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Hemoabzymes
Different Strategies for Obtaining Artificial Hemoproteins Based on Antibodies

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ABSTRACT

Besides existing models of chemical or biotechnological origin for hemoproteins like peroxidases and cytochromes P450, catalytic antibodies (Abs) with a metalloporphyrin cofactor represent a promising alternative route to catalysts tailored for selective oxidation reactions. A brief overview of the literature shows that, until now, the first strategy for obtaining such artificial hemoproteins has been to produce antiporphyrin Abs, raised against various free-base, N-substituted, Sn-, Pd-, or Fe-porphyrins. Four of them exhibited, in the presence of the corresponding Fe-porphyrin cofactor, a significant peroxidase activity, with k_{cat}/K_m values of 10^2 to $5 \times 10^3/M/s$. This value remained low when compared to that of peroxidases, probably because neither a proximal ligand of the Fe, nor amino acid residues participating in the catalysis of the heterolytic cleavage of the O—O bond of H_2O_2, have been induced in those Abs. This strategy has been shown to be insufficient for the elaboration of effective models of cytochromes P450, because only one set of Abs, raised against *meso*-tetrakis(*para*-carboxyvinylphenyl)porphyrin, was reported to catalyze the nonstereoselective oxidation of styrene by iodosyl benzene using a Mn-porphyrin cofactor, and attempts to generate Abs having binding sites for both the substrate and the metallopor-

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phyrin cofactor, using as a hapten a porphyrin covalently linked to the substrate, were not successful. A second strategy is then proposed, which involves the chemical labeling of antisubstrate Abs with a metalloporphyrin. As an example, preliminary results are presented on the covalent linkage of an Fe-porphyrin to an antiestradiol Ab, in order to obtain semisynthetic catalytic Abs able to catalyze the selective oxidation of steroids.

Index Entries: Hemoabzymes; catalytic antibodies; peroxidase; cytochromes P450; metalloporphyrins; oxidation; steroids.

INTRODUCTION

Hemoproteins play a fundamental role in many natural processes. They are not only involved in the transport (hemoglobin), storage (myoglobin), and activation of dioxygen (cytochrome P450), the most abundant element of our environment, but they also participate in electron transport (cytochromes b and c), reduction of peroxides (peroxidases), and oxidation of endogeneous and exogenous substrates (cytochromes P450 and peroxidases). As a consequence, the elaboration of systems that are able to mimic those enzymes is of great interest, not only to better understand their in vivo mechanism, but also to build up catalytic systems that exhibit, under mild conditions, the efficiency and high selectivity typical of the enzymatic processes. In particular, the design of model systems for peroxidases and cytochromes P450, of chemical or biotechnological origin, which are easier to handle than the enzymes themselves, should provide very important tools for two main purposes *(1):* the development of new catalysts for important reactions in industrial and fine chemistry, such as the selective oxidation of alkanes and the stereoselective epoxidation of alkenes; and the study and prediction of the oxidative metabolism of new biologically active molecules such as drugs.

REQUIREMENTS FOR THE ELABORATION OF MODELS OF HEMOPROTEINS

Considering the structure of hemoproteins (Fig. 1), the elaboration of model systems of these enzymes must take into account several elements that are involved, at least in part, in their functioning: First, the prosthetic group, an heme or iron (Fe) (III)-protoporphyrin IX, which is responsible for the binding of ligands such as O_2 (hemoglobin, cytochromes P450) *(2,3)*, electron transfer (peroxidases) *(4)*, or oxene transfer (cytochrome P450) *(1,3)* reactions; second, the apoprotein that binds and site-isolates heme, preventing its aggregation and its bimolecular self-oxidation, and its active-site amino acids, which not only provide an hydrophobic environment for the substrate and control its access to the heme (cytochromes

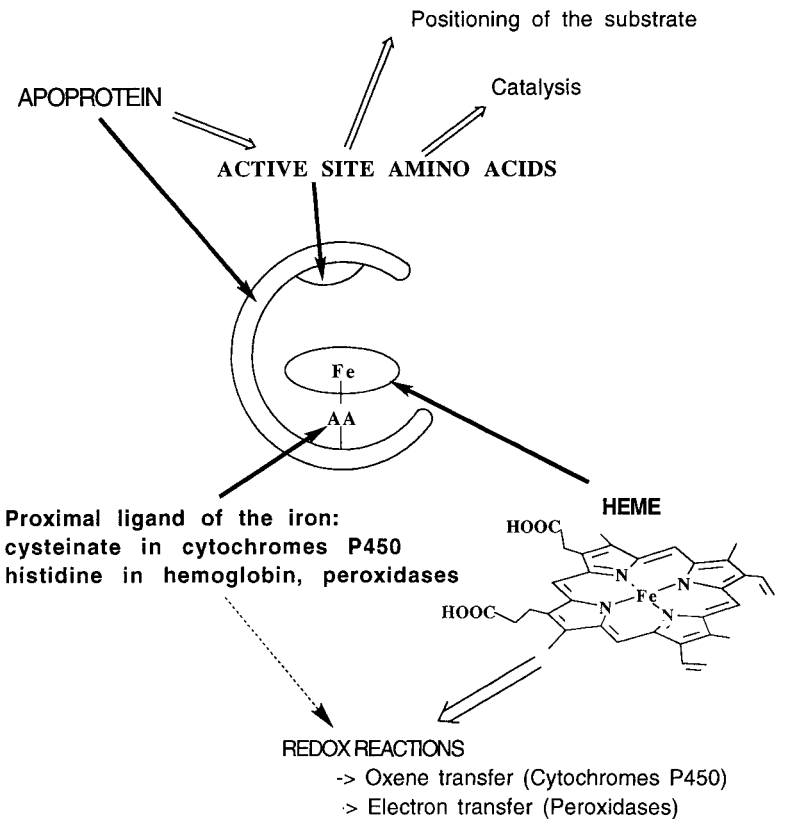


Fig. 1. Schematic view of the structure of hemoproteins.

P450), but also participate in some cases in catalysis (peroxidases) *(4)*; and third, the proximal axial ligand of the Fe atom, histidine in peroxidases or cysteinate in cytochromes P450, which has a role in controlling the heme redox potential and the reactivity of the high-valent Fe-oxo intermediates involved in the catalytic cycle of these enzymes *(5)*.

RECENT ADVANCES IN THE ELABORATION OF MODELS OF HEMOPROTEINS

In the past decade, three models able to mimic hemoproteins have been obtained. The first are homogeneous, purely chemical systems, based on Fe(III)- or manganese (Mn)(III)-porphyrins, soluble in organic solvents or in water. Second are supported catalysts consisting of a metalloporphyrin bound to organic or inorganic polymers. The third class of catalysts, more recently obtained by using the tools and concepts of molecular biology, are microorganisms in which mammalian cytochromes P450 have been expressed under a catalytically active form.

Homogeneous Metalloporphyrin Systems Mimicking Hemoproteins

Numerous recent reviews *(6–19)* have been devoted to the considerable effort made by several groups to develop such systems. Earlier models, simply obtained by binding a thiolate or an imidazole in the fifth axial coordination site of the Fe atom of Fe(III)-porphyrins, to mimic the proximal ligand of the Fe atom in hemoproteins, have provided very useful tools for the study of hexacoordinate Fe metabolite complexes of cytochrome P450 *(6,8)*, and for the study of the binding of O_2 to hemoglobin, myoglobin, and other hemoproteins *(6)*. The first functional systems have been obtained by associating Fe(III)- or Mn(III)-tetra-arylporphyrins as catalysts with various oxidants such as O_2 in the presence of a reductant like borohydride, H_2/Pt or ascorbic acid, or oxygen atom donors: iodosylarenes, NaOCl, alkylhydroperoxides, H_2O_2, peracids with or without imidazole *(20)*. Those systems have been able to reproduce, at least qualitatively, most reactions performed by cytochromes P450, such as the epoxidation of alkenes and the hydroxylation of alkanes *(7–19)*.

Starting from this first generation of very simple systems, efforts have been directed toward better efficiencies and selectivities. The first goal was reached by using as catalysts new generations of tetra-arylporphyrins, polyhalogenated on the phenyl and pyrrole rings *(1,21)*. The second goal has still to be reached. Indeed, first attempts to mimic the apoprotein of hemoproteins have been based on the use of superstructured porphyrins, such as picket-fenced, strapped, and capped porphyrins, sterically hindered at least on one face of the heme *(6)*. Porphyrins substituted by an arm or a handle bearing an imidazole or a pyridine have been used as mimics of hemoglobin *(6)*. Porphyrins substituted by bulky and/or asymmetric substituents, positioning the substrate with respect to the heme ion, have been used as catalysts for the regioselective and stereoselective hydroxylation of alkanes and stereospecific epoxidation of alkenes *(1,8,19)*. Unfortunately, two major disadvantages limited the use of those systems: The increase of selectivity was generally obtained at the detriment of reactivity; the superstructured catalyst was often destroyed by oxidation during the course of the reaction.

Supported Metalloporphyrin Models for Hemoproteins

Three major difficulties, encountered in the homogeneous metalloporphyrin models of hemoproteins described above, had to be overcome: catalyst recovery, reuse, and selectivity. One approach to this has been to anchor the metalloporphyrin on a solid surface, which should first allow an easy separation of the catalyst from reactants and products *(22)*. Second, the

support should provide a local environment for the metalloporphyrin, thus mimicking the protein matrix of heme enzymes. It should then ensure the site isolation of the metalloporphyrin molecules, preventing their aggregation and intermolecular self-oxidation, and its surface structure, rigidity, and polarity should influence the behavior and selectivity of the catalyst.

Early work in this field was directed to mimicking the chemistry of hemoglobin and myoglobin *(23)*. Models involving metalloporphyrins anchored on poly-4-vinylpyridines, poly-N-vinylimidazoles, or poly-L-lysine supports were used for studying the reversible binding of dioxygen to hemoglobin and myoglobin *(23)*. This work has been extended to obtain models for peroxidases *(24)* and cytochromes P450 *(25)*. Models for peroxidases have been obtained by associating Fe-, Mn-, or cobalt (Co)-porphyrins supported on amberlite *(26)* or poly-(mono-O-methylethyleneglycol) *(27)* as catalysts, with H_2O_2 as oxidant. Those systems have been able to oxidize peroxidase substrates, such as o-phenylenediamine, *N,N*-diethylaniline, with activities comparable to that of horseradish peroxidase. Models of chloroperoxidase and ligninase *(28)* have also been obtained using Mn(III)- or Fe(III)-tetra-arylporphyrins supported on cation exchange resin and polyvinylpyridine. In the models for cytochromes P450, Fe(III)- or Mn(III)-porphyrins have been supported either on polymers, such as polyisocyanide, anion exchange resins, polystyrenes, poly-(vinylpyridines) or albumin, or on zeolites and clays. The materials obtained have been able to catalyze the epoxidation of alkenes, such as styrene and cyclooctene, and the regioselective hydroxylation of alkanes, mostly by sodium hypochlorite and iodosylbenzene, and to a lesser extent by H_2O_2 or alkylhydroperoxides *(22)*. In the best system reported to date, Mn^{III}-*tetra*-*N*-methylpyridiniumporphyrin adsorbed in the interlamellar space of montmorillonite, was found able to catalyze with high yields the epoxidation of cyclooctene and the chemo- and regioselective oxidation of alkanes *(29)*. Moreover, the catalyst was found reusable and stable for at least 30,000 turnovers.

Overall, as expected, metalloporphyrins supported on inorganic solids or rigid inert polymers have appeared to be the best catalysts, limiting porphyrin oxidative self-destruction and oxidation of the support. Those properties should be further improved by the use of robust sterically hindered porphyrins. Metalloporphyrins, intercalated in clays, or silica-supported, have appeared promising for the selective hydroxylation of alkanes. More work will be needed to improve the chemo- and regioselectivity of those reactions, using clays, templated silica, or shape-selective polymers as supports. In addition, the production of enantioselective systems will require the development of supported chiral oxidation catalysts, involving either chiral supports or chiral metalloporphyrins, or both.

Biotechnological Catalysts Based on Yeasts Expressing Cytochromes P450

In the past 10 years, cytochromes P450 have been expressed in various kinds of cells or microorganisms *(30)*. One of them in particular, *Saccharomyces cerevisiae*, has been used for several reasons. First, this kind of yeast is easy to handle and can be grown on a large scale. Second, it contains all the necessary components for the expressed P450 apoprotein to be catalytically active: the ability to incorporate the heme in the expressed apoprotein; the existence of electron-transfer proteins, like cytochrome P450 reductase, to transfer electrons to P450; and the presence of membranes similar to those of human liver endoplasmic reticulum, which are necessary for the organization of the proteins in the monooxygenase cluster. Several cytochromes P450 have been expressed in yeast under a catalytically active form *(1)*. The corresponding yeasts have been used as very interesting tools for regioselective oxidations of complex molecules and for studies of drug metabolism. For instance, the major P450 of human liver, cytochrome P450 3A4, has been expressed at a high level in yeast *(31)*. This has allowed direct UV-visible studies, on yeast microsomes, of the interactions of this cytochrome P450 with its substrates and inhibitors. The yeast microsomes have also been able to catalyze the oxidation of several typical substrates of cytochrome P450 3A4: lidocaine, nifedipine, erythromycine, and quinidine, with the same regioselectivities as those previously found in human liver microsomes.

The monooxygenase activities of the yeasts have recently been increased by co-expressing, with cytochrome P450 3A4, some other components of the monooxygenase cluster like cytochrome P450 reductase and cytochrome b_5 *(32)*. This strategy, when applied to other forms of cytochromes P450 or to mutated forms of cytochrome P450, or to other hemoproteins such as peroxidases, should provide powerful tools to realize, in vitro, any kind of selective oxidation reaction.

CATALYTIC ANTIBODIES AS MODELS FOR HEMOPROTEINS?

As early as 1946, Pauling proposed that the ability of an enzyme to catalyze a chemical reaction was caused by the complementarity of the enzyme's active site structure to the activated complex *(33,34)*. Several years later *(35)*, people thought about using the remarkable ability of the immune system to generate immunoglobulins that possess high affinities and specificities against virtually any molecule, to produce catalytic antibodies (Abs) or abzymes. For that, monoclonal antibodies (MAbs) have been elicited against haptens carefully designed to mimic the transition state of a given reaction, to induce in the binding site of the Ab an imprint

that should be complementary in shape and charge to the hapten, and consequently to the transition state. Using this strategy, MAbs that are able to catalyze a wide range of reactions, such as ester and amide hydrolysis and synthesis, ether and phosphate hydrolysis, isomerization, Claisen rearrangement, reductions, Diels-Adler and redox reactions, had been reported *(36–39)*. A few examples of catalytic Abs modelling enzymes that require a cofactor, such as flavins *(40)*, metal ions *(41–44)*, or metal complexes *(45)*, have been described. Those Abs were designed either to bring into close proximity the cofactor and the substrate, or to bind tightly the cofactor to enhance its reactivity.

Thus, besides the three kinds of systems briefly reviewed above, catalytic Abs with metalloporphyrin cofactor appeared as an alternative approach to provide a route to catalysts tailored for specific oxidation reactions. In such models of hemoproteins, the heme should be mimicked by a synthetic metalloporphyrin, and the apoprotein should be mimicked by the Ab protein. The chief difficulty thus consisted in designing the ideal porphyrin hapten, which could induce in the binding site of the Ab, not only a binding site for the metalloporphyrin, but also an axial ligand of the Fe and a hydrophobic pocket to accommodate the substrate.

CATALYTIC ANTIBODY MODELS OF PEROXIDASES

At first sight, peroxidases appeared to be some of the easiest hemoproteins to be mimicked. Indeed, their active site, which contains an heme bound to the apoprotein by an histidine residue (Fig. 2), is relatively narrow *(4,46)*. Little space is left over the plane of the porphyrin, so that only the oxidant, H_2O_2 or ROOH, can enter the active site and interact with the Fe atom; the reducing co-substrates only interact with the edge of the porphyrin *(4,46)*. Thus, to induce such characteristics in the binding site of Abs one simple strategy was to use as haptens quite unsophisticated porphyrins.

Therefore, Abs have been elicited against various free-base *(47,51,56)*, nitrogen (N)-substituted *(48,49,55,57,58)*, tin (Sn)-*(50,52)*, or palladium (Po)-porphyrin *(53,54)*. Only three of the obtained Abs have shown a significant peroxidase activity *(49,55,56)*. The first one was obtained by Cochran and Schultz *(49)*, using *N*-methylmesoporphyrin IX (N-CH_3-MPIX) (Fig. 3), an inhibitor of ferrochelatase, as a hapten. The corresponding Fe(III)-MPIX-Ab complex was shown to catalyze the oxidation of several typical peroxidase co-substrates, such as *o*-dianisidine, 2,2′-azinobis (3-ethylbenzothiazoline-6-sulfonic acid) (ABTS), and pyrogallol, by H_2O_2. Later, Feng et al. *(55)* used *N*-hydroxymethylmesoporphyrin IX (N-CH_2OH MPIX) (Fig. 3) as a hapten, in which the oxygen atom of the hydroxymethyl group was supposed to mimic the oxygen atom of H_2O_2 when coordinated to the Fe atom of heme in peroxidase. They obtained two MAbs, 9A5 and 11D1, which were able to

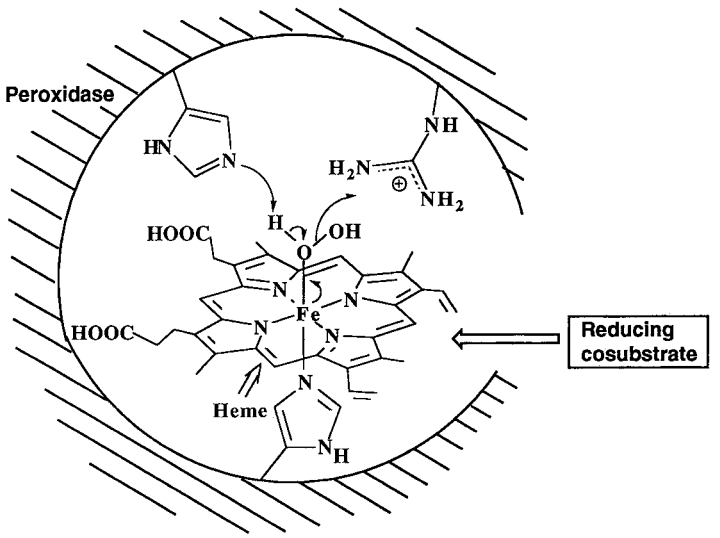
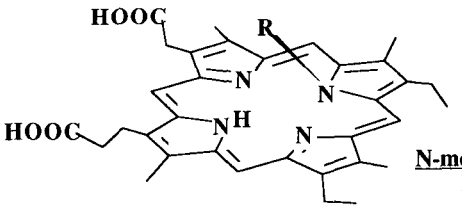


Fig. 2. Mechanism of the heterolytic cleavage of the O—O bond of hydroperoxides catalyzed by peroxidases.

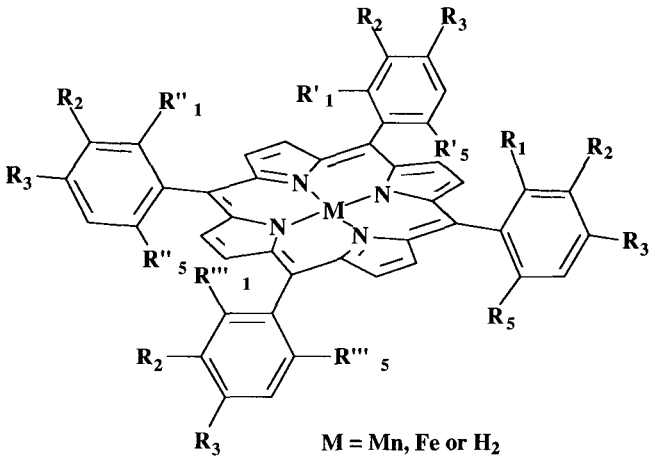
catalyze, in the presence of Fe(III)-mesoporphyrin IX (Fe[III]-MPIX) the oxidation of pyrogallol by H_2O_2 *(55)*. A third set of Abs was obtained by Takagi et al. *(56)* using *meso*-tetrakis(*para*-carboxyphenyl)porphyrin ($TpCPPH_2$) (Fig. 3) as a hapten. Not only did the corresponding Fe-TpCPP–Ab complexes catalyze the oxidation of ABTS and pyrogallol by H_2O_2, but the best catalyst for this reaction was found to be the complex associating Fe(TpCPP) with a recombinant Ab light (L) chain 13-1-L, which they named L-zyme.

Table 1 summarizes the kinetic parameters reported for the oxidation of various co-substrates by H_2O_2, catalyzed by Fe-porphyrin–Ab complexes or by the corresponding free Fe(III)-porphyrins. In the case of Fe-porphyrin–Ab complexes, the k_{cat} values range between 86 and 667/min, the best value being obtained in the case of the Fe(TpCPP)-13-1-L complex *(56)*. Those values are 2.4–6.3 × higher than those reported for Fe(III)-porphyrins, which range from 21 to 166/min, which shows that Ab-porphyrins are better catalysts than the Fe(III)-porphyrins. Fe(III)-porphyrin–Ab complexes also had a better affinity for H_2O_2 than free Fe(III)-porphyrins, as shown by the K_m values for H_2O_2, which range between 2.3 and 35 m*M* for Fe-porphyrin-IgG complexes, when compared to values of 43–100 m*M* for free Fe-porphyrins. As a consequence, Fe(III)-porphyrin–Ab complexes exhibited higher efficiencies k_{cat}/K_m, ranging between 63 and 4833/*M*/s, than the corresponding free Fe(III)-porphyrins, which led to k_{cat}/K_m values ranging between 4 and 64/*M*/s. It is noteworthy that the best efficiency was obtained for the L-zyme, which exhibited both the higher k_{cat} value, 667/min, and the lower K_m value, 2.3 m*M* *(56)*. Unfortunately, those values



N-methyl-mesoporphyrin IX (N-CH_3-MPIX)
R = CH_3

N-hydroxymethyl-mesoporphyrin IX (N-CH_2OH-
R = CH_2OH



M = Mn, Fe or H_2

meso-tetrakis(*ortho*-carboxyphenyl)porphyrin (ToCPP)
α,α,α,α isomer: $R_1 = R'_1 = R''_1 = R'''_1$ = COOH, other substituents
α,α,α,β isomer: $R_1 = R'_1 = R''_1 = R'''_5$ = COOH, "
α,α,β,β isomer: $R_1 = R'_1 = R''_5 = R'''_5$ = COOH, "
α,β,α,β isomer: $R_1 = R'_5 = R''_1 = R'''_5$ = COOH, "

meso-tetrakis(*ortho*-carboxymethylphenyl)porphyrin (ToCMePP)
α,α,α,β isomer: $R_1 = R'_1 = R''_1 = R'''_5 = COOCH_3$, other substituer

meso-tetrakis(*meta*-carboxyphenyl)porphyrin (TmCPP)
R_2 = COOH, other substituents = H

meso-tetrakis(*para*-carboxyphenyl)porphyrin (TpCPP)
R_3 = COOH, other substituents = H

meso-tetrakis(*para*-carboxyvinylphenyl)porphyrin (TpCVPP)
R_3 = CH=CH-COOH, other substituents = H

meso-tetrakis(*ortho*-dichlorophenyl)porphyrin (TDCPP)
$R_1 = R'_1 = R''_1 = R'''_1 = R_5 = R'_5 = R''_5 = R'''_5$ = Cl, $R_2 = R_3$ = H

meso-tetraphenylporphyrin (TPP)
all substituents = H

Fig. 3. Structure and nomenclature of the various porphyrins used as haptens for the generation of antiporphyrin Abs.

Table 1
Comparison of Kinetic Parameters for Oxidation of Various Co-substrates by H_2O_2 Catalyzed by Iron-Porphyrin–Antibody Complexes

Complex	Hapten	Substrate	k_{cat} (min^{-1})	$K_m(H_2O_2)$ (m*M*)	k_{cat}/K_m *M*/s	Ref.
Fe^{III}-MPIX[a]						
7G12-A10-G1-A12	N-CH_3-MPIX	*o*-dianisidine	394	24	274	
		ABTS[d]	–	–	233	*49*
		pyrogallol			122	
Fe^{III}-MPIX	–	*o*-dianisidine	166	43	64	*49*
Fe^{III}-MPIX-9A5	N-CH_2OH-MPIX	pyrogallol	132	35	63	
Fe^{III}-MPIX-11D1	N-CH_2OH-MPIX	pyrogallol	86	13	110	*55*
Fe^{III}-MPIX	–	pyrogallol	21	100	4	
Fe^{III}-TpCPP[b]-13-1 L	$TpCPPH_2$	pyrogallol	667	2.3	4833	*56*
Fe^{III}-ToCPP[c]-13G10	Fe(ToCPP)	ABTS	100	16	105	
Fe^{III}-ToCPP-14H7	Fe(ToCPP)	ABTS	63	9	119	*60*
Fe^{III}-ToCPP	–	ABTS	51	42	20	

[a]MPIX, mesoporphyrin IX; [b] TpCPP, *meso*-tetrakis(*para*-carboxyphenyl)porphyrin; [c] ToCPP, *meso*-tetrakis(*ortho*-carboxyphenyl)porphyrin; [d] ABTS, 2,2-azinobis (3-ethylbenzothiazoline-6-sulfonic acid).

cannot be compared to those corresponding to Fe(TpCPP) alone, which have not been reported. However, this k_{cat}/K_m value of about $5 \times 10^3/M/s$ remains far below those observed for peroxidases, which are among the most efficient enzymes, and exhibit k_{cat}/K_m values of about $10^7/M/s$ *(59)*. Therefore, the authors tried to get better peroxidase-like abzymes, using a more sophisticated hapten, the Fe(III)-α,α,α,β-*meso*-tetrakis(*ortho*-carboxyphenyl)porphyrin (α,α,α,β-Fe [ToCPP]) (Fig. 3).

Antibodies Elicited Against Iron(III)-α,α,α,β-*meso*-tetrakis (*ortho*-carboxyphenyl)porphyrin

The key step in the mechanism of the peroxidase reaction is the heterolytic cleavage of the O—O bond of H_2O_2 or ROOH assisted by two amino acid residues, a histidine and an arginine (Fig. 2) *(46)*, which leads to a highly reactive Fe(V)-oxo-species. This complex is further reduced to Fe(III) by two successive transfers of one electron from the reducing co-substrate occurring through the porphyrin ring. α,α,α,β-Fe(ToCPP) was then chosen as a hapten not only to generate in Abs a binding site for an Fe-porphyrin, but also in the hope of generating, opposite to the *ortho*-carboxylate substituents of the phenyl rings, amino acids such as histidine or arginine, which would assist the heterolytic cleavage of the O—O bond of

H_2O_2. In addition, the authors used the metal-porphyrin, expecting that it could induce in the Ab an amino acid residue that could bind the metal and mimic the histidine proximal ligand of Fe in peroxidases.

As reported recently *(60)*, three MAbs were found to recognize α,α,α,β-Fe(ToCPP). Two of them, 13G10 and 14H7, were able to bind it with K_d values of 2.9×10^{-9} *M* and 5.5×10^{-9} *M*, respectively. Those values are the best ones ever reported for the binding of an Fe-porphyrin to an Ab *(40–49)*, and are in the range of K_d values generally observed for natural heme-protein complexes (10^{-12}–10^{-8} *M*) *(53)*.

The two corresponding α,α,α,β-Fe(ToCPP)–IgG complexes catalyzed the oxidation of ABTS by H_2O_2, and the authors took care to demonstrate *(60)* that the reaction did occur in the binding site of the Abs. The measured kinetic parameters are included in Table 1, together with those of the reaction catalyzed by free α,α,α,β-Fe(ToCPP). Both porphyrin–IgG complexes are better catalysts than the Fe-porphyrin alone, as shown by k_{cat} values of, respectively, 100 and 63/min, which are better than the one observed for Fe(ToCPP) alone: 51/min. They also lead to lower K_m values for H_2O_2, respectively, 16 and 9 m*M*, than Fe(ToCPP) (K_m = 42 m*M*), which shows that H_2O_2 has a better affinity for the porphyrin–IgG complexes than for the free Fe-porphyrin. Accordingly, both 13G10 and 14H7-porphyrin complexes exhibit efficiencies of 105 and 119/*M*/s, respectively, which are about 5× higher than that obtained with Fe(ToCPP) (k_{cat}/K_m = 20 /*M*/s). Another major advantage has been observed in the case of porphyrin–antibody catalyzed reactions: the protecting effect of the Ab protein toward the oxidative degradation of the porphyrin. Indeed, although the rate of oxidation of 0.2 m*M* ABTS by 1 m*M* of H_2O_2 remained constant in the presence of 0.2 μ*M* 13G10-Fe(ToCPP) as catalyst, until complete conversion of the ABTS (1000 turnovers), it slowed down after 15 min in the presence of 0.2 μ*M* Fe(ToCPP), and the reaction stopped after only 40% conversion of ABTS.

From Table 1, it appears that the kinetic parameters calculated for the peroxidase reaction catalyzed by the authors' Ab–Fe(ToCPP) complexes, k_{cat} and k_{cat}/K_m are in the range of those already published for other Fe-porphyrin–Ab complexes *(49,55,56)*, and remain far below those of peroxidases. Thus, it is clear that the authors' hapten was not more adapted than others to induce the presence in the Ab of catalytic amino acids such as histidine and guanidine. Preliminary studies have been undertaken to determine the binding site topology of the author Abs, using the determination by competitive ELISA of the apparent dissociation constants (K_ds) for various porphyrins (Table 2) and UV-visible spectroscopy.

Several conclusions can be drawn from Table 2. First, the central metal atom of the hapten is not recognized by the Ab since α,α,α,β-Fe- and -Mn-ToCPP, as well as the free-base α,α,α,β-ToCPPH$_2$, all bound to 13G10

Table 2
Apparent Dissociation Constants for Various 13G10-Porphyrin Complexes

Porphyrin[a]	Binding constant (*M*)
α,α,α,β-ToCPPH$_2$	1.0×10^{-8}
α,α,β,β-ToCPPH$_2$	0.8×10^{-8}
α,β,α,β-ToCPPH$_2$	2.4×10^{-8}
α,α,α,α-ToCPPH$_2$	2.5×10^{-8}
α,α,α,β-Fe(ToCPP)	1.3×10^{-8}
α,α,α,β-Mn(ToCPP)	1.1×10^{-8}
α,α,α,β-ToCMePPH$_2$	3.0×10^{-6}
TmCPPH$_2$	3.0×10^{-5}
Fe(TPP)	$>2.0 \times 10^{-5}$
Fe(TDCPP)	$>2.0 \times 10^{-5}$
Hemin	$>2.0 \times 10^{-5}$
Protoporphyrin IX	$>2.0 \times 10^{-5}$

[a] For nomenclature, *see* Fig. 3.

with K_ds of 1–1.3 $\times 10^{-8}$ *M*. This shows that the authors did not induce any amino acid residue in the Ab protein that was able to coordinate the Fe atom of the hapten. UV-visible studies *(60)* confirmed that both 13G10- and 14H7-Fe(ToCPP) complexes were high-spin hexacoordinate complexes, in which two H_2O molecules and no amino acid residues were bound to the Fe atom. Indeed, only minor changes were induced by the insertion of Fe(ToCPP) into the Ab protein: a 5-nm shift and a slightly greater absorbance of the Soret band *(51)*, which were consistent with the binding of Fe(ToCPP) in a hydrophobic pocket, with no change in the Fe(III) spin state. It is noteworthy that similar phenomena were observed by Feng et al. *(55)* upon binding of Fe(MPIX) to Abs 9A5 and 11D1, and that a spectrum typical of a high-spin ferric porphyrin, with a large increase in the Soret band around 396 nm, was also described by Cochran and Schultz *(49)* for the 7G12-A10-G1-A12-Fe(MPIX) complex.

The second important observation from Table 2 is that the *ortho*-carboxylate substituents of the phenyl rings of the hapten play a key role in the recognition of Fe(ToCPP) by the Ab. When these substituents are esterified (α,α,α,β-Fe[ToCMePP]) or shifted to the *meta* position (TmCPPH$_2$), K_d is increased, respectively, by a factor of 10^2 and 10^3. In addition, *meso*-tetraarylporphyrins bearing no carboxylate substituents, TPP and TDCPP, are poorly recognized ($K_d > 2 \times 10^{-5}$). However, the tetra-aryl-porphyrin moiety is necessary for the recognition, because hemin and protoporphyrin IX, which both possess carboxylate substituents, but no *meso*-phenyl substituents, lead to K_ds higher than 2×10^{-5} *M*.

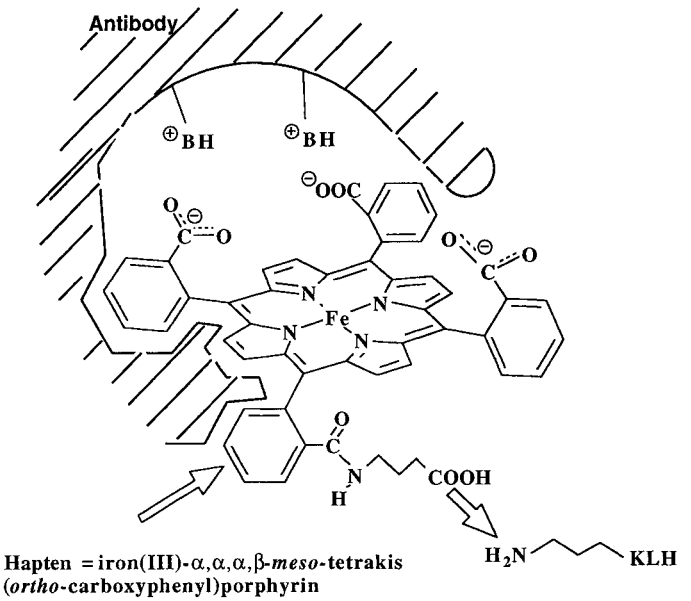


Fig. 4. Possible topology of the active site of anti-Fe(ToCPP) Abs.

Finally, K_ds for the four atropoisomers of $ToCPPH_2$ can be compared. K_d for the α,α,α,β-isomer, 1.0×10^{-8} *M*, is almost similar to that of the α,α,β,β isomer, 0.8×10^{-8} *M*, which shows that changing the third α-carboxylate substituent to β does not alterate the binding of the porphyrin to the Ab. On the contrary, both α,β,α,β and α,α,α,α isomers lead to a K_d of about 2.5×10^{-8} *M*, 3 × higher than that of the α,α,α,β isomer, which shows that both the first α-carboxylate and the fourth β-carboxylate are involved in the binding of the α,α,α,β-Fe(ToCPP) to the Ab. To explain those results, the authors propose a possible topology of the binding site of their Abs (Fig. 4). In this model, roughly two-thirds of the porphyrin macrocycle could be inserted in the binding pocket, with two adjacent α,α-carboxylates being more specifically bound to the protein. One α-carboxylate could be outside the binding pocket, but the β-carboxylate, presumably the one bearing the linker to the carrier protein during immunization, could be on the edge of the pocket.

Consequently, the authors' results show that the use of α,α,α,β-Fe(ToCPP) has led to the production of Abs having a peroxidase activity with no destruction of the catalyst by H_2O_2, even after complete conversion of the co-substrate. The attempt to generate an axial ligand of the iron failed, but the authors succeeded in generating amino acid residues opposite to some of the carboxylates of the porphyrin. Unfortunately, it seems unlikely that those residues could be involved in the catalysis of the heterolytic cleavage of the O—O bond of H_2O_2.

CATALYTIC ANTIBODIES AS MODELS OF CYTOCHROMES P450

Structure of Cytochromes P450

As already mentioned (*see* Introduction), monooxygenases are widely distributed enzymes that catalyze dioxygen activation, using two electrons and two protons coming from nicotinamide adenine dinucleotide phosphate (NADPH), with the insertion of one O atom from O_2 into a substrate and the formation of water (Eq 1).

$$RH + O_2 + 2H^+ + 2e^- \rightarrow ROH + H_2O \quad (1)$$

A great number of these enzymes contain cytochrome P450, which is the site for dioxygen activation. These cytochrome P450-dependent enzymes are involved in many steps of the biosynthesis and biodegradation of endogenous compounds, such as steroids, fatty acids, prostaglandins and leukotrienes, and arginine *(8)*. They also play a key role in the oxidative metabolism of exogenous compounds, such as drugs and other environmental products, allowing their elimination from living organisms.

Cytochromes P450 have been the subject of many studies in the past 10 yr, and much is known about their structure and functions *(3,8,61–64)*. Indeed, more than 500 P450 sequences are now available *(3)*, and the X-ray structure of four cytochromes P450 have now been published *(64)*. The active site of cytochromes P450 is divided in two parts: the heme-binding site, in which the heme Fe is linked to the 50-kDa apoprotein by a proximal cysteinate, and, above the plane of the heme, a hydrophobic site for the binding of substrates (Fig. 1).

Mechanism of Reactions Catalyzed by Cytochromes P450

The mechanism of the reactions catalyzed in vivo by cytochromes P450 has now been elucidated *(1,3,8,61–64)*. Under those conditions, the catalytic cycle involves the formation of a very reactive intermediate, a high-valent P450 $Fe^V{=}O$ species derived from the binding of one oxygen atom of O_2 to the Fe, and a two-electron oxidation of the Fe (III) (Eq 2). This highly oxidative species then transfers its O atom to the substrate (Eq 3).

$$P450\ Fe^{III} + O_2 + 2H^+ + 2e \rightarrow P450\ Fe^V = O + H_2O \quad (2)$$

$$P450\ Fe^V = O + SH \rightarrow P450\ Fe^{III} + SOH \quad (3)$$

This complex can be directly obtained in vitro by reaction of cytochrome P450 with various single oxygen atom donors (AO), such as C_6H_5IO, H_2O_2, or $NaIO_4$ *(3,8,61–63)*; (Eq 4).

$$P450\ Fe^{III} + AO \rightarrow P450\ Fe^{V} = O + A \quad (4)$$

Accordingly, systems associating cytochromes P450 and AO are able to perform the same reactions as those catalyzed by cytochromes P450 in vivo, such as hydroxylation of alkanes and epoxidation of alkenes. The catalytic cycle thus described is called the short catalytic cycle.

Antiporphyrin Antibodies as Models of Cytochromes P450

It appeared to be a very complex task to design models of cytochrome P450 using O_2 as oxidant, because the full in vivo catalytic cycle requires the presence not only of O_2, but also of a reductant, NADPH, and the participation in the monooxygenase cluster of electron transfer proteins and cytochrome P450 reductase to transfer electrons from NADPH to the heme Fe. On the contrary, it seemed much easier to design models using AO, because the in vitro short cycle only requires the one pot association of cytochrome P450 and AO, and model systems, simply associating an O atom donor and an Fe(III)- or Mn(III)-porphyrin, have been found to be able to perform all the reactions typical of cytochrome P450, without the need for an axial ligand of the metal to mimic the cysteinate proximal ligand of heme in cytochrome P450 *(1,8)*. Associating AO donors and antiporphyrin Abs complexed with Fe(III)- or Mn(III)-porphyrins thus appeared as a reasonable strategy to build up models of cytochromes P450.

However, there is only one report available in the literature, by Green et al. *(50)*, describing an application of this simple strategy to models of cytochromes P450. MAbs were raised against Sn(IV)-*meso*-tetrakis (*para*-carboxyvinylphenyl)porphyrin (Sn-TpCVPP). Five of those Abs that bound the hapten with binding constants, ranging between 2.5×10^{-8} *M* and 1.6×10^{-7} *M*, were selected. The catalytic activity of the Ab–Mn(III)–TpCVPP complexes was then investigated, using as a model reaction the epoxidation of styrene by iodosylbenzene. Three biphasic media were studied for this reaction: reverse micelles, microemulsions, and solid catalyst in organic solvent. The best results were obtained with a solid catalyst, obtained by lyophilization of Ab–Mn–TpCVPP complexes, in CH_2Cl_2 at room temperature. Under those conditions, the five Ab-Mn(III)–TpCVPP complexes produced turnover numbers ranging between 424 and 537 turnovers per 17 h of reaction; solid Mn(III)–TpCVPP alone was not catalytic *(50)*. However, those turnover numbers were only 30–60% higher than that measured in the presence of a lyophilized mixture of a nonrelevant Ab and Mn(III)–TpCVPP (300 turnovers/17 h). In addition, because no asymmetric induction was observed in any of these experiments, it seems unlikely that the reaction occurred inside the binding site of the Abs. It is most probable that the catalysis was caused by Mn-porphyrin molecules bound unspecifically on the surface of the Ab protein.

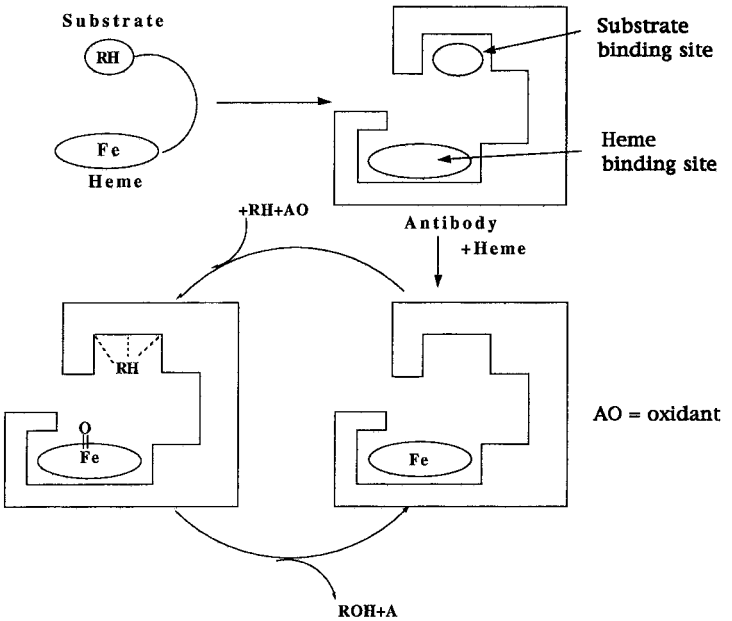


Fig. 5. One possible strategy to obtain catalytic Abs mimicking cytochrome P450, based on the generation of Abs raised against a porphyrin covalently linked to a substrate or an analog of substrate.

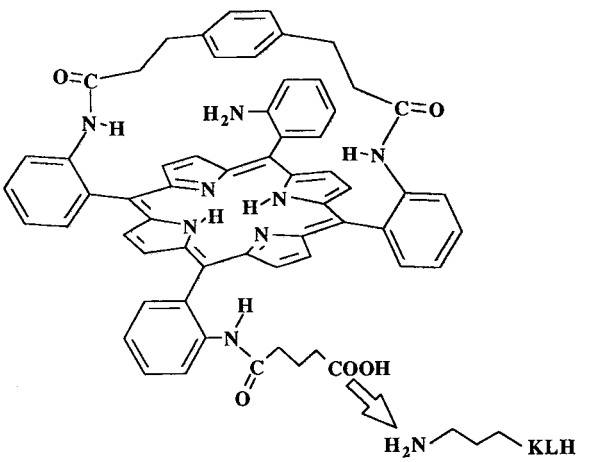


Fig. 6. Structure of the basket-handle porphyrin used as a hapten to obtain cytochrome P450-like Abs.

The authors initially thought about designing antimetalloporphyrin Abs, using the strategy described in Fig 5. The idea was to raise Abs against a heme covalently attached by an arm or a handle to the substrate or an analog of the substrate. The authors thus expected to induce in the

Abs both a binding site for the substrate and a binding site for the heme (Fig. 5), to obtain Ab–heme complexes that would be able to catalyze the selective oxidation of the substrate by oxygen. As an application of this strategy, mice were immunized with a basket-handle porphyrin bearing over the plane of the porphyrin a phenyl ring (Fig. 6; *65*). This hapten revealed to be poorly immunogenic and the few Abs obtained did not possess any cytochrome P450-like activity.

The primary reason for this failure is probably that the binding site of the Abs is not large enough to swallow big macromolecules such as basket-handle porphyrins. The authors then turned to another strategy, which aims at modifying antisubstrate Abs by covalent linkage of the Fe(III)-porphyrin, in order to obtain artificial hemoproteins able to catalyze the selective oxidation of the substrate.

SEMISYNTHETIC CATALYTIC ANTIBODIES AS MODELS OF CYTOCHROMES P450

The general strategy presented in Fig. 7 has been derived from the one used by Pollack and Schultz *(66,67)* to modify antiphosphocholine Abs by covalent linkage of an arm ending in an imidazole, in order to enhance their ester-hydrolyzing activity. It involves four key steps: (1) the incubation of the antisubstrate Ab with the substrate modified by an arm including a disulfide bridge and ending with a reactive group such as aryl chloride, acid, or aldehyde; (2) the chemical reaction between a lysine residue of the Ab and the terminal reactive group of the modified substrate; (3) the reductive cleavage of the disulfide bridge by dithiothreitol (DTT), which leads to the Ab labeled with an arm ending with a free thiol group close to the binding site; and (4) the covalent coupling of the Fe-porphyrin to the thiol group. Such a strategy should lead to the positioning of the Fe-porphyrin close enough to the substrate binding site to catalyze its regioselective oxidation.

The authors intended to use this strategy to transform an antiestradiol Ab into an artificial hemoprotein covalently linked to its Fe(III)-tetraarylporphyrin cofactor, which could be able to catalyze, in the presence of a bulky imidazole, the regioselective oxidation of steroids by oxygen donors such as PhI = O or H_2O_2 (Fig. 8).

For that, testosterone was chosen as a substrate, because it had a good affinity for the antiestradiol Ab ($K_d = 1.7 \times 10^{-7}$ *M*). In addition, its 3-keto function could be used as the starting point for the three step synthesis of an arm, including a cleavable disulfide bridge and ending in a reactive bromoacetyl group, as depicted in Fig. 9. Testosterone first reacted with carboxymethoxylamine, leading to the 3-O-carboxymethyl-oxime derivative. The cleavable disulfide bridge was then introduced by reaction with

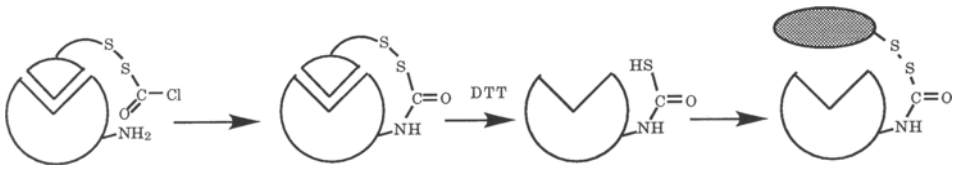


Fig. 7. General strategy for the production of semisynthetic catalytic Abs mimicking cytochrome P450.

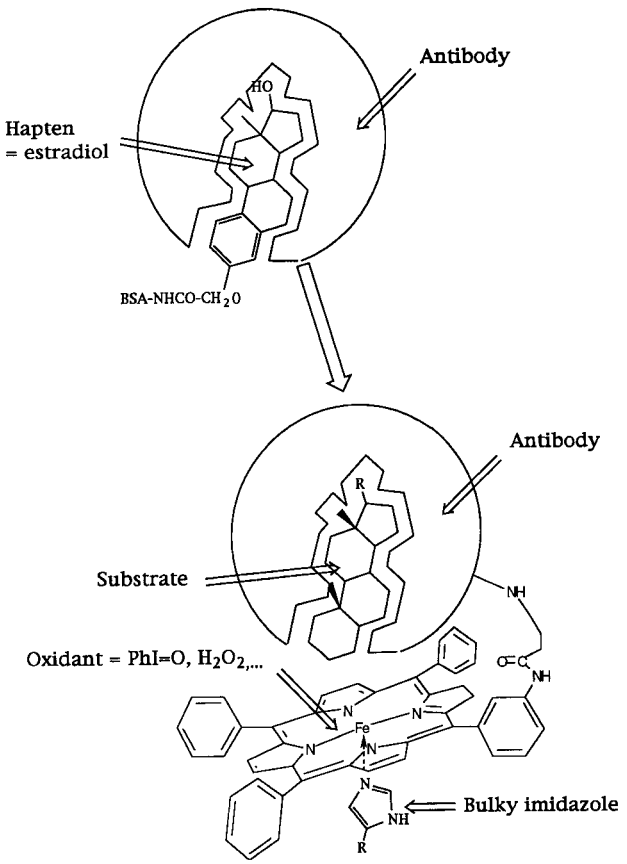


Fig. 8. Modification of an antiestradiol Ab into an artificial hemoprotein able to catalyze the selective oxidation of steroids.

cystamine, and a subsequent reaction with the succinimidyl ester of bromoacetic acid afforded the terminal bromoacetyl function (Fig. 9). Purified antiestradiol Abs were then incubated with the modified testosterone, and the covalent linkage of the arm was carried out under slightly basic conditions (Fig. 10). The disulfide bridge was further cleaved by reduction by DTT under nitrogen, and the testosterone moiety was eliminated by extensive dialysis under nitrogen (Fig. 10). Those two steps were controlled by competitive ELISA, using plates coated with the BSA–testosterone conju-

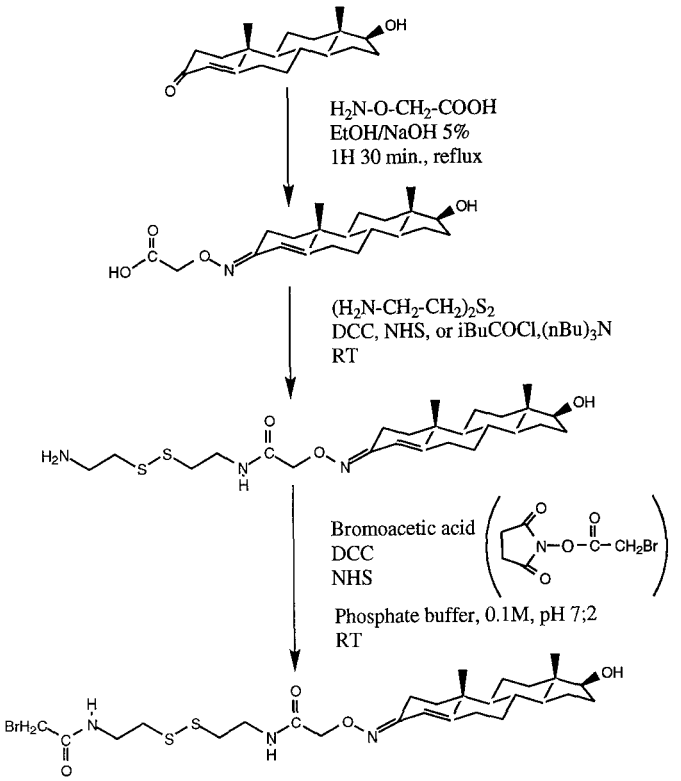


Fig. 9. Three-step modification of testosterone with an arm including a disulfide bridge and ended by an acetyl-bromide group. All the products were identified by ^{1}H NMR and mass spectroscopies.

gate. Indeed, after the former step, antiestradiol Abs only retained 30% of affinity for the immobilized testosterone, compared to that observed for the starting unmodified Abs. This was in agreement with the presence in, or close to, the binding pocket of a testosterone molecule covalently attached to the Ab protein, which prevented the binding of the Abs to the immobilized testosterone. Accordingly, after cleavage of the S—S bond by DTT and removal of the bound testosterone by dialysis, the affinity for the immobilized testosterone was almost totally restored, which showed that the binding site of the modified Abs had recovered its ability to bind the testosterone–BSA conjugate.

In parallel, an Fe(III)-tetra-arylporphyrin, specially designed to be coupled with the modified antiestradiol Abs was prepared in four steps from *meso*-mono(*ortho*-aminophenyl)triphenylporphyrine ($MAPTPPH_2$) (Fig. 11: *65, 68*). The successive reactions of $MAPTPPH_2$ with succinic anhydride and 1,4-diaminobutane led to the tetra-arylporphyrin bearing one 11-atom arm in *ortho* position of one of the *meso*-phenyl substituents. The Fe atom was then inserted into the macrocycle by a classical method

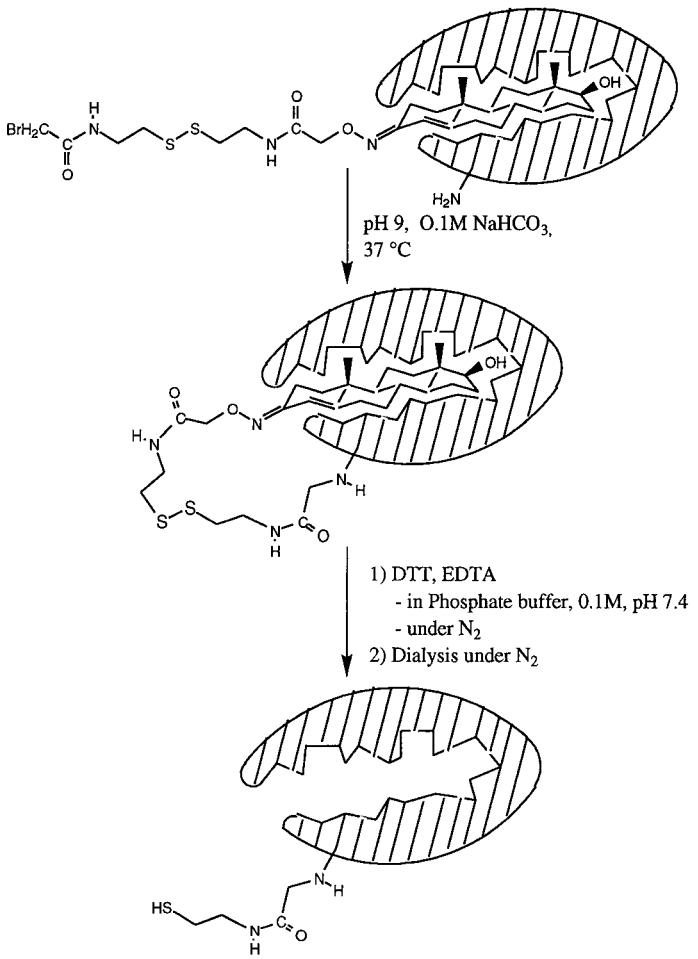


Fig. 10. Chemical labeling of an antiestradiol Ab with an arm ending in a thiol group.

(69), and the arm was activated by reaction of the terminal amino group with *N*-succinimidyl-3-(2-pyridyldithiopropionate) (SPDP) (Fig. 11). The final reaction of this activated porphyrin with the previously prepared Ab (Fig. 12) was carried out under nitrogen, at room temperature, in 0.1 *M* phosphate buffer, pH 7.6. This reaction was followed by UV-visible spectroscopy, which allowed monitoring of the formation of 2-pyridine-thiol absorbing at 343 mn and of the Ab–porphyrin complex absorbing at 426 nm. After purification on a P10 column, the isolated Ab–porphyrin complex exhibited a UV-visible spectrum typical of heme-proteins, with maxima at 280 and 426 nm (data not shown). Approximately 1.5 porphyrin was bound per Ab protein. Further work is underway to better characterize this new artificial hemoprotein and assay its catalytic activity.

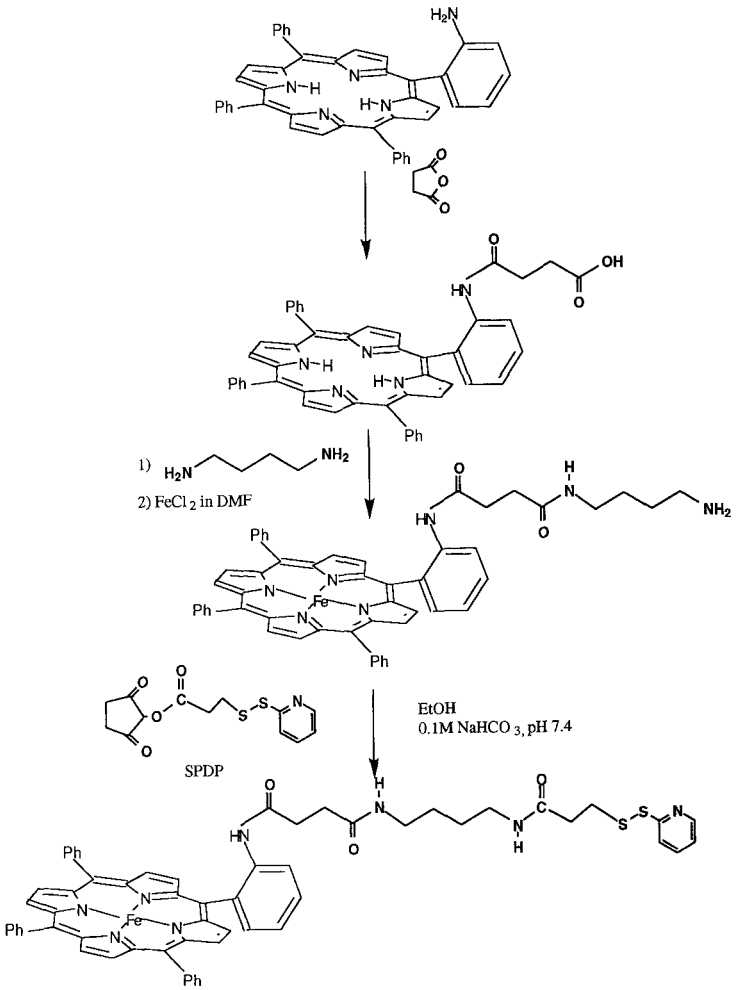


Fig. 11. Synthesis and activation by *N*-succinimidyl-3-(2-pyridyldithiopropionate) of an Fe(III)-tetra-arylporphyrin bearing in the *ortho* position of one *meso*-phenyl substituent an 11-atom arm. All the products were characterized by UV-visible, mass, and ^{1}H NMR spectroscopies.

CONCLUSION

The elaboration of catalysts based on the association of antiporphyrin Abs with metalloporphyrin cofactors appears to be a promising strategy for obtaining models of peroxidases. Indeed, Abs raised against N-substituted *(49,55)* or *meso*-aryl-carboxy-substituted *(56,60)* porphyrins have shown, in the presence of the corresponding Fe(III)-porphyrin cofactors, a significant peroxidase activity characterized by k_{cat}/K_m efficiencies ranging between 10^2 and $5 \times 10^3/M/s$. Those values are about 10 × higher than those observed for the free Fe(III)-porphyrins, but they still remain low

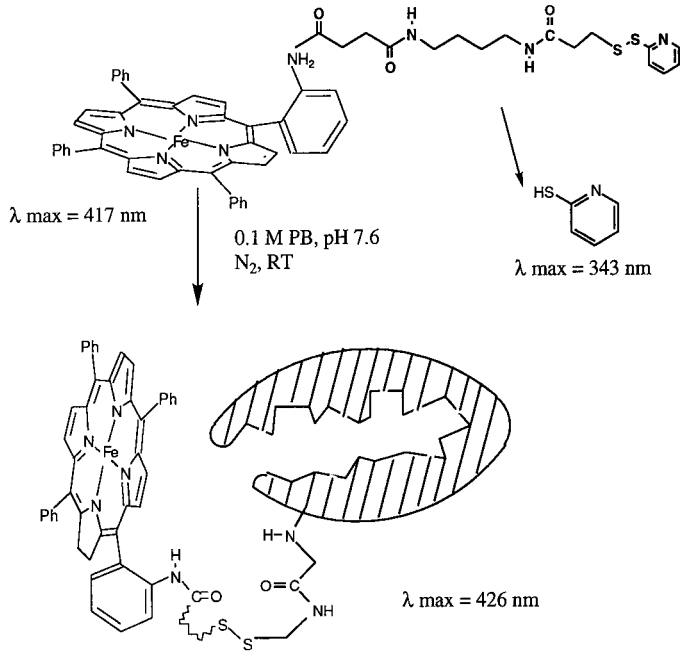


Fig. 12. Covalent binding of an Fe(III)-tetra-arylporphyrin to an antiestradiol Ab.

when compared to those of peroxidases, which are among the most efficient catalysts known, with k_{cat}/K_m values approaching $10^7/M/s$ *(59)*. This shows that the Ab contributes little to the catalytic efficiency of those systems, which could be explained by the fact that, whatever the porphyrin hapten used, neither amino acid residues participating in the catalysis of the heterolytic cleavage of the O—O bond of peroxides nor axial ligands of the Fe atom have been generated in the Ab binding site. Actually, the catalytic Abs described up until now as models of peroxidases provide two chief advantages: They site-isolate the Fe(III) porphyrin, preventing its bimolecular oxidative self-degradation, and they control the diffusion of H_2O_2 to the Fe-porphyrin. It is now time to think about the way to improve those models and to find catalysts with better efficiencies. This could probably be achieved either by modification of the already existing Abs using molecular biology, or through the design of new haptens. In that case, metalloporphyrins—including a metal ion having a high enough affinity for the macrocycle so that it could not be removed during the immunization process, or porphyrins N-substituted with a carefully chosen substituent—are likely to be the best candidates for generating in the Ab amino acids participating in catalysis and/or in binding the Fe atom.

It is not surprising that the strategy based on antiporphyrin Abs is particularly adapted to obtain models of peroxidases. Indeed, in those enzymes, the reducing co-substrate never enters their narrow active site,

but only interacts with the *meso* edge of the heme, which is exposed to the solvent. This is probably what occurs in the Ab–metalloporphyrin complexes. The results of Green et al. *(50)*, as well as the authors' preliminary results on the study of the binding site of the 13G10 antibody *(60)*, show that in those Abs about two-thirds of the porphyrin is included in the binding site (including the Fe) leaving one-third of the porphyrin exposed to the solvent. On the contrary, in the case of cytochromes P450, the substrate enters the active site and binds in a hydrophobic pocket, over the plane of the porphyrin close to the Fe(V)-oxo species. Thus, the strategy based on antiporphyrin Abs does not seem appropriate to obtain models of cytochromes P450, since not only Abs raised until now against rather flat porphyrins do not have any space over the plane of the porphyrin to bind the substrate, but also it has not been possible to obtain Abs having binding sites for both the substrate and the porphyrin, using as a hapten a porphyrin covalently linked to the substrate. Again, the modification of already existing antiporphyrin Abs by molecular biology could perhaps provide the Ab with a binding site for a substrate. However, the best strategy could probably be the one involving the modification of antisubstrate Abs by covalent linkage of an Fe(III)-porphyrin close to the binding site of the substrate, to obtain an artificial hemoprotein able to catalyze its regioselective oxidation.

Returning to the comparison with other models of hemoproteins mentioned at the beginning of this article, catalytic Abs have provided an interesting advantage, because they site-isolate the metalloporphyrin cofactor and protect it against oxidative degradation. However, since the initial goal from the catalytic Ab strategy was to elaborate efficient catalysts tailored for the selective oxidation of substrates, it is clear that the future work in this field will be directed towards improving the reactivity and introducing into the antiporphyrin Abs the elements necessary for the selectivity in the oxidation reactions.

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