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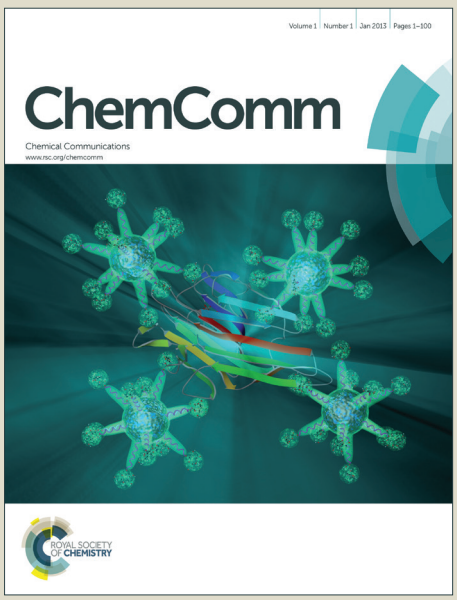

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ARTICLE

From "hemoabzymes" to "hemozymes": towards new biocatalysts for selective oxidations

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The design of artificial hemoproteins that could catalyze selective oxidations using clean oxidants such as O_2 or H_2O_2 under ecocompatible conditions constitutes a real challenge for a wide range of industrial applications. *In vivo*, such reactions are performed by heme-thiolate proteins, cytochromes P450, that catalyze the oxidation of substrates by dioxygen in the presence of electrons delivered from NADPH by cytochrome P450 reductase. Several strategies were used to design new artificial hemoproteins that mimic these enzymes. The first one involved the non-covalent association of synthetic hemes with monoclonal antibodies raised against these cofactors. This led to the first generation of artificial hemoproteins or "Hemoabzymes" that displayed a peroxidase activity and, in some cases catalyzed the regioselective nitration of phenols by H_2O_2/NO_2 and the stereoselective oxidation of sulfides by H_2O_2. The second one involved the non-covalent association of easily affordable non-relevant proteins with metalloporphyrin derivatives, using either the "Trojan Horse strategy" or the "host-guest" strategy. This led to a second generation of artificial hemoproteins or "Hemozymes", some of which were found able to catalyze the stereoselective oxidation of organic compounds such as sulfides and alkenes by H_2O_2 and $KHSO_5$.

1. Introduction

The importance of selective catalytic oxidations in modern chemistry has been crystallized by the attribution in 2002 of the Nobel Prize to Knowles, Sharpless and Noyori.[1,2,3] These processes have a wide spectrum of applications in areas with considerable economic potential, and wherever clean oxidations under mild conditions are needed: gas and petroleum industry - the large scale conversion of relatively cheap and easily available alkanes into more valuable oxidized products such as alcohols - decontamination and detoxification of industrial wastes, fine chemistry - synthesis of drugs and oxidation of molecules of pharmaceutical interest.

Considering fine chemistry, in most cases, drugs and their metabolites are obtained *via* total synthesis using processes that are long and only economically viable for products with a high added-value. Concerning the large scale conversion of relatively cheap and easily available alkanes into more valuable oxidized products such as alcohols, the major pathways involve stoichiometric oxidations and harsh conditions[4,5] that lead to low selectivities and are ecologically hostile.

Considering also the current economical and ecological context, the questions of setting up clean and eco-efficient processes as well as saving our energy resources appear of fundamental importance. There is thus a real need for developing reactions that would adress the problem of the selective oxidation of chemicals, even the poorly reactive hydrocarbons, under mild conditions.

In natural systems, such reactions are catalyzed by metal containing proteins, under conditions that perfectly fit to the current concept of "green chemistry".[6] This includes reactions taking place in water at room temperature under atmospheric pressure, the use of molecular dioxygen as a cheap, clean and naturally occurring reagent, and most of the time, catalysis at an iron active site, an ecologically friendly metal. The two main classes of metalloenzymes that process these reactions are non-heme iron such as, for example, Methane MonoOxygenase (MMO),[7] and heme enzymes such as cytochrome P450 dependant monooxygenases (Cyt. P450),.[8]

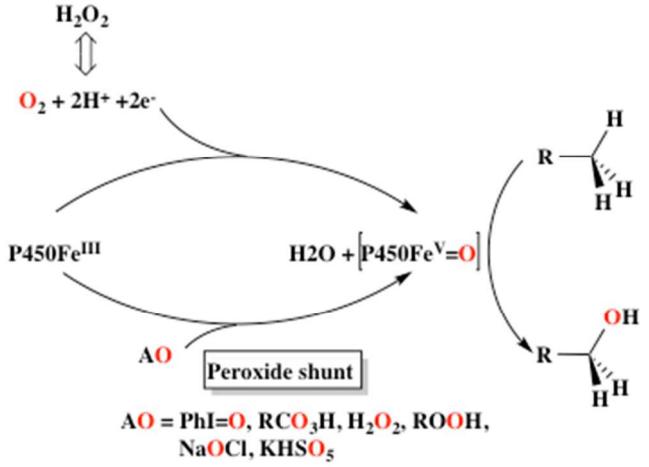
In the last 15 years, many teams have addressed the question of clean oxidation by elaborating artificial metalloproteins; a new kind of biocatalysts that would be able to perform efficiently, under mild conditions, regio- and stereoselective oxidation reactions and use when possible dioxygen as oxidant.[9] Several strategies have then been developed, either to increase or to modify the reactivity of already existing metalloproteins, or to induce a new reactivity by transforming a non-metal protein into a metalloprotein.

They include the introduction of metal binding sites in vacant crevasses of a protein whether by site-directed mutagenesis,[10-15] the insertion into binding sites of natural ligands,[16-19] the chemical modification of prosthetic groups,[20-23] or the covalent attachment of metal cofactors.[24-27] Several reviews have recently been published that describe the recent progress in the design of artificial metalloenzymes.[28-31]

Here we focus on artificial hemoproteins which we and others have built up in the hope to obtain new biocatalysts that would be able to reproduce the reactivity of Cytochrome P450s enzymes. A first generation of artificial hemoproteins which we named "Hemoabzymes", was obtained by the non-covalent association of a synthetic iron-porphyrins with monoclonal antibodies raised against these cofactors. A second generation of artificial hemoproteins which we named "Hemozymes", was obtained by the non-covalent association of a non-relevant protein with a tetraarylporphyrin.The different strategies developed in our group,the catalytic properties of the resulting artificial enzymes and their comparison with synthetic biocatalysts design by others are reported. Moreover, novel strategies for the design of biocatalysts able to use dioxygen as an oxidant are presented in conclusion.

2. Requirements for the elaboration of biomimetic systems for monooxygenase-like hemoproteins.

The cytochromes P-450 super family still remains one of the most investigated class of metalloenzymes that catalyze oxidation reactions under atmospheric conditions.[32] They are ubiquitous but a large number of them are mainly located in hepatocytes where they are able to oxidize a wide range of endogenous as well as exogenous substrates, such as drugs and pollutants (Scheme 1).



Scheme 1. oxidation of substrates catalyzed by Cyt. P450s.

In the catalytic cycle of Cyt. P450s, dioxygen is activated in the presence of 2 protons and 2 electrons that are provided by NADPH. One oxygen atom is then reduced to water and the other gives rise to an highly reactive iron(V)-oxo species[33,34] that is responsible for the hydroxylation of the substrates (Scheme 1). The nature of this powerful oxidizing intermediate has been deduced from cross-linked investigations using a broad array of spectroscopic techniques. [35,36]
However, activation of dioxygen is a complex process that involves sophisticated molecular machines, in which Cyt. P450 monoxygenases are associated to a cytochrome P450-reductase that transfers electrons from the two-electron donating cofactor NADPH to molecular dioxygen.[37,38] The electrons are then not directly transferred from NADPH to the iron center, but delivered one by one thanks to the two reductase flavin cofactors, FAD and FMN (Fig. 1).

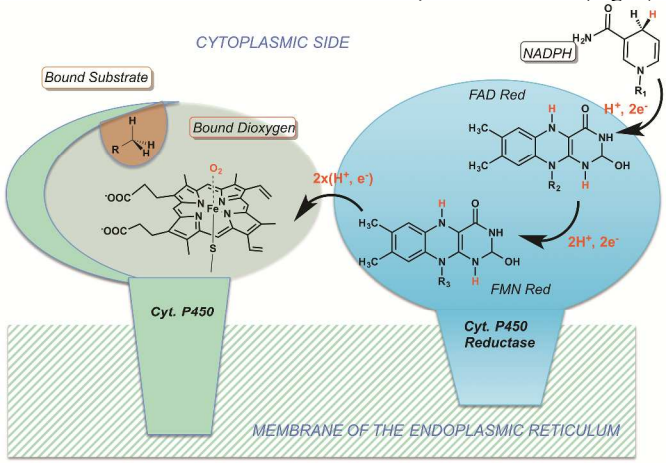


Fig. 1: Electron transfer through the cytochrome P450-cytochrome P450 reductase complex.

In vitro, high-valent iron-oxo species can directly be obtained by the so-called "peroxide shunt", from the reaction of iron(III)-P450 with various oxygen donating agents (AO) such as iodosylbenzene (PhI=O), peracids (RCO_3H), hydrogen peroxide (H_2O_2), alkylhydroperoxides (ROOH) or inorganic oxidants such as sodium hypochlorite (NaOCl), oxone ($KHSO_5$) (Scheme 1).[39] Model systems, associating an oxygen atom donor AO and an iron(III)- or manganese(III)-porphyrin, have been found to be able to perform all the reactions typical of cytochrome P450.[40,41] It thus seemed much easier to design biomimetic systems that used single oxygen atom donors (AO) as oxidant. In the results reported here hydrogen peroxide (H_2O_2) was most of the time used as oxygen atom donor since, first, it is a water soluble ecofriendly oxidant that leads to water as a by product and, second, it is a perfect equivalent of dioxygen activated with 2 electrons and 2 protons (Scheme 1).
The active site of P450s is a wide hydrophobic pocket that contains the prosthetic group, an iron(III)-protoporphyrin IX, which is bound to the apoprotein through a cysteinate axial ligand of the iron,[33,34] and a binding site for the substrate (Fig. 1). The apoprotein then controls the positioning of the substrate with respect to the heme, inducing the stereoselectivity of the reaction, modulates the redox potential of the heme, and protects the heme from oxidative degradations. Consequently, 3 structural elements have to be mimicked to elaborate artificial hemoproteins that possess the same characteristics than Cyt. P450 dependant monooxygenases: (i) The heme prosthetic group, that is responsible for the oxene-transfer reactions,[42,43] (ii) The apoprotein that binds and site-isolates heme, preventing its aggregation and its oxidative degradation, and its active-site amino acids, that provide an hydrophobic environment for the substrate and control its access to the heme, and (iii) The cysteinate proximal axial ligand of the iron atom, that has a role in controlling the heme redox potential and the reactivity of the heme iron.[44]
To reach this goal, monoclonal antibodies raised against porphyrin derivatives were first used, which, when associated with the corresponding metal-porphyrins, led to artificial hemoproteins named "Hemoabzymes". Second, other proteins of known sequence and 3D structure, that were cheap and available in large amounts and, in addition, that were heat and pH resistant and possessed a wide enough cleft to be able to incorporate the metal cofactor and a subtrate were used. This gave rise to a new kind of artificial hemoproteins named "Hemozymes". We used two proteins, xylanase 10A (Xln 10A) from *streptomyces Lividans* and neocarzinostatin (NCS), whereas other groups in the world used proteins such as Human and Bovine serum albumin[40,45-47] and apo-myoglobin[48-55] in which metalloporphyrins, metallochlorins, metallocorroles or metal-salen complexes were inserted.

3. Design of new artificial hemoproteins based on monoclonal antibodies "hemoabzymes"

The production of monoclonal antibodies against transition state analogs was a successful strategy for the production of antibodies that were able to catalyze a wide range of reactions.[56-67] However, most of these catalytic antibodies had modest catalytic efficiencies, which led researchers to envision other strategies such as the association of antibodies with inorganic cofactors,[68,69] natural cofactors,[70] metal ions,[71-73] or metal cofactors.[74-101] In this strategy, the antibodies were designed either to bring into close proximity the cofactor and the substrate, or to bind tightly the cofactor to enhance its reactivity. Antibodies then appeared as host-proteins of choice to build artificial hemoproteins by supramolecular anchoring of a metalloporphyrin structurally close to the antigen. To fulfil the previously mentioned requirements, the main difficulty then consisted in designing the ideal porphyrin hapten, which could induce in the paratope of the antibody, not only a binding site for the metalloporphyrin, but also a hydrophobic pocket to accommodate the substrate and, if possible, an axial ligand of the iron.

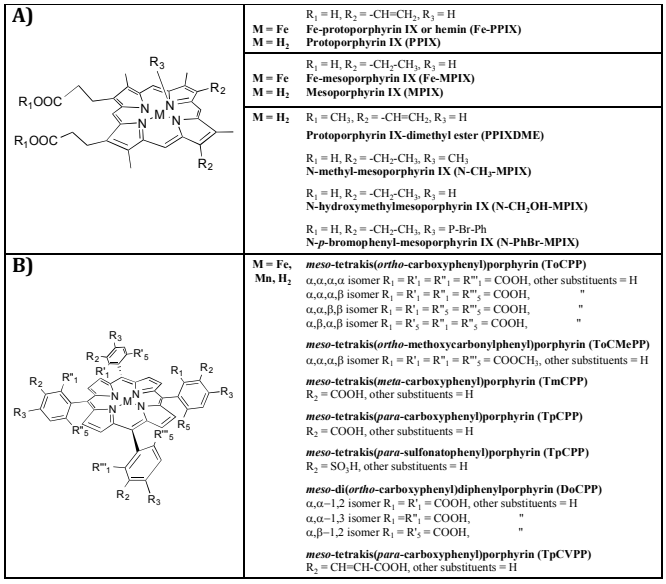


Fig. 2: Structure and nomenclature of A) the various natural heme derivatives and B) synthetic tetraarylporphyrins mentioned in this article.

Monoclonal antibodies, raised against specifically designed natural heme derivatives (Fig. 2A) or synthetic tetraarylporphyrins (Fig. 2B), have been obtained and were found to be able to bind non-covalently metalloporphyrin cofactors with a high affinity, and the corresponding metalloporphyrin-antibody complexes displayed either a Cyt. P450 like or a peroxidase-like activity.

3.1. Antibody-porphyrin complexes with a peroxidase-like activity.

Cochran and Schultz first reported in 1990 a metalloporphyrin-antibody complex catalytically active in oxidation.[77,78] It was obtained by associating iron mesoporphyrin IX (Fe-MMP) (Fig. 2A) as metal cofactor, with a monoclonal antibody 7G12 that was generated against N-methylmesoporphyrin IX (*N*-MMP) initially designed as an analogue of the transition state in the metallation of porphyrins by ferrochelatases. X-ray diffraction studies of the 7G12-Fe-MMP complex[94] (Fig. 3) showed that approximately 2/3 of the porphyrin were interacting with the antibody pocket, 3 pyrrole rings being packed tightly against residues of the V_H domain and two pyrrole rings packed against tyrosine residues of the V_L domain.

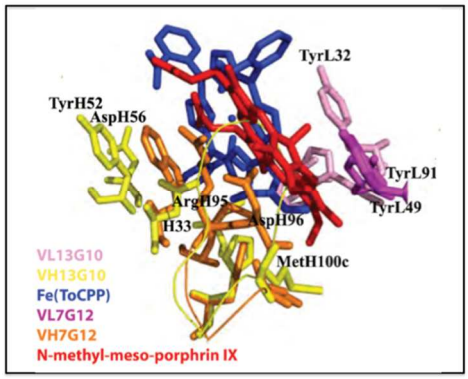
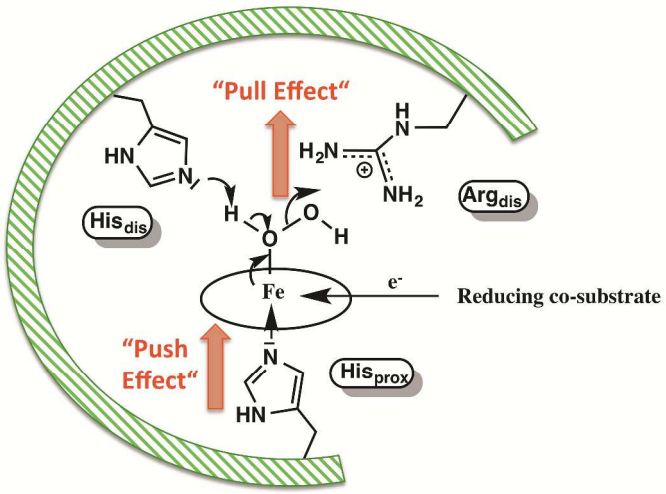


Fig. 3. Comparison of the 3D structures of the 7G12-NMPP and 13G10-Fe(ToCPP) complexes.

A methionine, H100c, interacted specifically with one pyrrole ring and forced it to adopt a tilted conformation which should be favorable for the insertion of metal ions in the porphyrin ring and could explain the ferrochelatase activity of antibody 7G12.[77]

The 7G12-Fe-MMP complex showed a peroxidase activity, catalyzing the oxidation of *o*-dianisidine, pyrogallol and 2,2'-azinobis(3-ethylbenzothiazoline-6-sulfonic acid) (ABTS) by H_2O_2, with a catalytic efficiency more than four time higher than with Fe-MMP alone ($k_{cat}/K_M(H_2O_2)$ = 1.6x10^4 M^{-1}.min^{-1} and 3.8x10^3 M^{-1}.min^{-1} resp.).[78]

We ourselves produced hemoabzymes with a peroxidase activity. For this, we first considered the so-called "push-pull" mechanism of heme peroxidases, that are able to catalyze the reduction of hydroperoxides H_2O_2 or ROOH, by electrons that are delivered by various reducing co-substrates (Scheme 2).[102,103] The push effect is provided by the axial proximal ligand on the iron His_{prox} that reinforces electron density on the iron atom. An iron-porphyrin was thus used as a hapten, expecting that the iron would induce in the antibody pocket an amino acid residue that would mimic the His_{prox} ligand of the iron in peroxidases. On the distal face of the heme, the key step of the mechanism, the heterolytic cleavage of the O-O bond, is assisted by the pull effect of the distal histidine His_{dis}, that acts as a general base and deprotonates the peroxide and a distal arginine Arg_{dis}, that polarizes the O-O bond (Scheme 2). Fe^{III}-α,α,α,β□*meso*-tetrakis(*ortho*-carboxyphenyl)porphyrin, α□β□Fe(ToCPP) (Fig. 2B) was chosen as a hapten in the hope of generating, opposite to the *ortho*-carboxylate substituents of the phenyl rings, amino acid residues such histidine or arginine that would mimic the His_{dis} and Arg_{dis} of peroxidases.



Scheme 2: schematic view of peroxidases and mechanism of the heterolytic cleavage of the O-O bond of hydroperoxides catalyzed by peroxidases.

Two antibodies, 13G10 and 14H7, that recognized α□β□Fe(ToCPP) with the best affinities found until now for anti-porphyrin antibodies, (K_D values of 2.9 nM and 5.5 nM resp.) were produced.[88] UV-visible spectroscopy studies, associated with the determination by competitive ELISA of the apparent dissociation constants (K_D) for various porphyrins suggested first that no amino acid residue was induced in the antibody pocket that was able to coordinate the iron atom of the hapten. Second, a comparison of the K_Ds for the four atropoisomers of $ToCPPH_2$ (Fig. 2B) showed that the *ortho*-carboxylate substituents of the phenyl rings of the hapten were crucial for the recognition of α□β□Fe(ToCPP).[93] Both studies allowed us to propose a binding site topology of antibodies 13G10 and 14H7 in which roughly two-thirds of the porphyrin macrocycle could be inserted in the binding pocket with mainly three carboxylates, in α,α,β positions, being recognized by amino acids of the antibody, one □-carboxylate substituent being outside the binding pocket.

These assumptions were recently confirmed by the determination of the 3D structure of the α□β□Fe(ToCPP)-13G10 and -

14H7complexes (Fig. 4) using X-ray diffraction studies, associated to molecular modeling studies.[104] It appeared that the two λ-light chain anti-porphyrin antibodies, 13G10 and 14H7, possess shallow hapten binding pockets compared with the other structurally characterized catalytic antibodies. The structural complementarity of $\alpha_2\beta_2$Fe(ToCPP) to the hydrophobic binding pocket of antibodies 13G10 and 14H7 thus leads to a remarkable thermostability of the $\alpha_2\beta_2$Fe(ToCPP)-antibody complexes. Molecular modeling further indicates that the recognition of various porphyrins with different carboxy-phenyl substituents is achieved mainly by stacking interactions but also by crucial hydrogen bonds with two or three carboxylate groups and that no proximal ligand of the iron is induced in 13G10 and 14H7 in agreement with the binding site topology reported above.

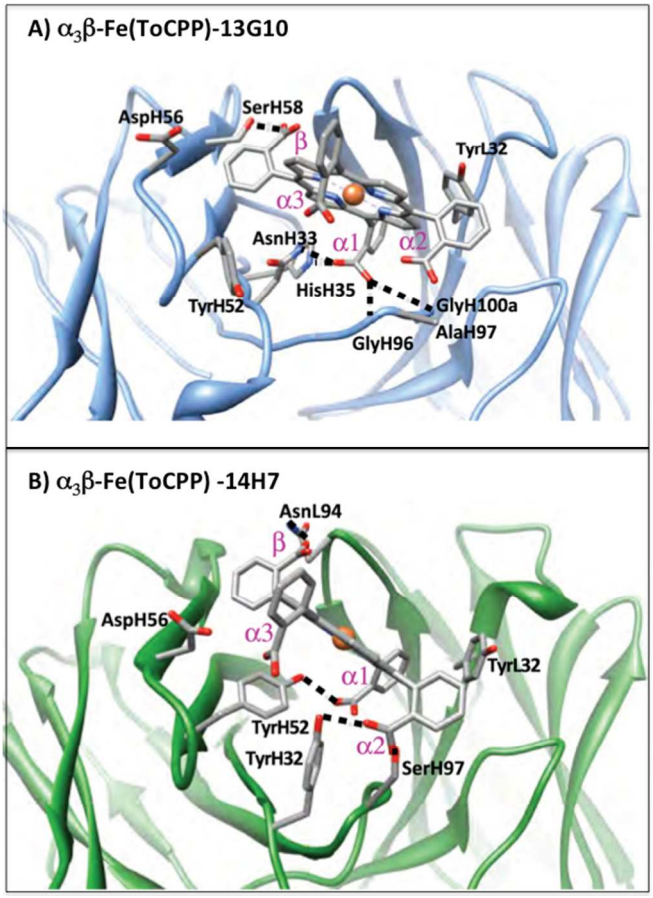


Fig. 4. 3D-structure of the 13G10- and 14H7-Fe(ToCPP) complexes.

The two $\alpha_2\beta$-Fe(ToCPP)-13G10 and -14H7 complexes catalyzed the oxidation of ABTS by H_2O_2 with a five time better efficiency than ToCPPFe alone (Table 1).[88] In both cases, the protein exerted a protecting effect toward the oxidative degradation of the porphyrin. In addition, $\alpha_2\beta$–Fe(ToCPP)-13G10 was found able not only to catalyze the oxidation of a variety of co-substrates such as o-dianisidine, pyrogallol, ABTS and TMB (Tetramethyl-benzidine) by H_2O_2 but also to use a wide range of hydroperoxides as substrates, including alkyl-, aryl- and fatty acid hydroperoxides.[95] pH dependence studies showed that the peroxidase activity of $\alpha_2\beta$-Fe(ToCPP)-13G10 was optimal at pH 4.6, with a k_{cat} value of 560 min^{-1} (Table 1) which led us to propose that a carboxylic acid side chain of the antibody could participate in the reaction mechanism.[95]
When examining the 3D structure of the $\alpha_2\beta_2$Fe(ToCPP)-13G10 and -14H7 complexes it appeared that neither a histidine nor a arginine residue correctly positioned to participate in the heterolytic cleavage of the O-O bond of peroxides, like in peroxidases, was present in the binding pocket of 13G10 or 14H7 (Fig. 4). In addition, contrary to what kinetic studies suggested, no COOH side-chain of the 13G10 antibody was correctly localized to act as a general acid-base catalyst. The only carboxylic acid side-chain in the combining site of 13G10 that could play a role in catalysis was that of AspH56 (Fig. 4), but its nearest oxygen atom is positioned 10.6 Å away from the iron, a distance that necessitates a mediating water molecule for it to act as a general acid catalyst in the peroxidase reaction. The increase of the peroxidase activity of $\alpha_2\beta_2$Fe(ToCPP), when bound to the antibodies, could then be explained by a loss of entropy due to accessibility of H_2O_2 to only one of the two faces of the porphyrin ring, and possibly by the stacking of several aromatic groups onto the porphyrin ring that would stabilize the [iron(IV)oxo-porphyrin]$^{+\cdot}$ intermediate in the peroxidase reaction. Moreover, one of the buried carboxylic group of the porphyrin substituents may also participate as a general acid catalyst, with the implication that H_2O_2 would bind on the sheltered face of the porphyrin ring. The carboxylic group would facilitate the heterolytic cleavage of the O-O bond, with the release of a water molecule and formation of the highly reactive iron (V)-oxo intermediate.
Figure 3 compares the structure of 13G10 with that of antibody 7G12 reported by Cochran and Schulz.[77] Both structures share some common general features. Both antibodies present shallow binding sites due to a bulky residue preceding Met H101 that is positioned at the bottom of the cavity, the one of 13G10 being shallower in comparison than in the 7G12 system (Fig. 3). As mentioned above, the porphyrin haptens are then positioned at the surface of the combining site with about 2/3 of the porphyrin interacting with the antibody pocket in both cases. However, concerning the coordination of the iron atom, in 7G12, the carboxylate side-chain of AspH96 positioned 1.9 Å from the center of the porphyrin ring (Fig. 3) is correctly positioned to act as a proximal ligand for the iron atom of Fe^{III}-mesoporphyrin IX whereas in 13G10 no residue is correctly positioned to bind the iron of Fe(ToCPP).
Concerning catalysis, in 7G12 like in 13G10, no distal ligands susceptible to enhance the peroxidase activity of Fe^{III}-mesoporphyrin IX is revealed. Thus, it is reasonable to think that H_2O_2 approaches the non-obstructed side of the porphyrin ring, opposite to AspH96, which is surrounded only by hydrophobic residues such as TyrL49 and TyrL91 (Fig. 3). Since these residues are involved in π-stacking interactions with the porphyrin ring, it was proposed like in 13G10, that π-cation interactions between those aromatic groups and the [iron(IV)porphyrin]$^{+\cdot}$ intermediate could help to stabilize the radical cation on the porphyrin ring and yield to higher peroxidase activity.[105]

3.2. Comparison of the peroxidase activity of various antibody-Fe-porphyrin complexes

In addition to the above mentioned 7G12-, 13G10- and 14H7-Fe-porphyrin hemoabzymes, several other Fe-porphyrin-antibody complexes also showed a peroxidase activity. These antibodies were elicited against synthetic porphyrins such as *meso*-carboxyaryl substituted-,[75,80,85,88-91,93,95,96] N-substituted-, [78,79,84,86,87,91,97] tin-[79,81] or palladium-porphyrins.[82,83]
N-methylmesoporphyrin IX was thus also used as a hapten by two other groups[84,91] who respectively got 2 monoclonal antibodies, 2B4 and 9A5. The 9A5- and 2B4-Fe^{III}-MPIX complexes catalyzed respectively the oxidation of pyrogallol,[84] *o*-dianisidine and ABTS[91] by H_2O_2. Feng et al.[84] further raised a monoclonal antibody 11D1 against N-hydroxymethy1-mesoporphyrin IX (N-CH_2OH MPIX) (Fig. 2A), in which the oxygen atom of the hydroxymethyl group was supposed to mimic the H_2O_2 oxygen atom that coordinates to the

iron atom of heme peroxidases. The obtained 11D1-Fe^{III}-MPIX complex catalyzed the oxidation of pyrogallol by H_2O_2. Finally, Takagi et al.[85] used meso-tetrakis(para-carboxyphenyl)porphyrin ($TpCPPH_2$) as hapten (Fig. 2B). The corresponding Fe-TpCPP-antibody complexes catalyzed the oxidation of ABTS and pyrogallol by H_2O_2, and the best catalyst for this reaction was found to be the complex associating Fe(TpCPP) with a recombinant antibody light (L) chain 13-1-L, which they named L-zyme.

A comparison of the kinetic parameters for the oxidation of various co-substrates by H_2O_2, catalyzed by Fe^{III}-porphyrin-antibody complexes and by the corresponding free Fe^{III}-porphyrins with those reported for horseradish peroxidase[91,106,107] is presented in Table 1.

From table 1, iron-porphyrin-antibody complexes appear to have a better peroxidase activity than Fe-porphyrins alone. Indeed, the former complexes display k_{cat} values ranging between 86 and 667 min^{-1}, the best value being observed for the oxidation of pyrogallol by H_2O_2 catalyzed by Fe(TpCPP)-13-1-L.[85] Those values are 2.4-6.3 fold higher than those reported for Fe^{III}-porphyrins, that range from 21 to 169 min^{-1}. Fe-porphyrin-antibody complexes also had a better affinity for H_2O_2, as shown by K_M values ranging respectively between 2.3-35 mM for Fe-porphyrin-antibody complexes and 43-100 mM for free Fe-porphyrins. As a consequence, Fe^{III}-porphyrin-antibody complexes exhibited higher catalytic efficiencies with k_{cat}/K_M values ranging between $3.7x10^3$ and $2.9x10^5$ $M^{-1}min^{-1}$, than the corresponding free Fe-porphyrins, with k_{cat}/K_M values ranging between $2.4x10^2$ and $3.8x10^3$ $M^{-1}min^{-1}$. The L-zyme exhibited both the higher k_{cat} value, 667 min^{-1} and the lower K_M value, 2.3 mM[85] and consequently the highest k_{cat}/K_M value, 2.9 x 10^5 $M^{-1}s^{-1}$ that exhibit k_{cat}/K_M values of about $6x10^8$ $M^{-1}min^{-1}$,[91,106,107] which is mainly due to the very good affinity of the enzyme for H_2O_2 with a K_M value of 0.5 μM.

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})	$K_M(H_2O_2)$ (mM)	k_{cat}/K_M ($M^{-1}min^{-1}$)	ref.
HRP		leucomalachite green	306	0.0005	$6.1x10^8$	102
		o-dianisidine	394	24	$1.6x10^4$	
Fe^{III}-MPIX[a]7G12	N-CH_3-MPIX	ABTS[d]	-	-	$1.4x10^4$	78
		pyrogallol	-	-	$7.3x10^3$	
Fe^{III}-MPIX		*o*-dianisidine	166	43	$3.8x10^3$	78
Fe^{III}-MPIX-9A5	N-CH_3-MPIX	pyrogallol	132	35	$3.7x10^3$	84
Fe^{III}-MPIX-11D1	N-CH_2OH-MPIX	pyrogallol	86	13	$6.6x10^3$	84
Fe^{III}-MPIX		pyrogallol	21	100	$2.4x10^2$	84
Fe^{III}-TpCPPb-13-1 L	$TpCPPH_2$	pyrogallol	667	2.3	$2.9x10^5$	85
Fe^{III}-ToCPP[c]-13G10	Fe(ToCPP)	ABTS	560	16	$3.7x10^4$	88
Fe^{III}-ToCPP-14H7	Fe(ToCPP)	ABTS	63	9	$7.1x10^3$	88
Fe^{III}-ToCPP		ABTS	51	42	$1.2x10^3$	88
Fe-MPIX-2B4	N-CH_3-MPIX	*o*-dianisidine	330	43	$7.7x10^3$	91
Fe-MPIX		*o*-dianisidine	77	25	$3.1x10^3$	91
MP8-3A3	MP8	*o*-dianisidine	885	0.45	$2x10^6$	101
MP8		*o*-dianisidine	590	0.4	$1.45x10^6$	101

[a]for the abbreviations of porphyrins see figs 2 and 3.

3.3. Hemoabzymes with a monooxygenase-like activity.

The first attempt to get hemoabzymes that would be able to catalyze mono-oxygenation reactions was reported by Keinan et al. in 1990.[79] Monoclonal antibodies that bound Sn^{IV}-meso-tetrakis(*para*-carboxyvinylphenyl)porphyrin (Sn^{IV}-TpCVPP) (Fig. 2B) with K_D values ranging between 25 and 163 nM, were first produced, and the ability of the corresponding antibody-Mn^{III}(TpCVPP) complexes to catalyze the epoxidation of styrene by iodosylbenzene was investigated. Lyophilized antibody-Mn(TpCVPP) complexes were used as catalysts, in CH_2Cl_2 at room temperature. Under those conditions, between 424 and 537 turnovers were produced per 17 h of reaction, whereas lyophilized Mn(TpCVPP) alone was not catalytic.[79] However, no asymmetric induction was observed and, in addition, a lyophilized mixture of a non relevant antibody and Mn(TpCVPP) led to about the same result (300 turnovers/17 h). This could indicate that the reaction was catalyzed by Mn-porphyrin molecules bound unspecifically on the surface of the antibody rather than inside its binding site.

In a second report by Liu et al.[97] N-4-bromophenyl-mesoporphyrin IX was used as a hapten in the hope of generating in the antibodies binding site a hydrophobic pocket to accomodate aromatic substrates. Two complexes, associating antibodies 7C7 and 4D4 with ferric mesoporphyrin IX, Fe^{III}-MPIX, catalyzed the epoxidation of styrene by NaOCl with respective turnovers of 0.48 and 0.98 min^{-1} and the 4D4-Fe-MPIX catalyzed aminopyrine N-demethylation by H_2O_2 with a k_{cat}/K_M of 1419 $M^{-1}.s^{-1}$.

Finally, Nimri and Keinan in 1999[98] used as a hapten a stable water soluble tin^{IV}-porphyrin bearing an axial α-naphtoxy ligand (Fig. 5A), that was designed to mimic the postulated transition state of the reaction of S-oxidation of aromatic sulfides, as well as the approach of iodosylbenzene, used as the oxidant, towards the metal cation (Fig. 5B). The complex associating one of the selected monoclonal antibodies (SN 37.4) and a Ru(II) porphyrin cofactor, catalyzed the stereoselective sulfoxidation of aromatic sulfides such as thioanisole by iodosylbenzene, the S-enantiomer of the sulfoxide being obtained with a 43% enantiomeric excess (Fig. 5C).

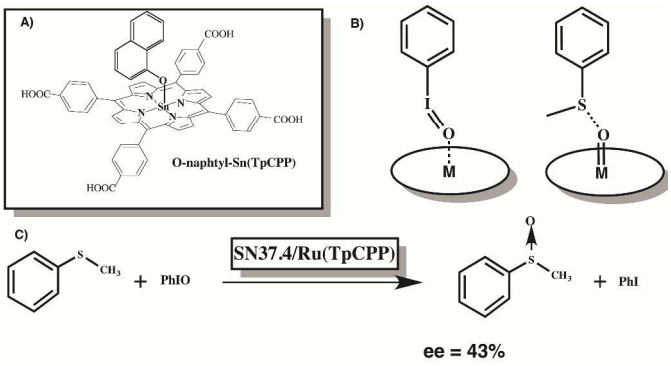


Fig. 5. A) Tin(IV)-*meso*-tetrakis(para-carboxyphenylporphyrin used as hapten, with an α-naphtoxy ligand. B) Approach of iodosylbenzene and thioanisole withrespect to the metal heme. C)Sulfoxidation of thioanisole by iodosylbenzene catalyzed by the SN 37.4-Ru(TpCPP) complex.

3.4. Improving hemoabzymes? : providing the iron atom of the antibody-porphyrin complexes with an axial ligand

The hemoabzymes described above are not as efficient catalysts as their natural hemoprotein counterparts. k_{cat}/K_M values three to four orders of magnitude lower than those for natural peroxidases have been reported for those with a peroxidase-like-activity (Table 1) whereas hemoabzymes with a monooxygenase-like activity do not catalyze very efficiently the oxidation of substrates such as alkenes. This could be due to the absence, in those antibodies, of an axial donating amino-acid ligand of the iron, such as a histidine (peroxidases) or a cysteinate (cyt. P450), that would facilitate the heterolytic cleavage of the O-O bond of H_2O_2 (push effect, Scheme 2).[48,108] Two strategies were then developped to obtain hemoabzymes with better activities: (i) exogenous imidazole was added to 13G10-Fe^{III}(porphyrin) complexes, as an axial coordinating ligand of the iron,[96] (ii) antibodies were produced against a new hapten, microperoxidase 8 (MP8), a heme octapeptide obtained by

hydrolytic digestion of horse heart cytochrome c,[109] that contained in particular an axial ligand of the iron, the imidazole of its His 18 residue (Fig 7).

3.4.1. Iron(III)-imidazole-porphyrin-antibody complexes. The influence of imidazole on the activity of 13G10-Fe^{III}-porphyrin complexes was first investigated. All of them only bound one imidazole ligand on the iron atom, but, whereas imidazole inhibited the peroxidase activity of the $\alpha_3\beta$-Fe(ToCPP)-13G10 complex, it enhanced that of the complexes of antibody 13G10 with less hindered iron(III)-α,α- and α,β-1,2-*meso*-di(ortho-carboxyphenyl)porphyrin (DoCPP) by a factor 8-9, leading thus to nice mimics of heme peroxidases.[96]

Molecular docking experiments allowed explaining this difference in reactivity of both 13G10-Fe-porphyrin complexes. Indeed, imidazole was predicted to bind to the solvent-exposed face of Fe^{III}(ToCPP) (Fig. 6A), which might prevent H_2O_2 from binding to the sheltered face of the cofactor and lead to the inhibition of the peroxidase activity. On the contrary, imidazole was predicted to bind on the opposite face of α,α□ or α,β-Fe^{III}(DoCPP) (Fig. 6B) in a higher affinity cavity formed between 13G10 and the Fe-porphyrin macrocycle, which could explain that the affinity for imidazole of both 13G10-Fe^{III}(DoCPP) complexes was 2–3 fold higher than that of 13G10-Fe^{III}(ToCPP). Imidazole would thus be located appropriately to act as the proximal histidine and H_2O_2 would bind on the solvent-exposed face of the porphyrin. In addition, TyrL32 could act as an acid catalyst and facilitate the cleavage of the O-O bond of hydroperoxide. Alternatively, in the case of α,β-Fe^{III}(DoCPP), the β carboxylate of the meso-carboxy-phenyl substituent could also play this role.

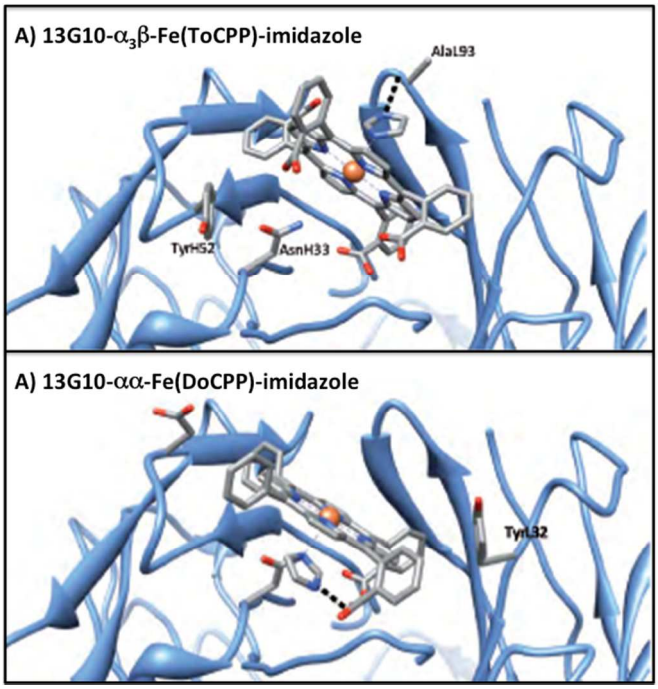


Fig. 6. 3D-structure of (A) the 13G10-Fe(ToCPP)-imidazole and (B)13G10-Fe(DoCPP)-imidazole complexes determined by molecular docking experiments.

3.4.2. Microperoxidase 8-antibody complexes. MP8 is a heme octapeptide that contains the amino acid residues 14 to 21 of horse cytochrome c, including His 18 whose imidazole acts as the axial ligand of the iron (Fig. 7). It also contains four carboxylate substituents, two from the propionate side chains of the heme, and two from the C-terminal glutamate (Glu 24) of the octapeptide, which could help anchoring it in the antibody binding site.

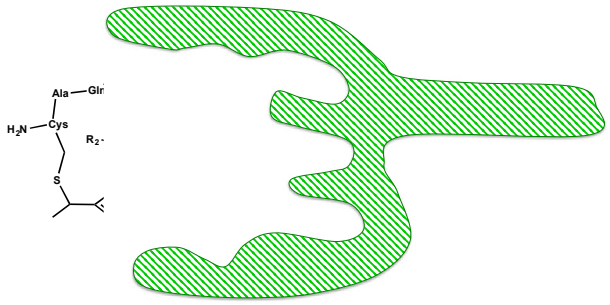


Fig. 7. Schematic view of anti-microperoxidase 8 (MP8) antibodies.

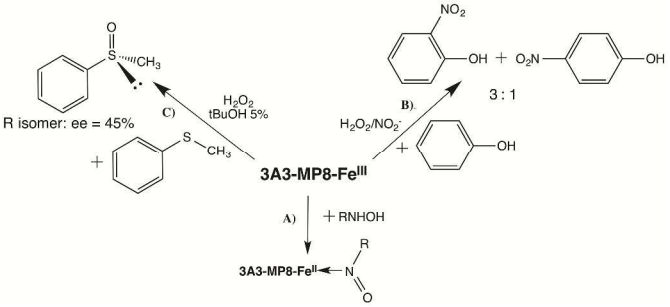
MP8 first possesses a peroxidase-like activity and is able to perform the oxidation of several typical peroxidase co-substrates like *o*-dianisine,[110] ABTS,[111] guaiacol.[112]It also displays a monooxygenase like activity and catalyzes the para-hydroxylation of aniline,[113] the S-oxidation of sulfides,[114] the monooxygenation of polycyclic aromatic compounds,[115] and the N- and O-dealkylation of aromatic amines and ethers.[116] MP8 was also shown to catalyze the oxidation of N-monosubstituted hydroxylamines with formation of very stable iron(II)-nitrosoalkane complexes,[117] and the nitration of phenols by nitrite in the presence of H_2O_2.[118] Unfortunately, two main drawbacks limit the use of MP8 as a catalyst: it dimerizes and aggregates at concentrations higher than 2 μM,[109] and is de-activated by oxydative degradation of the heme moiety.[112] It was then reasonable to expect that the binding of MP8 to an antibody receptor would allow its site-isolatation consequently keeping it under a monomeric catalytically active form and to protecting its heme against oxidative degradation. An additional the objective was of course to induce a selectivity in the oxidation reactions catalyzed by MP8.

Seven monoclonal antibodies were found to recognize MP8, and the best of them, IgG1 3A3, bound it with an apparent K_D of 10^{-7} M, which was in the range of the apparent K_D values already described for IgG-metalloporphyrin complexes.[83,119] A typical high-spin hexacoordinate iron(III) UV-visible spectrum, very similar to that of MP8-Fe^{III}, was recorded for the 3A3-MP8 complex, with only a lower intensity of the soret band at 396 nm. This could be the sign of a simple hydrophobic interaction between MP8 and the protein without any amino-acid side chain acting as a sixth axial ligand of the iron. Accordingly, the determination by competitive ELISA of the apparent dissociation constants (K_D) for various porphyrins suggested that the central iron atom of the hapten was not recognized by the antibody whereas the carboxylate substituents of the MP8 hapten played a key role in its recognition by the antibody. Additionally, coordination chemitry of the iron, through a comparison of the affinities of the tert-butyl-isonitrile ligand for the iron of MP8 or for that of 3A3-MP8, in the reduced and in the oxidized state, clearly indicated that a steric hindrance was brought by the protein on the distal face of MP8.[120] A possible binding site topology was then proposed for antibody 3A3, in which MP8 binds to the antibody thanks to interactions of the carboxylates from the heme propionates and from the C-terminal glutamate, opposite to the octapeptide chain, and the protein brings a partial steric hindrance of the distal face of MP8 (Fig. 7).

The 3A3-MP8 first displayed a peroxidase activity that was higher than that of MP8 alone as shown by the larger initial reaction rates measured for the oxidation of *o*-dianisidine, ABTS, guaïacol, and p-cresol.[101] As expected, the antibody protected the heme from oxidative degradations, and the percentage of co-substrate oxidized at the end of the reaction was 15-70 % larger with 3A3-MP8. Similar

results were obtained by Kawamura-Konishi et al. with the 2B4-MPIX-Fe complex as catalyst.[91] The kinetic parameters for the oxidation of *o*-dianisidine by H_2O_2, catalyzed by either 3A3-MP8 or MP8, were compared to those already reported for the oxidation of various co-substrates by H_2O_2 catalyzed by other hemoabzymes (Table 1). Both 3A3-MP8 and MP8 had a similar affinity for H_2O_2 as shown by respective K_M values of 0.45 and 0.4 mM, but 3A3-MP8 exhibited slightly higher k_{cat} (885 min^{-1}) and k_{cat}/K_M values (2 x 10^6 M^{-1} min^{-1}) than MP8 alone (k_{cat} = 590 min^{-1} and k_{cat}/K_M = 1.45 x 10^6 M^{-1} min^{-1}). Such a slight difference in the catalytic ativity of both complexes was in agreement with the absence, on the distal face of MP8, of an amino acid residue from the antibody participating to the catalysis of the heterolytic cleavage of the O-O bond of H_2O_2 (pull effect). However, when compared to the already reported values, the k_{cat} and k_{cat}/K_M values obtained in the case of 3A3-MP8 constitute the best ever observed for the oxidation of cosubstrates by H_2O_2 catalyzed by hemoabzymes (Table 1). Overall, k_{cat}/K_M values 2 to 3 orders of magnitude higher than those observed for other hemoabzymes were obtained, which arises both from a 100 fold better affinity of 3A3-MP8 for H_2O_2 than other Fe-porphyrin-antibody complexes and from an about 2-3 fold higher k_{cat} value due to the push effect brought by the proximal His 18 ligand of the iron atom of MP8.

The monooxygenase like activity of 3A3-MP8 was further investigated. This led us to highlight a new reaction of hemoabzymes: the oxydation of N-monosubstituted hydroxylamines in the presence of 3A3-MP8 by the first example of fully characterized antibody-porphyrin-iron(II)-metabolite complex, 3A3-MP8-Fe^{II}-RNO (Scheme 3A). The formation of such complexes was more difficult with the bulky and hydrophobic N-(1-*p*-chlorophenyl-propyl)hydroxylamine than with small and hydrophilic hydroxylamines such as isopropylhydroxylamine, which showed that, as expected, a partial steric hindrance was brought by the antibody on the distal face of MP8. Consequently the induction of selectivity in oxidation reactions catalyzed by the 3A3-MP8 hemoabzyme was investigated.



Scheme 3. A) Formation of iron(II)–nitrosoalkane complexes by oxidation of N-substituted hydroxylamines by the 3A3-MP8 complex, B) Nitration of phenol by H_2O_2/NO_2^- catalyzed by the artificial metalloenzyme 3A3-MP8. C) Enantioselective sulfoxidation of thioanisole by 3A3-MP8.

The 3A3-MP8 complex was then shown to catalyze efficiently the nitration of phenol into 2- and 4-nitrophenol by NO_2^- in the presence of H_2O_2 (Scheme 3B).[100] The inhibition of the reaction by cyanide and radical scavengers suggested a peroxidase-like mechanism, involving the reduction of high-valent iron-oxo species by NO_2^- and phenol producing respectively $NO_2^{\cdot}$ and phenoxy radicals which then reacted to give nitrophenols. pH dependence studies confirmed that no amino-acid from the host participated in the heterolytic cleavage of the O-O bond of H_2O_2, indicating that in those reactions again, the antibody appeared to have two major roles: the protection of MP8 towards oxidative degradations and the induction of the regioselective formation of 2-nitrophenol. The S-oxidation of sulfides was further examined. Both isolatedMP8 and the 3A3-MP8 complex were found to catalyze the oxidation of thioanisole by H_2O_2, following a peroxidase-like "two step oxygen-transfer mechanism" involving a radical-cation intermediate.[120] In the presence of 3A3-MP8 as catalyst, the S-oxidation of thioanisole was stereoselective with a 45% enantiomeric excess in favour of the R enantiomer (Scheme 3C),[120] whereas the opposite S-enantiomer was obtained by Keinan et al.[98] with almost the same enantiomeric excess using the SN 37.4-Ru(II)-porphyrin complex as catalyst. This constitutes the highest enantiomeric excess reported to date for the oxidation of sulfides catalyzed by hemoabzymes and validates the use of 3A3-MP8 both as a catalyst for the selective oxidation of substrates and as a model system for hemoproteins.

3.5. The fate of "hemoabzymes": looking towards "hemozymes"?

Overall, the association of monoclonal anti-porphyrin antibodies with metalloporphyrin cofactors has appeared as a successful strategy for obtaining new hemoabzymes with a peroxidase activity. At least two advantages arise from this association: first the antibody site-isolates the Fe^{III}-porphyrin, preventing its bimolecular oxidative self-degradation, and, second, it controls the diffusion of H_2O_2 to the porphyrin. Molar efficiencies ranging between 3.7 x 10^3 and 2.9 x 10^5 $M^{-1}min.^{-1}$ were then measured for the hemozymes associating antibodies raised against N-substituted[78,85,91] or meso-carboxyaryl-substituted[85,88,93] porphyrins and the corresponding iron(III)-porphyrin cofactor. Even if those values are about 10 fold higher than those observed for the Fe^{III}-porphyrins alone, they still stand 3 to 5 orders of magnitude lower than those of heme peroxidases, that are among the most efficient catalysts known, with k_{cat}/K_M values of 6 x 10^8 $M^{-1}min^{-1}$.[91] This difference may be due to the fact that neither a distal amino acid participates in the catalysis of the heterolytic cleavage of the O-O bond of peroxides ("pull effect") nor a strong donating axial ligand of the iron ("push effect") were generated in the antibodies produced to favor the formation of the highly reactive radical-cation Fe^{IV}-oxo species. In this case, catalysis may then arise thanks to the stabilization of such species by interactions with the π electrons of aromatic residues of the antibody binding site.

In line with the aforementioned observation, providing the iron with an axial ligand which could have the "push effect" led to a clear improvement of the catalytic efficiencies of hemoabzymes. Indeed, adding an exogenous imidazole ligand to get Fe^{III}-imidazole-porphyrin-antibody complexes[96] or raising antibodies against microperoxidase 8, a heme octapeptide that contains a distal histidine ligand on the heme iron,[101] caused a 5-6 fold increase of the peroxidase activity of the hemoabzymes. An antibody-microperoxidase 8 complex even led to the best k_{cat}/K_M value ever reported for hemoabzymes that was 2 orders of magnitude only lower than that reported for horseradish peroxidase itself.[96] Getting an efficiency closer to that of the enzyme, would require the modification of the obtained antibodies by molecular engineering, to provide the distal face of the porphyrin with amino-acids such as histidine and arginine that would improve the affinity for H_2O_2 and provide the "pull effect".

The active site of heme peroxidases is relatively narrow. Then, to deliver the necessary electrons to reduce the intermediate iron-oxo species into the iron(III) resting state, the reducing cosubstrate interacts with the solvent exposed meso edge of the heme, but never enters the active site. Such a situation also occurs in the antibody-metalloporphyrin complexes since, as shown by the 3D structure of Fe^{III}-MP9-7G12[94] and Fe^{III}(ToCPP)13G10 complexes,[104] about two-thirds of the porphyrin are included in the binding site, leaving one-third of the porphyrin exposed to the solvent and then accessible to reducing co-substrates. It is thus not surprising that the

anti-porphyrin antibody strategy leads to hemoabzymes with a good peroxidase activity.

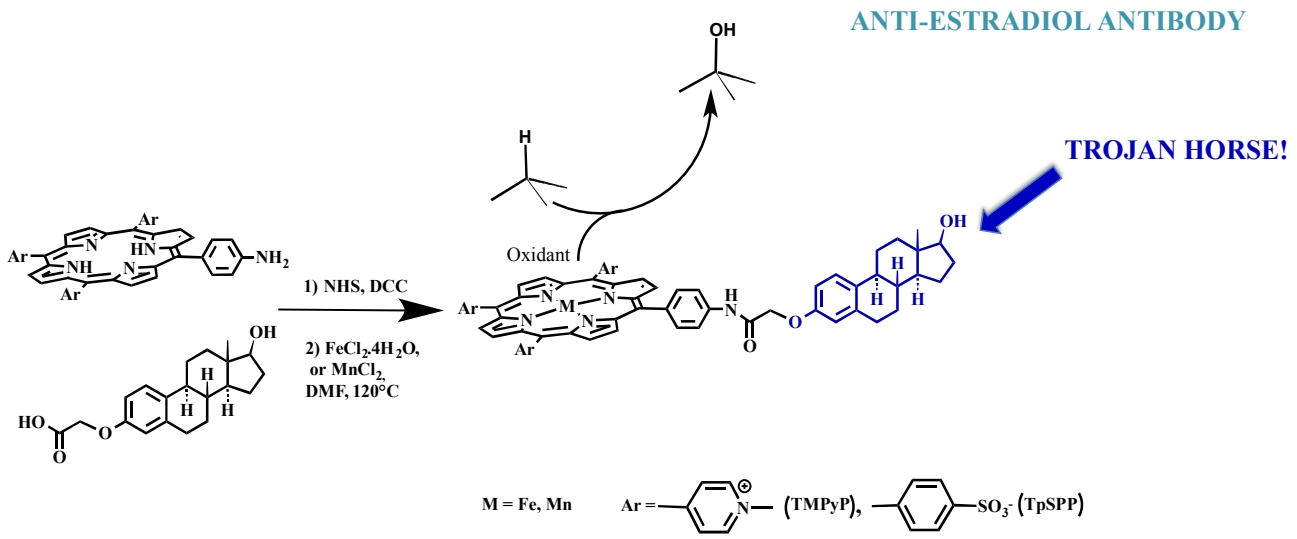


Fig. 8. Generation of a new "hemozyme" built up according to the "Trojan Horse" strategy by insertion of an Fe^{III}-porphyrin-estradiol conjugate into an anti-estradiol antibody 7A3.[148]

On the contrary, cytochromes P450 monooxygenases possess a wide hydrophobic active site, in which the substrate enters and binds selectively over the plane of the porphyrin close to the iron-oxo species. It is then not surprising that the anti-porphyrin antibody strategy appears to be less appropriate to obtain hemoabzymes with a Cyt. P450 like activity, as it is unlikely to obtain antibodies with a wide enough binding pocket that would be able to swallow both the porphyrin and the substrate.[119] Accordingly, those antibodies obtained that were obtained against porphyrins that display geometry mainly planar do not have any space over the plane of the porphyrin to bind the substrate.[101,119] Although the stereoselective sulfoxidation of aromatic sulfides by hydrogen peroxide[120] and iodosylbenzene[98] as well as the selective nitration of phenol derivatives[100] catalyzed by hemoabzymes has been observed, no efficient selective oxidation of substrates like alkanes and alkenes has been reported so far. We then decided like other teams in the world, to turn to other strategies to elaborate new artificial hemoproteins which we named "hemozymes".

4. Design of new artificial hemoproteins by insertion of synthetic metalloporphyrins into proteins: "hemozymes"

As already mentioned in the introduction of this paper, various strategies have been described to obtain artificial metalloenzymes or "Artzymes". The direct insertion of a metal ion into proteins has led artzymes that catalyze the selective bis-hydroxylation and hydroformylation of alkenes, the sulfoxidation of sulfides, the epoxidation of styrene derivatives, as well as Diels-Alder reactions.[121-129] "Artzymes" that catalyze a wide range of enantio-selective transformations such as hydrolysis of esters, sulfoxidation, epoxidation, hydrogenation and Diels Alder reaction, have also been obtained by covalent attachment of a metal cofactor to proteins, most often by a covalent bond with an active site cysteinyl residue.[25,52,130-134] Finally, the most widely explored method to obtain "artzymes" until now is the supramolecular anchoring of a cofactor in the cavity of a protein, using either the "host-guest" or the "Trojan-horse" strategies. The former was used to generate artificial metalloenzymes that catalyzed the stereoselective sulfoxidation of thioanisole,[40,47-51] stereoselective Diels-Alder reactions,[47] dihydrogen production,[135] or that displayed a peroxidase activity[53,54,136-138] and O_2 binding properties.[139-143] Only two strategies have been employed so far for the elaboration of artificial hemoproteins or "Hemozymes", the "host-guest strategy" and the "Trojan-horse strategy" that both involve the supramolecular anchoring of metalloporphyrin derivatives in a protein.

4.1. Elaboration of new Hemozymes using the "Trojan Horse" strategy.

Mainly developed and optimized by Ward and coworkers[144-146] the "Trojan-horse strategy" was historically introduced by Whitesides and coworkers.[147] It is based on the strong affinity of (strept)avidin for biotin ($K_D = 10^{-14}$ M) that is used as a "Trojan Horse" to anchor non covalently metal complexes inside its binding site, giving access to new enzymatic activities. This way, new avidin-biotin-rhodium complex biocatalysts were elaborated and found able to catalyze the stereoselective reduction of acetamidoacrylic acid by H_2.[144-146] Following these remarkable results, we decided to use the "Trojan Horse strategy" to build new "hemozymes", but taking advantage of the strong affinity of anti-steroid monoclonal antibodies for their antigen. The non-covalently anchoring of metalloporphyrin-steroid conjugates into the cavity of the antibody, which could provide an asymmetric environment to this metal cofactor, was then envisaged (Fig. 8).

On the one hand, the monoclonal anti-estradiol antibody, 7A3, constituted an excellent candidate for this strategy, since it had a very strong affinity for its estradiol antigen ($K_D \approx 10^{-9}$ M).[148] On the other hand, the metalloporphyrin cofactors to be chosen had to be efficient catalysts of oxidation reactions, the more stable as possible under oxidation conditions and water-soluble for a better interaction with the antibody. Fe(III) and Mn(III) complexes of ionic *meso*-aryl-substituted porphyrins, bearing three methyl-pyridiniumyl (TMPyP), or three sulfonato (TpSPP) moieties were synthetized (Fig. 9) fulfilled these 3 requirements.[149,150] Finally, since antibody 7A3 had been generated by immunization of mice with an antigen obtained by covalent linkage of estradiol in 3-position to BSA, these

metalloporphyrins were covalently linked to the estradiol through the same 3-O of its phenol moiety (Fig. 8).[149]

The four metalloporphyrin-estradiol conjugates bound to the antibody 7A3 with a 2/1 stoechiometry, showing that, despite the bulky porphyrin, the estradiol anchor was still well accommodated by the antibody.[150,151]The dissociation constants for the 4 metalloporphyrin-estradiol-7A3 complexes remained quite good, when compared to the affinity of the antibody for estradiol ($K_D \approx 10^{-9}$ M), with respective K_D values of 4 x 10^{-7} M and 2 x 10^{-6} M for the Fe- and Mn(TMPyP)-estradiol-7A3 complexes and 2 x 10^{-6} M and 9 x 10^{-6} M for the Fe- and Mn(TpSPP)-estradiol-7A3 complexes. The nature of the porphyrin (cationic or anionic), as well as that of the metal inserted had thus little influence on the association of the cofactor with the antibody.

The binding of the Fe^{III}(TMPyP)-estradiol conjugate to 7A3 had two effects. First, the dimerization of the iron-porphyrin, which could occur under basic conditions was avoided. Second, the iron remained able to coordinate two axial imidazole ligands, with a C_{50} value of 23 mM that was 7 fold higher than that measured in the absence of the antibody (3.4 mM)[149] which showed that the protein provided a steric hindrance around the porphyrin that penalized the complexation of the iron(III) by imidazole but did not prevent it. Taken altogether, the above-mentioned results confirmed that the anchoring of the metal cofactors did occur thanks to the specific recognition of the estradiol anchor by 7A3. However, the porphyrin seemed to remain very near to the entrance of the cavity, with a moderate interaction with the protein.

4.1.1. Catalytic Activity of the Metalloporphyrin-estradiol-7A3 complexes. The peroxidase activity of the Fe^{III}(TMPyP)-estradiol-7A3 complex was first assayed and compared to that of the cationic Fe^{III}(TMPyP)-estradiol cofactor alone. Both Fe^{III}(TMPyP)-estradiol and its complex with 7A3 catalyzed the oxidation of ABTS by H_2O_2 following a pseudo first-order kinetics regarding the catalyst or the H_2O_2 concentration. Kinetic studies showed that the antibody caused the acceleration, by a factor 2, of the first step of the reaction which consists in the activation of hydrogen peroxide by the Fe^{III}-porphyrin complex.[151]

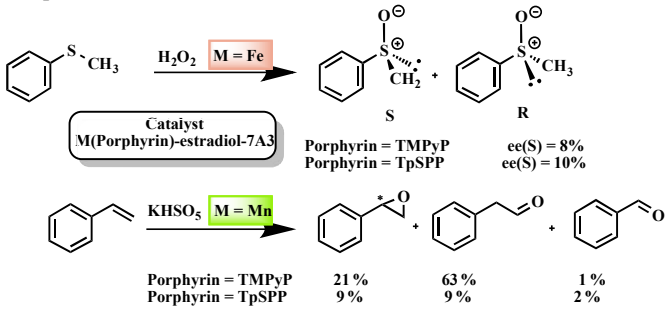


Fig. 9. Oxidation of thioanisole by H_2O_2 and of styrene by $KHSO_5$ catalyzed by Metal-TMPyP-estradiol-7A3 complexes.

The sulfoxidation of thioanisole by H_2O_2, catalyzed by iron^{III}(porphyrin)-estradiol cofactors, in the presence or not of 7A3, was then studied as a typical reaction to evaluate the induction of stereoselectivity by the antibody. First of all, up to 5 times higher amounts of sulfoxide were obtained in the presence of 7A3, which showed a protecting effect of the antibody against the self-oxidation of Fe^{III}(TMPyP)-estradiol in the presence of H_2O_2. Kinetic studies showed an increase in the initial rate of the reaction by a factor 2 in the presence of 7A3, which was consistent with the results obtained with ABTS.[149] In addition, whereas the product of the reaction was found racemic with Fe^{III}(TMPyP)-estradiol alone, an enantiomeric excess of about 8 % was measured in the presence of 7A3 (Fig. 9). Use of the anionic Fe^{III}(TpSPP)-estradiol and its complex with 7A3 as a catalyst led to very similar results, as a twice higher amount of sulfoxide and an enantiomeric excess of 10 % in favor of the S isomer were observed in the presence of 7A3 (Fig. 9), whereas racemic sulfoxide was obtained with the free Fe^{III}(TpSPP)-estradiol as catalyst.[150] These enantiomeric excesses are rather low, but close to those obtained by the teams of Watanabe (4%)[49] and Lu (12%)[52] in their first attempts to build artificial metalloenzymes by non-covalent insertion of a cofactor in a protein.

The Mn^{III}(porphyrin)-estradiol cofactors did not catalyze the sulfoxidation of thioanisole by hydrogen peroxide, but displayed a catalase activity, which led to decomposition of H_2O_2. Manganese porphyrins, however, are well known to catalyze the epoxidation of alkenes in the presence of various oxidants,[39-41] and the oxidation of styrene by oxone was chosen to test the influence of the antibody on the selectivity of this reaction catalyzed by Mn^{III}(porphyrin)-estradiol cofactors. In the presence of the four manganese-porphyrin catalysts, 2-phenyloxirane and 2-phenylacetaldehyde were formed as major product, with traces of benzaldehyde (Fig. 9).[150] It is likely that 2-phenylacetaldehyde was formed by over-oxidation of the epoxide, as previously reported with Ru(porphyrins).[152] The nature of the porphyrin had an important effect on the efficiency of the cofactor used. With the cationic Mn^{III}(TMPyP)-estradiol, the conversion of styrene was almost quantitative, whereas much lower yields were observed with the anionic Mn^{III}(TpSPP)-estradiol. The protein had little influence on the reaction as, whatever the cofactor, the global yields of oxidation products remain unchanged in the presence of 7A3 (Fig. 9) and no stereoselectivity in the epoxidation of styrene could be observed.

This first attempt to build artificial hemoproteins by the Trojan-horse strategy, using the high affinity of an anti-estradiol antibody for its antigen, was sucessful in the sense that the metalloporphyrin-estradiol cofactors were well recognized by the antibody through their estradiol anchor, that really played its role as a "Trojan Horse". Unfortunately, the porphyrin appeared to be mainly located at the entrance of the cavity of the protein, contrary to what was observed by Ward et al.[148,149] for the insertion in (strept)avidin of biotin-metal complex conjugates that were much smaller than our metalloporphyrin-estradiol conjugates. Consequently, even if the insertion of metalloporphyrin-estradiol conjugates in 7A3 caused an enhancement of the peroxidase and sulfoxidation activities, it led to a moderate enantioselectivity in the sulfoxidation of thioanisole and did not induce any stereoselectivity in the epoxidation of styrene.

Therefore, two modifications to the "Trojan Horse" strategy were envisaged in order to get hemozymes that would be able to induce better enantioselectivities. First, in order to drive the metalloporphyrin closer to the antibody binding site, so as to optimize the protein-substrate-metalloporphyrin interactions that would induce a higher enantioselectivity in the reactions catalyzed, the synthesis of new metalloporphyrin-estradiol conjugates containing a shorter spacer between the estradiol anchor and the metalloporphyrin is under development. Second, the search for another protein scaffold, that would possess a high affinity for its "Trojan Horse" substrate and a wide enough binding cavity to accommodate the metalloporphyrin cofactor was led. Our choice fell first on a 113 amino-acid protein, neocarzinostatin (NCS), the most studied member of a family known as chromoproteins, secreted by *Streptomyces*. This protein tightly and specifically binds a nine membered enediyne "chromophore" that is responsible for the powerful cytotoxic and antibiotic activities of the chromoprotein complexes. It has thus been intensively studied for its antitumor properties and has also been engineered as a potential drug-carrying scaffold.[153-156] In previous studies, directed evolution techniques were used to remodel the ligand-binding pocket of NCS and create a new binding site for a target unrelated to its natural ligand. The NCS-3.24 variant, that contains 7 mutations in the binding site with

respect to wild type NCS, was then found to bind testosterone with a good affinity (K_D = 13 x 10^{-6} M) and its X-ray structure was solved.[155] It was thus decided to associate this NCS-3.24 variant with a water-soluble anionic Fe^{III}-tetrarylporphyrin-testosterone conjugate (Fig. 10)[157] that was synthetized after the peptide coupling of 17'-succininimidyltestosterone with the para-aminophenyl-tri-para-sulfonatophenyl-porphyrin and further reaction with excess Fe^{II}-chloride.[158]

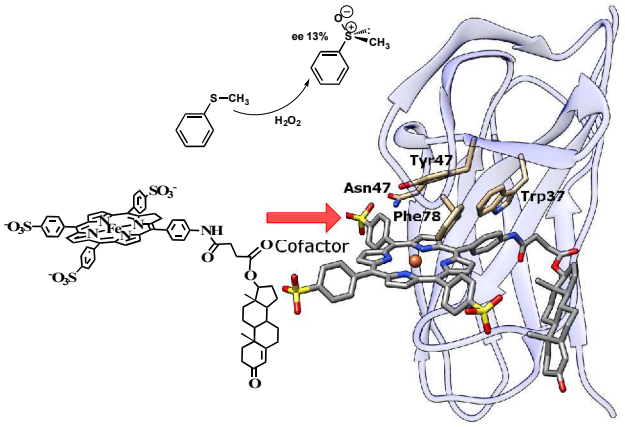


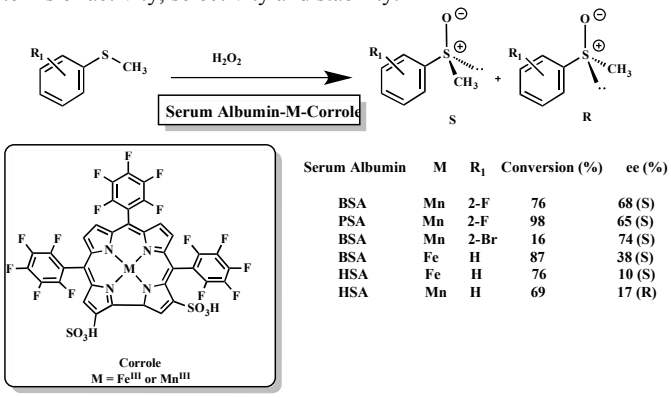
Fig. 10. Structure of the water-soluble anionic iron(III)-porphyrin–testosterone conjugate that was synthetized and structure of its complex with NCS-3-24.

NCS-3.24 bound the Fe^{III}-porphyrin–testosterone conjugate with an even better affinity (K_D = 1.6 µM) than testosterone (K_D = 13 µM) and testosterone-hemisuccinate (K_D = 40 µM). This could be explained by a synergy between the binding of the steroid moiety and that of the porphyrin macrocycle into the protein binding site as revealed by our molecular modeling studies which mainly show important hydrophobic complementarities between the "Troyan Horse" ligand of the NCS mutant and the testosterone binding sites. Moreover, the new Fe^{III}-Testo-porph-NCS-3.24 hemozyme was found to catalyze the chemoselective and slightly enantioselective (ee = 13 %) sulfoxidation of thioanisole by H_2O_2 (Fig. 10). This moderate enantioselectivity was also explained thanks to protein-ligand docking. Although the Fe^{III}-porphyrin complex was sandwiched between the two subdomains of the protein, providing with good complementarities the large dimension the artificial cofactor entirely filled the cavity but provide most of it widely exposed to the solvent and its reactive metal hanging out of the pocket.[157]

4.2. Elaboration of new Hemozymes by Non-covalent Association of Porphyrin Cofactors and proteins according to the "Host-Guest" strategy.

Finally, the so-called "host-guest" strategy was used to elaborate new "hemozymes". It involves the non-covalent insertion of a guest metalloporphyrin derivative inside the cavity of a non-related host protein, chosen not only to bring a chiral environment around the cofactor, but also, in some occasions, for its particular thermodynamic properties: thermoresistance, work under a wide range of pH. Several host proteins were commonly used as shown in the following paragraph.

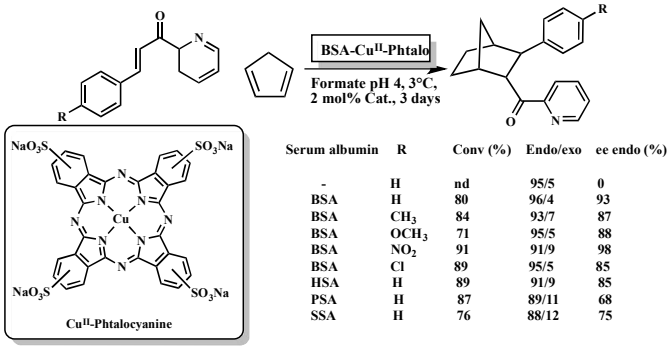
4.2.1. Serum albumins. Serum albumins are transport proteins that accept a broad range of substrates. For example, Human serum albumin (HSA), has a strong affinity for hemin (K_D < 10 nM), and the structure of the corresponding 1:1 complex in the presence of myristate was described in 2003 by Zunszain *et al.*[45] Taking advantage of this property, Gross and coll. inserted into HSA compounds with structures close to that of a porphyrin such as a bis sulfonated metal corrole (Fig. 11). This led to a stable 1:1 complex, the corrole being accommodated in a specific binding site of strong affinity (micromolar K_D).[46] New artificial metalloenzymes were then prepared, inserting the same corrole, containing iron or manganese, into several serum albumins; human, bovin, porcin, rabbit and sheep serum albumines.[40] These new "hemozymes" did actually catalyze the sulfoxidation of thioanisole and some analogs with conversions up to 98 % and enantiomeric excesses up to 74 % (Fig. 11). The manganese corrole was always superior to its iron counterpart, in terms of activity, selectivity and stability.



Serum Albumin	M	R_1	Conversion (%)	ee (%)
BSA	Mn	2-F	76	68 (S)
PSA	Mn	2-F	98	65 (S)
BSA	Mn	2-Br	16	74 (S)
BSA	Fe	H	87	38 (S)
HSA	Fe	H	76	10 (S)
HSA	Mn	H	69	17 (R)

Fig. 11. Metal-corrole cofactor and sulfoxidation of thioanisole derivatives catalyzed by the Serum Albumin-metal-corrole artificial metalloenzymes.

Reetz and coworkers also used serum albumins to prepare artificial metalloenzymes that catalyze the enantioselective Diels-Alder reaction.The insertion of copper phtalocyanine in BSA led to a new "artzyme" that catalyzed the cycloaddition of azachalcones to cyclopentadiene, with conversion yields above 80 %, and enantiomeric excesses up to 98 %(Fig. 12).[47]



Serum albumin	R	Conv (%)	Endo/exo	ee endo (%)
-	H	nd	95/5	0
BSA	H	80	96/4	93
BSA	CH_3	84	93/7	87
BSA	OCH_3	71	95/5	88
BSA	NO_2	91	91/9	98
BSA	Cl	89	95/5	85
HSA	H	89	91/9	85
PSA	H	87	89/11	68
SSA	H	76	88/12	75

Fig. 12. Copper phtalocyanine used as a cofactor and Diels Alder reaction catalyzed by several serum albumin-copper phtalocyanine complexes.

Similar results were obtained with other serum albumins (HSA, Porcin SA, Sheep SA, Rabbit SA and Chicken SA) although for the two last ones display lower *ees* (7 % and 1 %) and that the authors planned to improve by directed evolution.

4.2.2. Myoglobin. Several teams have also used the oxygen-binding protein, myoglobin, to build artificial metalloenzymes. The oxygen-binding site consists in a 10Å diameter cavity, with an iron-containing heme prosthetic group that is non-covalently linked to the protein, by hydrophobic interactions, electrostatic interactions via its two carboxylate moieties, and a coordination of the iron by the imidazole of the histidine His 93.[48]

Modification of myoglobin by molecular engineering is by far the most explored strategy to build up new "hemoabzymes". Mutation of active site amino-acids have thus allowed to provide myoglobin with unusual activities:[159] nitrite reductase,[160] peroxidase[161] and peroxygenase[162] activities as well as a catalytic activity in the selective oxidation of organic subtrates such as styrene and thioanisole by H_2O_2.[163-165] However, since the non-covalent anchoring of the heme in apo-myoglobin allows its rather easy removal to yield a free cavity able to accommodate another metal cofactor, several teams have used this property to prepare new "artzymes". Metal complexes of non-heme ligands such as salophens, salens[49-52] and phenantrolines (Fig. 13A),[54] and of tetrapyrrolic ligands such as porphycenes[54,166] and hemes modified with arms bearing several anionic carboxylates (Fig. 13B) have been incorporated in apo-myoglobin.[53]

Salophens are well fitted to the cavity of the apo myoglobin as they share several properties with heme: size, hydrophobicity and coordination of metals. Taking profit of these properties, Watanabe and coll. have inserted synthetic chromium salophens into apo-myoblogin and mutants to obtain "artzymes" that stereoselectively catalyzed the sulfoxidation of thioanisole, but with rather low turnovers ($\leqslant$ 0.13 TO.min^{-1}) and enantiomeric excesses (ee $\leqslant$ 33 % (S)).[49] The 3D structure of two complexes formed by association of Mn- and Cr- salophens with the A71G mutant of apo-myoglobin was determined. It showed that the complexes were bound to the protein by coordination of the metal by the imidazole moiety of His 93 and also that the bulky phenylenediamine moiety of the salophen ligand lowered the accessibility of the substrate to the active site of the "artzyme". Replacement of these complexes by smaller salens complexes led to an increase of the turnover number, to about 0.21 TO.min^{-1} with the Cr-salen complex, and to a much more noticeable increase with the Mn-salen cofactor, to reach 2.7 TO.min^{-1}. The stereoselectivity of the reaction could also be modulated as a function of the nature of the R_1 and R_2 subsituents of the salen (Fig. 13A).[50-51]

Artificial metalloenzymes were also built by Lu and coll. by covalent attachment of a manganese salen into the cavity of apo-myoglobin mutants.[52] The Mn-salen cofactor beared thiosulfonate methane moieties (Fig. 13A) that allowed its covalent binding to the protein, after reaction with cysteinyl residues of the apo-myoglobin mutants that were designed after modelling of the position of the Mn-salen in the cavity.

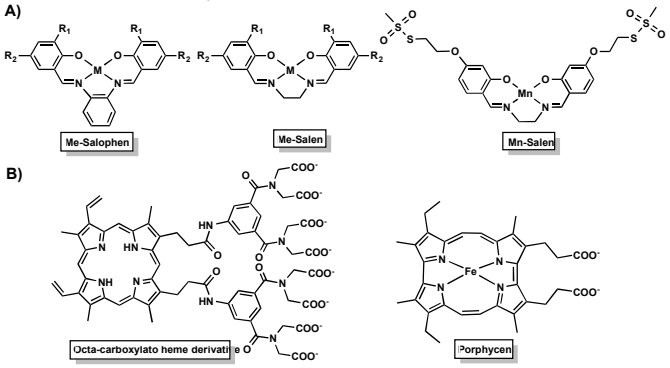


Fig. 13. Metal complexes ligands inserted into apo-myoglobin in order to build up new artificial metalloenzymes: A) metal complexes of salophen[49] and salen[50-52], B) Anionic porphyrin[53] and Fe^{III}-13,16-dicarboxyethyl-2,7-diethyl-3,6,12,17-tetramethylporphycen[54] inserted in apo-Mb by Hayashi and Hiseada.

The covalent attachment of the Mn-salen complex by a single position to the Y103C mutant of apo-myoglobin first led to an artificial metalloenzyme catalyzing the sulfoxidation of thioanisole with a rather low enantiomeric excess (12 %), similar to those obtained with a non-covalently bound Cr-salen. Reaction of the same Mn-salen complex with the L72C/Y103C double mutant allowed the dual covalent linkage of this cofactor to the protein, which stabilized the interaction between both moties and led to a significant improvement of the enantiomeric excess induced in the sulfoxidation of thioanisole that reached 51 %.[52]

The native heme of the myoglobin has also been replaced by another porphyrin, bearing eight anionic carboxylate moieties (Fig. 16), by Hayashi's group. The new hemozyme obtained had a peroxidase activity which catalyzed oxidation reactions, with rates up to 11 fold higher than native myoglobin in the case of catechol.[53]

The same group also replaced the heme of myoglobin by Fe^{III}-13,16-dicarboxyethyl-2,7-diethyl-3,6,12,17-tetramethyl-porphycen (Fig. 13B), which led to an artificial metalloenzyme that displayed peroxidase activity.[54] Recently, they reported that myoglobin reconstituted with a manganese-porphycen cofactor led to a new biocatalyst that was able to catalyze the regioselective hydroxylation of ethylbenzene into 1-phenylethanol by H_2O_2. Isotope effect experiments suggested that the rate limiting step of the reaction was hydrogen atom abstraction by an high-valent manganese-oxo species followed by an oxygen rebound step identical to that observed in the cytochrome P450 catalyzed hydroxylation reactions.[166]

Finally, very recent studies by Fasan et al,[167] indicated that myoglobin also constituted a promising scaffold for the design and development of catalysts for the direct conversion of aliphatic C-H bonds into C-N bonds. Indeed, a series of engineered and artificial myoglobin variants containing active site mutations and non-native Mn- and Co-protoporphyrin IX cofactors were prepared. Reactivity studies showed that they constitute viable C-H amination catalysts, revealing a distinctive reactivity trend as compared to synthetic metalloporphyrin counterparts. Mutations of amino acids in the heme binding improved the stereo- and enantioselectivity of these Mb-catalyzed reactions. Here also, studies involving kinetic isotope effect experiments indicate that C-H bond cleavage is involved in the rate-limiting step of the reaction.[167]

4.2.3. Non-covalent Insertion of Fe^{III}-porphyrins into Xylanase A (XlnA) from Streptomyces Lividans. We have ourselves chosen as a protein scaffold to elaborate new hemozymes a thermostable β-1,4-endoxylanase or xylanase A (XlnA) from *Streptomyces Lividans,* which function is to hydrolyze β-1,4 bonds in the main chain of xylan. Our choice was driven first by reports from Nakamura, Tsushida and coll., who produced heat resistant hemoproteins that were able to bind and release O_2 in aqueous medium by incorporating Fe^{II}-α_4-tetra-*o*-pivalamidophenylporphyrin into a thermo-resistant xylanase[168,169] Other crucial properties also guided our choice: (i) XlnA is available at low cost and in large quantities, (ii) several 3D structures of its catalytic domain have been elucidated, some of them up to a resolution of 1.2[170] andpresents a standard $(\alpha/\beta)_8$ TIM barrel fold that contains the two amino acids that are essential for catalysis, Glu 128 and Glu 236, (iii) this domain has a globally positively charge and wide enough pocket to accommodate metalloporphyrins that possess negatively charged substituents hence expecting to favor recognition processes involving charge-charge interactions. Accordingly, we decided to use the simple strategy that involved the non-covalent incorporation of Fe^{III}-α_4-tetra-*o*-carboxyphenylporphyrin (Fe^{III}(ToCPP)), Fe^{III}-

tetra-*p*-carboxyphenyl-porphyrin (Fe^{III}(TpCPP)), Fe^{III}-tetra-4-sulfonatophenyl-porphyrin (Fe^{III}(TpSPP)) (Fig. 2B) and MP8 (Fig. 7) into XlnA.

Coordination chemistry of the iron and molecular modelling studies showed that only the complexes para-phenyl substituted porphyrins Fe^{III}(TpCPP) and Fe^{III}(TpSPP) were able to insert deeply into XlnA, with K_D values in the micromolar range (Fig. 14).[171] UV-visible spectroscopy studies suggested that the position of both iron-porphyrins inside the binding pocket of XlnA was slightly different, as in the case of Fe^{III}(TpCPP) alone, no amino acid side-chain of Xln10A was coordinating the iron, whereas Fe^{III}(TpSPP)-XlnA displayed a typical spectrum of a low-spin hexacoordinate Fe^{III}-porphyrin, which could be due to its binding to one or two amino acid side-chains.[172, 173] It is noteworthy however, that even in the former case, the protein induced a steric hindrance around the iron, as Fe^{III}(TpCPP)-XlnA bound only one imidazole ligand, whereas Fe^{III}(T*p*CPP) alone was able to bind two. [171]

Molecular modeling analysis allowed to propose an explanation to these results. Fe^{III}(TpCPP) and Fe^{III}(TpSPP) (Fig. 14) were docked into the XlnA binding site with the program Gold (version 3.2).[172,173]

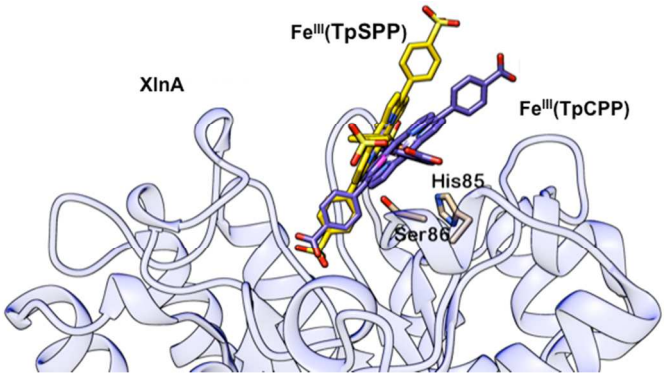
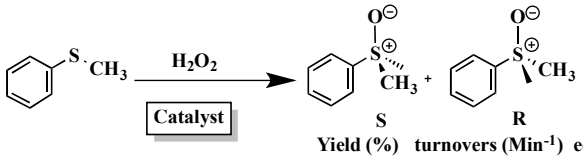


Fig. 14. Structure of the XlnA-Fe(TpCPP) and XlnA-Fe(TpSPP) hemozymes established from molecular modelling studies.

The main polar contacts occur between the negatively charged substituents of the phenyl groups of the ligands and the patches {His80, Lys48, Trp266} and {Ser212, Asn208, Asn173} of the receptor.[173] Owing to the larger size of the sulfonato substituents with respect to the carboxylato ones, Fe^{III}(TpSPP) is less buried than Fe^{III}(TpCPP) in the Xyln10A binding pocket. In the case of Fe^{III}(TpSPP), two residues, Ser 86 and His 85, though lying respectively, at 6.19 and 6.35 Å from the iron ion, could coordinate it after a structural rearrangement of the protein, which could be the origin of the change in UV-visible spectrum observed when Fe^{III}(TpSPP) was added to Xln10A.

Fe^{III}(TpCPP)-XlnA was found to have a peroxidase activity and catalyzed the oxidation of guaiacol and *o*-dianisidine by H_2O_2.[171] The K_M values measured for these two co-substrates with Fe^{III}(TpCPP)-XlnA were higher than those observed with free Fe^{III}(TpCPP), probably because of the steric hindrance and the increased hydrophobicity brought by the protein around the iron atom of the porphyrin. The peroxidase activity was inhibited by imidazole, and pH dependence studies on the oxidation of *o*-dianisidine suggested that an amino acid with a pK_A of 7.5 was participating in the catalysis. Finally, the protein provided an interesting protecting effect against oxidative degradation of the porphyrin.[171]



	Yield (%)	turnovers (Min^{-1})	ee(%)
Fe(TpSPP)	45	0.56	-
Fe(TpSPP)-Xln10A	24	0.30	24 (S)
Fe(TpCPP)	33	0.41	-
Fe(TpSPP)-Xln10A	24	0.30	36 (S)
Fe(TpSPP)-Xln10A + 100 eq. ImH	85	1.09	40 (S)

Fig. 15. Structure of the Xln10A-Fe(TpSPP) hemozyme established from molecular modelling studies and results obtained for the sulfoxidation of thioanisole catalysed by Fe-Porphyrins and their complexes with Xln10A.

Taken altogether, the properties pointed out by the first studies on the Fe^{III}(TpCPP)- and Fe(TpSPP)-XlnA hemozymes suggest that Xylanases are were excellent candidate to support hemo-like homogenous catalysts for the enantioselective oxidation of substrates. Their catalytic activity was then first assayed in the oxidation of thioanisole by H_2O_2[172,173] and the influence of the XlnA protein on the yield and selectivity was then studied (Fig. 15).

In both cases, the incorporation of the iron-porphyrin in XlnA led to decrease in the yield of sulfoxidation but it also induced a stereoselectivity in favor of the S-sulfoxide. With Fe^{III}(TpSPP) as a catalyst, racemic sulfoxide was produced in a 45 % yield (with respect to H_2O_2) with a turnover of 0.56 min^{-1}, and its insertion into XlnA led to a decrease in the yield (24 %) and in the turnover (0.30 min^{-1}), but an enantiomeric excess of 24% was observed (Fig. 15). Similarly, better yields and turnovers were obtained with Fe^{III}(TpCPP) (33 % and 0.41 min^{-1}) than with Fe^{III}(TpCPP)-XlnA (24 % and 0.30 min^{-1}), but in the latter case, an even larger enantiomeric excess of 36 % was observed. According to the molecular modeling studies, this is mainly due to the deeper insertion of Fe^{III}(TpCPP) than Fe^{III}(TpSPP) into the cleft of XlnA. Better yield and turnovers (85% and 1.09 min^{-1}) could be obtained in the presence of 100 eq. imidazole as co-catalyst as well as a better enantiomeric excess in favor of the S sulfoxide (ee = 40%) (Fig. 15) in agreement with the previous statement.

The yields and enantiomeric excesses obtained for the S-oxidation of thioanisole catalyzed by various artificial metalloenzymes are compared in Table 2. Fe(TpCPP)-XlnA appears to be best hybrid biocatalyst obtained by insertion of an iron-complex into a protein. Indeed, with the exception of the abzyme 3A3-MP8, that leads to a 45% enantiomeric excess in favor of the R sulfoxide but with a lower yield (45%),[120] all other iron-artzymes induce a lower enantiomeric excess in favor of the S sulfoxide. Indeed, apomyoglobin- and BSA-Fe-tetrapyrrolic[40] complexes lead to enantiomeric excesses ranging from 5 to 38%, whereas the new hemozymes, obtained recently by insertion of an iron-porphyrin into an anti-steroid antibody, using the "Trojan horse" strategy, only lead to a 8% enantiomeric excess.[152] Better enantiomeric excess could be obtained however, after covalent anchoring of metal complexes into apomyoglobin and mutants [49,52,174] or by exchanging the metal, for example iron to manganese.[40,174] The best system is obtained by direct incorporation of the $VOSO_4$ ion into (strept)avidin, which leads to an ee value of 93%.[154]

Table 2. Enantiomeric excesses, yields or turnovers obtained for the sulfoxidation of sulfides by H2O2 catalysed by artificial metalloenzymes.

Protein	Metal complex	Anchoring	ee (%)	Yield (%)	Conf.	Ref.
Xylanase A	**1-Fe**	SM[c]	40	85	S	173
Antibody 7A3	Estradiol-Fe-Porphyrin	SM[c]	8	-[a]	S	152
Apomyoglobin and mutants	Cr-Salen	SM[c]	5-13	54	S	49
	Mn-Salen	C[c]	51	-[b]	S	52
	Mn-Salen	SM[c]	60	49.4	S	174
Antibody 3A3	MP8	SM[c]	45	49	R	120
BSA	Fe-corrole	SM[c]	38	87	S	40
	Mn-corrole	SM[c]	74	16	S	40
Phytase	VO_4^{3-}	-	66	100	S	124
Streptavidin	$VOSO_4$	-	93	53	S	154

[a] yield not mentioned, turnover : 0.3 min^{-1} [b] yield not mentioned, turnover : 0.39 min^{-1}, [c] SM: supramolecular, [d] C: covalent

Further work has been developed to apply these strategies and expand the use of our new hemozymes constructed from XlnA to the selective oxidation of other substrates such as alkenes.[175] Unfortunately the Fe^{III}(TpCPP)-Xln10A was found unable to catalyze the oxidation of alkenes such as styrene by several oxidants including hydroperoxides (H_2O_2 and t-BuOOH), sodium hypochlorite (NaOCl), sodium periodate ($NaIO_4$) and oxone® ($KHSO_5$). We thus turned to manganese complexes, namely Mn^{III}(TpCPP) and the Mn^{III}-N,N'-Ethylenebis(2-hydroxybenzylimine)-5,5'-dicarboxylic acid (Mn^{III}-salen) complex, that were inserted into XlnA. The two hybrid biocatalysts were first characterized by UV-visible spectroscopy, circular dichroism and molecular modelling and then assayed as catalysts for the oxidation of various substituted styrene derivatives by the oxidants mentionned above.[175] Only Mn^{III}(TpCPP)-XlnA was found able to catalyze the oxidation of styrene by $KHSO_5$, with the highest catalytic activity reported so far for the oxidation of styrene catalyzed by artificial metalloproteins. A total yield of 26% in oxidation products was obtained, with a 17% yield in styrene oxide.

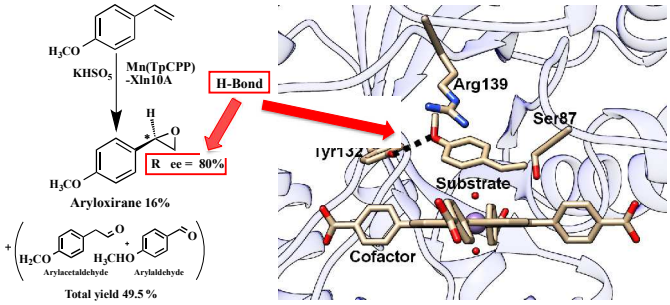


Fig. 16. Control of the styrene oxidation by the Xln10A protein: stereoselective formation of the R-sulfoxide upon oxidation of p-methoxystyrene by $KHSO_5$ catalyzed by the Mn(TpCPP)-Xln10A.

However only a 8.5 % ee for the S epoxide was observed which despite confirming the asymmetric environment of the protein upon the reaction still shows a limited effect. Rather low chemo- and enantio-selectivities were also observed for the epoxidation of poorly activated styrenes, substituted by electron withdrawing substituents such as Cl and NO_2 or a poorly donating substituent such as CH_3. This was probably due to the bleaching of the artificial metalloprotein by $KHSO_5$ during the course of the reaction. It is noteworthy that in all these cases a low stereo selectivity in favor of the S epoxide (3% < ee < 25%) was found. On the contrary, the epoxidation by $KHSO_5$ of *para*-methoxystyrene where the double bond was activated by the *para*-electron donating methoxy substituent of the aryl ring, a higher yield (49,5 %) and a high reverse enantioselectivity (80 % in favor of the R isomer) were observed (Fig. 16). This constitutes until now the best enantioselectivity ever reported for epoxidation reactions catalyzed by artificial metalloenzymes.[175]

Docking experiments helped explaining this stereoselectivity. With the less reactive styrene derivatives, insertion of the molecule in the active site of XlnA in a position that favours the formation of S-epoxide was energetically slightly more favourable by less than 0.5 $kJmol^{-1}$. On the contrary, with *p*-methoxystyrene, insertion of the molecule in a position that favours the formation of the R-epoxide was energetically more favourable by about 0.7 $kJmol^{-1}$ owing to a stabilizing H-bond between tyrosine 172 and the oxygen atom of the *p*-methoxy substituent (Fig. 16).

A comparison with other artzymes (Table 3) shows that Mn(TpCPP)-XlnA is the best one for the epoxidation of styrene derivatives. Indeed, although it only leads to an 8,5 % ee in favor of the S epoxide (with a 17% yield), it leads to the highest *ee* ever reported for the epoxidation of *p-methoxystyrene* with a 16 % yield whereas all other artificial Mn-proteins induce a lower enantiomeric excess in favor of the R epoxide and in addition with very low yield or conversion.

Table 3. Stereoselective oxidation of styrene derivatives catalyzed by artificial metalloproteins.

substrate	catalyst	oxidant	Epoxide yield (%)	*ee* (%)	Ref.
styrene	Mn-salen-Papain	tBuOOH	-	<10	176
styrene	Mn-carbonic anhydrase	H_2O_2	4	67(R)	126
4-chloro-styrene	Mn-carbonic anhydrase	H_2O_2	9	56(R)	127
styrene	Mn(TpCPP)-Xln10A	$KHSO_5$	17	8.5(S)	175
4-methoxy-styrene	Mn(TpCPP)-Xln10A	$KHSO_5$	16	80(R)	175

Mn-salen compounds into papain only lead to an enantiomeric excess lower than 10% for the epoxidation of styrene by tBuOOH[176] whereas insertion of a Mn^{III} cation into apo-carbonic anhydrase leads to artificial metalloproteins that catalyze the epoxidation of styrene with good enantiomeric excesses, 52 %[127] and 67 %[126] but with a very low yield (9%)[127] and conversion (4%).[126]

Further work is in progress to improve the efficiency of our new hemozymes constructed from XlnA and to extend their use to the selective oxidation of other substrates including phenol derivatives, aliphatic alkenes and alkanes.

Conclusions

As reviewed above, the elaboration of artificial metalloenzymes constitutes is a growing field of interest in bioinorganic chemistry and many teams in the world are developing original strategies to build up such new hybrid biocatalysts that cumulate the advantages of enzymes, that is regio and/or chemo-selectivity of the reaction catalyzed, mild operating conditions.

With the advent of catalytic antibodies, several teams first thought about using monoclonal antibodies raised against porphyrin cofactors,[75-96] to build up metalloporphyrin-antibody complexes that we named "Hemoabzymes". Indeed, associating those monoclonal antibodies with the corresponding Fe^{III}-porphyrin cofactor did afford artificial peroxidases that displayed catalytic efficiencies about 10 fold higher than those observed for the free Fe^{III}-porphyrins. These values still remained 3 to 5 orders of magnitude lower than those of peroxidases, which stand among the most efficient catalysts known and display k_{cat}/K_m values around 6 x 10^8 $M^{-1}min^{-1}$.[91,106,107] This difference may by due to the absence in most of the hemoabzymes, (i) of any distal amino acid residue that could participate in the heterolytic cleavage of the O-O bond of peroxides, or (ii) of any

axial proximal ligand of the iron. Several optimization attempts were made incorporate those effects in hemoabzymes. One consists in the use of an exogenous imidazole to act as a proximal ligand of the iron Fe-porphyrin-antibody complexes. This led to a 5-6 fold increase in the peroxidase activity.[96] Second, monoclonal antibodies were produced against microperoxidase 8, a heme octapeptide that contains a proximal histidine bound to its iron atom.[101] This led to antibody-MP8 complexes with the best peroxidase activity ever reported for antibody-porphyrin complexes, characterized by a k_{cat}/K_M value only 2 orders of magnitude lower than that reported for horseradish peroxidase itself.[101]

The strategy based on anti-porphyrin antibodies seems less appropriate to obtain models of cytochromes P450.Until now, the Antibodies have been raised against rather flat porphyrins and do not allow enough space over the plane of the porphyrin to bind the substrate,[101,119]. It has not been possible to obtain antibodies displaying binding sites for both the substrate and the porphyrin, using as a hapten a porphyrin covalently linked to the substrate.[119] Then, although some positive results concerning the selective oxidation of substrates catalyzed by antibody porphyrin complexes: (i) monoclonal antibodies raised against a water soluble tin^{IV}-porphyrin containing an axial α-naphtoxy ligand were found able, in the presence of a Ru^{II}-porphyrin cofactor, to catalyze the stereoselective sulfoxidation of aromatic sulfides by iodosylbenzene,[98] (ii) antibody-microperoxidase 8 complexes, have already proven to be able to catalyze the selective nitration of phenol derivatives[100] and the stereoselective sulfoxidation of aromatic sulfides by hydrogen peroxide,[120] it is clear that some other strategies have to be envisioned.

The first one, that we name the "Trojan Horse" strategy, is based on the high affinity of a protein for its substrate. In this strategy, the substrate, covalently linked to the catalytic metal cofactor, is used to anchor it non-covalently, but with a very high affinity, inside the binding site, the protein being responsible for the selectivity of the reaction catalyzed. This strategy has already been developed by Ward et al.,[144-146] who initially used the high affinity of avidin for biotin (K_D = 10^{-14} M) to elaborate new avidin-biotin-rhodium complex biocatalysts that were found able to catalyze the stereoselective reduction of acetamidoacrylic acid by H_2. We have applied Such a strategy to the design of new biocatalysts for selective oxidations using the insertion of water soluble metalloporphyrin-steroid conjugates into anti-steroid antibodies[150,151] and into a neocarzinostatin variant, NCS-3-24.[157] Incorporation of a Fe^{III}-tetra-para-sulfonato-phenyl- or -4-methyl-pyridiniumyl-porphyrin steroid conjugates in either anti-steroid antibodies or NCS-3-24 led to hemozymes that were found able to catalyze the stereoselective oxidation of sulfides by H_2O_2.[149,157] Incorporation of the corresponding Mn^{III}-porphyrins afforded artificial hemoproteins that catalyzed the epoxidation of styrene by $KHSO_5$.[150] However, in the former case low enantiomeric excesses in sulfoxide were obtained (10-23%) whereas no assymetric induction in the epoxidation of styrene was observed. This was mainly due to the fact that the metalloporphyrin cofactor was not deeply enough inserted in the protein. For comparison, the deep insertion of smaller biotin-rhodium complexes into avidin led to new biocatalysts that were found able to catalyze the stereoselective reduction of acetamidoacrylic acid by H_2 with enantiomeric excesses (up to 94 %).[144-146] It is then likely that to improve the enantioselectivity of the oxidation reactions catalyzed by artificial hemoproteins will require either the use of new conjugates in which the steroid will be directly connected to the metalloporphyrin whithout any linker arm, or the use of smaller macrocyclic derivatives either a metalloporphyrin bearing a non substituted meso-position or a metallocorrole.

Finally a second strategy, the so-called "host-guest" strategy has been used. It involves the non-covalent insertion of a guest metal cofactor inside the cavity of a host protein. Such a strategy has already used for the covalent insertion of a Rhodium complex inside human serum albumin (HSA), to build up an hybrid metalloprotein that is able to catalyze the stereoselective hydroformylation of styrene with CO and H_2.[122] Several teams have thus chosen, to build up new hemozymes, a simple strategy which involves the non-covalent incorporation of metalloporphyrin derivatives in various proteins. Association of Mn- and Fe-corroles afforded hemozymes that catalyzed the sulfoxidation of thioanisole derivatives with yields up to 98 % and enantiomeric excesses up to 74 %,[40] whereas the association of and copper-phtalocyanines with BSA, actually catalyzed the Diels-Alder reaction, with yields above 80 %, and enantiomeric excesses up to 98 %.[47]

Hemes modified with arms bearing several anionic carboxylates[53] and Metalloporphycenes[54,166] have been inserted in apo-myoglobin. The former hemozymes obtained had a peroxidase activity that catalyzed oxidation reactions, with rates up to 11 fold higher than native myoglobin with catechol[53] whereas myoglobin reconstituted with a manganese-porphycen cofactor led to a new biocatalyst that was able to catalyze the recioselective hydroxylation of ethylbenzene into 1-phenylethanol by H_2O_2. We ourselves non-covalently inserted Fe^{III}- and Mn^{III}complexes of anionic porphyrins, tetra-p-carboxyphenylporphyrin (TpCPP) and tetra-p-sulfonatophenylporphyrin (TpSPP) into thermostable β-1,4-endoxylanase or xylanase A (Xln10A) from *Streptomyces Lividans*. The Fe^{III}(TpCPP)-Xln10A hemozyme displayed a good peroxidase activity,[171] and the Fe^{III}-porphyrin based catalysts, Fe^{III}(TpCPP)- and Fe^{III}(TpSPP)-Xln10A, were found to catalyze the stereoselective oxidation of thioanisole in the presence of imidazole with enantiomeric excesses around 40%.[172,173] The best result was undoubtly observed for the stereoselective oxidation of styrene derivatives by oxone, catalyzed by the Mn^{III}(TpCPP)-Xln10A hemozyme, that led, in the particular case of p-methoxystyrene, to an 80% ee infavor of the R isomer. However, weak enantiomeric excesses were observed with other derivatives, bearing other substituents than methoxy, and there is still a need for new catalysts that would lead to higher ees whatever the substrate. For this, several solutions can be envisioned. First, the insertion of the metalloporphyrin catalyst into the host protein can be improved. Accordingly, mutants of Xln10A as well as new architectures such as β sheet (Arabinofuranosidase A), 5 fold β propeller (Arabinofuranosidase B) or jelly roll (Xylanases B, C) will be investigated in order to determine which one will be the best host candidate to accommodate metallopoprhyrin guests and lead to stereoselective oxidations. Alternatively, less hindered tetrapyrrole macrocycles, such as porphyrin bearing a non substituted meso-position or a corroles, that would insert deeper in the protein and possess a good catalytic activity in oxidation, will be assayed. Indeed, already published results show that the insertion of smaller complexes such as vanadate in the phytase of *Aspergillus ficuum*[177] led to enantiomeric excesses up to 66% in the Sulfoxidation of thioanisole by H_2O_2.

Finally a third strategy, involving the covalent anchoring of the metalloporphyrin porphyrin cofactor into the protein will be envisioned. In this respect, the work done by Lu and coll.,[52] who obtained up to 51% in the oxidation of by after covalent double linkage of a manganese salen into the cavity of an apo-myoglobin mutant, has clearly pointed out one essential condition to induce reproducible and noticeable stereoselectivity: the cofactor has to be firmly attached to the protein, so that the second coordination sphere of the metal is well defined. For example, the synthesis of porphyrins bearing an arm with a maleimide function or a

chloromethyl ketone will be be synthetized for their covalent anchoring into Xylanase mutants containing a cysteinate.

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