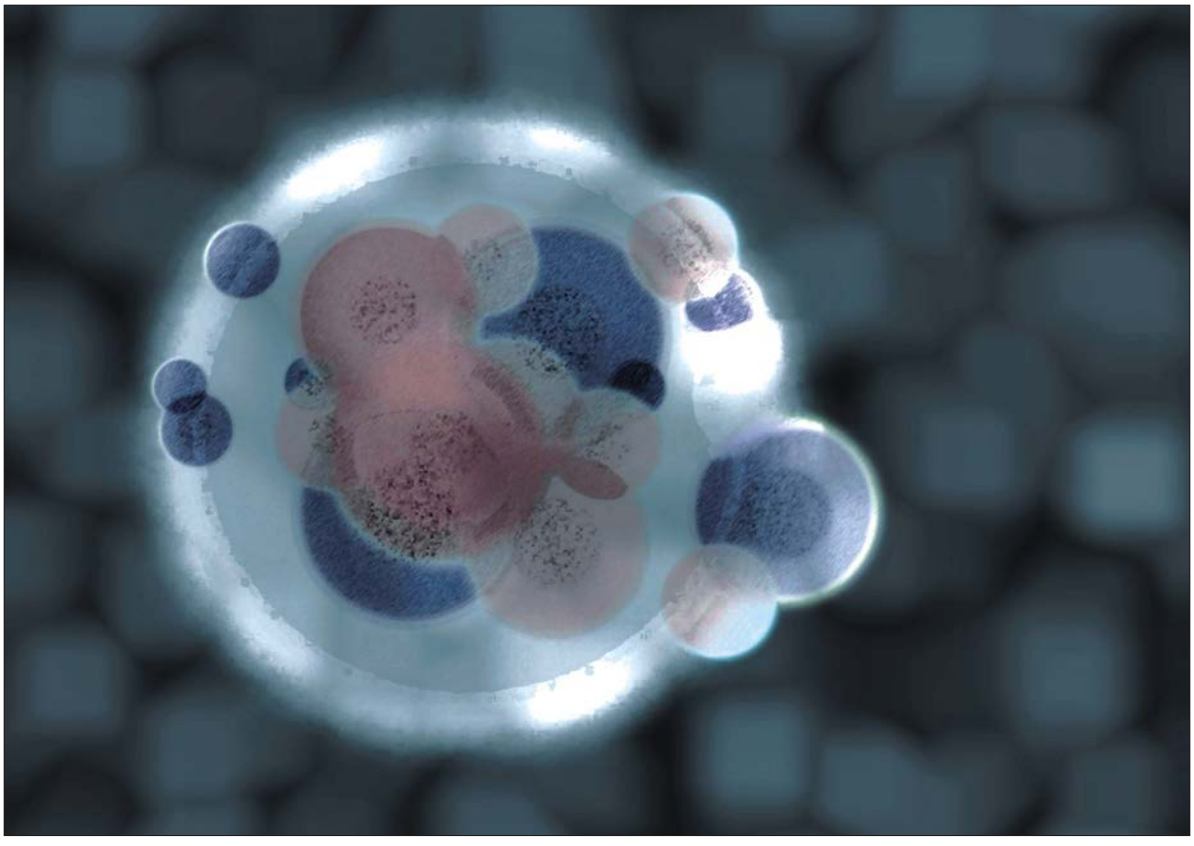

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Microperoxidase 8 as a Powerful Tool for Biological Applications

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Abstract: Microperoxidase 8 is a heme octapeptide obtained by hydrolytic digestion of cytochrome which contains the heme prosthetic group together with the amino acid residues 14 to 21 of horse cytochrome c, including His 18, whose imidazole group acts as the fifth axial ligand of the iron. It is a very good model for the coordination properties and the reactivity of hemoproteins like peroxidases and cytochromes P450. Indeed, it is first able to bind a wide variety of ligands on the 6th axial coordination position of the iron. Second, it is able to perform peroxidase-like reactions, such as the nitration of phenol by H_2O_2/NO_2^-, the oxidation of N-hydroxyguanidines with formation of nitrogen oxides. Third, it is also able to catalyze monooxygenase-like reactions, such as the N-dealkylation of aromatic amines and the O-dealkylation of aromatic ethers, the para-hydroxylation of aniline, the monooxygenation of polycyclic aromatic compounds and the S-oxidation of sulfides. In the later case no selectivity was observed with MP8 alone, whereas when MP8 was associated with a monoclonal antibody raised against it, the oxidation was stereoselective with a 45% enantiomeric excess in favor of the R isomer. This association constituted a new generation of artificial hemoproteins based on a monoclonal antibody, which we named "hemoabzyme" and which appeared as a promising biocatalyst for selective oxidation reactions. Finally, a new biomedical application has recently been found for MP8 as a biosensor for molecules of biological interest. For this, several strategies were envisioned to immobilize MP8 at the surface of an electrode, in such a way that the 6th coordination position of the iron may be accessible to ligands. Detection of the target molecule will then be realized upon measurement of the variations of the redox potential and of the current intensity induced by its binding on the iron of MP8.

Key words: Microperoxidase 8, catalytic antibodies, biosensors, artificial metalloenzymes

INTRODUCTION

Until now, heme protein studies which aim at understanding structure/function relationships between the heme and the protein matrix have relied heavily on investigating the properties of chemically synthesized porphyrins in organic solvents. These chemical models are able to reproduce the coordination properties and/or the catalytic functions of hemoproteins[1], but they lack the capacity to provide information on protein matrix/heme interactions. In this context, a water soluble hemoprotein model, based on the association of a natural heme and an octapeptide has been developed:microperoxidase 8 (MP8) (Fig.1).

Microperoxidase 8 is obtained by hydrolytic digestion of cytochrome c: it contains the heme prosthetic group together with the amino acid residues 14 to 21 of horse cytochrome c, including His 18, whose imidazole group acts as the fifth axial ligand of the iron. The presence of this ligand makes microperoxidase 8 an attractive model for studying hemoproteins.

Like many iron-porphyrins, MP8 is monomeric only at low concentrations in aqueous solution[2]. This does not represent a major problem in catalytic studies for which only sub-micromolar concentrations are required. But, on the contrary, this can constitute a major drawback for physical and spectroscopic studies of MP8 which require significantly higher concentrations. Indeed, aggregation by inter-molecular coordination of the N-terminal amino group of the peptide of one molecule to the iron atom of the other molecule, as well as π-interactions, lead to the formation of low spin dimers and higher aggregates[3], complicating the interpretation of the results. This aggregation can be minimised by use of surfactants[4] or alcohol-water mixtures[3], but more satisfactory is the blocking of the N-terminal amino group of the polypeptide by acetylation[5], which leads to species that are sufficiently monomeric in aqueous

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solution for studies by conventional spectroscopic methods.

MP8 as a good model for the coordination and reactivity studies on hemoproteins: The H_2O molecule coordinated in the sixth axial position of the iron atom of MP8 can readily be replaced by a variety of exogenous ligands. In fact, most studies on the substitution reactions of this ligand have focussed on the ferric state of microperoxidase. Several ligands like cyanide[6], imidazole and its derivatives[7], pyridine and its derivatives[8], ammonia and primary amines[9], thiolates[10] and N-aryl-N'-hydroxyguanidines[11] bound to the iron and form low spin $MP8Fe^{III}$ complexes. The weaker field ligands like N_3^-[12] lead to an equilibrium between two high-spin (S=5/2) and low-spin (S=1/2) complexes. Finally anions such as fluorine[13], thiocyanate[14] and the alcohol group of quinine[15] lead predominantly to high spin complexes.

In the case of ferrous microperoxydase 8 only a few complexes have been reported so far with: CO[16], NO[17], imidazole[18], lysine and N-acétylméthionine[19], which all are low-spin hexacoordinate iron (II) complexes. In addition, MP8 has also recently been shown to be able to oxidize N-monosubstituted hydroxylamines with formation of very stable iron (II)-nitrosoalkane complexes[20].

Furthermore the distal axial H_2O ligand of the heme iron can easily be also exchanged for an oxygen donor such as H_2O_2, leading to the formation of highly oxidized intermediates responsible for two types of catalytic reactions, a peroxidase-like and a monooxygenase-like reaction. Indeed, first of all, MP8 is able to perform the oxidation of several typical peroxidase co-substrates like ortho-dianisine[21], 2,2'-azinobis(3-ethylbenzothiazoline) -6-sulfonic acid (ABTS)[22], guaiacol[23] and to catalyze the nitration of phenol[24] by H_2O_2/NO_2^- as well as the formation of nitrogen oxides from the oxidation of N-hydroxyguanidines[25]. Second, MP8 also catalyzes the para-hydroxylation of aniline[26], the S-oxidation of sulfides[27], the monooxygenation of polycyclic aromatic compounds[28] and the N-dealkylation of aromatic amines and the O-dealkylation of aromatic ethers[29].

Finally the aforementioned paragraph shows that, thanks to the ease of exchange of the endogenous water molecule with a large variety of ligands, MP8 not only displays a very rich coordination chemistry both of iron (II) and iron (III) but (Fig. 2) is also able to catalyze numerous oxidation reactions which are usually catalyzed by hemoproteins such as peroxidases or monooxygenases. This minienzyme thus appears as a very good water soluble mimick for hemoproteins.

MP8 as an interesting cofactor for selective and efficient biocatalyst: catalytic antibodies: With the advent of catalytic antibodies, the association of a metalloporphyrin cofactor with monoclonal antibodies to obtain new artificial hemoproteins which we named " hemoabzymes", appeared as a promising route to models for hemoproteins like peroxidases and to catalysts tailored for selective oxidation reactions. The first strategy for obtaining such artificial hemoproteins has been to produce antiporphyrin antibodies raised against various free-base, N-substituted-, Sn-, Pd, or Fe-porphyrins[30]. Five of them exhibited in the presence of the corresponding Fe-porphyrin cofactor a significant peroxidase activity with K_{cat}/K_M values ranging between 3.7×10^3 and 2.9×10^5 $M^{-1}min^{-1}$[31-35]. This value remained however low when compared to that of peroxidases ($K_{cat}/K_M = 6.1 \times 10^8$ M^{-1} min^{-1})[36]. The relatively low efficiency of those porphyrin-antibody complexes was probably due, at least in part, to the fact that no proximal ligand of the iron has been induced in those antibodies. To avoid this problem we decided to use as a hapten microperoxidase 8 in which the imidazole group of His 18 acts as the fifth axial ligand of the iron. A set of 6 monoclonal antibodies was thus obtained and the best peroxidase activity, found in the case of the complex of MP8 with one of those antibodies, 3A3, was characterized by a K_{cat}/K_M value of 2×10^6 $M^{-1}min^{-1}$, which constituted the best one ever reported for an antibody-porphyrin complex[37] .

Active site topology studies suggested that the binding of MP8 occurred through interactions of its carboxylate substituents with amino acids of the antibody and that a partial steric hindrance of the distal face of the heme of MP8 was brought by the antibody protein[37]. This observation suggested that the 3A3-MP8 complex could be of interest for the selective catalysis of oxidation reactions. Since in addition, it was shown that the antibody protein protected MP8 against oxidative degradations, the 3A3-MP8 complex was assayed as catalyst for the regioselective nitration of phenol derivatives by NO_2^-/H_2O_2 and for the stereoselective oxidation of sulfides. It was first shown that 3A3-MP8 was a more efficient catalyst for the nitration of phenol by NO_2^-/H_2O_2 than MP8 alone and that it also induced a regioselectivity of the reaction in favor of the formation of 2-nitrophenol[38]. Furthermore, the influence of the antibody on the stereoselectivity of the S-oxidation of sulfides was examined more recently. Our results showed that MP8 alone and the antibody-MP8 complex catalyzed the oxidation of thioanisole by H_2O_2 and tert-butyl hydroperoxide, following a peroxidase-like two-step oxygen-transfer mechanism involving a radical-cation

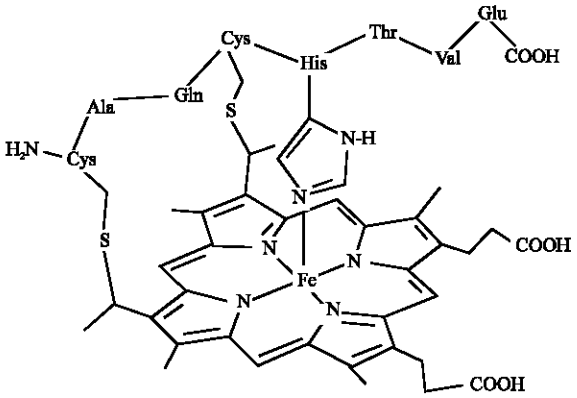

Fig. 1: Structure of microperoxidase 8

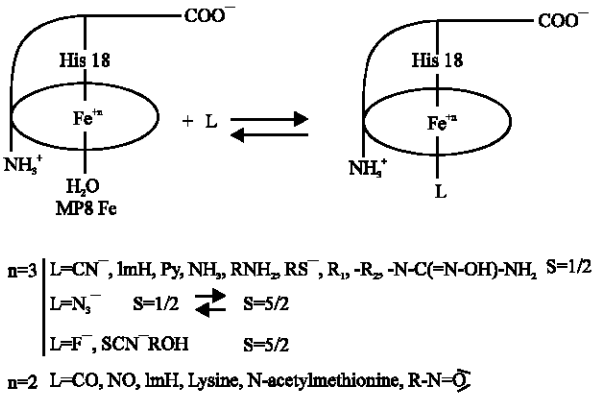

Fig. 2: Complexes of iron(II) and iron(III)-microperoxidase 8 with various ligands.

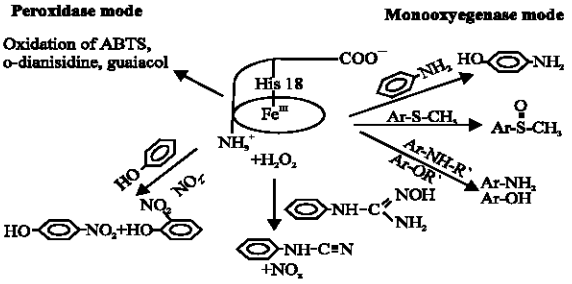

Fig. 3: Oxidation of various substrates by H_2O_2 catalyzed by microperoxidase 8.

intermediate (Fig. 3 and 4). The best system, associating H_2O_2 as oxidant and 3A3-MP8 as a catalyst, in the presence of 5% tert-butyl alcohol, led to the stereoselective S-oxidation of thioanisole with a 45% enantiomeric excess in favour of the R isomer[39]. This constitutes the highest enantiomeric excess reported to date for the oxidation of sulfides catalyzed by catalytic antibodies with a peroxidase activity.

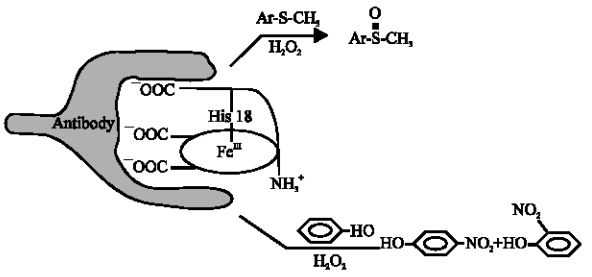

Fig. 4: Selective oxidation of substrates by H_2O_2 catalyzed by MP8-antibody complexes.

MP8 as a biosensor for the detection of molecules of biological interest at the surface of electrode: Thanks to the binding properties of its iron atom described in the first paragraph of this manuscript, a new biomedical application has been found for MP8 as a biosensor for molecules of biological interest. The principle of this method is based on the immobilization of MP8 at the surface of an electrode, in such a way that the 6th coordination position of the iron may be accessible to ligands and, then, on the measurement of the variations of the redox potential and of the current intensity induced by the binding of a ligand on the iron. At least three strategies may be envisioned to immobilize MP8 at the surface of an electrode (Fig. 5) : a simple adsorption at the surface of a roughened electrode, the covalent binding on a monolayer of functionalized thiols at the surface of a gold electrode and the covalent binding through an amide bond to a polypyrrole film grown at the surface of a carbon or a gold electrode.

The first strategy was already used and microperoxidase 8 was adsorbed at the surface of a roughened silver electrode in order to provide a new supported biomimetic system for hemoproteins[40] (Fig. 5A). A combination of two techniques was used to study its redox and coordination properties: electrochemistry and Surface Enhanced Resonance Raman (SERR) spectroscopy. This allowed to show that microperoxidase 8 could be adsorbed as a monolayer at the surface of the roughened silver electrode, where it could undergo a reversible electron transfer. Under those conditions, a redox potential of –0.4 V vs. SCE (-0.16 V vs. NHE) was measured for MP8, which was almost identical to that reported for N-acetyl-MP8 in aqueous solution. In addition, whereas MP8 appeared to aggregate in solution and led to a mixture of high spin penta-coordinated (5cHS)- and low spin hexa-coordinated (6cLS) iron (III) or iron (II) species, it was recovered almost exclusively as a monomeric high-spin penta-coordinated species at the surface of the electrode,

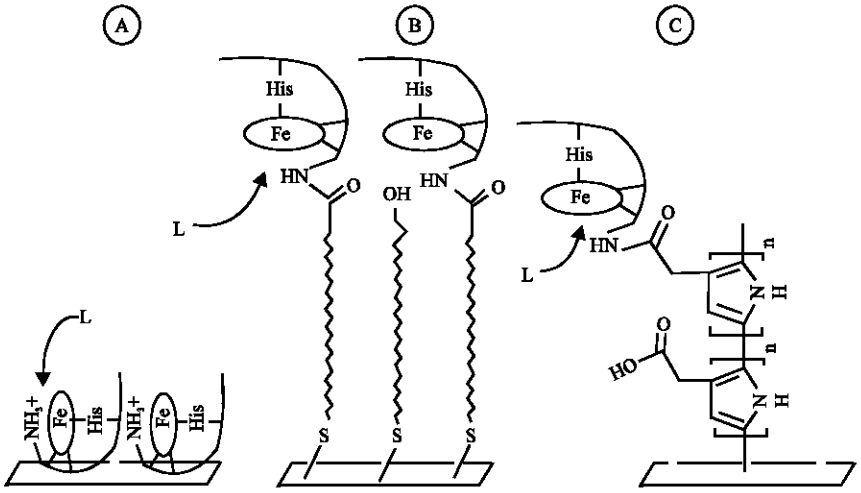

Fig 5: Various ways for immobilizing MP8 at the surface of an electrode: (A) adsorption, (B) covalent binding to Self Assembled Monolayers (SAM) of thiols (C) covalent binding to functionnalized polypryrrole.

both in the reduced and in the oxidized states. To validate the use of MP8 as a biosensor under those conditions, the binding of cyanide was realized and led almost exclusively to the low-spin hexacoordinated $MP8Fe^{III}$-CN species[40]. It is then reasonable to think that MP8 adsorbed at the surface of a roughened electrode will be able to bind a wide range of ligands such as CN^-, imidazole, oxidants like O_2 or H_2O_2, which will open the way to the biocatalysis of oxidation reactions at the surface of an electrode or drugs such as miconazole, ketoconazole, clotrimazole which will lead to the design of new biosensors for molecules of biological interest.

The only limitation to the use as a biosensor of MP8 adsorbed at the surface of a silver electrode is the slow desorption which occurs when a potential is applied to the electrode for a long time. To avoid this problem, two other methods which are aiming at binding more tightly MP8 at the surface of a silver electrode are under development. In the first method, MP8 has been immobilized on a self-assembled monolayer of thiols at the surface of a silver or a gold electrode. For this purpose, the monolayer was made of thiols of various lengths functionalized with an activated ester and MP8 was subsequently covalently attached through an amide bond after reaction with the terminal amino group of its octapeptide (Fig. 5B). Spacer thiols, functionalized with methyl or hydroxymethyl substituents were also added to control the hydrophilicity of the surface to insure an adequate conductivity[41]. ATR mode Fourrier Transform IR spectroscopy allowed to detect the vibrations due to the formation of the amide bond between MP8 and the activated thiol, thus proving that the covalent binding did occur. Then, cyclic voltametry studies coupled with SERR spectroscopy allowed to show that MP8 was attached as a very electrochemically reactive pentacoordinate high-spin iron (III) species that will be available for its use as a biosensor.

In the second method, MP8 has been covalently attached to a film of polypyrrole at the surface of a gold electrode (Fig. 5C). A copolymer of pyrrole substituted with an alcohol group and pyrrole substituted with an activated ester group was first grown electrochemically at the surface of the electrode. The subsequent covalent attachment of MP8 occurred through an amide bond, which was obtained by reaction of the terminal NH_2 of the octapeptide and the activated ester of the polypyrrole. An electrochemical analysis of these electrodes shows that, under those conditions, MP8 is characterized by a rather low redox potential and a fast and reversible electron transfer, which seems adequate for its further use as a biosensor.

As a conclusion, microperoxidase 8 which can easily be obtained from cytochrome c, represents not only a powerful tool for exploring the coordination chemistry of iron-porphyrins but also provides a good model for studying a variety of reactions catalyzed by hemoproteins. Furthermore, the association of MP8 with a monoclonal antibody constitutes a new generation of artificial hemoproteins having monooxygenase and peroxidase-like activities and appears as a promising biocatalyst for selective oxidation reactions. Finally, a new field of biomedical applications has recently been found for MP8 as a biosensor for molecules of biological interest. Now that the potential of biosensors based on MP8 is starting to be better understood one can anticipate many studies on these systems to be reported in future.

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