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Towards antibody-mediated metallo-porphyrin chemistry

E. Keinan[a], S. C. Sinha[a], A. Sinha-Bagchi[a], E. Benory[a], M.C. Ghozi[b], Z. Eshhar[b] and B.S. Green[c]

[a]Department of Chemistry, Technion - Israel Institute of Technology, Technion City, Haifa 32000, Israel, [b]Department of Chemical Immunology, The Weizmann Institute of Science, Rehovot 76100, Israel and [c]Department of Pharmaceutical Chemistry, The Hebrew University Medical Center, Jerusalem 91120, Israel.

Abstract - An attempt was made to mimic cytochrome P-450-like activity using antibodies elicited against metallo-porphyrins. Monoclonal antibodies raised against a water-soluble Sn(IV) porphyrin complex (**1**) exhibited specificity for a variety of monomeric metalloporphyrins, as well as for the μ-oxo-Fe(III) porphyrin dimer **2**. Some antibodies were found to be more selective for the monomer **1** than for the dimer **2**, suggesting an "edge-on" recognition of the planar porphyrin molecule. The catalytic activity of the antibody-metalloporphyrin complexes was investigated using the epoxidation of styrene by iodosobenzene as a model reaction. Three biphasic media were studied for this reaction: reverse micelles, microemulsions, and solid catalyst in organic solvent. The most promising results were obtained with solid catalyst (obtained via lyophilization of equimolar amounts of Mn(TCP)Cl and specific antibody) in dry CH_2Cl_2 at room temperature, as indicated by the high turnover numbers of the catalyst. A difference in the relative activity of the various monoclonal antibodies (MABs) was noted. The anti-**1** antibodies displayed ca. 30-60% higher activity compared to a nonrelevant MAB.

INTRODUCTION

Catalysts with tailored specificities and high activities remain one of the most challenging goals of organic chemistry. Much activity and ingenuity has been devoted to the development of general strategies that might afford such catalysts. Enzymes represent the most important family of specific catalysts that are being increasingly used in the laboratory and in industry to achieve high reaction rates and increased selectivity. However, for many applications, natural enzymes may be unstable, difficult to isolate, or may never have been identified. A revolutionary development in the biocatalysis area has been the exploitation of the exquisite specificity and unlimited diversity of the immune system to create high affinity molecules having catalytic activity.[1]

Some of the attractive features of the immune system that make it suitable for the production of high-selectivity catalysts include the following. 1) The immune system is the most prolific known source of specific binding molecules, called antibodies. It is estimated that the diversity of the immune system can produce 10^{12} different molecules. 2) Using currently available techniques, it is possible to use this system to make high affinity antibodies against virtually any molecule and any chemical structure. 3) Antibodies utilize the same kinds of interaction to bind their ligands (haptens or antigens) as do enzymes; these include hydrophobic interactions, hydrogen bonding, electrostatic and dispersion forces. 4) The estimated surface area of the combining site of an antibody is 500-700Å2. 5) Antibody-hapten interactions may be very strong, with typical dissociation constants ranging from 10^{-6} to 10^{-14} M. Such dissociation constants certainly represent enough binding energy to do chemical work.

Pauling postulated that enzymes achieve catalysis by virtue of their active sites being complementary, in shape and charge to the high energy species, the transition state, of the reactions they catalyze.[2] Jencks then proposed that antibodies raised against stable analogs of the transition state might act as enzyme-like catalysts.[3] This should occur if the antibody, like the enzyme, stabilizes the transition state relative to the ground state, thus lowering the energy barrier of the reaction when carried out on the surface of the protein compared to that in free solution.

Successful catalytic antibody systems have now been demonstrated for a relatively large number of different reactions. Although, in comparison with enzymes, the catalytic activity of these antibodies appear modest, these reactions generally show high reaction selectivity and high product stereoselectivity; since the hallmark of antibody recognition is molecular selectivity this is not surprising. In some cases enzyme-like catalytic efficiency has been observed.[4]

The successful eliciting of catalytic antibodies following immunization with stable transition state analogs has been largely dependent on three factors: the choice of immunizing hapten; the availability of the hybridoma technology; and the screening technique by which the catalytic monoclonal antibody (catMAB) is chosen. Earlier attempts to demonstrate catalysis using polyclonal antibodies were unsuccessful due, in large part, to the large amount of accompanying, non-specific antibody molecules (some of which may have been anti-catalysts, stabilizing the substrates). Similarly, since the hybridoma technique provides a very large number of potential binding antibodies, it is critical to be able to choose the catalytic antibody-secreting hybridomas from the larger number of noncatalytic counterparts.

In order to expand the range of chemical reactions that may be mediated by an antibody it would be useful to consider the approaches that nature has chosen to effect such a wide scope of enzymic activity. These features include the following: 1. Chemical catalysis; 2. Strain/distortion; 3. Proximity; 4. Desolvation; 5. Metal ion or other cofactors. The reported catMABs have generally been based on transition state stabilization; the use of cofactors or metal ions has thus far been little explored.[5,6]

The use of antibodies which bind metalloporphyrins is related to several of the enzymic aspects noted above. In addition, since the oxidation reaction of nature which has been extensively studied and modelled, namely the cytochrome P-450 catalyzed oxidations, involves metalloporphyrin, this appeared to be an ideal cofactor for an antibody-based system: the antibody may exerts its 'natural', exquisitely selective molecular differentiation and the metalloporphyrin acts as a cofactor, providing the chemical activation site.

Cytochrome P-450 enzymes represent an extraordinarily versatile class of biological oxidation catalysts.[7] As monooxygenases they are responsible for the hydroxylation of steroids and epoxidation of unsaturated fatty acids, the first step in the metabolism of these compounds. Biochemical studies and recent X-ray crystal structure analysis[8] have provided significant understanding of the structure and mode of action of P-450 cytochromes. Characteristic structural features for these enzymes include: 1) One side of an iron porphyrin molecule binds both the oxygen and the substrate. This cofactor is deeply buried in the protein. 2) No additional catalytically active groups have been found at this active site.[9] For monooxygenase activity, molecular oxygen is bound by the heme iron(II). In a sequence of redox processes, the bound oxygen is cleaved with formation of water and a high-valent iron-oxo complex believed to be Porphyrin(.+)Fe(IV)=O. This iron-oxo complex subsequently transfers its oxygen to the complexed substrate, leading to the oxidized product.

In recent years, the mechanism and the synthetic potential of olefin epoxidation and alkane hydroxylations catalyzed by simple cytochrome P-450 model systems have been explored.[10] In the majority of these studies, the natural process of oxygen activation has been circumvented, and the catalytically active iron-oxo intermediate has been generated by direct reaction of iron(III) porphyrinates with oxygen-transfer agents, such as iodosobenzene.[11]

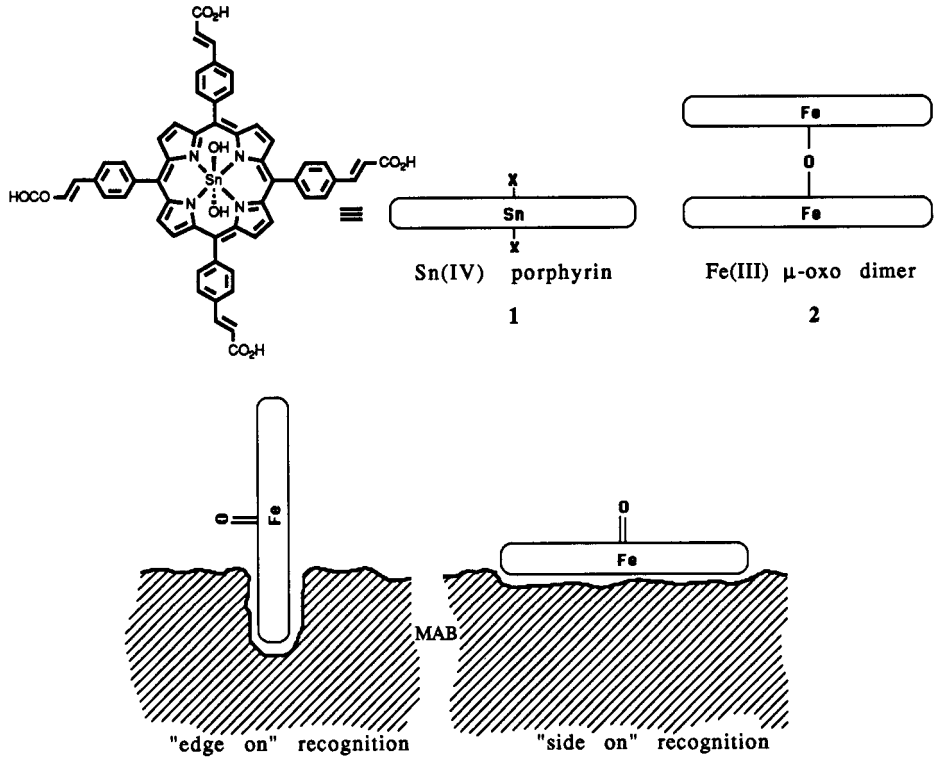
RESEARCH PLAN

In an initial approach to obtain Cyt P450-like activity we sought for antibodies that will specifically bind metalloporphyrins. The general idea is shown in Scheme 1. The hapten is an octahedral Sn(IV) porphyrin complex **1** which contains four cinnamic acid substituents. Tin porphyrins are extremely stable and do not release the metal even under harsh conditions.[12] This structure with four carboxylic groups was designed in order to achieve water solubility and high immunogenicity.

Coupling of **1** to a protein carrier must take place via one of the carboxyl groups and this allows a variety of different orientations of **1** to be recognized by an antibody, including the two extreme cases of "edge-on" recognition and "side-on" recognition (Scheme 1). By suitable screening procedures (see below) it might be possible to select the more interesting "edge-on" antibodies; these, for example, should bind an iron-porphyrin ligand and prevent its rapid dimerization to the catalytically inactive μ-oxo-dimer, **2**.

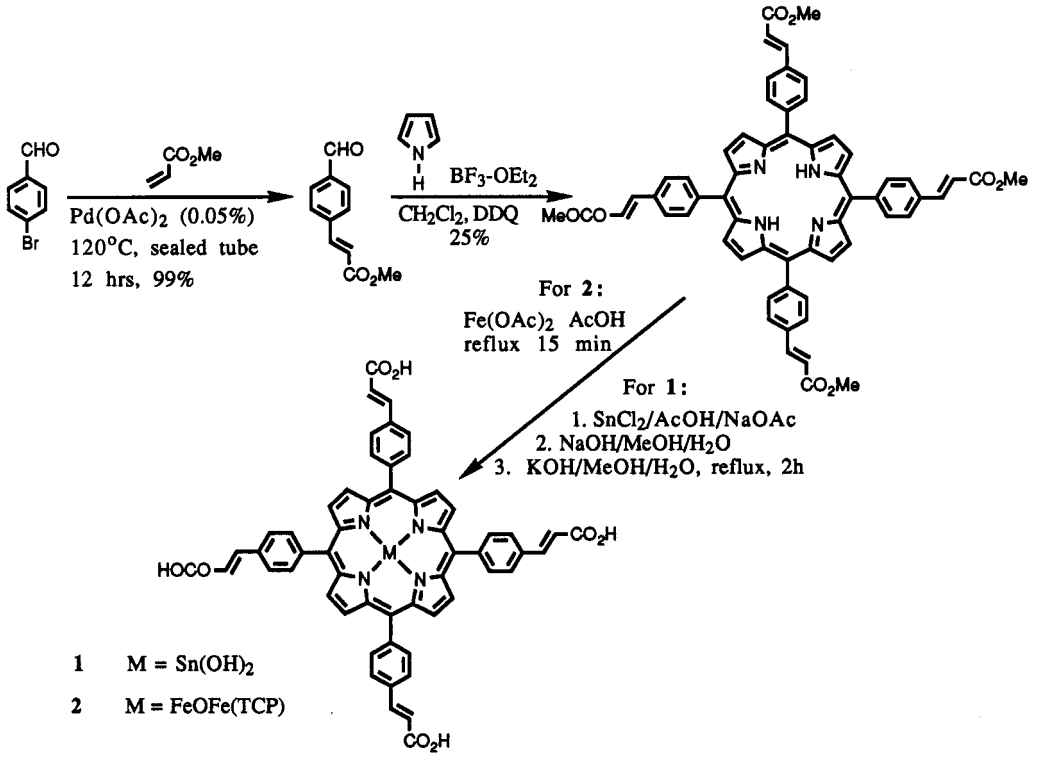
Such porphyrins are expected to show enzyme-coenzyme-like behavior, where the metalloporphyrin cofactor is sterically protected by the protein against undesired degradation and dimerization processes. Such an antibody-metalloporphyrin complex may serve as a catalyst for olefin epoxidation and hydroxylation of saturated hydrocarbons. Moreover, asymmetric induction is expected if the substrate approaches the oxo-metal reactive center in a pathway which involves interaction with the surface of the protein.

Scheme 1



Synthesis of 1 and 2

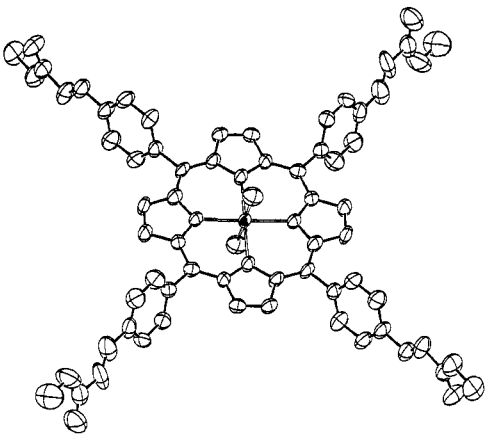
Scheme 2



PRODUCTION OF ANTIBODIES

meso Tetrakis (4-carboxyvinylphenyl) porphinato tin(IV)dihydroxide (**1**) was synthesized by conventional methods as outlined in Scheme 2. Methyl 4-formylcinnamate was prepared from 4-bromobenzaldehyde and methyl acrylate.[13] Reaction with pyrrole afforded *meso* tetrakis (4-methoxycarbonylvinylphenyl) porphyrin.[14] Stannation was carried out by heating the porphyrin with the stannous chloride under basic conditions.[15] Insertion of an iron atom to give the m-oxo-dimer **2** was carried out with ferrous acetate in refluxing acetic acid.[16] The structure of all metalloporphyrins was confirmed by NMR, microanalysis, and, in the case of **1**, by a single crystal X-ray determination of the tetramethyl ester (Fig. 1).

Figure 1: ORTEP drawing of 1-tetramethyl ester



The hapten **1** was linked to the proteins keyhole limpet hemocyanin (KLH) and bovine serum albumin (BSA) using carbonyldiimidazole. A short immunization protocol was adopted with mice receiving KLH conjugate using lymph node cells for fusion.[17,18] Draining lymph node cells were found to yield a greater repertoire of clones than spleen cells using a short immunization protocol.[19] The resulting hybridomas were first screened for binding to the BSA conjugate of **1**, as well as to BSA alone[17] in order to choose all antibodies that selectively recognize the hapten **1** and then for inhibition of binding of the conjugate **1**-BSA using the free Sn(IV)porphyrin, **1**. In order to eliminate those antibodies that bind the Fe(III) μ-oxo-dimer, this substance was also included in the screening procedure. The results are presented in Table 1.

Antibodies of selected clones were purified from ascites fluid by protein A (Pharmacia) affinity chromatography[20] and dialyzed against 30 mM TBS, pH 8. Protein concentration was determined by measuring optical density at 280 nm. Homogeneity of antibody was judged by sodium dodecylsulfate-polyacrylamide gel electrophoresis (SDS-PAGE), which yielded only heavy and light chains under reducing conditions, using Coomassie blue staining.

Out of the 1880 independent hybridoma wells taken for the first screening, 136 hybridomas (7.2%) produced antibodies with appreciable binding to the hapten **1** (dissociation constants smaller than 10^{-4}M). Further screening against both **1** and **2** resulted in the best five clones whose dissociation constants with respect to **1** and **2** are given in Table 1.

Table 1: Binding/Inhibition Constants of different MABs.

Antibody clone / Metalloporphyrin	130	26.2	104.1	135.2	76.1
Sn(TCP)$(OH)_2$ (1)	$10^{-6.8}$	$10^{-7.2}$	$10^{-7.3}$	$10^{-7.5}$	$10^{-7.6}$
Fe(TCP)-dimer (2)	$10^{-4.9}$	$>10^{-4}$	$10^{-5.6}$	$10^{-7.3}$	$10^{-7.4}$

Different concentrations of hapten were incubated with the various MABs and the residual activity was tested by ELISA. For each MAB the working concentration was predetermined to provide maximal sensitivity in the competitive inhibition studies. The binding/inhibition constants given, Ci50, are the concentration of free hapten required to inhibit 50% of MAB binding to immobilized antigen.

As is clearly seen from the Table, all five clones bind the tin porphyrin **1** well (Ci50=$10^{-6.8}$-$10^{-7.6}$). However, there are significant variations with respect to the iron dimer **2**. With MABs 130, 26.2 and 104.1 there are differences of 100-1000 fold in binding the two species. These five MABs also display size selectivity. For example, MAB 76, showed no significant recognition for either the smaller or larger analogs **3** and **4** (Scheme 3). The synthesis of the latter two Sn(IV)

porphyrin complexes was carried out analogously to **1**. Selective MABs to metalloporphyrins have previously been raised; chemical studies with these antibodies have not yet been reported.[6]

Scheme 3

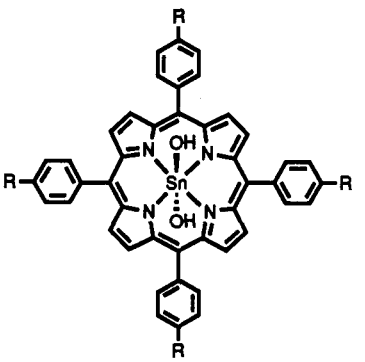


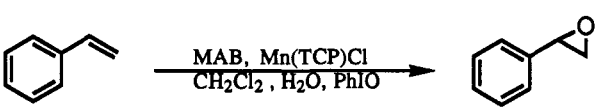
Table 2

R	C_i50 with BEH-76
CO_2H	$>10^{-3}$
CO_2H	$10^{-7.5}$
CO_2H	$>10^{-3}$

CHEMICAL REACTIVITY

The large differences in binding constants displayed by the various MABs suggested that differences in chemical reactivity of the antibody-metalloporphyrin complex might be observed. The epoxidation of styrene, using iodosobenzene as the source of oxygen, was chosen as the model reaction (Scheme 4). Obviously, this choice of water insoluble reactants required the employment of an appropriate biphasic medium. Three such techniques were studied: 1) reverse micelles,[21] 2) microemulsions,[22] 3) solid (lyophilized) MAB in organic solvent.[23]

Scheme 4



Reactions in reverse micelles were carried out in CH_2Cl_2 containing bis(2-ethylhexyl) sodium sulfosuccinate (AOT, 50 mM), styrene (0.43M) and iodosobenzene (0.01M). An aqueous solution of Mn(TCP)Cl with or without equimolar quantities of the antibody was injected into the organic phase. An appropriate amount of water was used to keep the water pool size at the level of 22-28. Reverse micelles have already been shown to be useful in this range of W_0 values for antibody-catalyzed hydrolysis of esters.[24] Although this approach was proved useful in our case as well, displaying a range of 100 turnovers per hour, no rate enhancement was caused by the presence of the antibodies. Approximately the same reaction rates were observed using the Mn porphyrin with or without antibodies. Moreover, in both cases racemic styrene oxide was formed (as verified by ^1H NMR spectroscopy using europium chiral shift reagent; data not shown).

Using the above described conditions with increased W_0 values (150) we attained heterogeneous media that required vigorous stirring. Although this microemulsion approach provided higher yields than with reversed micelles, the styrene oxide product was accompanied by significant amounts of side products. Again, in terms of reaction rates and optical yield, no difference could be observed between reactions with or without antibodies.

The most promising results were obtained with solid catalyst in dry organic solvent. A solution containing equimolar amounts of Mn(TCP)Cl and specific antibody was lyophilized and the resulting solid was added to a stirred CH_2Cl_2 solution of styrene and iodosobenzene at room temperature. Reactions were monitored by both GC and NMR using internal standards. Reactions were carried out with all of the five MABs as well as with a nonrelevant antibody CNJ-111 (Table 3). This approach works well, as indicated by the high turnover numbers of the catalyst. In addition, reactions are much cleaner than with the other two methods, with essentially no side products being formed. No catalysis was observed in a control experiment where solid Mn(TCP)Cl without any protein was used.

The notable feature of Table 3 is the difference in relative activity of the various MABs, as expressed by different turnover numbers. All anti-1 antibodies caused approximately 30-60% higher conversions when compared to the nonrelevant MAB (CNJ-111). One explanation for the small difference in relative activity is that there are many nonspecific interactions of the porphyrin molecules with the surface of the proteins, allowing porphyrin-catalyzed reactions outside of the antibody combining site. Again, as in the case of reverse micelles, no asymmetric induction was observed in any of these experiments (using NMR and chiral shift reagents which would only detect up to 10%e.e.). Interaction of the metalloporphyrin with the protein during the bond forming stage is expected to lead to asymmetric induction.[25]

Table 3: Styrene epoxidation catalyzed by solid antibody-porphyrin complex in organic solvent.

Antibody	Turnovers	Relative reactivity
BEH-76	424	1.27
BEH-104	457	1.37
BEH-135	487	1.46
BEH-130	549	1.64
BEH-26	537	1.61
CNJ-111	334	1.00

All reactions were carried out at 25°C for 17 h in stirred CH_2Cl_2 suspensions containing, Mn(TCP)Cl (1.5×10^{-6}M), BEH-MAB (1.5×10^{-6}M), styrene (0.43M) and iodosobenzene (0.01M). The turnover numbers presented are net values obtained after subtracting the background level of noncatalyzed oxidation, which was estimated by control experiments performed in the absence of protein.

These results led us to suspect that our initial selection of MABs was based on a premature interpretation of the difference in binding of compounds **1** and **2**. We assumed that the main difference arises from the difference in the shape of these molecules (monomer vs. dimer), neglecting the functional group difference (the fact that 1 bears two axial hydroxyl while 2 does not have any axial group in addition to the μ-oxo-bridge). In order to check the validity of these assumption we measured the dissociation constants of our five clones with Ni(TCP). The latter is a square planar Ni(II) complex without any axial ligands (Table 4). Indeed, it is clearly seen from the dissociation constants obtained that the MABs which do not bind 2 too well have even lower affinity for the Ni analog **5**. Apparently, the functional group selectivity is much more important than what we initially expected.

Table 4: Binding/Inhibition Constants.

Antibody clone / Metalloporphyrin	130	26.2	104.1	135.2	76.1
Sn(TCP)$(OH)_2$ (1)	$10^{-6.8}$	$10^{-7.2}$	$10^{-7.3}$	$10^{-7.5}$	$10^{-7.6}$
Fe(TCP)-dimer (2)	$10^{-4.9}$	$>10^{-4}$	$10^{-5.6}$	$10^{-7.3}$	$10^{-7.4}$
Ni(TCP) (5)	$>10^{-3}$	$>10^{-3}$	$>10^{-4}$	$10^{-5.3}$	$10^{-5.2}$

For details, see Table 1.

One might have expected that tight binding of the metalloporphyrin catalyst would have prevented any catalyzed reaction from occurring because the metalloporphyrin would be inaccessible to the iodosobenzene oxidant and/or styrene substrate. Antibody-binding has been observed to exert this effect in some cases. For example, antibodies which bind tightly to a p-nitrophenyl-substituted hapten stabilized p-nitrophenyl acetate towards hydrolysis.[26] Similarly, anti-2,4-dinitrophenyl antibodies significantly protect 2,4-dinitrophenyl ligand from chemical reduction.[27] The above results show that the monoclonal antibody-bound porphyrin is approximately 30% more reactive than is the non-relevant antibody. We ascribe the enhanced reactivity to the improved accessibility of the metalloporphyrin in the presence of the antibody. Perhaps this is also due to prevented aggregation and precipitation of the metalloporphyrin during the lypholization.

CONCLUSIONS

In this work we have shown that specific MABs can be obtained for various metalloporphyrins. We have also demonstrated that increased catalytic activity is achieved by porphinato Mn(III) complex upon interacting with specific MAB. We demonstrated for the first time that organic reactions are mediated by solid MABs in dry organic solvents. Relatively minor modifications in the porphyrin molecule can lead to major differences in binding of various antibodies. Thus, by appropriate screening procedures with a library of metalloporphyrins it is possible to select a MAB with desired binding properties. This approach underlies our present and future efforts in this field.

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