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Nitrite increases the enantioselectivity of sulfoxidation catalyzed by myoglobin derivatives in the presence of hydrogen peroxide

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Abstract—The effect of nitrite in the sulfoxidation of organic sulfides catalyzed by myoglobin (Mb) in the presence of hydrogen peroxide has been investigated. A general improvement in enantioselectivity was found for the reaction catalyzed by horse heart metMb and a series of sperm whale metMb derivatives including the wild type protein, the active site mutants T67K Mb, T67R Mb, T67R/S92D Mb, and the T67K Mb derivative reconstituted with the modified prosthetic group protohemin-L- histidine methyl ester.

1. Introduction

Recent studies show that many proteins can provide low levels of activity in reactions different from those involved in their normal biological function.[1] Activity can be improved by engineering the proteins, particularly in the active site features. Such a strategy has become popular in the case of metalloproteins, where the metal-binding sites have been redesigned in different ways, to introduce structural features which are specific to other proteins or enzymes.[2] Among these proteins is myoglobin (Mb), the protein of storage and intracellular transfer of molecular oxygen,[3] which is widely used as a catalyst in peroxide-dependent one-electron oxidation, such as oxidation[4] and nitration[5] of phenolic substrates, and in reactions involving formal two-electron oxidation, such as sulfoxidation of organic sulfides and epoxidation of alkenes.[6] The latter type of reactions, termed peroxygenations, can proceed with a certain degree of stereoselectivity. Both enantioselective sulfoxidation[7] and epoxidation[8] are catalyzed much more efficiently by chloroperoxidase, but in general, commercial applications of peroxidases are limited by the high cost and the low stability of the enzyme, due to the rapid inactivation by the oxidant.[9] For this reason, other proteins such as Mb and its derivatives are explored as potential biocatalysts to perform asymmetric transformations. Single or double mutations can provide substantial contributions to the optimization of the new activity: by engineering the active site of Mb, the group of Watanabe obtained mutants that exhibit significant catalytic turnover with high stereoselectivity in the sulfoxidation and epoxidation of substituted aromatic substrates.[4a,6,10] A different strategy of Mb modification involves replacement of the natural prosthetic group with a synthetic hemin.[4b,11]

We have recently found that during the catalytic cycle of peroxidase-type reactions by Mb, nitrite, in the presence of hydrogen peroxide, produces two powerful nitrating and oxidizing species, nitrogen dioxide ($NO_2^{\cdot}$) and peroxynitrite ($ONOO^-$), depending on nitrite concentration.[5] Herein we report on the effect of nitrite in the oxidation of aromatic sulfides catalyzed by horse heart Mb (hh Mb), sperm whale Mb (WT Mb), the three active site mutants of the latter protein T67R Mb,[12] T67K Mb[13] and T67R/S92D Mb,[14] and the mutant T67K Mb reconstituted with the modified hemin obtained by covalently linking an L-histidine methyl ester residue to one of the propionate side chains of protohemin IX, T67K-His Mb.[4b,13] All the proteins are utilized in their met (Fe^{3+}) form. The aim of this investigation is to assess whether the highly reactive species generated in the presence of nitrite could participate in the sulfoxidation catalytic cycle of Mb and increase the efficiency and/or stereoselectivity of the reaction. Indeed, a general improvement in enantioselectivity was obtained both for horse heart Mb and sperm whale Mb mutants. In addition, we performed NMR studies on Mb-substrate complexes with the aim of gaining a picture of the interaction between the sulfides and the protein.

Keywords: Asymmetric oxidation; Myoglobin; Nitrite; NMR; Sulfoxidation.
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2. Results and discussion

2.1. Effect of nitrite in the sulfoxidation reaction

The sulfoxidation of thioanisole (**1**) catalyzed by hh Mb in the presence of hydrogen peroxide was chosen as a standard reaction to optimize reaction conditions. The effects of reaction time, temperature, amount of hydrogen peroxide, and concentration of Mb on the enantioselectivity and conversion of sulfoxide were taken into consideration in these preliminary experiments. It was also established that the non-catalyzed reaction, that is, the reaction carried out in the absence of Mb by simply mixing the sulfide with hydrogen peroxide (and nitrite when required) was negligible in the optimized conditions (see Section 4). The effect of nitrite concentration on sulfoxide yield and enantiomeric excess was then studied in order to investigate the eventual involvement of the two different active species derived from nitrite in the mechanism of sulfoxidation. Indeed, an improvement in enantioselectivity by nitrite was observed and the concentration of nitrite that maximizes the enantioselectivity was found to be 50 mM (Table 1). At this relatively low [NO_2^-] the only active species derived from nitrite which may be involved in the sulfoxidation is nitrogen dioxide.[5] On the other hand, the yield of sulfoxide decreased in the presence of nitrite, indicating that part of the hydrogen peroxide was consumed in the parallel, unproductive oxidation of nitrite to nitrate.

The increase of enantioselectivity in the sulfoxidation carried out in the presence of nitrite was confirmed with the other substrates (Table 2). We chose sulfides carrying a para substituent, methyl *p*-tolylsulfide (**2**), the *iso*-propyl *p*-tolylsulfide (**3**), and methyl 2-pyridylsulfide (**4**). Different reactions conditions were needed for compound **4** (see Section 4), because this substrate is more difficult to oxidize than the others, due to the electron withdrawing effect of the heteroatom. For this reason, it was necessary to increase the reaction time and add the oxidizing agent and nitrite in small aliquots in order to protect the Mbs from degradation.

In order to exclude the possibility that nitrite acted in the reaction through a simple salt effect, that is, by an electrostatic interaction with the protein, the sulfoxidation of **1** catalyzed by hh Mb was carried out in the presence of sodium nitrate. These experiments were made at different concentrations of nitrate and in every case the enantiomeric excess did not change with respect to the reactions carried out in the absence of nitrate.

2.2. Asymmetric sulfoxidation catalyzed by Mb mutants

In our previous studies, we prepared Mb mutants containing

Table 1. Effect of addition of nitrite in the sulfoxidation of thioanisole catalyzed by hh Mb in the presence of hydrogen peroxide (the *R* enantiomer is always the major sulfoxide product)

Conversion (%)	ee (%)	[NO_2^-] (M)
39±4	10±2	—
15±2	21±2	0.01
24±1	29±3	0.05
20±1	22±1	0.10
28±1	5±1	0.20

Table 2. Enhancement of the ee in the sulfoxidation of **2**–**4** catalyzed by hh Mb and hydrogen peroxide in the presence of nitrite (the *R* enantiomer is always the major sulfoxide product)

Substrates	[NO_2^-]=0 M		[NO_2^-]=0.05 M		Method
	Conversion (%)	ee (%)	Conversion (%)	ee (%)	
2	15±2	10±1	10±1	16±1	A
3	5±0.5	5±1	11±3	10±1	A
4	3±0.5	0	3±0.5	0	A
4	14±1	20±2	12±1	24±2	B

a basic residue in the heme distal pocket, by replacement of Thr67 with either Arg (T67R Mb)[12] or the more flexible Lys residue (T67K Mb),[13] and a double mutant where, in addition to the Thr67Arg mutation, the proximal Ser92 was substituted with an Asp residue (T67R/S92D Mb).[14] These modifications aimed at introducing into the Mb active site those amino acid residues which are critical for the activation of hydrogen peroxide in peroxidases.[4b] In addition, we prepared a derivative of T67K Mb reconstituted with protohemin-L-histidine methyl ester (T67K-His Mb).[13] The latter hemin contains only one free carboxylate group and therefore, reconstitution of Mb with the modified cofactor involves the loss of the interaction with one of the propionate groups which stabilize heme binding to the protein. This causes a relaxation of the protein fragment around the heme which was found to increase the accessibility of donor molecules and phenolic substrates to the active site.[4b]

All Mb mutants shared with hh Mb the positive effect of nitrite in the sulfoxidation of thioanisole (Table 3). Among the Mb mutants studied here, the best enantioselective catalyst was found to be T67R Mb, since it afforded the highest increase of enantiomeric excess with respect to WT Mb in the presence of nitrite (from 15 to 51%). The yield of sulfoxide was also significantly improved. The highest conversion to sulfoxide was actually obtained with the mutant T67K Mb, but in this case the enantiomeric excess did not increase with respect to WT Mb. The low asymmetric induction is possibly due to the fact that the lysine residue at position 67 is not rigid enough to control the preferential formation of one sulfoxide enantiomer. Apparently, the guanidinium group in the arginine side chain exerts stronger steric control on the binding of the sulfide to the protein, that leads to a better stereodifferentiation in the oxygenation process.

The T67R Mb derivative gives higher enantioselectivity and yield also in the oxidation of substrate **4** (Table 4). In this case, the reactions were only carried out in the presence of nitrite, since the enhancement of enantioselectivity by nitrite in the oxidation of **4** was already observed for the reaction catalyzed by hh Mb (Table 2). With both the substrates, the T67K-His Mb derivative produced racemic sulfoxides: the increased protein mobility around the heme is clearly not advantageous for the immobilization of the substrate and results in the formation of achiral sulfoxides.

2.3. Mechanism of sulfoxidation

Mechanistic investigations of oxygen transfer reactions by heme peroxidases resulted in a 'hydrogen abstraction oxygen-rebound' mechanism; the actual oxygen transfer reagent could be one of the high-valent oxoferryl intermediates known as compound I ($P^{\cdot+}$ Fe^{IV}=O, where $P^{\cdot+}$ indicates a porphyrin cation radical) and compound II (Fe^{IV}=O), depending on the nature of the enzyme used.[15] For Mbs, unlike peroxidases, the high-valent oxoferryl species resulting from oxidation of Fe^{3+} by hydrogen peroxide cannot be differentiated, because instead of a porphyrin cation radical the initial species contains the radical localized on protein residues.[16] However, the possibility to stabilize a 'compound I-like' species for the Mb mutants obtained upon replacement of the distal His64 residue, allowed Watanabe and co-workers to investigate the direct sulfide-induced reduction of different compound I species and compare the mechanism of sulfoxidation of these proteins with that of horseradish peroxidase (HRP).[17] Two distinct pathways were found: the reduction of HRP compound I by thioanisoles proceeds via electron transfer in the protein cage (followed by oxygen rebound from compound II), whereas the reduction of H64S Mb compound I proceeds via direct oxygen transfer (the intermediate compound II being not involved). Compound I was observed as the catalytic species for the peroxygenase activity of all His64 Mb mutants, whereas only compound II was observed with WT Mb.[18]

Considering that all the proteins investigated here contain the His64 residue, both the oxoferryl intermediates produced in the catalytic cycle, that we indicate as. $MbFe^{IV}$=O and $MbFe^{IV}$=O, are involved in the sulfoxidation. The catalytic scheme can be summarized as follows:

$$MbFe^{3+} + H_2O_2 \rightarrow^{\cdot} MbFe^{IV}{=}O + H_2O \quad (1)$$

$$^{\cdot}MbFe^{IV}{=}O + S \rightarrow MbFe^{3+} + SO \quad (2)$$

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

$$[MbFe^{IV}{=}O + S^{\cdot+}] \rightarrow MbFe^{3+} + SO \quad (4)$$

Table 4. Sulfoxidation of methyl 2-pyridyl sulfide catalyzed by Mb mutants and hydrogen peroxide in the presence of 50 mM nitrite (the *R* enantiomer is always the major sulfoxide product)

Mb	Conversion (%)	ee (%)
Horse heart	12±1	24±2
WT	39±4	19±3
T67R	76±5	33±3
T67K-His	40±4	0

Table 3. Sulfoxidation of thioanisole catalyzed by Mb mutants and hydrogen peroxide in the presence of 50 mM nitrite (the *R* enantiomer is always the major sulfoxide product)

	$[NO_2^-]$=0 M		$[NO_2^-]$=0.05 M	
Mbs	Conversion (%)	ee (%)	Conversion (%)	ee (%)
Horse heart	39±4	10±2	24±1	29±1
WT	30±4	13±2	22±2	15±1
T67R	61±4	20±2	54±4	51±2
T67K	27±3	10±2	75±5	15±3
T67R/S92D	1±0.5	[a]	25±2	9
T67K-His	12±1	0	40±3	0

[a] The low conversion does not allow a reliable estimate of the enantiomeric excess.

$$[MbFe^{IV}{=}O + S^{\cdot+}] \rightarrow MbFe^{IV}{=}O + S^{\cdot+} \quad (5)$$

$$MbFe^{IV}{=}O + S \rightarrow MbFe^{3+} + S^{\cdot+} \text{ (slow)} \quad (6)$$

$$2S^{\cdot+} + H_2O \rightarrow S + SO + 2H^+ \quad (7)$$

where $MbFe^{3+}$ is the initial metMb form, S indicates the sulfide, SO the sulfoxide and $S^{\cdot+}$ the sulfur cation radical, respectively.

The first protein intermediate, $^{\cdot}MbFe^{IV}{=}O$, can convert sulfide to sulfoxide with a certain degree of stereoselectivity by a direct O-transfer reaction (formally a two-electron process), reaction (2). $^{\cdot}MbFe^{IV}{=}O$ may also form a sulfur cation radical by one-electron oxidation in the protein cage (indicated with square brackets) according to reaction (3). The latter reaction converts the protein into the second intermediate, $MbFe^{IV}{=}O$, containing a $S^{\cdot+}$ cation radical in the active site. This species can evolve according to two competitive pathways: it can give the oxygen rebound to afford the sulfoxide with a certain degree of stereoselectivity according to reaction (4), or the sulfenium radical can diffuse from the protein cage according to reaction (5). The following reduction of $MbFe^{IV}{=}O$ by another molecule of sulfide produces with low efficiency (see below) a sulfur cation radical, reaction (6). Finally, the dismutation of sulfur cation radicals in the bulk of the solution by reaction (7) gives the racemic product. Therefore, the stereoselectivity in the sulfoxidation depends on the fraction of Mb that reacts *via* $^{\cdot}MbFe^{IV}{=}O$ (reaction (2)) or *via* oxygen rebound from $MbFe^{IV}{=}O$ in the protein cage (reaction (4)).

Reaction (6) is a slow, low efficiency, process. In fact, treating the $MbFe^{IV}{=}O$ intermediate of hh Mb with different amount of thioanisole did not significantly affect the rate of disappearance of the $MbFe^{IV}{=}O$ bands; that is, the formation of metMb and Mb dimers through self oxidation of the active species is faster than reaction with the sulfide.

In contrast, nitrite efficiently reduces the $MbFe^{IV}{=}O$ intermediate to the native state with a second order rate constant $k=(18.6\pm0.6)\ M^{-1}\ s^{-1}$ according to the reaction:[5]

$$MbFe^{IV}{=}O + NO_2^- \rightarrow MbFe^{3+} + NO_2^{\cdot} \quad (8)$$

This process is faster than reaction (6). Furthermore, the reduction rate of $MbFe^{IV}{=}O$ by nitrite is not affected by the presence of thioanisole in the reaction mixture (see Section 4), indicating that the sulfide does not inhibit reaction (8) to a significant extent.

According to this mechanism, nitrite efficiently competes with sulfide for reduction of $MbFe^{IV}{=}O$. As a result, in the presence of this anion the importance of reaction (6) is reduced and, along with this, the contribution of the pathway that produces the sulfoxide through the stereochemically unproductive coupling of sulfur cation radicals. The protein returning to its native form can be involved in another catalytic cycle. Therefore, the effect of nitrite is to increase the fraction of protein that proceeds via two-electron oxidation of the substrate and thereby increase also the stereoselectivity of sulfoxidation.

Nitrite could participate in the sulfoxidation reaction also through the oxidant species $NO_2^{\cdot}$ produced by reaction (8). Nevertheless, by reacting thioanisole with nitrogen dioxide gas, we did not observe appreciable formation of the sulfoxide during the reaction time of the catalytic experiments. As a result, nitrite acts only as a quencher of the intermediate $MbFe^{IV}{=}O$ in the present system. It is worth nothing that increasing the nitrite concentration above 50 mM, where the Mbs can generate peroxynitrite,[5] the enantiomeric excess in the sulfoxidation reaction drops (Table 1). Probably, the formation of $ONOO^-$ implies a decreased reactivity of $^{\cdot}MbFe^{IV}{=}O$ with the sulfide and, at the same time, the highly reactive $ONOO^-$ readily diffuses into the bulk solution, where its reaction with the sulfide produces racemic sulfoxide.

The formation of racemic sulfoxide in the reaction catalyzed by the T67K-His Mb both in the absence and in the presence of nitrite (see Table 3) confirms the effect of nitrite according to the proposed mechanism. In fact, if the oxygen transfer from $^{\cdot}MbFe^{IV}{=}O$ (or from $MbFe^{IV}{=}O$ in the protein cage) is not stereoselective, then quenching of $MbFe^{IV}{=}O$ by nitrite according to reaction (8) cannot increase the enantiomeric excess in the sulfoxidation.

Considering the fast reaction of nitrite with the intermediate $MbFe^{IV}{=}O$, also the rate of the sulfoxidation, and consequently the conversion into product, should increase in the presence of nitrite. However, a fraction of hydrogen peroxide is involved in the unproductive oxidation of nitrite to nitrate according to the reaction:

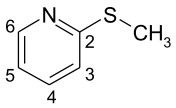
$$NO_2^- + H_2O_2 \rightarrow NO_3^- + H_2O \quad (9)$$

which is also catalyzed by Mb. As a result, the yields of sulfoxide in the presence of nitrite are smaller than those obtained without nitrite.

2.4. NMR relaxation measurements and ^{1}H NMR spectroscopy

To gain an understanding of the protein–substrate interaction, as this is the basis for the stereoselective effects in the sulfoxidation reaction, we performed ^{1}H NMR relaxation time measurements on substrate **4** in the presence of a representative set of proteins, including the best enantioselective catalyst (T67R Mb), the catalyst that produces racemic sulfoxide (T67K-His Mb), and WT Mb, for comparison purposes. In these experiments, the paramagnetic contribution to relaxation by the high spin Fe^{3+} center of the protein can be exploited to get an estimate of the distances of the protons of the bound substrate from the iron atom. The study had to be restricted to substrate **4**, because it is the only sulfide sufficiently soluble in aqueous buffer; attempts to obtain NMR relaxation data with other substrates gave unreliable data due to the very limited range of concentrations attainable. As shown by the data collected in Table 5, the iron-proton distances for protein bound **4** were in the range of 6.9–7.3 Å for WT Mb, 5.8–6.2 Å for T67R Mb, and 6.5–7.0 Å for T67K-His Mb. The similarity in the iron-proton distances for the substrate protons in each protein complex clearly indicates that the sulfide maintains a certain degree of mobility even when

Table 5. Substrate proton relaxation times and iron–proton distances for protein–substrate **4** complexes of Mb derivatives, in deuterated 0.2 M phosphate buffer pD 7.5, 25 °C



Proton (ppm)	WT Mb		T67R Mb		T67K-His Mb	
	T_{1M} (s)	r (Å)	T_{1M} (s)	r (Å)	T_{1M} (s)	r (Å)
H-3 7.29	$(2.6\pm0.1)\times10^{-3}$	6.9	$(9.3\pm0.9)\times10^{-4}$	5.8	$(1.9\pm0.1)\times10^{-3}$	6.6
H-4 7.66	$(3.2\pm0.2)\times10^{-3}$	7.2	$(9.8\pm0.4)\times10^{-4}$	5.9	$(1.8\pm0.2)\times10^{-3}$	6.5
H-5 7.10	$(3.5\pm0.2)\times10^{-3}$	7.3	$(1.16\pm0.06)\times10^{-3}$	6.1	$(2.1\pm0.1)\times10^{-3}$	6.7
H-6 8.27	$(3.1\pm0.1)\times10^{-3}$	7.1	$(1.09\pm0.05)\times10^{-3}$	6.0	$(2.3\pm0.1)\times10^{-3}$	6.8
CH_3 2.47	$(2.7\pm0.1)\times10^{-3}$	7.0	$(1.33\pm0.06)\times10^{-3}$	6.2	$(2.8\pm0.2)\times10^{-3}$	7.0

bound to the protein active site. The distance values agree with a disposition of methyl 2-pyridyl sulfide partially inside the distal cavity of the Mbs, as previously found for the binding to Mb of *p*-cresol, which has comparable size.[12] The sulfide can approach the heme in T67R Mb more closely than in WT Mb, suggesting that this can be a key feature for the enhancement of the enantioselectivity of the sulfoxidation reaction (see Table 4). In the complex between substrate **4** and T67K-His Mb, the iron-proton distances are larger than those observed for T67R Mb. Moreover, the data account for a different orientation of the substrate inside the cavity of T67R Mb and T67K-His with respect to WT Mb, the aromatic protons being more inside the heme cavity and the methyl group more outside in the former cases. We can speculate that the basic residue (Arg or Lys) present in the two mutants contributes to the binding by hydrogen bonding to the sulfur atom or the pyridyl nitrogen atom of the substrate. The interaction with the sulfur atom, however, would be the only polar interaction established by T67R Mb with thioanisole, which gives the best enantioselectivity in the sulfoxidation. The larger mobility of the polypeptide chain of T67K-His Mb around the heme would prevent the relative immobilization of the sulfides, which results in racemic products (Tables 3 and 4).

In order to complete the analysis of the interaction between the Mb derivatives and the sulfides, the paramagnetic ^{1}H NMR spectra of the proteins were studied in the presence of excess sulfide. The downfield region of the NMR spectrum of horse heart Mb is compared in Figure 1 with the spectrum obtained after the addition of substrate **1** to the protein. The assignment of the resonances of hh Mb are made on the basis of the data previously reported.[19] The most notable differences can be found in the chemical shifts of the signals of the heme propionate-7 protons, H_α and $H_{\alpha'}$: while the former undergoes an upfield shift of 1.7 ppm, the latter shifts 0.5 ppm downfield. Actually, this propionate group is localized in the solvent accessible side of the heme moiety. A slight shift of 0.3 ppm upfield can be detected also for the methyl group in position 1 of the porphyrin, probing the ability of thioanisole to deeply enter into the heme distal cavity.

The addition of substrate **4** to a solution of hh Mb produces appreciable changes in several of the downfield paramagnetic signals (Fig. 2); this is probably due to polar effects established by methyl 2-pyridyl sulfide within the protein active site. The largest shifts are again observed for the resonances of the propionate-7 protons H_α (from 72.7 to 71.0 ppm) and $H_{\alpha'}$ (from 31.0 to 31.9 ppm), and for methyl-1 (from 51.3 to 52.0 ppm), but here also methyl-5 is affected (from 83.8 to 84.5 ppm). The selective perturbation of the NMR signals accounts for a disposition of both the sulfides at least partially into the heme distal cavity, spanning the space above the substituents at positions 1, 7 and 8 of the porphyrin, and is in agreement with the range of distances of the substrate protons from the iron center obtained in the relaxation rate measurements.

3. Conclusion

We have shown that nitrite can be used as a reagent to improve the enantioselectivity of the sulfoxidation of organic sulfides catalyzed by several Mb derivatives in the presence of hydrogen peroxide. A model for the interaction between the substrates and the proteins has been obtained through NMR experiments. Although the sulfoxidation of thioanisole catalyzed by the double mutants H64D/V68A and H64D/V68S of Mb[6b,10c] has been reported to occur with enantioselectivities higher than those found here, the effect of nitrite is significant and may find other applications in catalytic oxidation reactions.

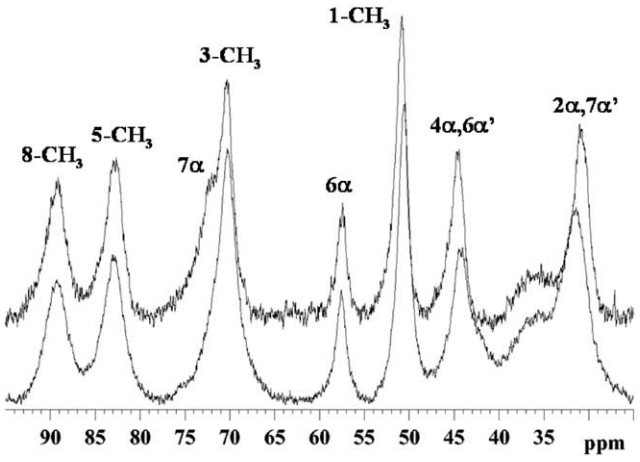


Figure 1. Downfield region of 400 MHz ^{1}H NMR spectrum of hh Mb (~0.1 mM) in deuterated 0.2 M phosphate buffer pD 7.5 in the presence of substrate **1** (0.5 mM) at 25 °C (lower trace), compared with the protein spectrum in the absence of substrate (upper trace). The assignment of the peaks, according to Ref. 19, is shown.

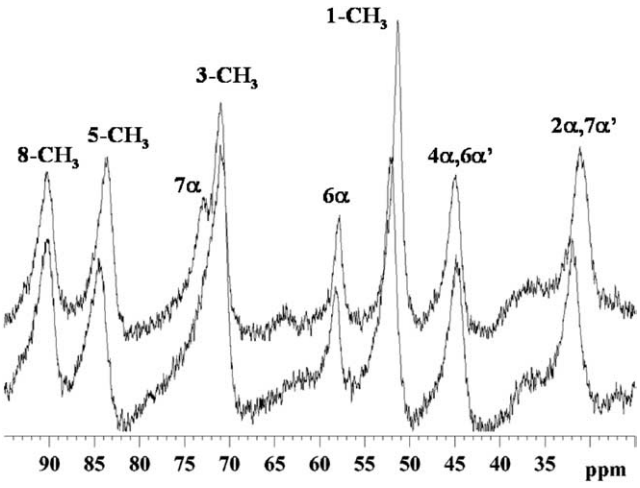


Figure 2. Downfield region of 400 MHz ^{1}H NMR spectrum of hh Mb (~0.1 mM) in deuterated 0.2 M phosphate buffer pD 7.5 in the presence of substrate **4** (12 mM) at 25 °C (lower trace), compared with the protein spectrum in the absence of substrate (upper trace). The assignment of the peaks, according to Ref. 19, is shown.

4. Experimental

4.1. Materials

The substrates **1** and **2** were from Aldrich, while **3** and **4** were synthesized as previously reported.[20] The sulfoxides were synthesized from the corresponding sulfide by oxidation with sodium metaperiodate. Horse heart Mb was obtained from Sigma as a lyophilized sample. The protein derivatives WT Mb, T67R Mb, T67K Mb and T67R/S92D Mb were produced, expressed and purified as previously reported.[12–14] The synthesis of hemin-L-histidine methyl ester and the reconstitution of T67K Mb with the modified hemin were performed as previously reported.[13]

The concentration of Mb solutions was determined from the extinction coefficients of the met forms in 100 mM phosphate buffer, pH 6.0, as follows: hh Mb, 1.88×10^5 M^{-1} cm^{-1} at 408 nm;[3] WT Mb, 1.57×10^5 M^{-1} cm^{-1} at 410 nm;[12] T67K Mb, 1.52×10^5 M^{-1} cm^{-1} at 408 nm;[13] T67R Mb, 1.49×10^5 M^{-1} cm^{-1} at 410 nm;[12] T67R/S92D Mb, 1.61×10^5 M^{-1} cm^{-1} at 408 nm;[14] T67K-His Mb, 1.33×10^5 M^{-1} cm^{-1} at 410 nm.[13] The other reagents were obtained from commercial sources at the best grade available.

4.2. Enzymatic sulfoxidation

Method A—The sulfides (**1–3**) (0.50 mM) were reacted, at 25 °C, under stirring, in 4 ml of 10 mM phosphate buffer, pH 7.5, containing hh Mb or the Mb mutants (5.0 μM) and H_2O_2 (0.50 mM). When required, $NaNO_2$ (0.010–0.20 M) in the usual phosphate buffer was added. After 5 min the reaction mixtures were extracted with dichloromethane (3× 10 ml) and the combined organic phase dried with Na_2SO_4. The solvent was removed under reduced pressure to give the crude product, which was then analyzed by HPLC.

Method B—Sulfide **4** (0.50 mM) was reacted, at 25 °C, under stirring in 4 ml of 10 mM phosphate buffer, pH 7.5, containing hh Mb or the Mb mutants (5.0 μM). H_2O_2 (0.50 mM) and $NaNO_2$ (0.05 M), when required, were added in 100 min in 10 aliquots at 10 min intervals. After 5 min additional time the reaction mixture was extracted with dichloromethane (3×10 ml) and the organic phase dried with Na_2SO_4. The solvent was removed under reduced pressure to give the crude product, which was then analyzed by HPLC.

The HPLC analyses were performed with a Merck–Hitachi L-7100 pump and a DAD 1050 HP detector on Daicel chiral columns OD and OB, at a flow rate of 0.8 ml/min. Elution conditions were as follows: column OD, 20% isopropanol, 80% hexane for **1**; column OB, 20% isopropanol, 80% hexane for **2**; column OB, 15% isopropanol, 85% hexane for **3** and **4**. Readings were made at 230 nm for **1–3** and at 210 nm for **4**. On Chiralcel OB the (*S*)-sulfoxides eluted first, whereas on Chiralcel OD the elution order was the opposite.[21] Standard curves prepared using synthetic sulfoxides were used for quantitative analysis and the value of % conversion and enantiomeric excess were determined on the basis of the peak areas of HPLC traces.

4.3. Reactions of $MbFe^{IV}$=O with thioanisole and with nitrite

The hh $MbFe^{IV}$=O intermediate was prepared by incubating metMb (4 μM) in 0.2 M phosphate buffer, pH 7.5 with 2 equiv. H_2O_2 for about 15 min, until the Soret band shifted from 410 to 420 nm and stabilized at this wavelength. The reaction with **1** was monitored observing the return of the Soret band from 420 to 410 nm with time, after the addition of the substrate at different concentrations (up to 0.5 mM). The spontaneous evolution of $MbFe^{IV}$=O to metMb was a slow process and was not influenced by thioanisole, since an appreciable increment in the rate of this process in the presence of the sulfide was not observed.

Moreover, the observed first-order rate constants for reduction of $MbFe^{IV}$=O by nitrite (followed spectrophotometrically under pseudo-first order conditions as previously reported)[5] were not affected by the presence of thioanisole (0.5 mM) in the reaction mixture.

4.4. Reaction of $NO_2^{\cdot}$ with thioanisole

$NO_2^{\cdot}$ was obtained by air oxidation of $NO^{\cdot}$. 200 μl of 1 atm gaseous $NO_2^{\cdot}$ (a slight excess with respect to the amount that gives a final concentration of 1 mM) were bubbled through a gas-tight syringe into a solution of substrate **1** (0.50 mM) in 4 ml of 0.2 M phosphate buffer, pH 7.5. After 5 min, the solution was extracted with CH_2Cl_2, the organic phase was dried under vacuum and then analyzed by HPLC as reported above (see Section 4.2).

4.5. NMR relaxation measurements and ^{1}H NMR spectroscopy

The T_1 relaxation time for the protons of substrate **4** in the presence of variable amounts of WT Mb, T67R Mb, or T67K-His Mb were determined at 25 °C with a Bruker AVANCE 400 NMR spectrometer operating at 400.13 MHz, using the standard inversion recovery

method.[22] The solutions of **4** were prepared in deuterated 0.2 M sodium phosphate buffer pD 7.5 and contained different amount of the proteins. In order to eliminate interferences by metal impurities, a small amount of EDTA was added to the solutions. The concentrations employed were the following: [**4**] = 12.0 mM and [WT Mb] = 0–6 μM; [**4**] = 14.0 mM and [T67R Mb] = 0–2 μM; [**4**] = 14.5 mM and [T67K-His Mb] = 0–4 μM. The relaxation rate of the protons of the substrate molecules interacting with the Mbs, T_{1b}, was calculated from experimental relaxation rate, T_{1obs}, through the equation:[23]

$$\frac{1}{T_{1obs}} = \left[\frac{1}{T_{1b}} - \frac{1}{T_{1f}}\right]\frac{E_0}{K_D + S_0} + \frac{1}{T_{1f}} \quad (10)$$

where T_{1f} is the T_1 value for free substrate, E_0 and S_0 are the initial protein and substrate concentrations, respectively, and K_D is the dissociation constant for the Mb-substrate complex. The major contribution to the T_{1b} value is the paramagnetic contribution (T_{1M}), which is correlated to the distance (r) of the nucleus from the Fe^{3+} center according to the Solomon–Bloembergen equation (assuming an electron relaxation time τ_s of 5×10^{-11} s).[12,24,25] The K_D values are not known and were neglected in the present calculations; however, the magnitude of the error associated with this approximation is identical for all the substrate protons. It is possible to estimate for r a maximum error of 10% assuming a dissociation constant equal to the substrate concentration.

The paramagnetic proton NMR spectra of hh Mb were recorded at 25 °C on solutions of the protein (~0.1 mM) in deuterated 0.2 M sodium phosphate buffer, pD 7.5. The interaction of hh Mb with the sulfides was studied by recording NMR spectra of the Mb solution containing substrate **1** (~0.5 mM) or **4** (12 mM). Spectra were recorded acquiring 5K scans, with a 80000 Hz spectral window, and suppressing the water signal by presaturation for 0.3 s.

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