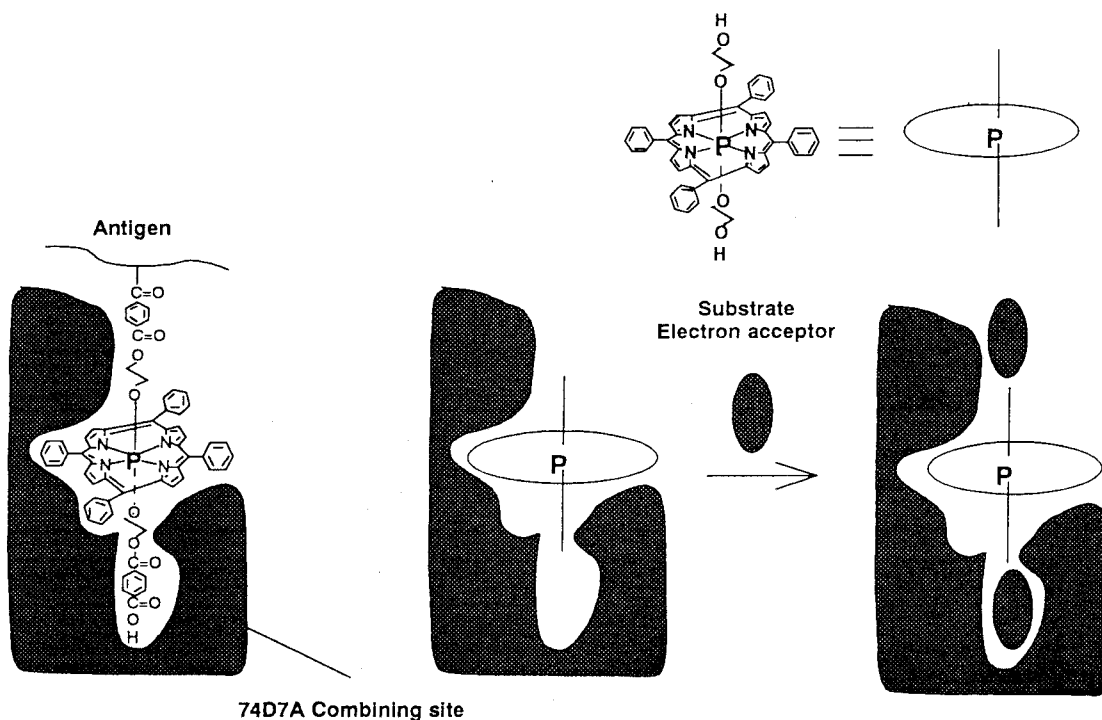

Scheme 5.2. Specific insertion of substrates or electron acceptors into antibody combining sites.

Table 5.3. Fluorescence quenching of porphyrins on addition of terephthalic acid or isophthalic acid in the presence of antibody 74D7A.

Porphyrin	Substrate	$(1 - I / I_0) \times 100\ (\%)$ [a]
2	HOOC–⟨⟩–COOH	20
2	HOOC–⟨⟩ COOH	2
TCPP	HOOC–⟨⟩ COOH	5

a) I_0 : Fluorescence intensity of P(V)porphyrin 2 or TCPP in the absence of antibody 74D7A and substrates.

I / I_0 : Relative fluorescence intensity.

2

TCPP
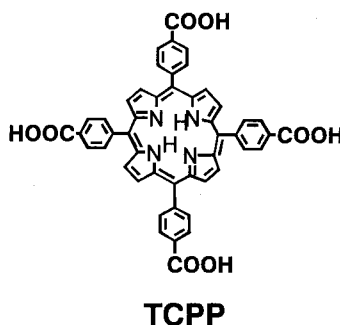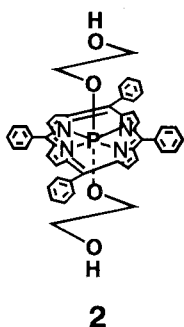
Conclusion

The binding ability of antibody 74D7A was studied by UV-Vis spectroscopy, fluorescence spectroscopy, and ELISA. The antibody has been found to bind phosphorus(V) porphyrin derivatives, such as dihydroxyphosphorus(V)tetraphenylporphyrin chloride, and the diethoxy derivative with different binding affinities. It is suggested that the different affinities of the antibody to these porphyrins are caused by the recognition of the axial ligands as well as the recognition of the structure of the porphyrin ring. Electron transfer takes place selectively to an acceptor which is used as a part of the antigen.

References

(1) Pollack, S. J.; Jacobs, J. W.; Schultz, P. G. *Science* **1986**, *234*, 1570-1573.

(2) Tramontano, A.; Janda, K. D.; Lerner, R. A. *Science* **1986**, *234*, 1566-1569.

(3) Schultz, P. G. *Angew. Chem., Int. Ed. Engl.* **1989**, *28*, 1283-1295.

(4) Cochran, A. G.; Schultz, P. G. *J. Am. Chem. Soc.* **1990**, *112*, 9414-9415.

(5) Keinan, E.; Sinha, S. C.; Sinha-Bagchi, B.; Benory, E.; Auditor, M.; Ghozi, M. C.; Eshhar, Z.; Green. B. S. *Inorg. Chem.* **1992**, *31*, 5433-5438.

(6) Schwabacher, A. W.; Weinhouse, M. I.; Auditor, M. -T.; Lerner, R. A. *J. Am. Chem. Soc.* **1989**, *111*, 2344-2346.

(7) Harada, A.; Okamoto, K.; Kamachi, M. *Chem. Lett.* **1991**, 953-954.

(8) Harada, A.; Fukushima, H.; Shiotsuki, K.; Kamachi, M. *Supramolecular Chem.* **1993**, *2*, 153-155.

(9) Harada, A.; Shiotsuki, K.; Fukushima, H.; Yamaguchi, H.; Kamachi, M. *Inorg. Chem.* **1995**, *34*, 1070-1076.

(10) Sayer, P.; Gouterman, M.; Connell, C. R. *J. Am. Chem. Soc.* **1977**, *99*, 1082-1087.

(11) Carrano, C. J.; Tsutsui, M. *J. Coord. Chem.* **1977**, *7*, 79-83.

(12) Marrese, C. A.; Carrano, C. J. *Inorg. Chem.* **1983**, *22*, 1858-1862.

(13) Segawa, H.;Kunimoto, K.; Nakamoto, A.; Shimidzu, T. *J. Chem. Soc., Perkin Trans. 1* **1992**, 939-940.

(14) Segawa, H.; Kunimoto, K.; Susumu, K.; Taniguchi, M.; Shimidzu, T. *J. Am. Chem. Soc.* **1994**,*116*, 11193-11194.

(15) Segawa, H.; Nakamoto, A.; Shimidzu, T. *J. Chem. Soc., Chem. Commun.* **1992**, 1066-1067.

(16) Friguet, B.; Chaffotte, A. F.; Ohaniance, L. D.; Goldberg, M. E. *J. Immun. Method.* **1985**, *77*, 305-312.

(17) Ichinose, N.; Kitamura, N.; Masuhara, H. *Macromolecules* **1993**, *26*, 2331-2339.

Chapter 6

Supramolecules
between Antibodies and Porphyrins

Introduction

The binding ability of one of monoclonal antibodies obtained from meso-tetrakis(4-carboxyphenyl)porphine (TCPP) has been investigated and its unique binding behavior has been observed. In addition, the antibody has been suggested to form not only one-to-one complexes but also higher order complexes with porphyrin derivatives. In order to confirm this suggestion, atomic force microscopy (AFM) and biosensor measurements of the system of porphyrins and antibody 03-1 have been carried out. Because AFM is revolutionizing the concept of microscopy and giving rise to a family of scanning probe microscopes.[1-8] More recently, to overcome this difficulty, Au-coating or flow cell methods, and cryo-AFM have been invented for observing molecular image of macromolecules, such as proteins and DNAs.[9,10] However, these measurements were carried out under unnatural conditions such as low temperature and indirect method. It is important to observe the molecular images of macromolecules under conditions close to their natural environments; namely, at room temperature and in an ambient atmosphere.

In this Chapter, the images of antibody molecules were obtained by AFM and, in addition, the images of complexes of antibodies and porphyrin derivatives suggested the formation of supramolecules. The

quantitative construction of supramolecules between antibody 03-1 and porphyrins has been studied by biosensors.

Experimental Section

Materials

Monoclonal antibody (03-1) for *meso*-tetrakis(4-carboxyphenyl) porphine (TCPP) was prepared according to the method described in Chapter 2.[11-13] It was purified by affinity chromatography using protein A (Amersham Ampure™ PA kit). The purity of antibody 03-1 was checked by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). Antibody solutions were stored in phosphate borate buffer with various ionic strength (10 mM and 100 mM)and pH (5.7 and 9.0).
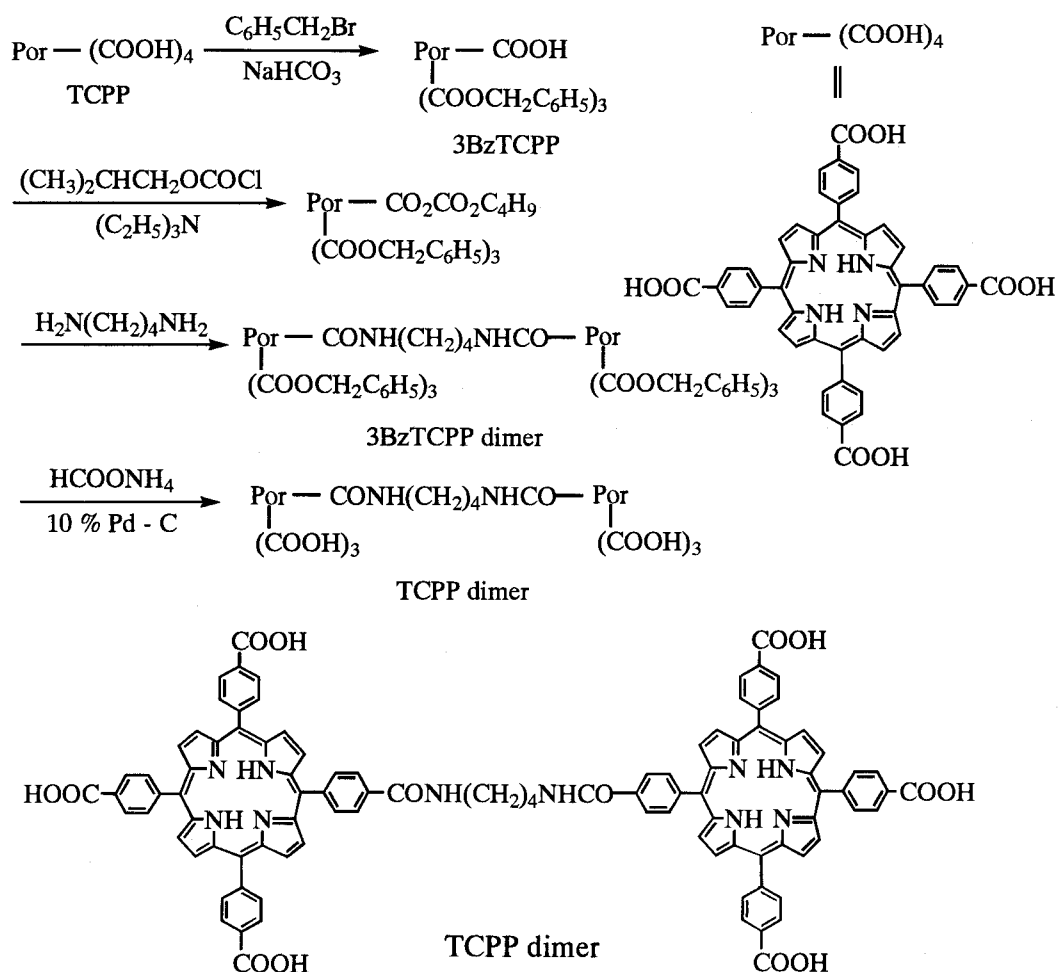
TCPP was prepared by refluxing a propionic acid solution of 4-carboxy benzaldehyde and pyrrole for 2 h and precipitated as purple crystals. They were recrystallized from MeOH-CHCl3 solution.

TCPP Dimer

Scheme 6.1 shows the synthesis of TCPP dimer.[14]

5,10,15-Tri-(4-benzenemethoxyphenyl)-20-(4-carboxy-phenyl)porphyrin (3BzTCPP). To a solution of TCPP (1.7 g, 2.1 mmol) in DMF (30 mL) was added sodium hydrogen carbonate (1.0 g, 12 mmol), followed by benzyl bromide (1.2 mL, 16 mmol). The mixture was stirred at room temperature for 41 h. The reaction mixture was concentrated and the sample was purified by silica gel chromatography with ethyl acetate as an eluent. Yield; 4.5 %. ^{1}H NMR (CDCl3) δ: 8.8 (8H, d, pyrrole), 8.5 (16H, m, ArH), 7.4-7.6

(15H, m, ArH), 5.6 (6H, s, CH$_2$), -2.8 (2H, s, pyrrole-NH). Anal. Calcd for C$_{69}$H$_{48}$N$_4$O$_8$: C, 78.10; H, 4.56; N, 5.28. Found : C, 77.70; H, 4.57; N, 5.27.

$$Por\!-\!(COOH)_4 \xrightarrow[NaHCO_3]{C_6H_5CH_2Br} Por\!-\!\underset{(COOCH_2C_6H_5)_3}{\overset{|}{COOH}}$$

3BzTCPP

$$\xrightarrow[(C_2H_5)_3N]{(CH_3)_2CHCH_2OCOCl} Por\!-\!\underset{(COOCH_2C_6H_5)_3}{\overset{|}{CO_2CO_2C_4H_9}}$$

$$\xrightarrow{H_2N(CH_2)_4NH_2} Por\!-\!\underset{(COOCH_2C_6H_5)_3}{\overset{|}{CONH(CH_2)_4NHCO}}\!-\!Por\!\underset{(COOCH_2C_6H_5)_3}{\overset{|}{}}$$

3BzTCPP dimer

$$\xrightarrow[10\;\%\;Pd\,\text{-}\,C]{HCOONH_4} Por\!-\!\underset{(COOH)_3}{\overset{|}{CONH(CH_2)_4NHCO}}\!-\!Por\!\underset{(COOH)_3}{\overset{|}{}}$$

TCPP dimer

Scheme 6.1. Synthesis of TCPP dimer.

3BzTCPP dimer. To a solution of 3BzTCPP (0.30 g, 0.28 mmol) in DMF (150 mL) were added under ice-salt cooling (-6 °C) first triethylamine (0.04 mL, 0.3 mmol) and then isobutyl

chloroformate (0.04 mL, 0.3 mmol) with exclusion of moisture. The mixture was stirred at -5 °C for 11 min, tetramethylenediamine (0.015 mL, 0.15 mmol) in DMF (0.20 mL) was added, and the reaction mixture was first stirred at 0 °C for 1 h at room temperature overnight. DMF was removed under reduced pressure. The residue was dissolved in $CHCl_3$. The solution was washed twice with water and once with saturated NaCl solution, then dried over anhydrous $NaSO_4$. The solvent was evaporated. The residue was separated by silica gel column chromatography using $CHCl_3$-EtOH. 3BzTCPP dimer was recrystallized from THF. Yield; 22 %. ^{1}H NMR ($CDCl_3$) δ: 8.8 (16H, d, pyrrole), 8.2-8.5 (32H, m, ArH), 7.4-7.6 (30H, m, ArH), 7.0 (2H, t, amide), 5.5 (12H, s, CH_2), 3.8 (4H, s, methylene), 2.0 (4H, s, methylene), -2.8 (4H, s, pyrrole-NH). Anal. Calcd for $C_{142}H_{104}N_{10}O_{14}$ $3.2H_2O$: C, 76.41; H, 4.99; N, 6.28. Found : C, 76.40; H, 4.90; N, 6.33. MS (FAB) m/z 2174 (M).

TCPP dimer. To a solution of 3BzTCPP dimer (50.6 mg) in THF (8.0 mL) - MeOH (0.8 mL) containing ammonium formate (54.0 mg, 0.86 mmol), 10 % palladium-on carbon (25.8 mg) was added the mixture allowed to stand for 3 h. The reaction mixture was filtered through celite and the filtrate evaporated to dryness. The residue was dissolved in THF (9.0 mL) - MeOH (1.9 mL), and ammonium formate and palladium-on-carbon were added to this solution again. After 3 h the reaction mixture was filtered and the filtrate evaporated to dryness. The next reaction was done in THF (8.0 mL) - MeOH (8.4 mL) and the last in THF (18 mL) - MeOH (25 mL). The last filtrate was concentrated and the precipitation was filtered. Yield; 22 %. ^{1}H NMR ($CDCl_3$) δ: 8.8-8.9 (16H, d, pyrrole), 8.2-8.4 (32H, m, ArH), 7.4-7.6 3.6 (4H, s, methylene), 1.8 (4H, s, methylene), -2.9 (2H, s, pyrrole-NH).

Measurements

Size Exclusion Chromatography. Size exclusion chromatography analyses were performed with Sephadex G150 column. HPLC analyses were performed with Shimadzu C-R4AX by using Shodex column.

Biosensor Technique. Biospecific interaction analysis (BIA) was performed by using a BIAcore X system from Pharmacia Biosensor, which was described in Chapter 2.

AFM Measurement. The AFM experiments were performed using an anti-tetracarboxyphenylporphyrin monoclonal antibody, whose structure belongs to IgG,[7] an anti-tetraphenylporphyrin monoclonal antibody (IgM). In addition, the AFM measurements of antibody-TCPP-dimer complexes were performed. In this experiment, these samples were air-dried onto the surface of freshly cleaved HOPG (highly oriented pyrolytic graphite, 1.2 cm x 1.2 cm) substrate. A total 2 μL of antibody solution (0.5 mg/mL in 0.1M phosphate borate buffer (pH 9.0)) was used for the experiment. The sample was allowed to stand for 1 day in a desiccator with $CaCl_2$ for drying sample before it was transferred into the AFM. Under the suitable conditions that any damage caused by the preparation or harsh conditions was minimized, the sample surface was observed by contact AFM. AFM measurements were carried out by using a Si_3N_4 tip with a radius of ca. 40 nm. All measurements were taken on a multimode Nanoscope IIIa (Digital Instruments, Santa Barbara, CA). All the images presented in this paper were obtained with cantilevers of a spring constant 0.03 N/m. The line scan speed was 2 Hz with 512 pixels per line. All scales were calibrated against a standard sample (5 μm x 5 μm grid) and rechecked with graphite.

Results and Discussion

In Chapter 4, it was shown that in the complex formation of antibody 03-1 and TCPP there are at least two binding species in antibody 03-1 and porphyrin complexes, one at low concentrations of TCPP which gives negative Cotton effects, and the other at high concentrations which gives positive induced Cotton effects. The following three models are considered to explain this phenomenon. (1) The first binding of TCPP to one of the binding sites of the antibody affects the second binding of TCPP to the same antibody by means of conformational changes or other effects. (2) There are two binding sites, a strong binding site which gives negative Cotton effects, and a weak binding site which causes positive Cotton effects. (3) One-to-one binding, giving positive Cotton effects and two-to-one (binding site : TCPP) binding, which gives negative Cotton effects. When the Fab fragment was used instead of the antibody itself, spectroscopic changes similar to the antibodies were observed both in the absorption and CD spectra. Therefore, the model (1) cannot explain the spectroscopic behavior. When the CD spectrum of TCPP was investigated in the presence of equimolar amount of the antibody, large positive Cotton effects were observed. If the model (2) is correct, negative Cotton effects should be observed. So the model (2) does not explain the behavior. Therefore, model (3) is most reasonable. Figure 6.1 shows a proposed structure of the one-to-one complex and the two-to-one complex.
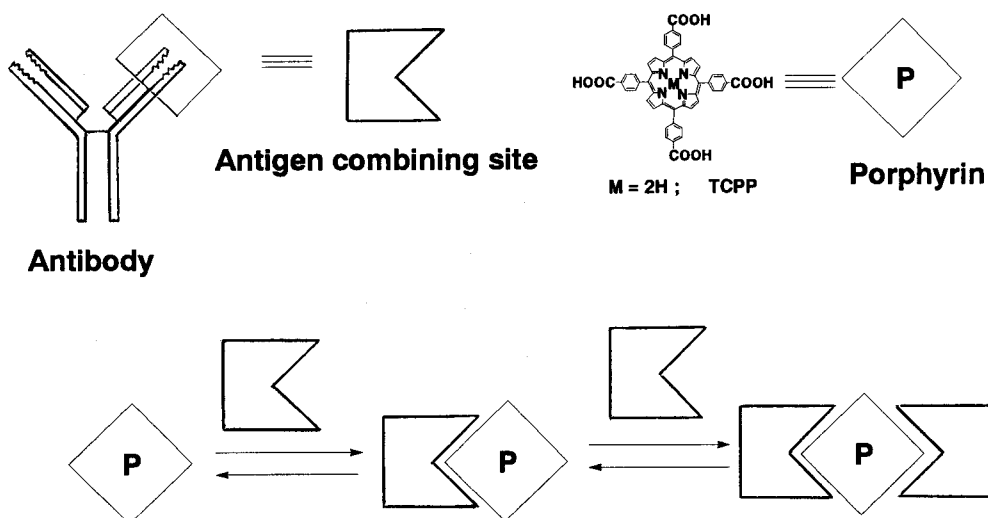
Figure 6.1. One-to-one complex and two-to-one complex formation between antibody 03-1 and TCPP.

Size Exclusion Chromatography

TCPP Monomer and Antibody 03-1. When TCPP and antibody 03-1 were mixed, precipitation of solids occurred. After the precipitates were removed by ultracentrifuge, the supernatant of antibody-TCPP mixture was analyzed by HPLC measurement. Figure 6.2 (a) and (b) show elution diagrams of the TCPP-antibody mixture and antibody 03-1 alone, respectively. In a solution of the TCPP-antibody mixture, there are fractions indicating that polymer whose molecular weight is 5.0×10^5 was formed in the presence of TCPP.
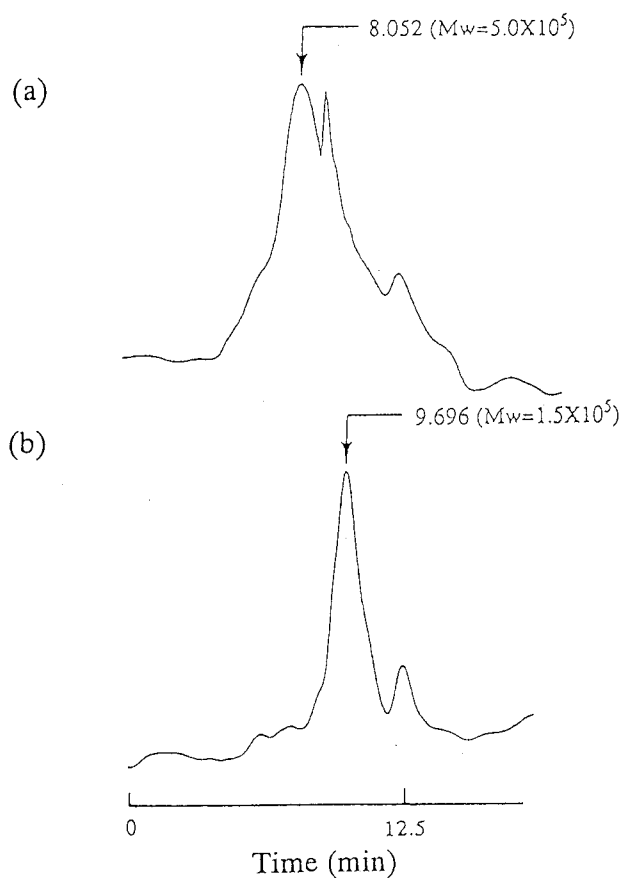
Figure 6.2. HPLC elution diagrams of antibody 03-1 (a) with and (b) without TCPP.

Furthermore, precipitates were obtained for the case of the antibody-TCPP complex. These precipitates are considered to have much higher molecular weights. Accordingly, supramolecules are considered to be formed in the mixture of antibody 03-1 and TCPP. Figure 6.3 shows the schematic structures of precipitates (a) and structures of supernatant (b) of antibody 03-1 and TCPP mixture.
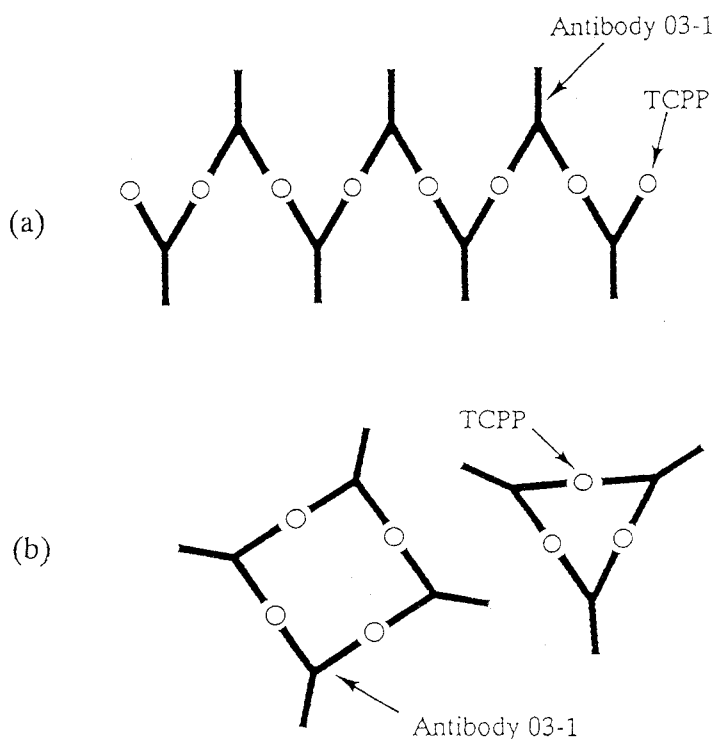
Figure 6.3. (a) Proposed structure of precipitation. Supramolecular polymer of antibody 03-1 and TCPP. (b) Proposed structure of supramolecules of antibody 03-1 with TCPP in solution.

TCPP Dimer and Antibody 03-1. Equivalent amounts of TCPP dimer and the antibody were mixed. The mixture was also separated into two phases: the precipitate and the supernatant. The supernatant was fractionated on a Sephadex G-150 column. Figure 6.4 (i) and (ii) show elution diagrams monitored at 280 and 423 nm, respectively. In the case of 280 nm, three fractions (a), (b) and (c) were detected, and the fraction (c) was considered to be a one-to-one complex between antibody 03-1 and TCPP dimer. Fractions (a) and (b) have higher molecular weights. Fractions (a) and (b) were assigned to that of the antibody oligomer and the antibody dimer, respectively.

The complexes between two antibody combining sites and one TCPP dimer have been found in a fraction of high molecular weight.
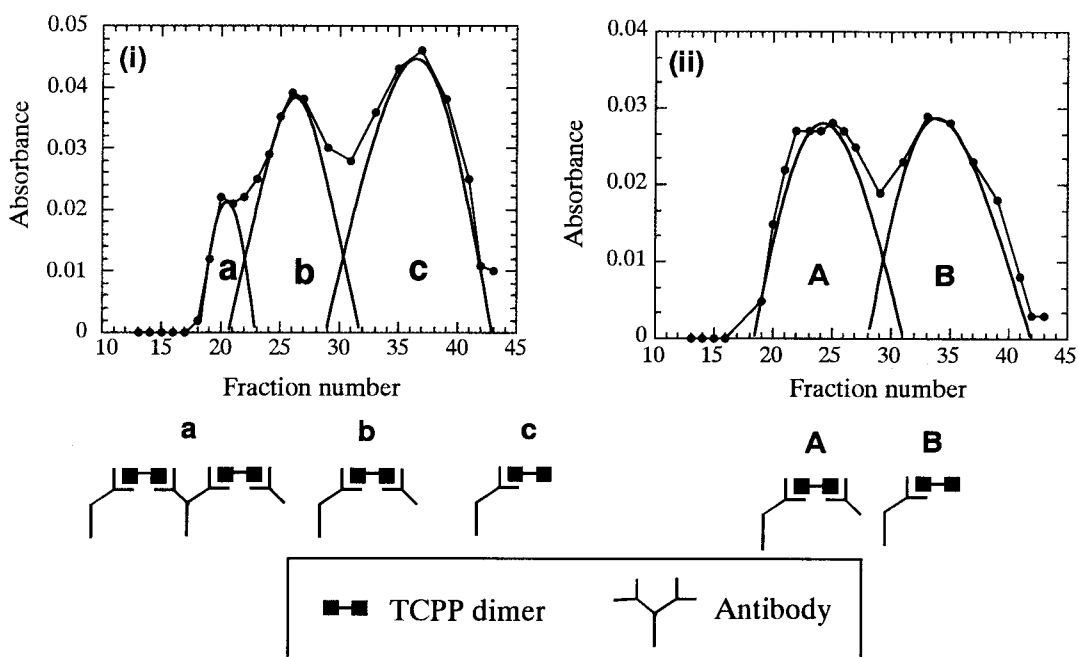

Figure 6.4. Elution diagrams of the complexes between TCPP-dimer and antibody 03-1 detected by the absorbance at 280 nm (i) and 423nm (ii). A Sephadex G-150 column with phosphate borate buffer (pH 9.0) was used.

Detection of Stepwise-Growth of Supramolecules between Antibodies and Porphyrins by Biosensor

Biosensors are generally used to examine molecular interactions between antibody molecules and ligands. Accordingly, we applied biosensors to the formation of supramolecules, which means polymer formation from antibodies and porphyrins. In this experiment, antibody 03-1 was immobilized on the surface of the sensor chip. The signal intensity in this biosensor is proportional to the mass of

porphyrins bound to the sensor chip. If porphyrins bind specifically to the antibody immobilized on the surface of the sensor chip, the amounts of bound porphyrins are observed as a remaining signal intensity after the surface is subsequently washed with buffer. On the other hand, if there are no interactions between antibodies and porphyrins, no increase in the signal intensity is observed. Figure 6.5 shows the changes in the signal with exposure time of surface with immobilized antibody 03-1 to a solution of antibody 03-1 in the presence of TCPP (a) and absence of TCPP (b). In Figure 6.5 (a), a solution of TCPP was
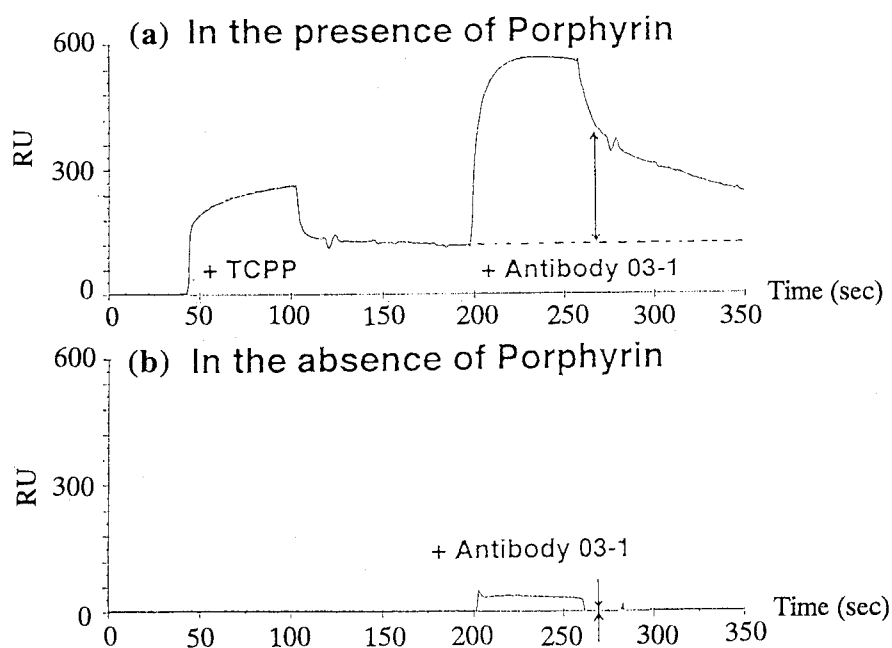

Figure 6.5. SPR signal enhancement by the addition of TCPP.

injected into the sensor chip at 40 sec after the start. Then the surface was washed with buffer at t=100 sec, the amount of bound TCPP was measured at 110 sec. The signal intensity increased by the injection of a solution of TCPP. In addition, a solution of antibody 03-1 was injected

into the sensor chip at 200 sec. The injection of an antibody solution caused a remarkable increase in the signal intensity at 270 sec in the presence of TCPP as shown in Figure 6.5 (a). While a remarkable increase in the signal intensity was observed in the presence of TCPP, there was no increase in the signal intensity at 270 sec in the absence of TCPP, as shown in Figure 6.5 (b). These results indicate that two-to-one complexes are formed on the surface of the sensor chip. The schematic representation of two-to-one complex between antibody 03-1 and TCPP was shown in Figure 6.6, the state (2).
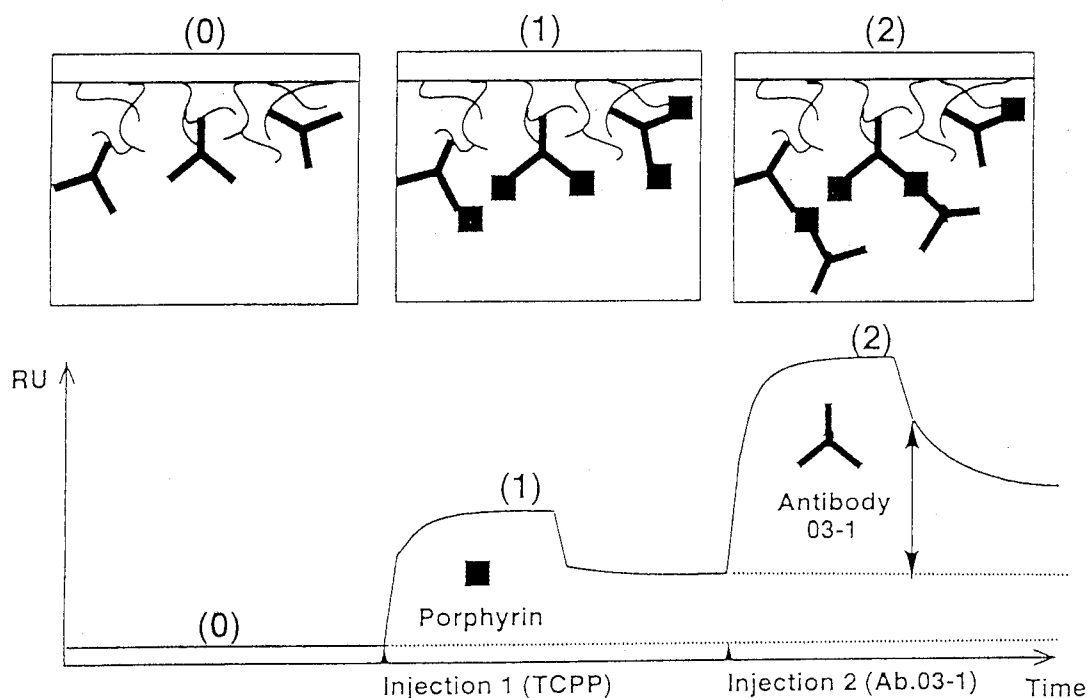

Figure 6.6. Schematic representations of the antibody-porphyrin complexes constructed on the surface of sensor chip. Specific interactions between TCPP and whole antibody 03-1 or its Fab fragment.

In order to examine where the binding site is, Fab fragment whose molecular weight is one third of antibody itself was used. A solution of the Fab fragment was injected into the sensor chip after the injection of a TCPP solution. Figure 6.7 shows the difference in sensorgrams between antibody itself and its Fab fragment in the presence of TCPP.
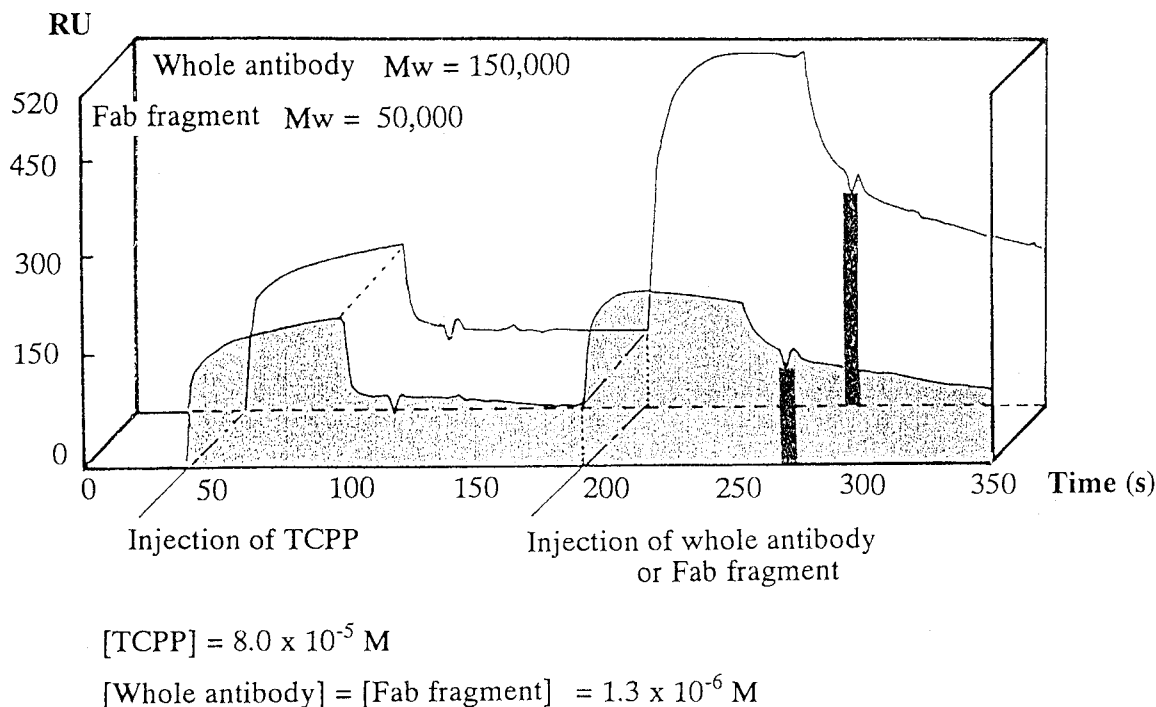
RU

Whole antibody   Mw = 150,000

Fab fragment   Mw = 50,000

[TCPP] = 8.0 x 10^{-5} M

[Whole antibody] = [Fab fragment]   = 1.3 x 10^{-6} M

Figure 6.7. Difference of SPR signal intensities between the antibody and its Fab fragment.

The signal intensity which indicates the amount of bound Fab is ca. one third of that by injection of the antibody. The Fab fragment of antibody 03-1 has one combining site specific for TCPP. Accordingly, the increases in the signal intensity by the injection of the antibody and its Fab fragment are probably ascribable to the interaction between TCPP and the antibody combining site. The correlations between

sensorgrams and structures of the complexes between the antibody and TCPP, or its Fab and TCPP were illustrated in Figure 6.8.
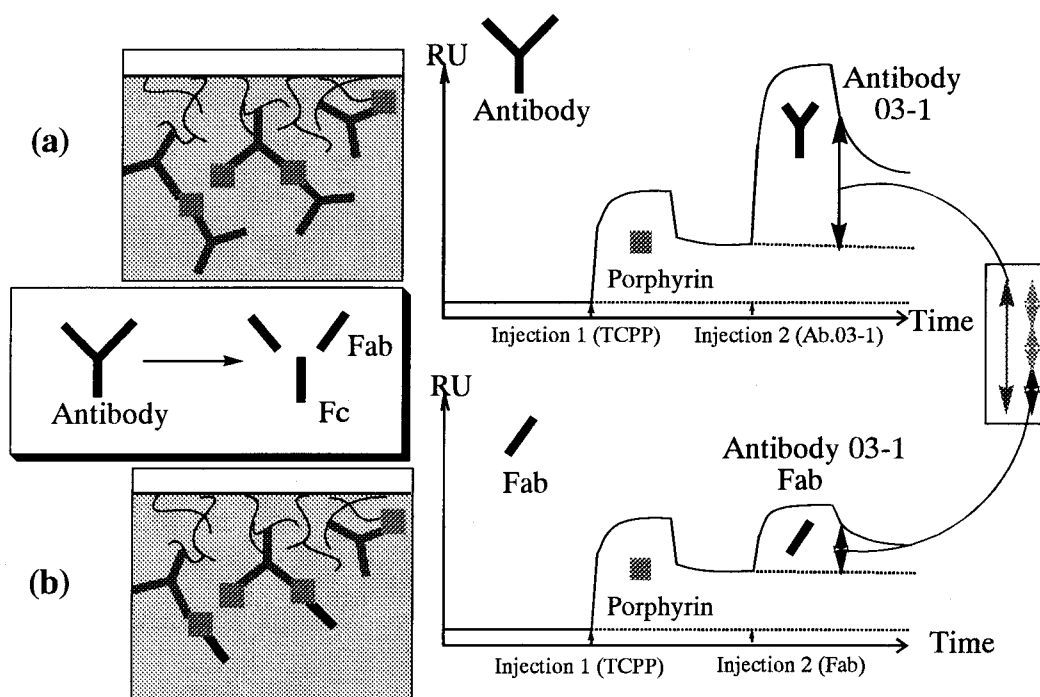

Figure 6.8. Specific interactions between porphyrins and antibody combining sites. (a) Whole antibody 03-1 and (b) Fab fragment of antibody 03-1.

In the extension of this study, repeated injection of TCPP and antibody 03-1 into the sensor chip, whose surface was already immobilized with antibody 03-1, was carried out. Figure 6.9 shows the sensorgram on repeated injection of TCPP and antibody 03-1. Each time, the signal intensity in the sensorgram increased. It is considered that these increases in the signal intensity are due to expanding complex formation of the antibody with TCPP. This result indicates that it is possible to construct step-wise supramolecules between antibodies and porphyrins. Figure 6.10 shows schematic structures of supramolecules in the second generation. An alternating injection of TCPP and the

antibody is considered to be indispensable in the step-wise formation of supramolecules.
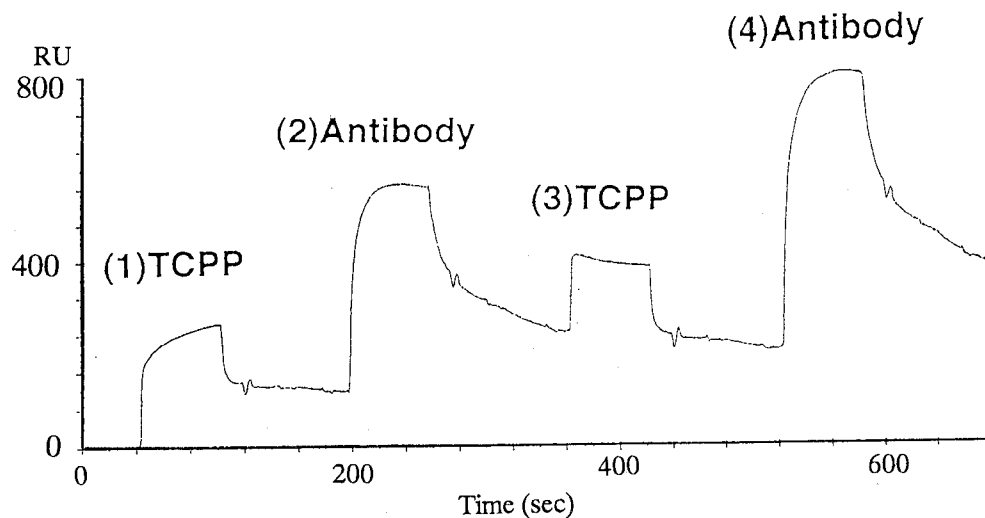

Figure 6.9. Step-wise enhancement of SPR signals by the addition of TCPP and antibody 03-1.
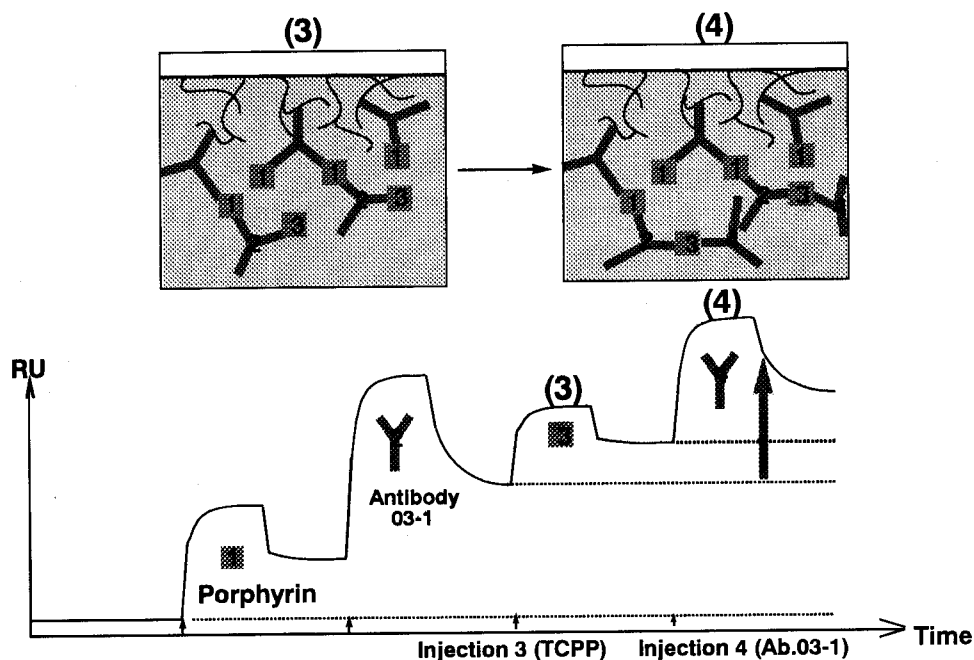

Figure 6.10. Step-wise growth of supramolecular structure constituted by antibody 03-1 and TCPP.

The sensorgram in lack of second injection of TCPP is shown in Figure 6.11. In this case, there was no increase in the signal intensity by the second injection of the antibody. Accordingly, it was found that the alternating injection of TCPP and antibody 03-1 was necessary to expand the formation of supramolecules. Consequently, step-wise preparation and observation of supramolecules between antibody 03-1 and TCPP were confirmed by using BIA.
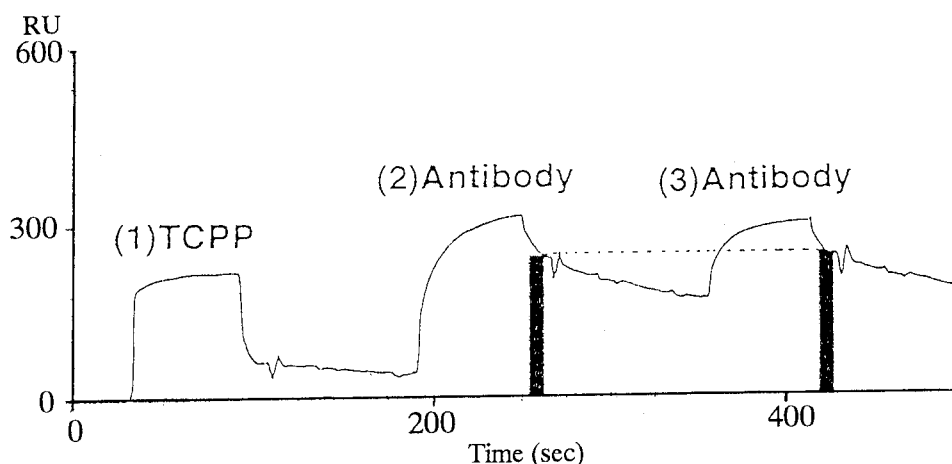

Figure 6.11. SPR signals without the second injection of TCPP solution.

AFM Measurement

(1) **Direct Observation of Antibody Molecules.** AFM allows a direct observation of biological samples without the need for shadowing with heavy metals or labeling with probes. Monoclonal antibodies (IgG and IgM) were observed by atomic force microscopy

(AFM) at room temperature depositing from a solution onto a freshly cleaved HOPG surface.

Figure 6.12 shows images of the anti-TCPP antibody which is composed of IgG structure of molecular weight 150,000. Characteristic T or Y shape molecules[15-17] can be seen. The overall lateral dimension is 40-50 nm as shown A, B, and C in Figure 6.12, which is in good agreement with the expected values.[18] Although these molecules are monoclonal antibodies, each antibody molecular image is not identical. There is two fragments whose height are the same and one fragment whose height is different with the other two fragments in each molecular image. Fab fragments shown as blue circles in Figure 6.12 and an Fc fragment shown as green circle can be differentiated by comparison of the height among three fragments. The angles between the Fab fractions of each immunoglobulin are different. Each antibody molecules takes somewhat different shape. This is probably due to the flexibility of its hinge region.

(2) **Direct Observation of Supramolecules between Antibodies and Porphyrin.** The fraction (a) obtained by size exclusion chromatography (in Figure 6.4) was measured by AFM. Result is shown in Figure 6.13, which suggests the presence of the dimer, trimer, pentamers, and so on. Accordingly, the molecular images of trimer and pentamer are clearly observed by picking up each image and expanding them as shown in Figure 6.14. It is indicated that the image (a) is assigned to a cyclic trimer and that the image (b) is assigned to a pentamer. In addition, Figure 6.14 (c) shows the 3D structure of the molecule (b). From the 3D image, it is suggested that this pentamer is a precursor of a cyclic pentamer. The proposed structure of the molecules (a) and (b) is shown in Figure 6.15.

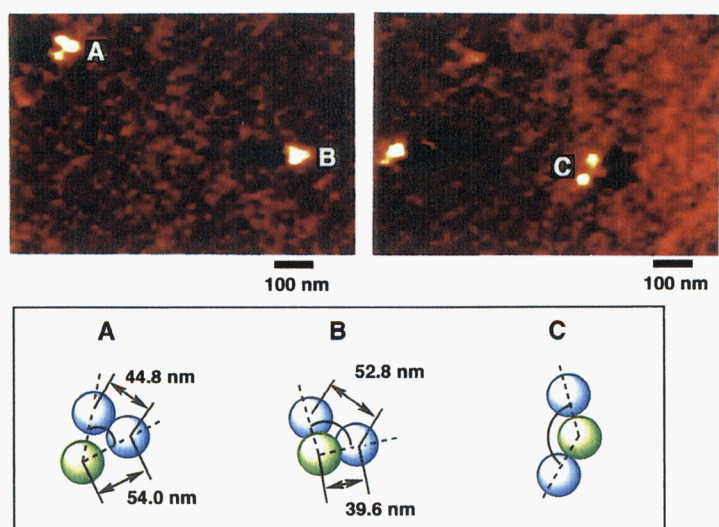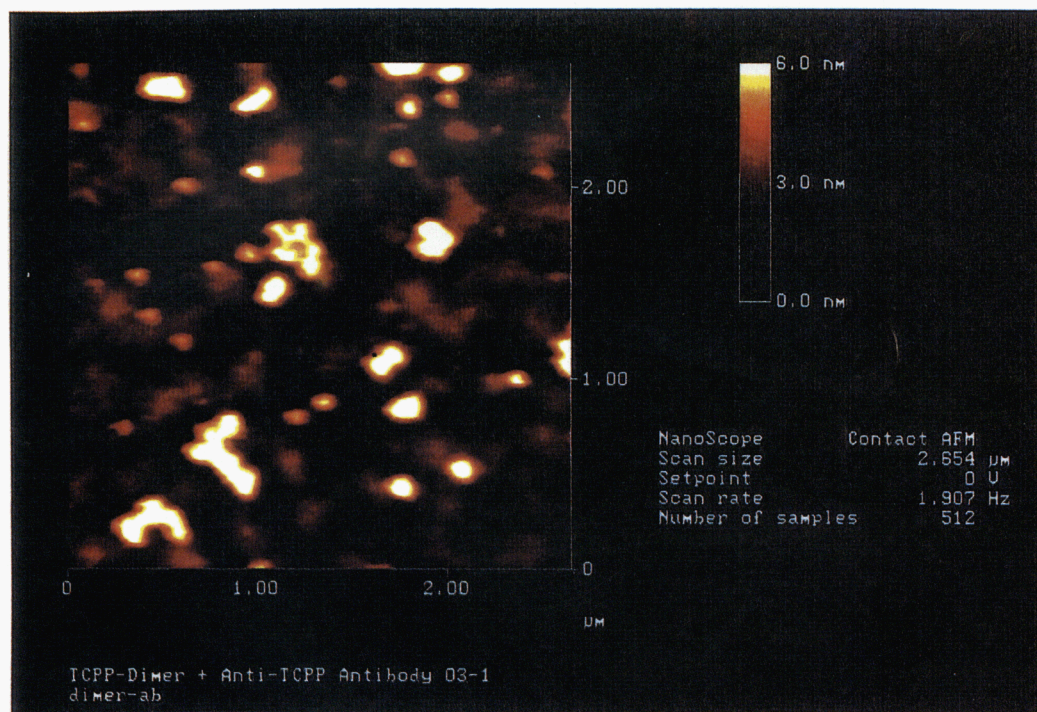**Figure 6.12.** AFM images of anti-TCPP antibody 03-1

Figure 6.13. Contact AFM images of antibody-TCPP dimer complexes.
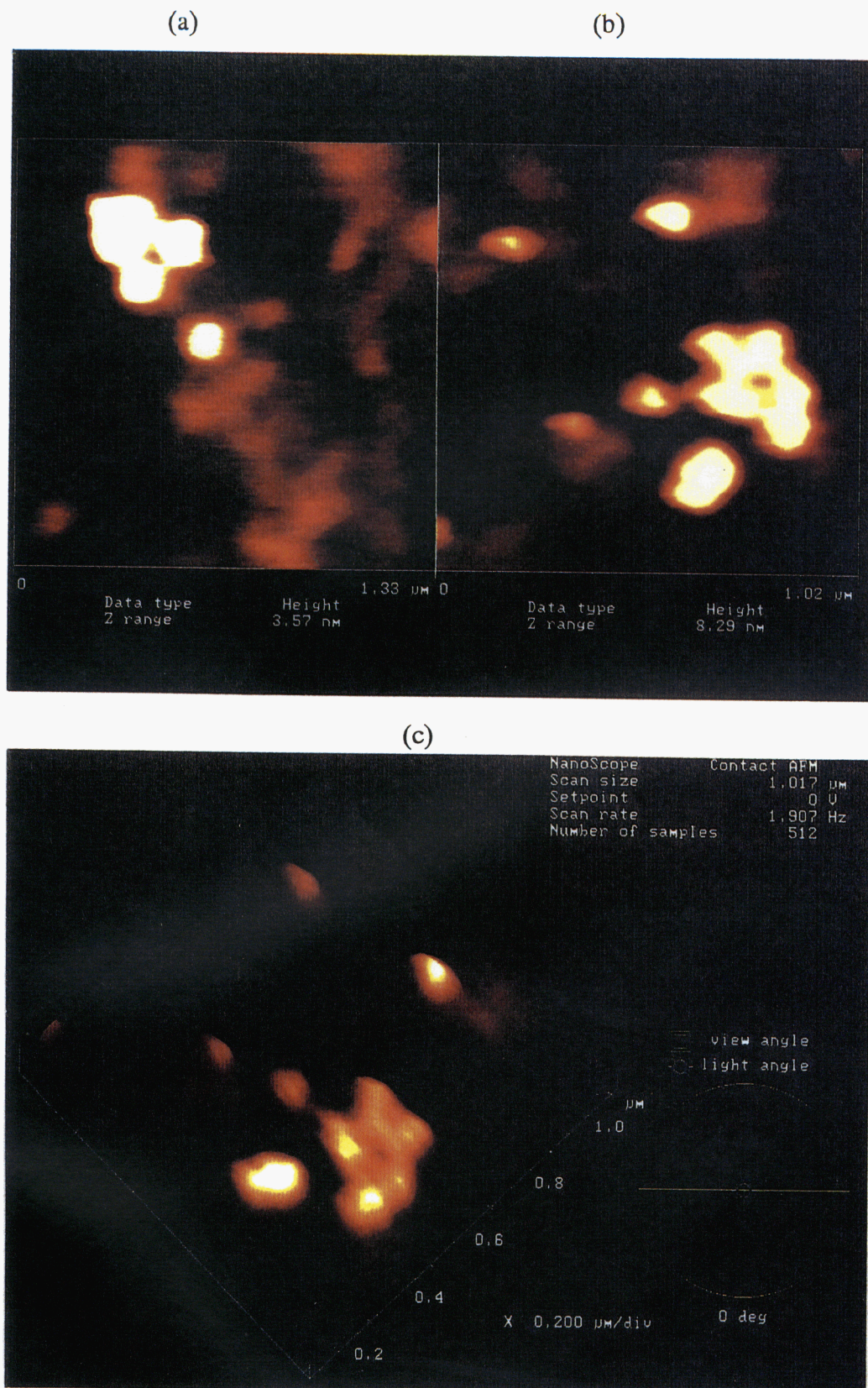
Figure 6.14. AFM images of the antibody-TCPP dimer complexes. (a) Trimer, (b) pentamer, and (c) pentamer (3D image).
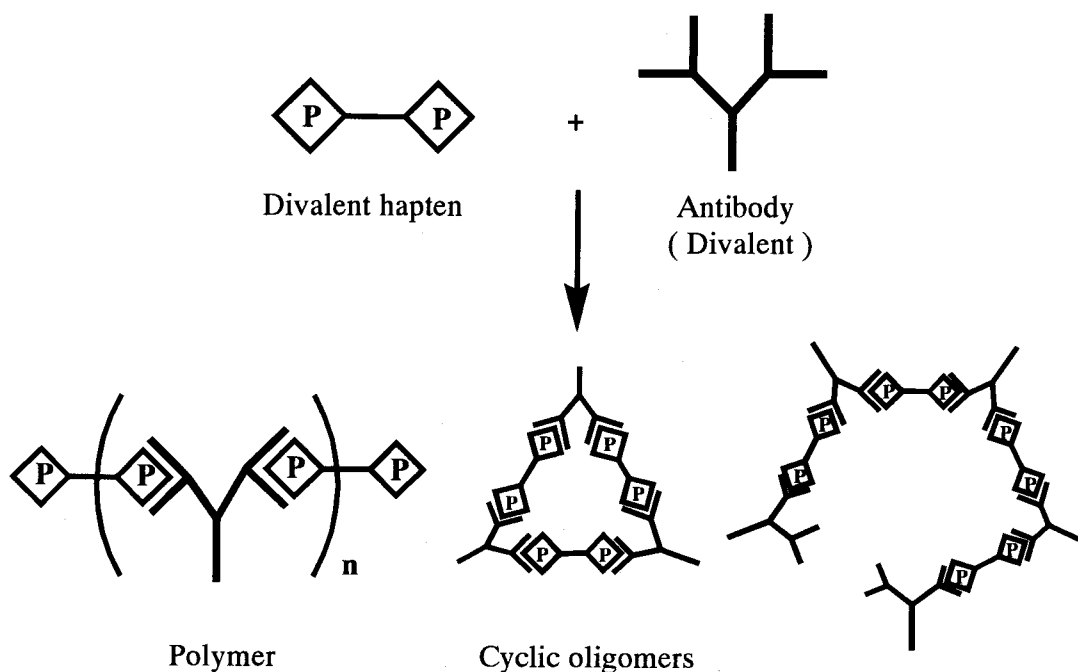
Figure 6.15. Complex formation between antibody 03-1 and TCPP dimer to give linear supramolecules and cyclic polymers.

Conclusion

The interactions between antibody molecules and a porphyrin were observed by biosensors. The step-wise preparation and observation of supramolecules between antibodies and porphyrins were made clear by a biosensor technique. Supramolecular images between antibodies and porphyrins were confirmed by AFM.

References

(1) Binning, G.; Rohrer, H.; Gerber, C.; Weibel, E. *Phys. Rev. Letter* **1982**, *49*, 57-61.

(2) Hansma, P. K.; Elings, V. B.; Marti, O.; Bracker, C. E. *Science* **1988**, *242*, 209-216.

(3) Blackford, B. L.; Jericho, M. H.; Mulhern, P. J. *Scanning Microscopy* **1991**, *5*, 907-918.

(4) Hansma, H. G.; Hoh, J. H. *Annu. Rev. Biophys. Biomol. Struct.* **1994**, *23*, 115-139.

(5) Hansma, H. G.; Weisenhorn, A. L.; Edmundson, A. B.; Gaub, H. E.; Hansma, P. K. *Clin. Chem.* **1991**, *37*, 1497-1501.

(6) Cullen, D. C.; Lowe, C. R. *J. Colloid and Interface Sci.* **1994**, *166*, 102-108.

(7) Lin, J. N.; Lea, A. S.; Hansma, P. K.; Andrade, J. D. *Langmuir*, **1990**, *6*, 509-511.

(8) Yang, J.; Mou, J.; Shao, Z. *Biocimica et Biophysica Acta* **1994**, *1199*, 105-114. (9) Lea, A. S.; Pungor, A.; Hlady, V.; Andrade, J. D.; Herron, J. N.; Voss, E. W. Jr. *Langmuir* **1992**, *8*, 68-73.

(9) Han, W.; Mou, J.; Sheng, J.; Yang, J.; Shao, Z. *Biochemistry* **1995**, *34*, 8215-8220.

(10) Zhang, Y.; Sheng, S.; Shao, Z. *Biophys. J.* **1996**, *71*, 2168-2176.

(11) Harada, A.; Okamoto, K.; Kamachi, M. *Chem. Lett.* **1991**, 953-954.

(12) Harada, A.; Fukushima, H.; Shiotsuki, K.; Kamachi, M. *Supramolecules* **1993**, *2*, 153-155.

(13) Harada, A.; Shiotsuki, K.; Fukushima, H.; Yamaguchi, H.; Kamachi, M. *Inorg. Chem.* **1995**, *34*, 1070-1076.

(14) Oka, F.; Yamaguchi, H.; Harada, A.; Kamachi, M. *Polymer Preprints, Japan* **1996**, *45*, 555.

(15) Davies, D. R.; Padlan, E. A. *Annu. Rev. Biochem.* **1975**, *44*, 639-667.

(16) Silverton, E. W.; Navia, M. A. and Davies, D. R. *Proc. Nat. Acad. Sci. U. S. A.* **1977**,*74*, 5140-5144.

(17) Leatherbarrow, R. J.; Stedman, M.; Wells, N. C. *J. Mol. Biol.* **1991**, *221*, 361-365.

(18) Mazeran, P. -E.; Loubet, J. -L.; Martelet, C.; Theretz, A. *Ultramicroscopy* **1995**, *60*, 33-40.

Chapter 7

Preparation and Properties of Antibody Polymers

Introduction

Recently, monoclonal antibodies have attracted much attention not only in the field of biology but also in the field of chemistry,[1,2] because they are homogeneous and have been extensively studied as catalytic antibodies and sensors. Although the importance of monoclonal antibodies has been recognized, their use has been limited to diagnoses and catalysts for laboratory use. In order to make monoclonal antibodies versatile, it is important to make antibody polymers. Antibody polymers may be useful not only for the affinity chromatography but also for the high sensitive immunosorbent assay and a DNA cloning method.

Antibody polymers are obtained by treatment of antibodies with cross-linking agents. Glutaraldehyde is one of the most widely used for the derivatization of proteins.[3-8] The advantages of this procedure, as compared with other coupling procedures, are its simplicity, the mild conditions of coupling, and its wide applicability. In a low degree of cross-linking, soluble polymers can be obtained. These soluble polymers may be useful for the high sensitive immunosorbent assay. An increase in the degree of cross-linking may make the protein polymers insoluble. This insoluble part of the polymer may be useful as a carrier of column chromatography.

112

In this Chapter, the preparation and properties of antibody polymers are described. In order to prepare antibody polymers, glutaraldehyde was used as a cross-linking agent. The molecular weights and the cross-linking sites of the soluble antibody polymers were determined by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The binding properties of the antibody polymers were evaluated by UV-Vis spectroscopy in the presence of a porphyrin. The antibody polymers were found to be useful for the high sensitive immunosorbent assay.

Experimental Section

Materials

Monoclonal antibody (03-1) against *meso*-tetrakis(carboxyphenyl) porphine (TCPP) was prepared according to the previous reports.[9-11] It was purified by affinity chromatography using protein A (Amersham Ampure™ PA kit). The purity of antibody 03-1 was checked by SDS-PAGE electrophoresis. Antibody solutions and cross-linked antibody solutions were stored and treated in 0.1 M phosphate borate buffer (pH 9.0) or 0.1 M phosphate buffer (pH 7.0).

Glutaraldehyde was purchased from Nacalai Tesque Inc., and stored in 25 wt% aqueous solution. 2-Mercaptoethanol was purchased from Wako Pure Chemical Ind., Ltd.
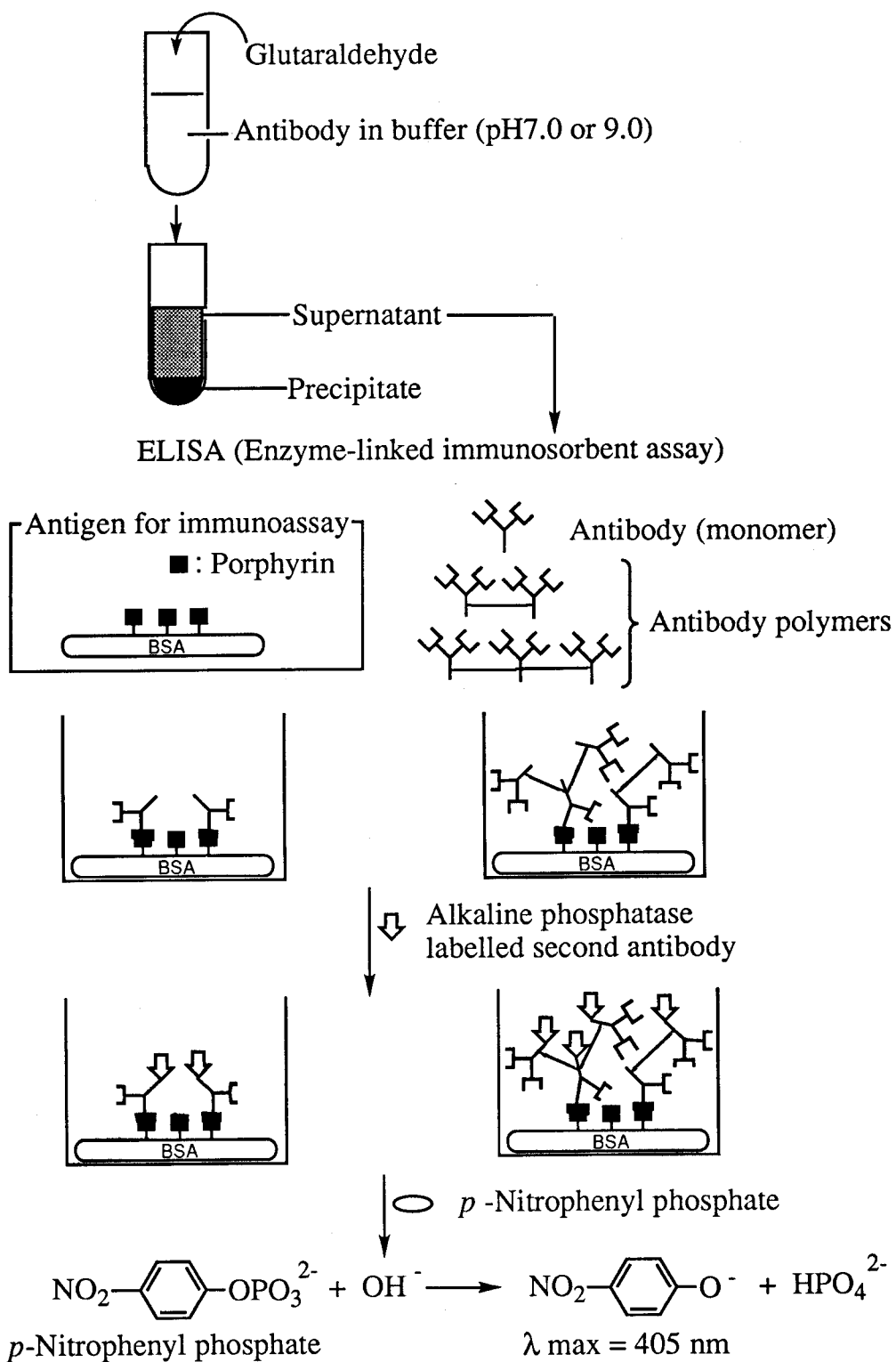
Preparation of Polymers.
To 1.0 mL of 0.1 M phosphate borate buffer or phosphate buffer containing 1.0 mg mouse anti-TCPP monoclonal antibody 03-1 (IgG), 15 μL of various concentrations of glutaraldehyde aqueous solution were added with gentle stirring.

Coupling reaction was carried out for 2 h at room temperature(25 °C): 0.05 mL of 2 M glycine was then added and the mixture was allowed to stand for 5 h. Precipitated antibody was removed by centrifugation for 30 min at 15000 x g. The supernatant was used for further experiments.

Characterization

SDS-PAGE. The molecular weights of antibody polymers were determined by SDS polyacrylamide gel electrophoresis without denaturing agent. SDS-PAGE was also carried out for the antibody polymers and reference antibody (IgG) with 2-mercaptoethanol. SDS-PAGE was carried out with PhastSystem™ (Pharmacia LKB Biotechnology).

UV-Vis Spectroscopy. The concentration of the antibody was determined by UV-Vis spectroscopy (absorbance of wavelength at 280 nm). Absorbance 1.4 contains 1.0 mg / mL antibody (IgG) in phosphate borate buffer. The solutions of polymers with various concentrations were added to the TCPP solution whose concentration was fixed at 1.1 μM. The ratios of the concentration of TCPP to that of antibody combining site were fixed at 1:0.5, 1:1, 1:2, respectively. Glutaraldehyde (0.25 wt% aqueous solution) 15 μL was added to the 3.0 mL of the antibody solution or the antibody-TCPP complex solution. After 1 hour, UV-Vis spectra of TCPP in the presence of the reaction products were recorded on a Shimadzu UV-2100 UV-visible spectrophotometer.

Glutaraldehyde

Antibody in buffer (pH7.0 or 9.0)

Supernatant

Precipitate

ELISA (Enzyme-linked immunosorbent assay)

Antigen for immunoassay

■ : Porphyrin

BSA

Antibody (monomer)

Antibody polymers

BSA

BSA

Alkaline phosphatase labelled second antibody

BSA

BSA

p-Nitrophenyl phosphate

$$NO_2 - \bigcirc - OPO_3{}^{2-} + OH^- \longrightarrow NO_2 - \bigcirc - O^- + HPO_4{}^{2-}$$

p-Nitrophenyl phosphate

λ max = 405 nm

Scheme 7.1.

115

ELISA. According to Scheme 7.1, the antibodies to be assayed are allowed to react with the immobilized antigen in the first step and in the second step the amount of antibody bound to the antigen is measured using a second antibody labeled with an enzyme. The antigen for immunosorbent assay was prepared by conjugating TCPP to bovine serum albumin (BSA). Alkaline phosphatase conjugated affinity purified goat anti-mouse immunoglobulin (polyclonal second antibody for IgA, IgG, IgM) was used as a second antibody. The absorbance of wavelength at 405 nm was monitored by ImmunoMini NJ-2300 (Inter Med).

Results and Discussion

Preparation of Antibody Polymers

Table 7.1 shows the reaction conditions for cross-linking and the percentage of the soluble part of antibody polymers. The cross-linking reaction at pH 7.0 gave insoluble products, while the reaction at pH 9.0 proceeds homogeneously. These results show that the reactions can be controlled by pH. This cross-linking reaction starts immediately after the addition of glutaraldehyde and proceeds very fast. Therefore, the reaction time and temperature do not influence the reaction kinetics.

Table 7.1. Effects of pH and glutaraldehyde on the cross-linking reaction of antibody 03-1.

[Glutaraldehyde] (wt%), 15 μl	pH [a]	Percentage of soluble antibodies (%)
25	7.0	0
2.5	7.0	2
0.25	7.0	9
0.025	7.0	10
0.25	9.0	100

Reaction time ; 120 min, temperature ; 20 °C, [antibody] = 7.2 μM, 1.0 ml.

a) pH = 7.0 ; in 0.1 M phosphate buffered saline.
   pH = 9.0 ; in 0.1 M phosphate borate buffer.

Molecular Weights and Cross-linking Sites of the Antibody Polymers

In order to determine the molecular weights and the cross-linking sites of the antibody polymers, we conducted SDS-PAGE for the antibody polymers in the presence and absence of 2-mercaptoethanol. In the absence of 2-mercaptoethanol, the spots show the molecular weights of the whole cross-linked antibodies (Figure 7.1 (a)). The use of a 0.25 wt% glutaraldehyde solution gave the products containing the cross-linked dimer, trimer, and oligomer. It is difficult to determine the molecular weights and the cross-linking sites of the antibody polymers prepared by using glutaraldehyde (>25 wt%), because the products were insoluble at pH 7.0. The result shows that the reactivity of glutaraldehyde is high enough to react with most of lysine residues including those in the antibody combining sites to form insoluble antibody polymers.

In the presence of 2-mercaptoethanol, the heavy chain fragment disappeared, and the fragments of higher molecular weights appeared

(Figure 7.1 (b)). It is considered that the cross-linking reactions occur mainly at a heavy chain of the antibody.
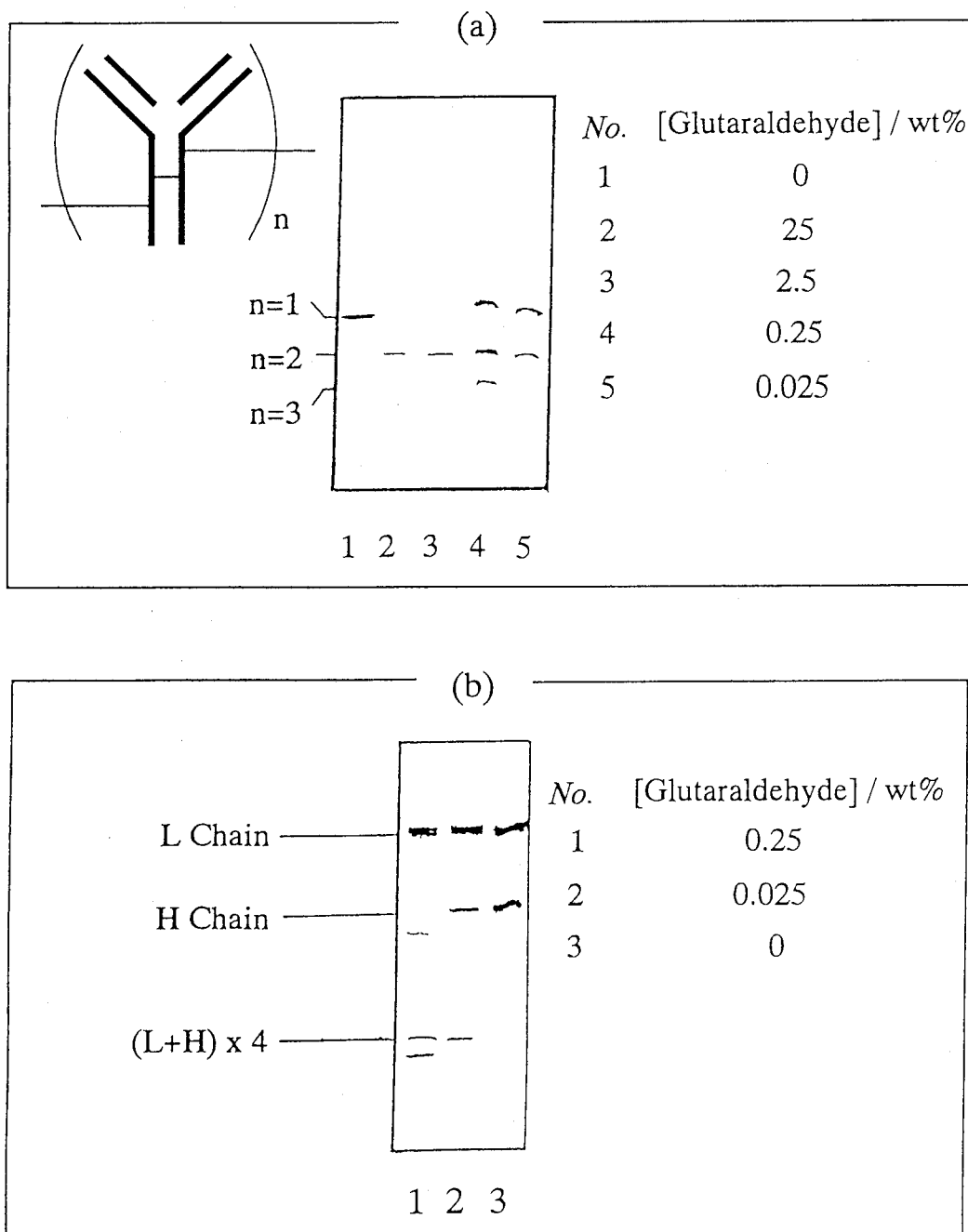

Figure 7.1. SDS-PAGE for the antibody polymers in the absence of 2-mercaptoethanol (a) and in the presence of 2-mercaptoethanol (b).

Binding Ability of the Antibody Polymers

Antibody polymers were prepared in the absence and presence of TCPP to examine the binding ability of antibody polymers. Antibody polymer **1** was prepared by cross-linking the antibody in the absence of TCPP, and antibody polymer **2** was obtained in the presence of TCPP. Figure 7.2 shows the UV-Vis spectra of TCPP in the presence of various concentrations of the antibody polymers. Figure 7.2 (a) shows the spectra of TCPP in the presence of antibody polymers **1** and Figure 7.2 (b) shows that in the presence of antibody polymer **2**. The shift of
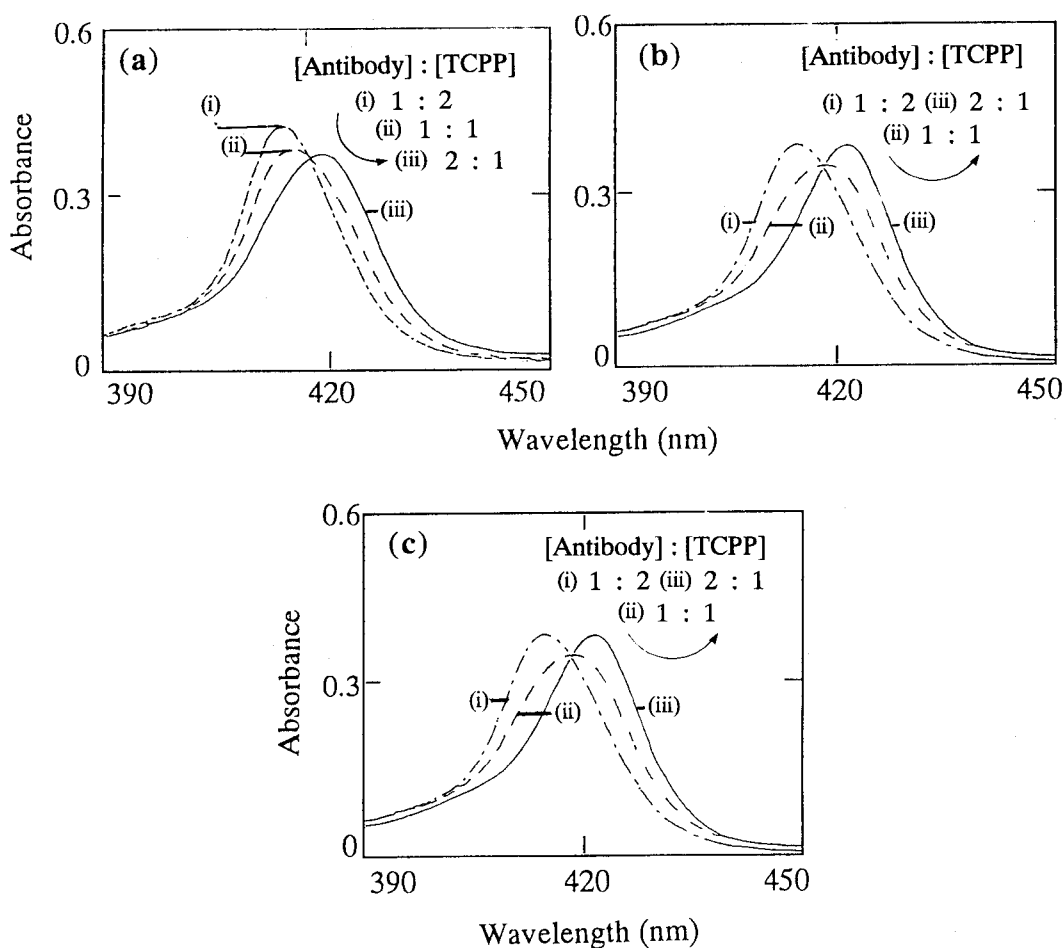

Figure 7.2. The UV-Vis spectra of TCPP in the presence of antibody polymer **1** (a), antibody polymer **2** (b) and antibody monomer (c).

wavelength and absorbance of TCPP in the presence of antibody polymer **1** were compared with those in the presence of antibody polymer **2**. It is suggested that antibody polymer **1** has lost their binding ability by ca. 20 % by cross-linking reactions. The deactivation of the combining site is probably ascribable to the reactions of the lysine residues in the combining site of the antibody with glutaraldehyde. The preparations of antibody polymers were carried out under mild conditions, nevertheless the antibody polymers lose their binding ability to some extent. The spectral changes of TCPP in the presence of antibody polymer **2** are similar to those in the presence of the antibody monomer as shown in Figure 7.2 (c). The cross-linking reaction in the presence of TCPP gave the antibody polymers which have the same binding property as the antibody monomer.

Effective Enhancement of Sensitivity in ELISA

ELISA was carried out using soluble antibody polymers to study the effects of cross-linking of antibodies on the sensitivity. Antibody polymers were prepared at pH 7.0, and the supernatant was applied to ELISA. Figure 7.3 shows the effects of the concentration of glutaraldehyde on ELISA. This graph shows the relationship between the absorbance of the hydrolytic product on ELISA and the concentration of the antibody. The number n shows the number of dilution, in which the corresponding concentration of the antibody is (7.2×2^{-n}) μM owing to 50 % dilution in each step (n). At 1.4×10^{-8} M of the antibody polymer solution (n = 9), the absorbance was 100-fold larger than that in the non-cross-linked antibody (monomer). This is partly due to the fact that a larger amount of labeled second antibody has been bound to the cross-linked antibody polymer. The results

indicated that antibody polymers brought about a much higher sensitivity in ELISA.
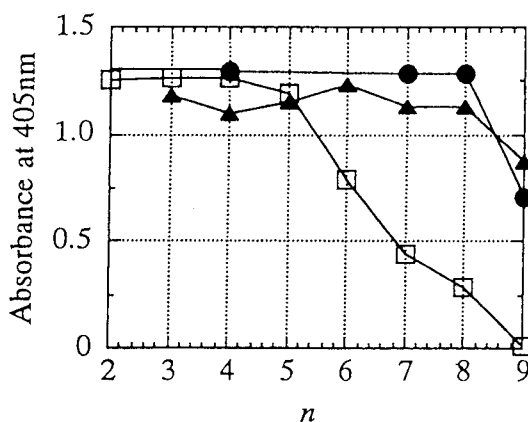

Figure 7.3. Effects of the concentration of glutaraldehyde on ELISA. The number n shows the dilution times, the corresponding concentration of the antibody is (7.2×2^{-n}) μM. Aqueous solutions of glutaraldehyde (15 μl) were added to antibody solution (1.0 ml) at room temperature. After 2 h, the reactions were stopped by the addition of 0.05 ml of 2 M glycine solution.

[Glutaraldehyde] = 2.5 wt% (●), 0.25 wt% (▲) and 0 wt% (□).

In all cross-linked antibodies, the enhancement of the value of the number which is constant ELISA coloration was observed. It is considered that these results are due to the cooperativity of the accumulated antibody.

Conclusion

Polymers of anti-TCPP antibody were prepared by using glutaraldehyde. The degree of the cross-linking was controlled by the concentration of glutaraldehyde and by pH. The reaction products were found to include antibody dimer, trimer, and some oligomers. The cross-linking reactions occur mainly at a heavy chain of the antibody.

Although the binding abilities of the antibody decreased by about 20 % during cross-linking reaction, this problem could be overcome by using TCPP as a blocking agent. The enhancement of the sensitivity in ELISA was observed by using antibody polymers. The antibody polymers were found to be useful for the high sensitive immunosorbent assay.

References

(1) Schultz, P. G.; Lerner, R. A. *Science* **1995**, *269*, 1835-1842.

(2) Li, T.; Lerner, R. A.; Janda, K. D. *Acc. Chem. Res.* **1997**, *30*, 115-121.

(3) Avrameas, S. *Immunochemistry* **1969**, *6*, 43-52.

(4) Avrameas, S.; Ternynck, T. *Immunochemistry* **1969**, *6*, 53-66.

(5) Avrameas, S.; Taubou, B.; Chuilon, S. *Immunochemistry* **1969**, *6*, 67-76.

(6) Engvall, E.; Perlmann, P. *Immunochemistry* **1971**, *8*, 871-874.

(7) Mannik, M.; Downey, W. *J. Immunol. Methods* **1973**, *3*, 233-242.

(8) Avrameas, S.; Ternynck, T.; Guesdon, J. -L. *Scand. J. Immunol.*, **1978**, *8(S7)*, 7-23.

(9) Harada, A.; Okamoto, K.; Kamachi, M.; Honda, T.; Miwatani, T. *Chem. Lett.* **1990**, 917-918.

(10) Harada, A.; Okamoto, K.; Kamachi, M. *Chem. Lett.* **1991**, 953-956.

(11) Harada, A.; Shiotsuki, K.; Fukushima, H.; Yamaguchi, H.; Kamachi, M. *Inorg. Chem.* **1995**, *34*, 1070-1076.

Summary and Conclusions

In this thesis, preparation of some antibodies for tetraphenylporphyrin derivatives toward functional antibodies and their characterizations have been described. The summary and the main conclusions of this thesis are as follows.

Chapter 2 described the preparation of anti-tetracarboxyphenyl-porphyrin (TCPP) antibodies and that of anti-phosphorus(V) tetraphenylporphyrin antibodies. Various complexes between porphyrins and antibodies were prepared and their binding affinities were characterized.

In Chapter 3, one of the antibodies prepared by immunization with TCPP conjugate has been found to bind strongly to Mn(III)-TCPP and Fe(III)-TCPP complexes. The complexes of the antibody 03-1 with Mn(III)-TCPP and Fe(III)-TCPP were found to catalyze oxidation of pyrogallol selectively.

In Chapter 4, the photoinduced electron transfer from Zn-porphyrin incorporated in an antibody combining site to an electron acceptor molecule (MV^{2+}) was demonstrated using antibodies against TCPP-Zn-TCPP noncovalent assembly system. The electron transfer from Zn-porphyrin to the acceptor molecule can be controlled by the antibody.

In Chapter 5, monoclonal antibodies for the phosphorus porphyrins have been prepared in order to induce the substrate binding space into the antibody combining site. One of the antibodies 74D7A binds not only a porphyrin part but also terephthalic acid and its monoester strongly and specifically. These results indicate that terephthalate, which was used as a part of an axial ligand for hapten,

was recognized specifically by the antibody. In the presence of antibodies, fluorescence quenching of porphyrins was observed by adding terephthalic acid, but there were no changes in the case of phthalate, or iso-phthalate. The selective electron transfer took place in the combining site of the antibody.

In Chapter 6, a supramolecular assembly of porphyrins and monoclonal antibodies was discussed. Absorption spectroscopy, size exclusion chromatography (SEC), biosensor measurements and atomic force microscopy (AFM) were applied to observe the supramolecular structures composed of antibodies and porphyrins.

In Chapter 7, cross-linked antibody polymers have been prepared by coupling reactions of anti-porphyrin antibody using glutaraldehyde. The antibody polymers were characterized by SDS-PAGE, UV-Vis spectroscopy, and enzyme-linked immunosorbent assay (ELISA). The reaction products were found to include the antibody dimer, trimer, and some oligomers cross-linked mainly in the heavy chains. The sensitivity in ELISA was enhanced by using the antibody polymers.

List of Publications

(1) Supramolecular Assembly of Porphyrins and Monoclonal Antibodies.

Harada, A.; Shiotsuki, K.; Fukushima, H.; Yamaguchi, H.; Kamachi, M. *Inorg. Chem.* **1995**, *34*, 1070-1076.

(2) Peroxidation of Pyrogallol by Antibody-Metalloporphyrin Complexes.

Harada, A.; Fukushima, H.; Shiotsuki, K.; Yamaguchi, H.; Oka, F.; Kamachi, M. *Inorg. Chem.* **1997**, *36*, 6099-6102.

(3) Photoinduced Electron Transfer from Porphyrin Incorporated in Antibody Combining Sites to Electron Acceptor Molecules.

Harada, A.; Yamaguchi, H.; Kamachi, M. *J. Inorg. Biochem.* **1997**, *67*, 114.

(4) Imaging Antibody Molecules at Room Temperature by Contact Mode Atomic Force Microscope.

Harada, A.; Yamaguchi, H.; Kamachi, M. *Chem. Lett.* **1997**, 1141-1142.

(5) Direct Observation of Supramolecules between Antibody and Porphyrins.

Yamaguchi, H.; Harada, A.; Kamachi, M. *Biophys. J.* to be submitted.

(6) Control of Photoinduced Electron Transfer by Anti-Porphyrin
Antibodies.
Yamaguchi, H.; Harada, A.; Kamachi, M. to be submitted

(7) Preparation of Anti-P(V) porphyrin Antibodies Having Substrate
Binding Sites for Electron Acceptors.
Yamaguchi, H.; Harada, A.; Kamachi, M. to be submitted.

(8) Preparation and Characterization of Antibody Polymers.
Yamaguchi, H.; Harada, A.; Kamachi, M. *Reactive & Functional
Polymers* **1997**, in press.

(9) Thermostable Peroxidase Activity with a Recombinant Antibody L
Chain-Porphyrin Fe(III) Complex.
Takagi, M.; Kohda, K.; Hamuro, H.; Harada, A.; Yamaguchi, H.;
Kamachi, M.; Imanaka, T. *FEBS Letters* **1995**, *375*, 273-276.

(10) Structural Characterization of Mouse Monoclonal Antibody 13-1
against a Porphyrin Derivative: Identification of a Disulfide Bond
in CDR-H3 of Monoclonal Antibody 13-1.
Akashi, S.; Kato, K.; Torizawa, T.; Dohmae, N.; Yamaguchi, H.;
Kamachi, M.; Harada, A.; Imanaka, T.; Shimada, I.; Takio, K.
BBRC **1997**, *240*, 566-572.