Engineering and Prosthetic-Group Modification of Myoglobin: Peroxidase Activity, Chemical Stability and Unfolding Properties

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The role of myoglobin (Mb) is not yet completely understood and recent evidence indicates it is involved in a variety of pseudo-enzymatic functions. Mb is also extensively used as a tool to redesign novel active-site features and introduce new activities into a protein. This review summarizes our approach to enhance the peroxidase-like activity of Mb toward phenolic compounds by combining two different strategies of protein modification, i.e. active-site engineering and cofactor replacement. The main objective of active-site engineering is to increase the rate of hydrogen peroxide activation upon reaction at the iron(III) center, while cofactor replacement facilitates substrate interaction at the protein active site, thereby increasing the efficiency of the catalytic process and stereoselective recognition of the substrate. The thermodynamic stability of the modified Mb derivatives has been evaluated through guanidinium chloride induced and thermal unfolding experiments. As expected, both protein mutation and cofactor replacement decrease the stability of the protein, the latter effect being somewhat more pronounced for the Mb derivatives studied here. For evaluating the catalytic efficiency of the protein in non-natural reactions, the chemical stability of the protein during catalysis is also important and therefore a new protocol was developed to evaluate the competitive degradation undergone by the protein upon substrate oxidation. It was found that autodegradation can occur through two different pathways involving oxidation of protein residues by the Mb active species or by a substrate-derived phenoxy radical. The peroxidase-like nitrating activity and the sulfoxidation reactions promoted by Mb, both involving the nitrite ion as a cosubstrate, have also been briefly described.

Introduction

Myoglobin (Mb), "the hydrogen atom of biology",[1] is a monomeric protein which gives muscles their red color. It has a globular structure consisting of eight α helices wrapping around a central hydrophobic pocket containing iron protoporphyrin IX as the prosthetic group. Since the early 19th century, Mb was believed to function in the storage of dioxygen and in facilitating its transport from the periphery of the cell to the mitochondria,[2] although, the situation has now changed and it appears that Mb may play additional roles. Four conserved cavities surrounding the heme group, termed xenon cavities because they can host Xe atoms, are lined with highly conserved hydrophobic residues and appear to be actively involved in the binding of small molecules like O_2, CO and NO.[3] This suggests that Mb is built like a miniature chemical reactor with the iron atom as active centers and the cavities as chambers in which reactants are concentrated and oriented. Since different molecules react at different sites of the protein, Mb could be considered as an allosteric enzyme.[1,3] In the case of NO, for example, binding of the ligand to the oxygenated form of Mb (oxyMb) is followed by rapid conversion into the nitrate.[4−6] Since NO is an inhibitor of cytochrome *c* oxidase, it has been suggested that the scavenging effect of Mb protects the cell from inhibition of cellular respiration.[3,7] Other recent observations support a multiple role for Mb. Studies using a gene disruption strategy to produce mice that lack Mb show that the protein acts as an important modulator of cardiac function.[8] In addition, in vitro Mb is active in oxidative phosphorylation, shows some peroxidase and P450-like catalytic activities,[9] and protects cells against oxidative damage.[10,11] In vivo Mb may be present up to a concentration of 0.2 mM within the cytosol of myocardial cells and exists prevalently as oxyMb. However, high concentrations of oxidants, such as those present under in-

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flammatory conditions, or acidic pH may induce autoxidation of the protein with formation of metMb. This is the state in which Mb can exhibit a peroxidase-like behavior.

The similarity that the Mb heme environment shares with other heme proteins, like cytochromes or peroxidases, makes Mb so widely employed for engineering novel metalloproteins by redesign of the existing metal-binding site.[12] Redesign of heme proteins gives insight into the factors governing structure and function within members of a closely related family and is invaluable for the developing area of de novo protein design. In this respect, Mb has several advantages because of its relatively small size, compact structure, and the extensively accumulated biochemical and biophysical characterization, including a large number of X-ray crystal structures available for both the native proteins from various sources and several mutants.[13] The current strategies for the modification of heme proteins follow four different approaches: i) rational design of heme enzymes; ii) directed evolution of heme enzymes; iii) exon shuffling of hemoglobin; and iv) replacement of the native heme prosthetic group (reconstitution).[14] For instance, the engineering of heme proteins is used as a strategy to develop new blood substitutes.[15,16] The availability of the expression system for recombinant Mb in *E. coli* and the relatively easy methods of site-directed mutagenesis make the engineering approach of extreme importance for investigating the role of critical residues and for introducing non-natural activities into the protein.[17,18] On the other hand, reconstitution of Mb with a modified prosthetic group (Figure 1) allows the introduction into the protein of a specific binding site for exogenous substrates or protein−protein interactions.[19,20] In this review, we summarize the results obtained in our group on the peroxidase-like activity of Mb upon modification of the protein by site-directed mutagenesis and by altering the prosthetic group. Combining the two approaches allowed us to develop a powerful method to enhance the reactivity and selectivity of Mb derivatives. Peroxidative processes mediated by Mb appear to be important in the oxidative degradation of lipid compounds in biological systems and foods.[21] Moreover, the transient protein radical, produced by the reaction between Mb and hydrogen peroxide, appears to be a key determinant of oxidative damage in ischemic heart.[10]

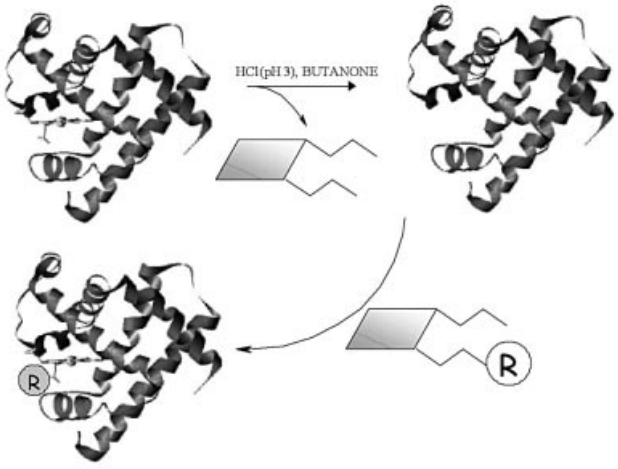

Figure 1. Two steps of the Mb reconstitution process: (i) extraction of the native heme with an organic solvent at acidic pH, and (ii) introduction of the chemically modified heme cofactor into apoMb

Characteristic Features of Peroxidases

Peroxidases are a class of ubiquitous heme enzymes that catalyze the oxidation of a wide range of substrates by hydrogen peroxide.[22,23] The stoichiometry of the reaction is normally as follows:

$$2\,SH + H_2O_2 \rightarrow 2\,S^{\cdot} + 2\,H_2O \quad (1)$$

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which emphasizes that the typical activity of these enzymes involves the one-electron oxidation of two substrate molecules by hydrogen peroxide or other peroxides. In their catalytic cycle, the resting state Fe^{III} center of peroxidases is oxidized by H_2O_2 to generate an intermediate containing an $Fe^{IV}=O$ group with a porphyrin or protein cation radical, which is known as Compound I. In the presence of a suitable substrate Compound I is reduced to a second ferryl intermediate, known as Compound II, and subsequently to the resting state by two sequential one-electron oxidations of substrate molecules.[23,24] The reaction with H_2O_2 is facilitated by a "push−pull" mechanism, to which suitably positioned residues located in both the heme proximal and distal sites contribute.[25,26] The distal arginine and histidine residues favor O−O bond polarization of the Fe-bound hydroperoxide and proton transfer from the Fe-bound oxygen atom to the other to form, after cleavage of the O−O bond, the $Fe^{IV}=O$ species and a water molecule (Figure 2). The proximal aspartate participates in the weakening of the peroxide O−O bond by increasing the electron-donating character of the proximal histidine through a hydrogen bond to the imidazole NH group, which enhances back-donation from Fe into the peroxide π* orbitals[27] (Figure 2).

Typical low molecular weight substrates processed by peroxidases according to reaction 1 are electron-rich organic compounds such as phenols or aromatic amines.[28] These bind to the enzyme near the heme edge but cannot enter into the heme distal pocket, the access to which is controlled by several aromatic residues.[29−31] The disposition of the substrate at the heme edge allows an efficient transfer of electrons to the enzyme active species through the extended porphyrin π system.[32] In the past decades we have extensively studied the kinetics and mechanisms of these reactions using a variety of phenolic compounds and enzymes from various sources.[33−36] In particular, by using paramagnetic NMR spectroscopic and relaxation rate measurements we could gain an understanding of the selectivity and stereoselectivity effects observed in the enzymatic reactions in terms of the interaction between the enzymes and the substrates.

In addition to the classical peroxidase reaction, phenolic substrates can be efficiently nitrated on the aromatic ring by peroxidases in the presence of nitrite and hydrogen peroxide.[37,38] This reaction is important in vivo because nitration of tyrosine, to give 3-nitrotyrosine, has been associated with a wide range of human and animal diseases.[39,40] In fact, nitrite is a major product of nitrogen monoxide metabolism[41] and accumulates upon activation of NO synthases under inflammatory and other disease conditions. Interestingly, we have recently found that both peroxidases[42,43] and myoglobin[44] perform nitration of tyrosine derivatives through two distinct pathways which involve nitrogen dioxide or peroxynitrite, respectively, as nitrating agents depending on the conditions.

Reconstitution of Myoglobin with Chemically Modified Hemins and Site-Directed Mutagenesis

Mb, with peroxidases, shares the same heme cofactor and the same heme distal and proximal His residues (His64 and His93, respectively, in Mb). However, its globular shape, the different functional oxidation state of the iron atom, and the differences in heme environment and overall polarity of the active site, make the catalytic activity of Mb in peroxidase-type reactions markedly lower than that of the enzymes. In particular, Mb lacks the amino acid residues that are essential for the activation of hydrogen peroxide, i.e. a distal arginine and a proximal aspartate (Figure 2), and a specific cleft for substrate binding near the heme. The scope of our modifications of Mb basically aimed at introducing these characteristic features into the protein. In our initial attempts an Arg−Ala residue was covalently linked to one of the propionate side chains of protohemin and the resulting complex, HM−RA (Scheme 1), was inserted into horse heart apoMb.[45] Insertion of a modified hemin into Mb affects the pre-existing network of hydrogen bonds be-

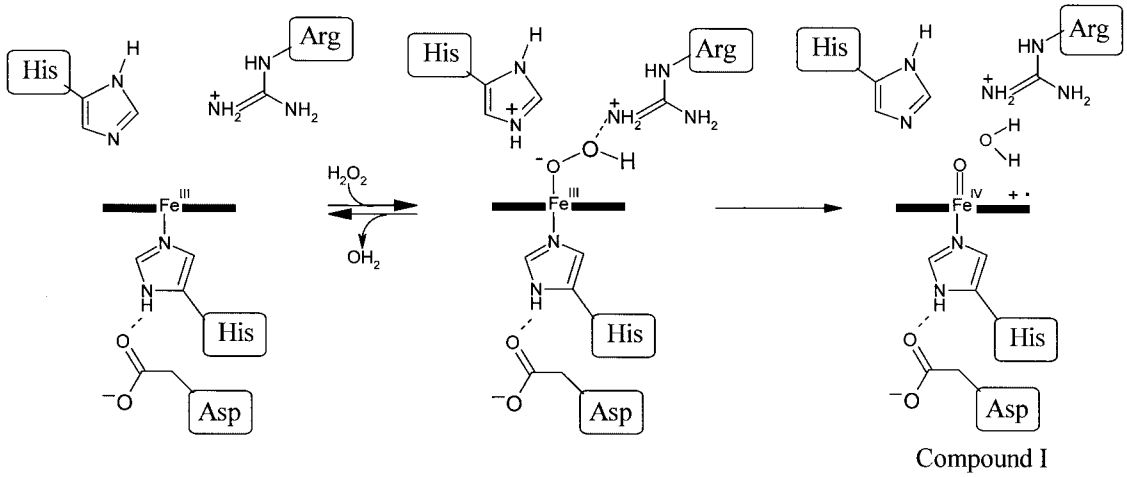
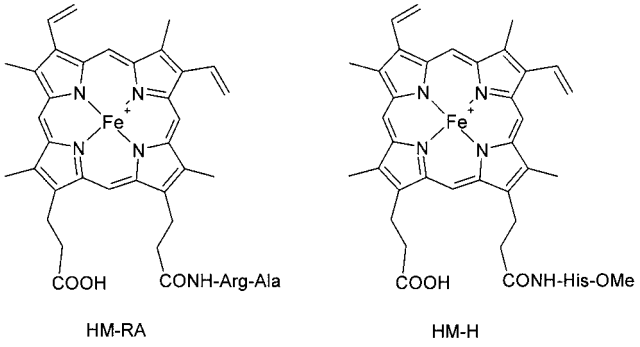


Figure 2. Mechanism of compound I formation in peroxidases promoted by general acid−base catalysis of polar residues in the active site

tween the propionate groups of the cofactor and side chain residues of the protein. In the case of HM−RA, the polypeptide fragment Phe43−Lys47 of Mb acquired sufficient mobility to allow collapse of the distal helix and binding of distal His to the sixth coordination position of the iron.[45] The presence of distal His as the sixth ligand hindered the binding of hydrogen peroxide to the iron and reduced the activity of the reconstituted protein. On the contrary, when the heme cofactor was modified by attachment of a histidine residue, HM−H (Scheme 1), the reconstituted Mb contained a significant portion of high spin form, which reacted normally with hydrogen peroxide. Since the His imidazole group of the HM−H prosthetic group is partially protonated at physiological pH, it could play a role similar to that of distal arginine in peroxidases.

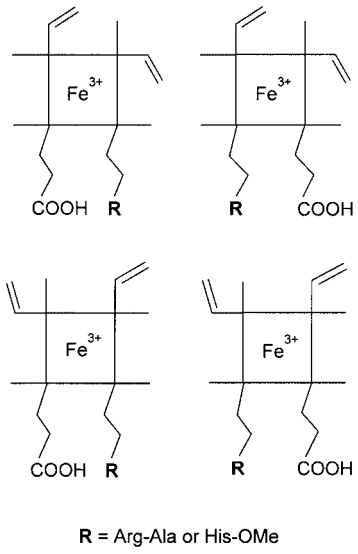


Scheme 1

Reconstitution of Mb with monosubstituted protohemins like HM−RA and HM−H unavoidably leads to a mixture of protein isomers. The hemin derivatives are in fact obtained as 1:1 mixtures of isomers containing the covalent modification at positions 6 or 7 of the porphyrin ring. Therefore, upon reconstitution of apoMb four protein isomers are obtained, depending on the type of substitution on the porphyrin and the mode of incorporation (“up” or “down”) of the prosthetic group into the protein (Scheme 2).[45] The ratio of these protein isomers is dictated by their relative stability and generally produces one major and one minor isomer for both 6- and 7-substituted HM−H derivatives, assessed by NMR spectroscopy.

The problem of Mb reconstitution isomers is reduced in the approach followed by Hayashi and Hisaeda, where Mb was reconstituted with synthetic protohemins bearing identical substituents at both the porphyrin propionates;[19,20] although, it is expected that the resulting protein derivatives will bear additional loss of stability because the interactions between the prosthetic group and the protein are further weakened.

In further developments we produced site-directed mutants of sperm whale Mb containing those amino acid residues critical for peroxide activation in peroxidases. In particular, we replaced the distal Thr67 with either Arg (T67R Mb)[46] or the more flexible Lys residue (T67 K Mb)[47] and obtained a double mutant where, in addition to the Thr67Arg mutation, the proximal Ser92 was substituted



Scheme 2

with an Asp residue (T67R/S92D Mb) (Figure 3).[48] The mutants T67 K Mb and T67R/S92D Mb were also reconstituted with HM−H, while with T67R Mb we could not obtain a stable derivative. The reconstituted sperm whale Mb derivatives will be indicated as WT Mb−H, T67 K Mb−H and T67R/S92D Mb−H.

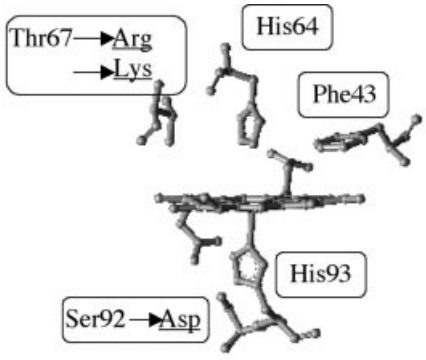


Figure 3. Engineering of the Mb active site: site-directed mutated residues are underlined

In general, point mutations do not appreciably affect the overall protein structure and only marginally perturb the heme environment.[46] The most significant change has been shown by the X-ray structure of T67R/S92D Mb to consist of an increase in the conformational mobility of the porphyrin propionate-7, which allows this group to span the heme proximal and distal sides through hydrogen-bonding interactions with water molecules.[46] The conformational mobility of propionate-7 is confirmed by marked shift of its signal in the ^{1}H NMR spectra of all Mb mutants with respect to the position of the signal in the spectrum of wild type Mb.[48] Reconstitution of Mb with synthetic hemins results in more substantial changes in the NMR spectra,

but the problem of the existence of several protein isomers makes a detailed analysis of the NMR spectra impossible.

Ligand Binding Data, Peroxide Activation and Catalytic Activity

Two representative ligands of different charge and size, azide and imidazole, were used as probes to investigate the effect of Mb modification on the affinity and accessibility of the iron site to external donor molecules. The affinity for azide is systematically increased upon introduction of a positively charged Arg, Lys or His residue into Mb by either engineering or reconstitution of the protein with HM−H, whereas with the bulky imidazole ligand a significant increase in the binding affinity is generally obtained only with the reconstituted proteins, due to the higher protein mobility at the entrance of the distal pocket (Table 1). The enhanced azide binding affinity exhibited by the modified Mb derivatives is expected to similarly stabilize the binding of H_2O_2 to the iron as a hydroperoxide ligand. As with peroxidases, this binding step is rapidly followed by cleavage of the O−O bond in the Fe^{III}−O−OH complex to produce a ferryl Mb species and a protein radical ($^{\cdot}MbFe^{IV}$=O). The positively charged residue introduced into the modified Mbs is expected to favor the heterolytic cleavage of the bound hydroperoxide, even though it cannot be suitably positioned to produce such a strong polarization effect of the O−O bond as that promoted by distal Arg in peroxidases. The rate constants for the formation of the Fe^{IV}=O Mb radical species (k_1) indeed show an appreciable increase for the modified Mbs, from a value of 760 ± 10 M^{-1} s^{-1} for WT Mb to 2100 ± 40 M^{-1} s^{-1} for the T67R/S92D Mb mutant.[46−48] Regarding the location of the protein radical, several studies indicate that it is distributed among several residues, including tyrosine, tryptophan and histidine residues,[49−53] with tyrosine being largely preferred, leading to the formation of Tyr−Tyr cross-links and Mb dimerization.[54]

To investigate the peroxidase activity of the Mb derivatives we followed a similar approach as in our previous studies with peroxidases.[33−35] We therefore selected a group of phenolic substrates which are related to tyrosine and contain substituents on the aromatic ring that can be useful for investigating selectivity effects in the enzymatic reaction. During activity, the phenolic substrates undergo one-electron oxidation to phenoxy radicals by the Mb active species, according to a reaction scheme analogous to that of peroxidases, which can be summarized as follows.

$$MbFe^{3+} + H_2O_2 \xrightarrow{k_1} {}^{\cdot}MbFe^{IV}{=}O + H_2O \quad (2)$$

$$^{\cdot}MbFe^{IV}{=}O + PhOH \xrightarrow{k_2} MbFe^{IV}{=}O + PhO^{\cdot} + H^+ \quad (3)$$

$$MbFe^{IV}{=}O + PhOH + H^+ \xrightarrow{k_3} MbFe^{3+} + PhO^{\cdot} + H_2O \quad (4)$$

$$2\ PhO^{\cdot} \longrightarrow \text{Dimers} \quad \text{(fast)} \quad (5)$$

The phenoxy radicals evolve independently of the protein catalyst in solution, giving a fast coupling reaction to dimeric products,[46] which subsequently yield complex mixtures of oligomers, as in the peroxidase-catalyzed reactions.[36,55] As discussed above, the rate constant k_1 can be determined by the reaction between Mb and hydrogen peroxide. The rate of reduction of the first protein intermediate ($^{\cdot}MbFe^{IV}$=O) by the substrate (k_2) is difficult to determine because this intermediate has optical features similar to the second intermediate ($MbFe^{IV}$=O), but the process is probably fast. Considering that $^{\cdot}MbFe^{IV}$=O does not accumulate in solution, the catalytic scheme can be reduced to a bimolecular ping-pong mechanism.[33] A further simplification in the in vitro kinetic scheme can be obtained by operating under saturating H_2O_2 conditions because in this case the rate equation reduces to conventional Michaelis−Menten kinetics, rate = k_{cat}[substrate]/(K_M + [substrate]).[46] Under these conditions, the third step, reduction of $MbFe^{IV}$=O by substrate, is rate limiting, and we can assume that the rate constant k_3 coincides with the turnover rate constant (k_{cat}).

The kinetic parameters for the catalytic oxidation of (*p*-hydroxyphenyl)propionic acid obtained with the various Mb derivatives exemplify how the catalytic performance of Mb is enhanced in an additive fashion by engineering and cofactor reconstitution (Table 2). The k_{cat} parameter, in particular, measures the electron transfer rate from the substrate to the $MbFe^{IV}$=O center. For a given substrate, this parameter depends on (i) the redox potential of the ferryl species, which is likely to increase by introduction of positively charged residues into Mb, and (ii) the mode of substrate interaction with the protein, which allows a closer approach to the heme, especially in the case of the reconstituted proteins. This interpretation is confirmed by the selectivity effects observed in the catalytic reactions, which could

Table 1. Ligand binding constants (K_B) for azide and imidazole by the Mb derivatives, in 0.2 M phosphate buffer pH 6.0 at 25 °C

Protein	Azide, K_B (M^{-1})	Imidazole, K_B (M^{-1})	References
WT Mb	(5.0 ± 0.1) × 10^4	25 ± 1	[46]
WT Mb−H	(5.6 ± 0.1) × 10^4	38 ± 1	[47]
T67 K Mb	(9.8 ± 0.5) × 10^4	32 ± 1	[47]
T67 K Mb−H	(27.0 ± 0.1) × 10^4	60 ± 1	[47]
T67R Mb	(6.0 ± 0.5) × 10^4	12 ± 1	[46]
T67R/S92D Mb	(9.0 ± 0.1) × 10^4	47 ± 1	[48]
T67R/S92D Mb−H	(15.0 ± 0.1) × 10^4	89 ± 2	[48]

Table 2. Kinetic parameters for the catalytic oxidation of (*p*-hydroxyphenyl)propionic acid by hydrogen peroxide in the presence of the Mb derivatives, measured in phosphate buffer pH 6.0 at 25 °C

Protein	k_{cat} (s^{-1})	K_M (mM)	k_{cat}/K_M (M^{-1} s^{-1})	References
WT Mb	1.0 ± 0.1	72 ± 5	14 ± 0.4	[46]
WT Mb−H[a]	−	−	−	[47]
T67 K Mb	2.3 ± 0.2	28 ± 5	83 ± 9	[47]
T67 K Mb−H	6.7 ± 0.4	26 ± 3	255 ± 18	[47]
T67R Mb	2.3 ± 0.1	12 ± 2	190 ± 25	[46]
T67R/S92D Mb	1.5 ± 0.6	20 ± 2	80 ± 6	[48]
T67R/S92D Mb−H	6.6 ± 0.3	20 ± 2	330 ± 20	[48]

[a] Biphasic kinetics.

be rationalized according to a structural model for the protein-substrate interaction deduced from paramagnetic ^1H NMR spectroscopic relaxation measurements. According to this technique it is possible to obtain an estimate of the distances between the protons of the bound substrate and the paramagnetic Fe center through the enhancement of the relaxation rate of the nuclei caused by the proximity of the paramagnetic, high-spin Fe center of the protein (Figure 4).[46,47]

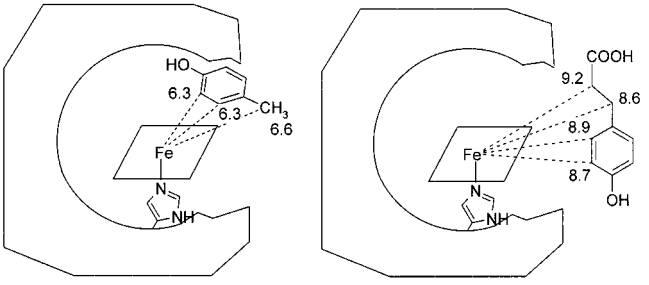


Figure 4. Distances (in Å), deduced by NMR spectroscopic relaxation measurements, between the paramagnetic Mb iron(III) center and the protons of protein-bound aromatic substrates: *p*-cresol (left) and (*p*-hydroxyphenyl)propionic acid (right)

(*p*-Hydroxyphenyl)propionic acid carries a side chain with a charge complementary to that of the residues introduced in the Mb heme environment and is therefore favored from the point of view of the protein-substrate interaction. Among the Mb derivatives, the highest catalytic activity (both in terms of k_{cat} and k_{cat}/K_M) is exhibited by T67 K Mb−H and T67R/S92D Mb−H (Table 2), because with these reconstituted proteins (*p*-hydroxyphenyl)propionic acid can reach closest to the Fe center and thus offer the most favorable arrangement for the electron transfer process.[47,48] The opposite trend holds for tyramine, which carries a positive charge in the side chain, while tyrosine is a very poor substrate for all Mb derivatives because it binds strongly to the protein but at a distance from the heme.[46−48]

The best performance by the Mb derivatives, in terms of k_{cat}/K_M, is obtained with *p*-cresol. The behavior of this substrate is unusual since its size is comparable with that of imidazole, and it can enter into the distal cavity of Mb, as shown by the short distance to the heme (Figure 4).[46] The reaction between *p*-cresol and the Mb active species is therefore unusually fast (almost similar to that of peroxidases),[46] and becomes limited by the reaction between the Fe^{3+} species and H_2O_2, making the comparison between the various Mb derivatives difficult.

Thermodynamic Stability and Stability of the Proteins during Catalysis

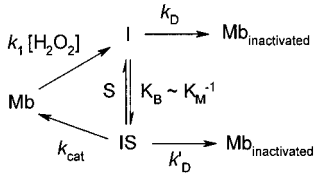
Many globular proteins can be reversibly unfolded in solution by addition of a denaturant, like urea or guanidinium chloride (Gdn−HCl), to the solution.[56,57] The stability of the protein, in terms of the unfolding free energy, can be determined by the analysis of a denaturant-induced unfolding curve as a function of the denaturant concentration. Unfolding of Mb has been studied intensively, and in the urea or Gdn−HCl denaturation experiments the behavior of the protein has been satisfactorily described by a two-state model (native/unfolded), without any defined intermediate state.[58−62] We obtained thermodynamic data on the stability of the various Mb derivatives through thermal and Gdn−HCl denaturation experiments, which were analyzed according to the two-state model.[62] The Gdn−HCl induced denaturation process was found to be completely reversible, indicating that the heme remains bound to the polypeptide chain even at high concentration of denaturant. On the contrary, the behavior of the proteins with temperature is different since they tend to precipitate at higher temperatures and the denaturation process becomes irreversible, possibly because, in this case, the heme is released by the protein. Nevertheless, the trend of thermal stabilities follows the same order as that found in the Gdn−HCl denaturation experiments.[47] As shown by the data reported in Table 3, both protein mutation and protein reconstitution decrease the stability of Mb, but in the latter case the effect is more pronounced. The observed trend can be accounted for in terms of the interactions involved in the binding of the heme to the protein, since heme binding gives the most prominent contribution to the stability of the holo protein. This contribution has been factored into that of the covalent bond between the iron atom and proximal His93 (25%), those of hydrophobic interactions between the heme and apolar residues in the pocket (50%), and those of specific interactions, which include hydrogen bonds and charge effects, between the protein and the propionate

Table 3. Thermodynamic parameters for unfolding induced by Gdn−HCl (ΔG^0_{N-U} and $-m$) and Gdn−HCl concentration causing 50% denaturation of the Mb derivatives ($[Gdn-HCl]_0$), obtained in 0.2 M phosphate buffer pH 6.0 at 25 °C

Protein	ΔG^0_{N-U} (kcal mol^{-1})	$-m$ (kcal mol^{-1} M^{-1})	$[Gdn-HCl]_0$ (M)	References
WT Mb	4.95 ± 0.13	3.45 ± 0.09	1.46 ± 0.01	[47]
WT Mb−H	3.99 ± 0.08	3.64 ± 0.07	1.09 ± 0.01	[47]
T67 K Mb	4.71 ± 0.12	3.50 ± 0.09	1.36 ± 0.01	[47]
T67 K Mb−H	3.35 ± 0.07	3.97 ± 0.08	0.83 ± 0.01	[47]
T67R Mb	4.08 ± 0.11	3.44 ± 0.09	1.20 ± 0.01	this work
T67R/S92D Mb	4.48 ± 0.09	3.79 ± 0.08	1.16 ± 0.01	this work
T67R/S92D Mb−H	1.10 ± 0.06	1.35 ± 0.05	0.88 ± 0.03	this work

groups (25%).[63] The specific hydrogen-bonding and electrostatic interactions involving the propionates in sperm whale Mb are with Leu89, Ser92, His93 and His97 for propionate-7, and with His64 and Arg45 for propionate-6.[64][65] The decrease in stability of Mb upon mutation of the Thr67 residue can be attributed mainly to a change in conformation of the propionate-7 group, which is shown by the NMR spectra of the Mb mutants[46−48] and is confirmed by the X-ray structure of T67R/S92D Mb.[48] The loss of the hydrogen-bonding structure can be partially compensated by new interactions established by the mutated residue. This is suggested by the observation that the most stable Mb mutant, T67 K Mb, is the one that contains the engineered residue with most flexible side chain that can more easily establish interactions with other groups in the protein. The parameter $-m$ that is related to the protein−water interaction and the surface roughness of the protein is reported in Table 3. Surprisingly, this parameter is much smaller for T67R/S92D Mb−H than the other Mb derivatives. This may indicate a significant difference in the solvent-exposed surface area of the reconstituted Mb double mutant or, more likely, the existence of some intermediate state in the unfolding process. The decrease in stability observed for the reconstituted Mb derivatives is largely due to the loss of one of the hydrogen-bonding networks maintained by the two heme propionates in the native protein. This increases the protein mobility around the heme, facilitating the interaction with the denaturant. The lowest free energy of unfolding is observed for the engineered and reconstituted Mb−H derivatives; here, an additional repulsive interaction occurs between the positively charged Lys or Arg residues and the His residue present in the HM−H cofactor. Although the reconstitution isomers of each Mb−H derivative may have different stabilities, the fact that, in every case, the various protein isomers are formed in similar amounts indicates that such differences in stability are limited.

Of particular interest is the problem of protein stability during catalysis since this is an important aspect for the assessment of biological catalysts. The Mb active species formed during turnover are not only able to oxidize exogenous substrates but can competitively react with amino acid residues, which ultimately leads to loss of catalytic activity. When the Mb derivative reacts with hydrogen peroxide in the absence of a substrate, a fast inactivation process occurs, which is characterized by an autodegradation rate constant k_D. The inactivation depends on the formation of radical species, produced at various protein residues, as discussed above.[49−54] In the presence of substrate the catalytic reaction competes with protein degradation and the possible pathways are summarized in Scheme 3. In this scheme, I is the Mb active species, S the substrate, IS the complex substrate−Mb active species, and $Mb_{inactivated}$ the degradation product (e.g. the protein derivative resulting from autoxidation of amino acid residues or the porphyrin) which can be formed directly or through a process mediated by the bound substrate. The latter process is characterized by rate constant k'_D.



Scheme 3

The competition between the catalytic reaction and protein degradation depends on substrate concentration but, at least with the phenolic substrates, inactivation can never be completely neglected. In fact, the substrate-bound by Mb can also promote protein inactivation, and the relative importance of this process with respect to substrate oxidation is governed by the ratio between the rate constants k'_D/k_{cat}. Different situations can be envisaged according to whether $k_D \approx k'_D$, $k'_D > k_D$, or $k'_D < k_D$ (Table 4 and Figure 5). Protein autodegradation may not be affected by the presence of the substrate ($k'_D < k_D$), as in the case of T67R Mb and T67R/S92D Mb, or only marginally affected by the presence of the substrate ($k_D \approx k'_D$), as in the case of WT Mb, WT Mb−H and T67 K Mb. This situation occurs when the active site is relatively closed and the phenoxy radicals produced by the Mb active species rapidly diffuse in the solution, thus preventing the substrate-induced degradation of the protein. The protecting effect exerted by the protein accounts for the longer lifetime of these biocatalysts with respect to simple hemins or microperoxidases where the active site is quite open and allows for a close approach of the substrate to the heme, which strongly facilitates the substrate induced degradation process.[66−68] A

Table 4. Protein autodegradation rate constants (k_D) and protein degradation rate constants in the presence of the substrate (*p*-hydroxyphenyl)propionic acid (k'_D) during catalysis for the Mb derivatives

Protein	k_D (s^{-1})	k'_D (s^{-1})	References
WT Mb	$1.93 \times 10^{-3} \pm 4 \times 10^{-5}$	1.9×10^{-3}	[47]
WT Mb−H	$8.31 \times 10^{-3} \pm 1 \times 10^{-5}$	8.3×10^{-3}	[47]
T67 K Mb	$0.94 \times 10^{-3} \pm 8 \times 10^{-5}$	0.9×10^{-3}	[47]
T67 K Mb−H	$2.40 \times 10^{-3} \pm 4 \times 10^{-4}$	6.1×10^{-3}	[47]
T67R Mb	$2.29 \times 10^{-2} \pm 3 \times 10^{-4}$	≈ 0	this work
T67R/S92D Mb	$8.16 \times 10^{-3} \pm 1 \times 10^{-4}$	1.9×10^{-3}	this work
T67R/S92D Mb−H	$1.18 \times 10^{-2} \pm 2 \times 10^{-4}$	1.2×10^{-2}	this work

similar situation is observed with T67 K Mb−H only, for which the inactivation process in the presence of substrate is faster than in the absence of substrate ($k'_D > k_D$). This is consistent with the already mentioned increased accessibility of the heme site of T67 K Mb−H to the substrate. In this case, the phenoxy radical produced by the Mb active species can attack the porphyrin before diffusing into the solution, thus causing protein inactivation. However, it is worth emphasizing that the closer binding of the substrate to the heme has the advantage of increasing the catalytic turnover, and we can see from the data in Table 2 and Table 4 that the increase in k_{cat} exhibited by T67 K Mb−H with respect to WT Mb is significantly larger than the increase in k'_D. An increase in the ratio k_{cat}/k'_D always improves the performance of the biological catalyst. In general, at least with phenolic substrates, the numerical values of the protein degradation rate constants k_D and k'_D are about two orders of magnitude smaller than the turnover rate constants (k_{cat}), indicating that all Mb derivatives perform a large number of catalytic turnovers before undergoing significant degradation.

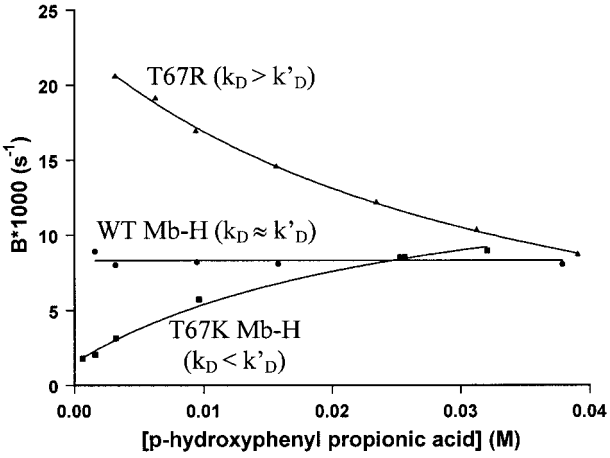


Figure 5. Types of behavior of Mb derivatives in the degradation processes undergone during catalysis; the dependence of parameter B [defined as $B = (k_D K_M + k'_D[S])/(K_M + [S])$][47] on (*p*-hydroxyphenyl)propionic acid concentration is shown under the various conditions that can affect the degradation process: $k_D \approx k'_D$, $k'_D < k_D$ or $k'_D > k_D$, depending on the way the substrate interacts with the protein; protein autodegradation may be only marginally affected ($k_D \approx k'_D$), unaffected ($k'_D < k_D$), or strongly affected ($k'_D > k_D$) by the presence of substrate

New Peroxidase-Like Reactions of Myoglobin Promoted by Nitrite Activation

Nitrite is a major product of nitrogen monoxide metabolism.[69,70] Although nitrite is considered a harmless metabolite, its accumulation is reported in some pathological conditions.[71] Actually, NO_2^- dramatically enhances hydrogen peroxide toxicity in the presence of heme proteins because these systems can generate powerful oxidizing and nitrating species.[39,40][72] We have recently demonstrated that enzymatic nitration of tyrosine derivatives[43] promoted by peroxidase/H_2O_2/NO_2^- proceeds through two distinct pathways which involve nitrogen dioxide or peroxynitrite, depending on the conditions, as nitrating agents. A similar behavior is exhibited by the metMb/NO_2^-/H_2O_2 system.[44] At low nitrite concentration Mb reacts through the classical peroxidase-like cycle, with two active intermediates which can either react with the phenolic substrate, according to reactions (2)−(4), or with nitrite to generate nitrogen dioxide in the competing reactions:

$$^{\cdot}MbFe^{IV}{=}O + NO_2^- \rightarrow MbFe^{IV}{=}O + NO_2^{\cdot} \quad (6)$$

$$MbFe^{IV}{=}O + NO_2^- \rightarrow MbFe^{3+} + NO_2^{\cdot} \quad (7)$$

The enzymatically produced phenoxy radical and $NO_2^{\cdot}$ can give then a fast recombination reaction producing the nitrophenol:

$$NO_2^{\cdot} + Ph{-}O^{\cdot} \rightarrow O_2N\text{-}Ph\text{-}OH \quad (8)$$

$$NO_2^{\cdot} + Ph{-}OH \rightarrow Ph{-}O^{\cdot} + NO_2^- \quad (9)$$

The phenoxy radical can also be produced by reaction of $NO_2^{\cdot}$ with phenol. At high nitrite levels this acts as a ligand for the iron center, and hydrogen peroxide reacts with the iron-bound nitrite producing an active species that we formulate as a protein-bound peroxynitrite, $MbFe^{3+}{-}N(O)OO^-$. This is capable of nitrating the phenol in a two-electron process:

$$MbFe^{3+} + NO_2^- \rightleftarrows MbFe^{3+}-NO_2^- \quad (10)$$

$$MbFe^{3+}-NO_2^- + H_2O_2 \rightarrow MbFe^{3+}-N(O)OO^- + H_2O \quad (11)$$

$$MbFe^{3+}-N(O)OO^- + PhOH \rightarrow MbFe^{3+} + O_2N\text{-}PhOH \quad (12)$$

In the absence of an exogenous substrate Mb catalyzes its own nitration. Mb contains several amino acid residues that can be nitrated. Upon treatment of Mb with peroxynitrite Bourassa et al.[73] found that nitration occurs at the exposed Tyr103, while Herold et al.[74] found that nitration occurs at Tyr146. By reacting Mb with nitrite and hydrogen peroxide, we found that the first and most important nitration site is the heme (30% to 50% yield depending on the conditions), while a secondary protein nitration site has been identified as Tyr146 (about 5% yield).[44] This is a surprising result considering that Tyr146 is an inner residue lined to the Xe1 cavity of Mb and it is farther from the heme iron center than Tyr103 (the closest C atom of the phenol ring is 9.7 Å from the iron atom for Tyr146 and 3.3 Å for Tyr103).[3][75] Therefore, the nitrating agent responsible for the self nitration of Mb does not come from the outside of the protein but diffuses from the heme to the Xe1 cavity and reacts through an intramolecular reaction.

Heme peroxidases catalyze the oxidation of organic sulfides to sulfoxides with a certain degree of stereoselectivity.[28,76] This type of reaction is catalyzed much more efficiently by chloroperoxidase.[77–79] Through the engineering of Mb, the group of Watanabe obtained mutants that exhibit catalytic turnovers and interesting stereoselectivity effects in the sulfoxidation of thioanisole.[80–82] We have studied the effect of nitrite in the oxidation of aromatic sulfides catalyzed by various Mb derivatives. As shown before, in the presence of hydrogen peroxide nitrite produces nitrogen dioxide or peroxynitrite, which are both powerful nitrating and oxidizing species. The idea was that these species could participate in the sulfoxidation catalytic cycle of Mb and increase the efficiency and/or selectivity of the reaction. Indeed, nitrite caused a general improvement in the enantioselectivity in the produced sulfoxide (with *ee* up to about 50% for sulfoxidation of thioanisole).[83] By analogy with peroxidases,[79] the catalytic scheme we propose for the sulfoxidation by Mb is the following:

$$MbFe^{3+} + H_2O_2 \rightarrow {}^{\cdot}MbFe^{IV}=O + H_2O \quad (12)$$

$${}^{\cdot}MbFe^{IV}=O + S \rightarrow MbFe^{3+} + SO \quad (13)$$

$${}^{\cdot}MbFe^{IV}=O + S \rightarrow MbFe^{IV}=O + S^{\cdot+} \quad (14)$$

$$MbFe^{IV}=O + S \rightarrow MbFe^{3+} + S^{\cdot+} \text{ (slow)} \quad (15)$$

$$S^{\cdot+} + S^{\cdot+} + H_2O \rightarrow S + SO + 2\,H^+ \quad (16)$$

where S indicates the sulfide and SO the sulfoxide. The first protein intermediate, ${}^{\cdot}MbFe^{IV}=O$, can convert sulfide to sulfoxide by a direct *O*-transfer reaction [formally a two-electron process, reaction (13)], or to a sulfur cation radical by a one-electron oxidation according to reaction (14). The second protein intermediate, $MbFe^{IV}=O$, can only produce a sulfur cation radical. The former reaction gives the sulfoxide with a certain degree of enantioselectivity, while dismutation of sulfur cation radicals by reaction (16) gives racemic sulfoxide.[79,84–86] Therefore, the observed enantioselectivity depends on the fraction of Mb that reacts by reaction (13). Nitrite efficiently reduces intermediate $MbFe^{IV}=O$ to the native state[44] according to:

$$MbFe^{IV}=O + NO_2^- \rightarrow MbFe^{3+} + NO_2^{\cdot} \quad (17)$$

and this process is faster than reaction (15). Therefore, the effect of nitrite is to reduce the contribution of the pathway that produces the sulfoxide through the stereochemically unproductive coupling of sulfur cation radicals.

Concluding Remarks

In this review we summarized our contribution to the search for new catalytic activities of Mb by combining site-directed mutagenesis with prosthetic-group modification. We focused on peroxidase-type reactions promoted by the activation of hydrogen peroxide because this reagent transforms the iron center into an active species that can, in principle, perform controlled reactions on exogenous substrates. Due to similarities between the active sites of Mb and peroxidases, the changes that are introduced in the protein are insignificant relative to those that are necessary for the simulation of the activities of other enzymes using Mb. In general, the results obtained so far clearly indicate that the strategies of active-site engineering and cofactor modification are cumulative in enhancing the pseudo-peroxidase activity of Mb, and can be further elaborated to introduce more sophisticated substrate recognition sites into the protein. We have also introduced a new experimental method to evaluate the relative stability of the Mb catalysts under operational conditions and identified the pathways by which the protein undergoes competitive degradation. Although the investigated reactivities are not natural for Mb, both peroxide and nitrite activation by this protein may bear particular importance under pathological conditions where bursts of nitric oxide and hydrogen peroxide are produced by the immune defense system.[87] Manipulating protein activities by chemical or genetic methods is an intellectual challenge that will strongly affect the way chemists devise new approaches to synthetic methods and applications.

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