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Easy Oxidation and Nitration of Human Myoglobin by Nitrite and Hydrogen Peroxide

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Abstract: The modification of human myoglobin (HMb) by reaction with nitrite and hydrogen peroxide has been investigated. This reaction is important because NO_2^- and H_2O_2 are formed in vivo under conditions of oxidative and nitrative stress, where protein derivatization has been often observed. The abundance of HMb in tissues and in the heart makes it a potential source and target of reactive species generated in the body. The oxidant and nitrating species produced by HMb/H_2O_2/NO_2^- are nitrogen dioxide and peroxynitrite, which can react with exogenous substrates and endogenous protein residues. Tandem mass analysis of HMb modified by stoichiometric amounts of H_2O_2 and NO_2^- indicated the presence of two endogenous derivatizations: oxidation of C110 to sulfinic acid (76%) and nitration of Y103 to 3-nitrotyrosine (44%). When higher concentrations of NO_2^- and H_2O_2 were used, nitration of Y146 and of the heme were also observed. The two-dimensional gel-electrophoretic analysis of the modified HMbs showed spots more acidic than that of wild-type HMb, a result in agreement with the formation of sulfinic acid and nitrotyrosine residues. By contrast, the reaction showed no evidence for the formation of protein homodimers, as observed in the reaction of HMb with H_2O_2 alone. Both HMb and the modified HMb are active in the H_2O_2/NO_2^--dependent nitration of exogenous phenols. Their catalytic activity is quite similar and the endogenous modifications of HMb therefore have little effect on the reactivity of the protein intermediates.

Keywords: myoglobin · nitration · nitrogen oxides · oxidation · protein modifications

Introduction

Although myoglobin (Mb) has been one of the most widely studied proteins, continuing interest in its study arises from the new activities and applications that have been reported through the years. Besides the classical functions of storage and intracellular transfer of molecular oxygen,[1] several other activities of this protein have been found.[2–5] Of particular interest in the present context is the fact that human myoglobin (HMb) differs from the other known mammalian Mbs in the presence of a cysteine residue at position 110. The abilities of HMb to preserve NO bioactivity through the formation of an *S*-nitrosothiol group at this C110 residue and to regulate NO in vivo either by the formation of an iron–nitrosyl HMb complex or by oxidation of NO to NO_3^- are the most important recent findings in the biochemistry of this protein.[6–8] Other reported activities of Mbs involve catalytic reactions on exogenous substrates; examples of these reactions are the peroxide-dependent oxidation[9,10] and nitration[11] of phenolic substrates, the sulfoxidation of organic sulfides,[12–16] and the epoxidation of alkenes,[13–16] although the catalytic efficiency of the protein in these processes is generally modest.

Chemical modification of proteins is systematically observed under pathophysiological conditions; the identification of specific residues involved and of the nature of the modification produced is therefore essential for the understanding of the mechanisms of development of the pathologies.[17] Reactive oxygen and nitrogen species are typically responsible for these modifications,[18–20] through the forma-

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tion of protein-centered radicals similar to those involved as intermediates in the mechanism of many redox enzymes.[21–26] For instance, the reaction of ferric human myoglobin (metHMb) with H_2O_2 yields protein radicals localized on tyrosine and/or tryptophan residues.[27,28] Of relevance for the present investigation is the finding by the group of Mauk that the reaction of HMb with hydrogen peroxide, peroxynitrite, or nitric oxide leads to modification of two specific residues, Y103 and C110, through radical mechanisms.[6,29–31] In particular, addition of H_2O_2 to HMb produces a C110 thiyl radical, through an intermolecular electron-transfer reaction from C110 to a Y103 phenoxy radical, which results in the formation of an intermolecular disulfide bond and a protein homodimer.[29,30] The same dimer is also obtained from the reaction of HMb with peroxynitrite, but the addition of physiological concentrations of carbonate results in the nitration of the protein at Y103.[31] The cysteine residue can also be derivatized to form an *S*-nitrosothiol group by the reaction of metHMb and NO.[6] Both protein and nonprotein thiol groups are potential targets of H_2O_2 and $ONOO^-$. Besides disulfide bridges, the oxidation of thiol groups can lead to sulfenic (RSOH), sulfinic (RSO_2H), or sulfonic (RSO_3H) acids, depending on the species involved.[32]

Herein we report on the modifications undergone by HMb in the reaction with nitrite and hydrogen peroxide in conditions of low and high reagent concentrations, which produce modified protein derivatives labeled as m-HMb and m′-HMb, respectively. In both cases, the major protein modification occurs at the cysteine residue, which is transformed into sulfinic acid, in addition to the expected tyrosine nitration. This extremely facile reaction is of some interest in view of the emerging role of Mb in the heart, where the protein is particularly abundant.[17,33] Cardiovascular pathologies like heart failure and ischemia-reperfusion injury are typically characterized by sudden formation of oxygen radicals and NO overexpression, which lead to extensive nitration of protein tyrosine residues through mechanisms that are still not completely understood.[17] While tyrosine nitration is the commonly accepted marker of these events, we show here that modification of the cysteine residue of Mb occurs to a much larger extent. Comparative studies show that the modified Mb derivative maintains the same ability to catalyze nitrative reactions on exogenous substrates as wild-type Mb.

Results

Cloning and expression of HMb: The fully successful expression of HMb in bacteria is reported here for the first time. All previous attempts gave the protein in inclusion bodies,[34,35] while we obtained the holoprotein in soluble form. In our hands, complete maturation of the apoprotein into its holo form, with the heme bound to the polypeptide, was observed only when bacterial growth was carried out under slow mechanical stirring.

Modification of HMb: Modification of HMb under mild conditions was obtained by reaction of the protein (50 μM), nitrite, and H_2O_2 in a molar ratio of 1:2:2. This reaction yields a protein derivative, m-HMb, with UV-visible spectral features basically indistinguishable from those of HMb. On the other hand, when HMb was treated with larger concentrations of NO_2^- (0.8 M) and H_2O_2 (1 mM), the solution turned from brown to greenish-brown and the reaction yielded the m′-HMb derivative. The change of color does not correspond to a shift of the Soret band, with the maximum remaining always at 408 nm. In a comparison of the UV-visible spectra of HMb and m′-HMb (Figure 1), only a slight broadening of the Soret band is observed. The color change is due to nitration of one of the heme vinyl groups and tyrosine residues (see below).

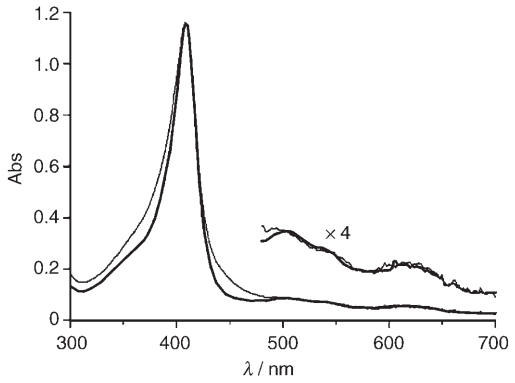


Figure 1. UV/Vis spectrum of m′-HMb (thin upper trace) compared with the spectrum of HMb (bold lower trace), both in 0.2 M phosphate buffer (pH 7.5) at 25 °C. The concentration of the proteins was 7.5 μM.

Tandem mass analysis of m′-HMb: The characterization of the modifications introduced into HMb by reaction with NO_2^- and H_2O_2 was initially carried out on m′-HMb, where the extent of the modifications was larger. To this end, the polypeptide fragments resulting from tryptic digestion of apoHMb and apo-m′-HMb were analyzed by HPLC–ESI-MS/MS. Comparison of the data showed the presence of two modified peptide fragments as bicharged ions with $m/z = 973$ (mass of 1944 amu) and $m/z = 995.5$ (mass of 1989 amu). These both correspond to modification of the 103–118 peptide (containing Y103 and C110, with a mass of 1912 amu). The peptide with a mass of 1944 amu contains two oxygen atoms more than the parent peptide, while the one with a mass of 1989 amu shows the further replacement of a proton with NO_2. Both modified peptides were analyzed by collision-induced dissociation (CID). The MS/MS spectrum of the latter is shown in Figure 2, where the y and b ion series are reported. A mass difference of 135 amu was detected between b_8 and b_7, a difference indicative of the presence of a cysteinyl residue modified by two oxygen atoms. In the y ion series, the mass difference of 117 amu between y_9^0 and y_8 corresponds to the same cysteine modifi-

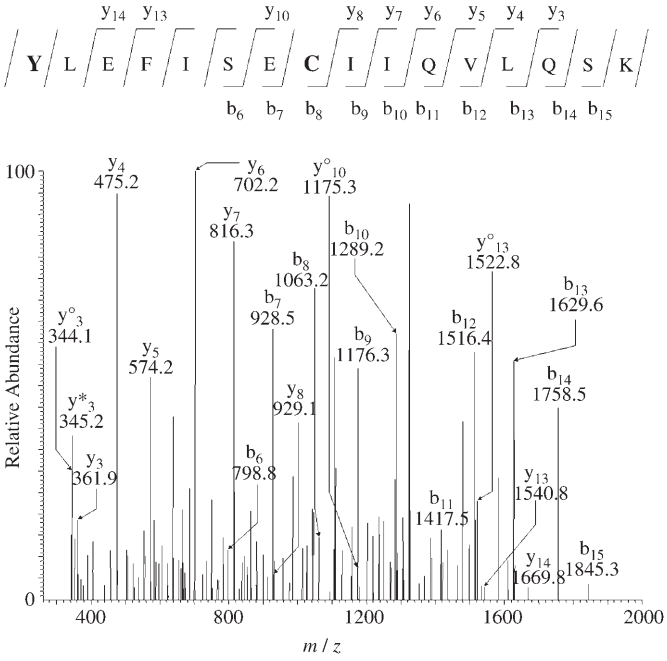


Figure 2. MS/MS spectrum of the $m/z = 995.5$ peak (mass of 1989 amu) assigned to the 103–118 peptide in a double-charged state and with two modifications: the oxidation of C110 to the corresponding sulfinic acid, C110-SO_2H, and the nitration of Y103 to 3-nitrotyrosine, Y103-NO_2. The assignment of the y and b ion series is shown. Above the spectrum, the sequence of the 103–118 peptide, with the two modified amino acids in bold and with the summary of the y and b ions found in the spectrum, is reported.

cation (with consideration of the loss of a water molecule in the y^0 series). These results allowed unequivocal assignment of the oxidation of C110 to the corresponding sulfinic acid. The mass of the peptide, together with the b ion series, is indicative of the presence of another modification, corresponding to a mass increment of 45 amu; the known reactivity of tyrosine residues under these conditions[11] allowed the assignment of the modification as the nitration of Y103 to 3-nitrotyrosine.

A quantitative analysis of the modified 103–118 peptide was obtained by comparing, in the extracted ion current (EIC) chromatograms, the areas of the peaks corresponding to the derivatized peptides ($m/z = 973$ and 995.5 for the bi-charged ions, with masses of 1944 and 1989 amu, respectively) with that of the starting peptide ($m/z = 957$ for the bi-charged ion, with a mass of 1912 amu). The relative amounts of the three peptides were as follows: unmodified peptide 5%, peptide containing only the C110-SO_2H modification 41%, and the peptide containing both the Y103-NO_2 and C110-SO_2H modifications 54%. The peptide nitrated only at Y103 was not found in the HPLC–MS/MS analysis, although it might be present in a very small amount. This result indicates that the cysteine residue is more reactive than tyrosine.

The occurrence of nitration at the other tyrosine residue of HMb, Y146, was also investigated. In the LC–MS spectrum of m′-HMb, three peptides containing the Y146 residue were found that are absent in the LC–MS spectrum of HMb and that had a mass difference of 45 amu with respect to the corresponding unmodified peptides. These peptides were 140–153 (mass difference from 1587 to 1632 amu), 141–153 (mass difference from 1459 to 1504 amu), and 141–147 (mass difference from 827 to 872 amu). These data indicate the presence of another endogenous nitration at Y146; this modification occurs to a lower extent with respect to the nitration at Y103 and the modified protein represents ≈10–20% of the initial HMb, as judged by relative peak areas in the EIC chromatograms.

The nitration of one heme vinyl group was reported to occur upon treatment of Mb at pH 5.5 with a large excess of nitrite; in this case, the reaction was also accompanied by a change of color of the protein solution to greenish-brown.[36] At pH 7.5 this reaction is much slower. We previously found the same heme modification in horse heart Mb after treatment with NO_2^- and H_2O_2 at pH 7.5.[11] To assess the occurrence of the modification of the heme prosthetic group in m′-HMb, an HPLC–ESI-MS/MS analysis was performed on the protein solution. The injected m′-HMb sample was denatured in the HPLC column and the released hemin was separated from the apoprotein; ions at $m/z = 616$ and 661, corresponding to hemin and to a modified hemin where a proton of the prosthetic group was replaced by a NO_2 group in the porphyrin, respectively, were detected in MS/MS mode. Integration of the peaks in the MS/MS chromatograms indicated that the nitration occurred with 8% conversion.

Tandem mass analysis of m-HMb: To assess whether the modifications taking place in HMb in the presence of high concentrations of NO_2^- and H_2O_2 could also be obtained under milder conditions, the polypeptide fragments resulting from tryptic digestion of apo-m-HMb were analyzed by HPLC–ESI-MS/MS. The modified 103–118 peptides corresponding to the oxidation of C110 to sulfinic acid (mass of 1944 amu) and to both the oxidation of C110 and the nitration of Y103 (mass of 1989 amu) were found. The latter peak was analyzed by CID obtaining an MS/MS spectrum analogous to that previously reported for m′-HMb (Figure 2). The relative amounts of the starting and modified 103–118 peptides resulting from quantitative analysis were as follows: unmodified peptide 24%, peptide containing only the C110-SO_2H modification 32%, and peptide containing both the Y103-NO_2 and the C110-SO_2H modifications 44%.

As in the case of m′-HMb, the peptide nitrated only at Y103 was also absent in the tryptic fragments resulting from the analysis of m-HMb. Furthermore, unlike m′-HMb, peptide fragments containing neither nitration at residue Y146 nor nitration at the heme prosthetic group (analyzed by HPLC–ESI-MS/MS on the protein solution) could be detected, a result indicating that both types of modification were at best present in very small, undetectable amounts. It should be noted that in the conditions employed here, that

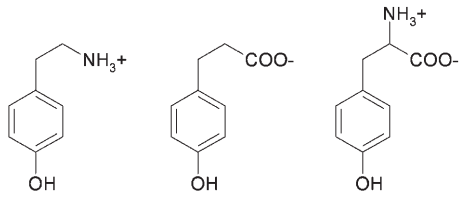
is, with only a slight excess H_2O_2 with respect to HMb, almost all of the peroxide was consumed for the modification of Y103 and C110, so only a small fraction was actually available for nitration of Y146 and the heme.

Gel electrophoresis analysis of modified HMb: The two-dimensional polyacrylamide gel of m′-HMb showed that the modified protein is not homogeneous: two bands with different isoelectric points and similar intensity could be observed in the gel. The comparison of the two-dimensional polyacrylamide gel of m′-HMb with that of HMb indicated that both the spots of m′-HMb where more acidic than the native protein. By considering the linear pH gradient from 6.5–8.5 that was used, it was possible to estimate pI shifts of 0.1 and 0.2 units for the two spots of m′-HMb from the 7.2 value for HMb (as reported by Varadarajan et al.).[34] We can assign the spot with pI 7.1 to the C110-SO_2H protein derivative (41% from MS/MS analysis) and the spot with pI 7.0 to the Y103-NO_2/C110-SO_2H protein derivative (54%). The increased acidity of the modified protein can be related both to tyrosine nitration and to sulfinic acid formation. In fact, the oxidation of one cysteine residue to sulfinic acid in peroxiredoxin I (an enzyme catalyzing the destruction of peroxides under oxidative stress conditions) was reported to cause a decrease in the isoelectric points of 0.5 pH units.[37]

Homodimers of wild-type HMb were formed through disulfide linkage involving C110 residues following the reaction with H_2O_2[30] or peroxynitrite.[31] However, no dimer was detected in the sodium dodecylsulfate (SDS) polyacrylamide gel of m′-HMb performed in nonreducing conditions: this gel was, in fact, equivalent to the gel of wild-type HMb. Thus, the HMb/NO_2^-/H_2O_2 system performs the endogenous modifications identified by the MS analysis without formation of protein homodimers (either by dicysteine or dityrosine linkages).

Binding of nitrite to HMb: The iron(III) center of HMb binds nitrite to form the low-spin HMbFe^{III}–NO_2^- complex; the reaction can be followed through the shift of the Soret band of the protein from 408 to 410 nm. From spectrophotometric monitoring of the changes of the Soret band as a function of nitrite concentration at pH 7.5, the binding constant $K_B = (76 \pm 2)\,M^{-1}$ was obtained. This value is comparable to that obtained with horse heart Mb ($K_B = 51\,M^{-1}$) under the same conditions.[11]

Phenol nitration catalyzed by HMb and m′-HMb: Like the met form of horse heart Mb,[11,38–40] HMb and m′-HMb also catalyze the nitration of phenols to *ortho*-nitrophenols in the presence of hydrogen peroxide and nitrite. The reactions studied here are the nitration of representative phenolic substrates **1–3** (Scheme 1). By considering that in the case of m′-HMb the extent of the modifications at residues Y103 and C110 was in excess of 50% and very close to 100%, respectively, the reactivity of the modified HMb deduced from the kinetic studies can be considered as an indication of the



1 **2** **3**

Scheme 1. Structures of the phenols employed in the kinetic studies.

reactivity of the Y103-NO_2/C110-SO_2H HMb derivative, while the contribution of the protein fraction containing partial nitration at Y146 and at the heme can be neglected. Therefore, m′-HMb could be a good probe for the analysis of the effect of Y103 and C110 modification on the reactivity of the protein in the presence of nitrite and hydrogen peroxide.

In the phenol nitration, each reactant affects the reaction rate in a way dependent on the concentration of the others. In this study, the concentration of the Mb derivatives was kept constant at 1 μM, because under these conditions the noncatalytic reaction could always be neglected.[11] The concentration of the phenols and nitrite were instead varied; in order to simplify the analysis, it was found convenient to vary the concentration of one reagent while maintaining that of the others constant at the value that maximizes the rate. The plots of rate versus [phenol] showed saturation behavior, a result suggesting the need for an interaction with the protein. The rate versus [NO_2^-] exhibited a slightly more complicated dependence; in this case, an initial sigmoidal phase, at low [NO_2^-], is followed by nitrite saturation. This behavior can be explained by considering for HMb and m′-HMb a mechanism similar to that observed with the protein from horse heart: two competing pathways are in fact operative in the nitration of the phenols, depending on nitrite concentration.[11] In the first one, which dominates at low [NO_2^-], the protein reacts through a peroxidase-like cycle involving two active intermediates, which can give one-electron oxidation of the substrates, thereby producing a phenoxy radical from the phenol and $NO_2^{\bullet}$ from nitrite. The other mechanism predominates at high [NO_2^-] and does not involve peroxidase-like intermediates; hydrogen peroxide reacts with the iron-bound nitrite to produce a nitrating active species that we assumed to be a protein-bound peroxynitrite, MbFe^{III}–N(O)OO.[11] With changes in [NO_2^-], the fraction of protein–nitrite complex changes and this necessarily affects the relative importance of the two paths (via $NO_2^{\bullet}$ or via $ONOO^-$) operating in the Mb/NO_2^-/H_2O_2-dependent nitration.[11] A simplified kinetic study of the nitration process can be carried out by assuming that the peroxynitrite path dominates at relatively high nitrite concentration.[11] The rate dependence on the phenol concentration at constant (high) nitrite level is then given by Equation (1). The rate dependence on nitrite concentration should instead consider that the $NO_2^{\bullet}$ path can be observed only at low nitrite concentration [Eq. (2)].[11]

$$\text{rate} = \frac{k_{cat}[Mb][PhOH]}{(K_M^{PhOH}+[PhOH])} \quad (1)$$

$$\text{rate} = \frac{k_{cat}[Mb]([NO_2^-]-b)}{(K_M^{nitrite}+[NO_2^-]-2b)} \quad (2)$$

In these equations, [Mb] is the total HMb or m′-HMb concentration, k_{cat} represents the maximum turnover number of the proteins, and $K_M^{nitrite}$ and K_M^{PhOH} give an indication of the dissociation constant of NO_2^- and PhOH, respectively, from the complexes with the proteins in the presence of the other reagent. The parameter b had to be introduced into Equation (2) to account for the change in the mechanism at increasing nitrite concentration; furthermore, the data at very low $[NO_2^-]$ were not considered in the interpolation of the experimental data. The values of k_{cat}, K_M^{PhOH}, and k_{cat}/K_M^{PhOH} obtained from the rate dependence on [PhOH] and those of k_{cat}, $K_M^{nitrite}$, and $k_{cat}/K_M^{nitrite}$ obtained from the rate dependence on $[NO_2^-]$ for the nitration of substrates **1**–**3** are reported in Table 1.

Table 1. Steady-state kinetic parameters for the HMb- and m′-HMb-dependent phenol nitration by NO_2^-/H_2O_2 in 0.2 M phosphate buffer (pH 7.5) at 25 °C. For comparison purposes, corresponding kinetic data for the lactoperoxidase (LPO) catalyzed reactions under the same conditions are included (from ref. [54]).

Phenol	K_M^{PhOH} [mM]	k_{cat} [s^{-1}]	k_{cat}/K_M^{PhOH} [$M^{-1}s^{-1}$]	$K_M^{nitrite}$ [M]	k_{cat} [s^{-1}]	$k_{cat}/K_M^{nitrite}$ [$M^{-1}s^{-1}$]
HMb						
1	0.35±0.07	2.1±0.1	6000±1000	0.30±0.04	2.1±0.1	6.9±0.6
2	0.12±0.02	0.67±0.03	5600±900	0.24±0.04	1.03±0.08	4.3±0.5
3	0.47±0.04	2.4±0.1	5200±200	0.25±0.04	1.7±0.1	6.9±0.8
m′-HMb						
1	0.40±0.07	1.26±0.06	3100±400	0.24±0.04	1.31±0.09	5.5±0.6
2	0.16±0.02	0.62±0.03	3800±400	0.23±0.04	0.68±0.05	3.0±0.4
3	0.5±0.1	1.3±0.2	2400±200	0.20±0.04	1.0±0.1	5.1±0.7
LPO						
1	0.12±0.01	380±10	3.2×10^6	48±5	380±15	7.9×10^3
2	0.14±0.02	130±5	9.4×10^5	30±3	135±5	4.5×10^3
3	0.11±0.01	75±2	6.8×10^5	16±1	80±2	5.0×10^3

The data show that the catalytic activity of HMb and m′-HMb in the phenol nitration is comparable to that of horse heart Mb, with HMb being slightly more efficient in the production of 3-nitrotyramine and 3-nitrotyrosine.[11] The relatively small differences observed in the k_{cat} parameters for the different substrates with different charges at pH 7.5 are probably connected to the high efficiency of the nitrating agent generated by the $Mb/H_2O_2/NO_2^-$ systems. As observed with horse heart Mb,[11] the K_M^{PhOH} values are below the millimolar range; this is compatible with a disposition of the substrate at the protein surface. It is interesting to note that the values of $K_M^{nitrite}$ are quite similar for the different substrates. The reciprocal of $K_M^{nitrite}$ value should give an indication, extrapolated from kinetic studies, of the nitrite binding constant to the protein ($K_B = 1/K_M^{nitrite} \approx 4\,M^{-1}$). This value differs significantly from the binding constant obtained independently for the $HMbFe^{III}$–NO_2^- complex ($K_B = 76\,M^{-1}$). The smaller value of $1/K_M^{nitrite}$ can be ascribed to the fact that the binding of the phenolic substrate near the heme site during catalysis hinders the interaction of NO_2^- at the iron center. Furthermore, it should be taken into consideration that the change in the mechanism of the reaction with changing $[NO_2^-]$ prevents the precise determination of $K_M^{nitrite}$. In general, however, the reactivity of the modified HMb in the nitration of phenolic substrates is quite similar to that of wild-type HMb. By comparing the parameters k_{cat} and k_{cat}/K_M^{PhOH} (obtained at saturating nitrite concentration) and k_{cat} and $k_{cat}/K_M^{nitrite}$ (obtained at saturating phenol concentration) for HMb and m′-HMb, a general slight decrease in the rate constants can be noted for the modified protein.

Discussion

In analogy with horse heart Mb,[11] HMb is able to use nitrite and hydrogen peroxide to generate the oxidant and nitrating species nitrogen dioxide and peroxynitrite. Both these species perform nitration of exogenously supplied phenols and the protein is thus an efficient catalyst in the nitration of substrates **1**–**3**. However, $NO_2^{\cdot}$ and $ONOO^-$ can also oxidize and nitrate protein residues. The tandem mass analysis of HMb modified with stoichiometric amounts of the reagents indicates the presence of two endogenous derivatizations: nitration of Y103 to 3-nitrotyrosine (at 44%) and/or oxidation of C110 to the corresponding sulfinic acid C110-SO_2H (at 76%). Upon reaction of HMb with high concentrations of NO_2^- and H_2O_2, the extent of oxidation of C110 (at 95%) and nitration of Y103 (at 54%) increased and nitration of Y146 (at ≈10–20%) and the heme (at ≈8%) was also observed. Although the ferric form studied here is not the physiological form of the protein, the presence of hydrogen peroxide and nitrite would rapidly convert ferrous or oxy Mb into the oxidized form.

Both $NO_2^{\cdot}$ and $ONOO^-$ may be responsible for the oxidation of the cysteine residue. A free-radical pathway can be proposed for the formation of sulfinic acid by nitrogen dioxide, according to the Equations (3)–(5), in analogy with the mechanism proposed for the oxidation of human serum albumin by peroxynitrite in the presence of carbon dioxide.[41]

$$HMb\text{-}SH + NO_2^{\cdot} \rightarrow HMb\text{-}S^{\cdot} + HNO_2 \quad (3)$$

$$HMb\text{-}S^{\cdot} + O_2 \rightarrow HMb\text{-}SOO^{\cdot} \quad (4)$$

$$HMb\text{-}SOO^{\cdot} \xrightarrow[H^+]{e^-} HMb-SO_2H \quad (5)$$

The thiol group of HMb is oxidized by nitrogen dioxide to the thiyl radical [Eq. (3)]; this radical can react with dioxygen to form a peroxy radical [Eq. (4)], which in turn produces the sulfinic acid after hydrogen-atom abstraction from a reductant, according to Equation (5). Peroxynitrite can also promote the oxidation of cysteinyl residues beyond sulfenic acid; this has been observed in the oxidation of bovine serum albumin, which has only one sterically isolated thiol group.[32] Both the oxidation of C110 and the nitration of Y103 were apparent in the two-dimensional polyacrylamide gel of m′-HMb, where the two spots that were more acidic than HMb and have similar intensity correspond to the modified proteins with both sulfinic acid at C110 and 3-nitrotyrosine at Y103 and with sulfinic acid at C110, respectively. Sulfinylation is not a rare event, given that 1–2% of the cysteine residues of soluble proteins from rat liver were detected as cysteine sulfinic acid.[42] Formation of sulfinic acid is a reversible reaction, as is the case for the oxidation of the active-site cysteine of peroxiredoxins.[43] Nitration of tyrosine residues (Y103 and Y146) can also be promoted by both nitrogen dioxide and peroxynitrite, according to the mechanisms previously described.[11]

Witting et al. reported that the reactions between HMb and H_2O_2 or $ONOO^-$ result in the formation of a protein homodimer through an intermolecular disulfide bond.[30,31] The first step of this process is the oxidation of the Y103 residue to the Y103 phenoxy radical (as a component of the compound I like species). In our case, where $NO_2^{\bullet}$ is also produced, the protein radical is intercepted in a fast process that leads to the formation of nitrotyrosine, according to Equation (6).

$$\text{HMb-Y103}^{\bullet} + NO_2^{\bullet} \rightarrow \text{HMb-Y103-NO}_2 \quad (6)$$

In this way, the electron transfer from C110 to Y103$^{\bullet}$ to produce a thiyl radical and hence the dicysteine linkage is prevented. In fact, in the SDS polyacrylamide gel of m′-HMb, the band corresponding to the HMb homodimer is absent or below the detection limit.

The MS analysis of m′-HMb (but not that of m-HMb) also revealed the presence of a modification on the heme prosthetic group that corresponds to the replacement of a proton with an NO_2 group; the modification probably occurs at one of the heme vinyl groups, in analogy to the heme nitration process by a large excess of nitrite reported for Mb at pH 5.5.[36] We previously found similar heme nitration in horse heart Mb upon treatment with NO_2^- and H_2O_2.[11] However, nitration of HMb occurs only to the extent of 8%, while for horse heart Mb conversion into the nitrated heme accounted for 50% of the starting protein under the same conditions. This difference clearly depends on the presence in HMb of the reactive cysteine residue that can capture the oxidant species and thereby protect the prosthetic group from nitration.

The three-dimensional structure of wild-type HMb is not available. A reference structure for localizing the residues subjected to modifications is the crystal structure of the K45R/C110A mutant of HMb (Figure 3).[44] In this structure, Y103 is the tyrosine residue nearest to the heme group and is exposed to solvent, while Y146 is an internal residue, as is also the case for horse heart Mb. Our previous studies showed that in the presence of nitrite and hydrogen peroxide the latter protein undergoes endogenous nitration only at the Y146 residue, not at Y103.[11] This result was of some importance because a controversy exists concerning the position of the tyrosine residue (Y103 versus Y146) in horse heart Mb that is nitrated by peroxynitrite.[31,45,46] The reaction of the latter species and HMb in the presence of carbonate results in nitration at Y103; in the presence of hydrogen peroxide, or peroxynitrite without carbonate, both residues Y103 and C110 of HMb undergo radical reactions which eventually lead to the formation of protein homodimers.[30,31] A different modification at the cysteinyl residue was only observed in the reaction of HMb with nitric oxide, which yielded an *S*-nitrosated protein derivative.[6]

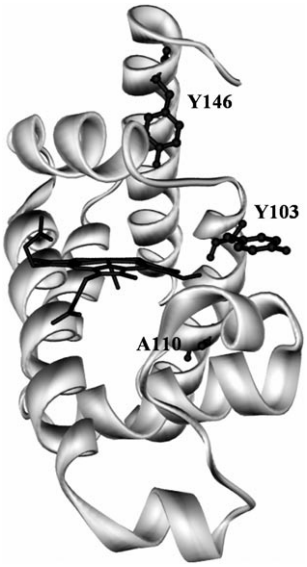


Figure 3. Structure of the K45R/C110A mutant of HMb.[44] The disposition of the side chains of Y103, A110, and Y146 is shown; HMb contains a cysteine residue at position 110.

In our system, once the oxidizing and nitrating species (that is, $NO_2^{\bullet}$ or $ONOO^-$) is produced near the heme, it performs derivatization at the nearest protein residues, Y103 and C110. According to the mechanism previously reported for the nitration catalyzed by horse heart Mb, at low [NO_2^-] (such as that employed for the production of m-HMb) the oxidant and nitrating species is $NO_2^{\bullet}$, while at high [NO_2^-] (employed for the production of m′-HMb) the reactive species is mainly a protein-bound peroxynitrite species. As discussed before, both $NO_2^{\bullet}$ and $ONOO^-$ may be responsible for oxidation of C110 and nitration of Y103 and the two modifications are actually observed in both the m-HMb and m′-HMb derivatives. The cysteine residue is always more re-

active than tyrosine: it is oxidized to a larger extent both in m-HMb (by $NO_2^{\bullet}$) and in m′-HMb (by $ONOO^-$).

In the presence of high nitrite concentration, the peroxynitrite species can also react, albeit with lower efficiency, with the prosthetic group and diffuse to the Xe1 cavity of HMb, where it performs the nitration of the Y146 residue, which is located in the proximity of this cavity.[3,35,47] As suggested by a comparison of the data on the catalytic activity of wild-type HMb and the m′-HMb derivative (Table 1), it is apparent that the endogenous modifications have little effect on the reactivity of the protein intermediates.

Conclusion

Myoglobin is abundant in tissues and particularly in the heart, where it can be present at concentrations up to 0.2 mM.[7] Experiments with transgenic mice deficient in HMb have recently shown that the protein exhibits a cardioprotective role against nitrosative[33,48] and oxidative stress.[49] However, HMb is itself a potential source and, at the same time, a potential target of the oxidant and nitrating species generated in the body in the presence of hydrogen peroxide and nitrite. This situation can occur during inflammatory processes in tissues and in cardiovascular pathologies, where H_2O_2 and nitrite are continuously produced.[17,50] The oxidant and nitrating species produced by the HMb/H_2O_2/NO_2^- system can react both with exogenous substrates and endogenous protein residues. In particular, we have found here that modification of the Y103 and C110 residues is a remarkably efficient process which occurs extensively on HMb in the presence of stoichiometric amounts of nitrite and hydrogen peroxide. Nitrite actually appears to potentiate the harmful effect of the peroxide on the protein and can completely depress the formation of myoglobin dimers, which are the most typical modification product of the protein with H_2O_2.[29,30] The present results show that the pattern of covalent modifications that HMb can undergo in pathological conditions is more complex than was previously thought.

Experimental Section

Materials: Hydrogen peroxide (30% solution), tyramine (**1**), 3-(4-hydroxyphenyl)propionic acid (**2**), and L-tyrosine (**3**) were obtained from Aldrich. Ampicillin was from Shelton Scientific, trypsin from Sigma, and *Bam*HI and *Nde*I from Amersham Biosciences. The other reagents were obtained at the best grade available. All the buffer solutions were prepared by using deionized, double-distilled water. The concentration of hydrogen peroxide solutions was controlled by monitoring the formation of the 2,2′-azino-bis(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) radical cation according to a standard method that we followed previously.[51] The concentration of HMb solutions was determined from the extinction coefficient of metHMb $\varepsilon = 1.60 \times 10^5\,\text{M}^{-1}\,\text{cm}^{-1}$ at 408 nm;[34] the same value was assumed for the extinction coefficient of the modified proteins m-HMb and m′-HMb. UV/Vis spectra were recorded on a Hewlett Packard HP 8452 A diode array spectrophotometer.

Cloning, expression, and protein purification: The open reading frame of the human myoglobin gene was amplified from the fagmide pDNR-LIB, clone IRALp962G1819Q2 (genome bank RZPD, Deutsches Ressourcenzentrum für Genomforschung GmbH, Heubnerweg 6, 14059 Berlin), by using the polymerase chain reaction (PCR) with the nucleotides 5′-CGC*GGATCC*TAGCCCTGGAAGCCCAG-3′ and 5′-GGGAATTC*CATATG*GGGCTCAGCGAC-3′ which contain the *Bam*HI and *Nde*I restriction sites (italicized). The *Bam*HI–*Nde*I fragment containing *hmb* was cloned in the *Bam*HI–*Nde*I sites of the expression vector pET-11a, thereby yielding pET*hmb*. The presence of the wild-type *hmb* gene was confirmed by DNA sequence analysis prior to protein expression in bacteria. *Escherichia coli* BL21(DE3) (*E. coli*) cells transformed with pET*hmb* were grown in flasks under shaking at 37 °C in Luria–Bertani medium supplemented with ampicillin (100 $\mu g\,mL^{-1}$) for the purpose of plasmid selection and maintenance. Cells were grown to the midexponential growth phase before induction with 1 mM isopropyl-β-D-thiogalactoside. Bacterial growth was carried out under slow stirring conditions. Cells were harvested after 24–28 h and analyzed for the presence of soluble HMb by SDS polyacrylamide gel electrophoresis (SDS PAGE) analysis.

All purification steps were performed at 4 °C except for HPLC runs. The *E. coli* cell paste was resuspended in 20 mM tris(hydroxymethyl)aminomethane–HCl (Tris-HCl)/1 mM ethylenediaminetetraacetate (EDTA) buffer (pH 8.0, 2 $mL\,g^{-1}$) containing 1 mM β-mercaptoethanol and 1 mM phenylmethylsulfonyl fluoride (PMSF) and disrupted by sonication. After removal of cell debris by centrifugation at 20000 g for 1 h, the dark-brown solution was brought to 60% ammonium sulfate saturation, the precipitate was discarded, and the soluble fraction was precipitated with 95% ammonium sulfate saturation. The solution was stirred overnight at 4 °C and then centrifuged (at 4 °C, 43000 g, for 1 h); the pellet was resuspended in 20 mM Tris-HCl/1 mM EDTA buffer (pH 8.0) containing 1 mM β-mercaptoethanol. The solution was dialyzed overnight (with several buffer changes) against buffer and then it was loaded onto a diethylaminoethyl (DEAE) CL-6B anion-exchange column, which was eluted with 20 mM Tris-HCl/1 mM EDTA buffer (pH 6.5) at a flow rate of 12 $mL\,min^{-1}$. The fractions containing the protein were collected and the purity was checked by monitoring the absorbance ratio A_{Soret}/A_{280} (which was above 2.5). The protein solution was loaded onto a Superdex G75 gel-filtration column (Amersham Biosciences) connected to a Jasco HPLC instrument with a MD-1510 diode array detector and it was eluted with 20 mM Tris-HCl/150 mM NaCl buffer (pH 8.0) at a flow rate of 0.5 $mL\,min^{-1}$. The fractions containing the protein were collected and the purity was checked as described above, with an A_{Soret}/A_{280} value >4.0 being achieved.

The expression of HMb yields the protein in the Fe^{II}–O_2 form; the met (Fe^{3+}) species was obtained by treating the protein with diluted H_3PO_4 until pH 3.5 was reached and then adding diluted NaOH until pH 7 was reached. In all the experiments described below, the proteins (wild-type HMb and modified proteins) are utilized in their met form.

Modification of HMb in the presence of NO_2^- and H_2O_2: The solution of metHMb (≈50 μM) was dialyzed against 20 mM phosphate buffer (pH 7.5). The samples of modified HMbs, m-HMb and m′-HMb, were prepared by adding sodium nitrite (final concentrations of 0.1 and 800 mM, respectively) and hydrogen peroxide (final concentrations of 0.1 and 1 mM, respectively) to the metHMb solution. The proteins were allowed to react at 20 °C for 10 min. While the color of the solution of m-HMb remained unchanged, the solution turned from brown to greenish-brown in the case of m′-HMb. Excess nitrite and oxidant were removed by overnight dialysis against 20 mM phosphate buffer (pH 7.5).

Analysis of protein fragments and heme modification: For the analysis of protein fragments, a portion of each sample (HMb, m-HMb, and m′-HMb, about 0.5 mg) was transformed into the apoprotein by the standard hydrochloric acid/2-butanone method[1] and subsequently hydrolyzed by trypsin. Digestion was performed with 1:50 (w/w) trypsin at 37 °C for 3 h in 20 mM phosphate buffer (pH 7.5). The samples were then analyzed by HPLC–MS/MS. The heme modification was studied by direct HPLC–MS/MS analysis on solutions of m-HMb and m′-HMb in 20 mM phosphate buffer (pH 7.5).

LC–MS and LC–MS/MS data were obtained by using an LCQ DECA ion-trap mass spectrometer equipped with ESI ion source and controlled by Xcalibur 1.3 software (Thermo-Finnigan, San Jose, CA). ESI experiments were carried out in positive-ion mode under the following constant instrumental conditions: source voltage 4.5 kV, capillary voltage 15 V, capillary temperature 250 °C, and tube lens voltage 15 V. The system was run in automated LC–MS/MS mode and by using a Surveyor HPLC system (Thermo Finnigan, San Jose, CA, USA) equipped with a Simmetry300 C18 column (3.5 μm, 2.1 × 100 mm, Waters, Milford, MA). The elution was performed with a 0–55 % linear gradient over 65 min with 0.1 % trifluoroacetic acid (TFA) in water as solvent A and 0.1 % TFA in acetonitrile as solvent B. MS/MS spectra obtained by CID were performed with an isolation width of 3 Th (m/z). The activation amplitude was around 35 % of the ejection RF amplitude that corresponds to 1.58 V.

For the analysis of protein fragments derived from m′-HMb, the mass spectrometer was set such that one full MS scan was followed by a zoom-scan and MS/MS scan on the most intense ion from the MS spectrum. The acquired MS/MS spectra were automatically searched against a protein database for human proteins (human.fasta) by using the SEQUEST algorithm to identify the modified residues. This algorithm has been incorporated into the Bioworks 3.0 software (ThermoFinnigan, San Jose, CA). For the analysis of protein fragments of m-HMb, the mass spectrometer was set in MS/MS mode for the parent bicharged ions at m/z = 957 (mass of 1912 amu), 995.5 (mass of 1989 amu), and 973 (mass of 1944 amu). To compare the amount of modified heme, the mass spectrometer was set in MS/MS mode for the parent ions at 616 and 661 amu

Two-dimensional and SDS PAGE analysis: Two-dimensional polyacrylamide gel electrophoresis was performed by using the immobilized pH gradient system.[52] The first dimension, isoelectric focusing, was performed on laboratory-made gels, cast on Gel-Bond (Amersham Biosciences) with a 6.5–8.5 linear immobilized pH gradient obtained with Acrylamido buffer solutions (Fluka), and the separation was run in the Multiphor II horizontal system (Amersham Biosciences). The protein solutions (HMb and m′-HMb) were diluted 1:2 with a solution containing 8 M urea, 4 % (w/v) 3-[(3-cholamidopropyl)dimethylammonio]-1-propanesulfonate (CHAPS), and 40 mM Tris. After centrifugation, 125 μL of each sample containing 30 μg protein were loaded. The gel strips were then equilibrated with SDS and placed on top of vertical 17 % gels, before the second dimension was performed by using a Mini PROTEAN II cell (BioRad). SDS PAGE (17 % gel) was performed by the method of Laemmli[53] and the gel was stained with Coomassie Blue.

Binding of nitrite: Increasing quantities of a concentrated nitrite solution in 200 mM phosphate buffer (pH 7.5, final concentration of 0.0019–0.53 M) were added to a solution of HMb (3.8 μM, 1600 μL) in the same buffer in a quartz cuvette with a pathlength of 1 cm thermostated at 25.0 ± 0.1 °C; UV/Vis spectra were recorded after each addition. Blank spectra were recorded in the same way but in the absence of protein. After subtracting the corresponding blank from each spectrum, the resulting spectra were corrected for dilution and then transformed into difference spectra by subtracting the native protein spectrum. A plot was constructed with the difference between the absorbance changes at 424 and at 406 nm, the wavelengths at which the difference spectra exhibit the maximum variations, versus the ligand concentration. The binding constant, K_B, was obtained by interpolation of the absorbance data with the binding isotherm for low-affinity binding of a single ligand: $\Delta A = \Delta A_\infty K_B[NO_2^-]_{total}/(1+K_B[NO_2^-]_{total})$, where $[NO_2^-]_{total}$ represents the total nitrite concentration.[11]

Kinetic studies of phenol nitration catalyzed by HMb and m′-HMb: The kinetic experiments were carried out in 200 mM phosphate buffer (pH 7.5) by using a quartz cuvette with a pathlength of 1 cm thermostated at 25.0 ± 0.1 °C and equipped with a magnetic stirrer. The initial solution containing protein (HMb or m′-HMb, 1 μM) and variable substrate and nitrite concentrations (final volume 1600 μL) was obtained by mixing solutions of appropriate concentration of the reagents in the buffer. The reaction was started by addition of the H_2O_2 solution and was followed by monitoring the absorbance change at 450 nm during the initial 10–15 s. The transformation of the rate data from absorbance per second into $[nitrophenol]_{produced}$ per second was done by using the extinction coefficient of the nitrophenols at 450 nm obtained from their absorbance spectra (in phosphate buffer at pH 7.5):[54] for 3-nitrotyramine ε_{450} = 2300, for 3-(4-hydroxy-3-nitrophenyl)propionic acid ε_{450} = 3350, and for 3-nitro-L-tyrosine $\varepsilon_{450} = 3100\ M^{-1}\ cm^{-1}$. The kinetic parameters were obtained from fitting the plots of experimental rates at different substrate/nitrite concentrations to the appropriate equations.

For each substrate, the rate dependence on the various reactant concentrations was studied through three series of steps: 1) finding a suitable $[H_2O_2]$ maximizing the rate but avoiding unwanted excess of the oxidant, and then using this $[H_2O_2]$ 2) to study the dependence of the rate versus [substrate], and 3) to study the dependence of the rate versus $[NO_2^-]$, by following the iterative procedure described previously in detail.[11] The protein concentration (HMb or m′-HMb) was kept at 1 μM in all the reactions, while the concentrations of the other reactants used in the kinetic studies were as follows:

For the phenol nitration catalyzed by HMb: 1) optimization of the peroxide concentration: for substrate **1** (2.0 mM): $[NO_2^-]$ = 1.3 M, $[H_2O_2]$ = 0.071–0.71 M; for substrate **2** (0.40 mM): $[NO_2^-]$ = 0.30 M, $[H_2O_2]$ = 0.053–0.36 M; for substrate **3** (0.3 mM): $[NO_2^-]$ = 0.8 M, $[H_2O_2]$ = 0.071–0.71 M; 2) dependence of the rate on phenol concentration: for substrate **1**: $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 1.3 M, [phenol] = 0.31–3.7 mM; for substrate **2**: $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 0.3 M, [phenol] = 0.062–1.6 mM; for substrate **3**: $[H_2O_2]$ = 0.53 M, $[NO_2^-]$ = 0.8 M, [phenol] = 0.013–0.71 mM; 3) dependence of the rate on nitrite concentration: for substrate **1** (3.1 mM): $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 0.075–2.0 M; for substrate **2** (0.62 mM): $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 0.062–0.92 M; for substrate **3** (0.71 mM): $[H_2O_2]$ = 0.53 M, $[NO_2^-]$ = 0.050–1.1 M.

For the phenol nitration catalyzed by m′-HMb: 1) optimization of the peroxide concentration: for substrate **1** (2.0 mM): $[NO_2^-]$ = 1.0 M, $[H_2O_2]$ = 0.071–0.71 M; for substrate **2** (0.40 mM): $[NO_2^-]$ = 0.80 M, $[H_2O_2]$ = 0.071–0.71 M; for substrate **3** (0.30 mM): $[NO_2^-]$ = 0.80 M, $[H_2O_2]$ = 0.071–0.71 M; 2) dependence of the rate on phenol concentration: for substrate **1**: $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 1.0 M, [phenol] = 0.062–3.1 mM; for substrate **2**: $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 0.80 M, [phenol] = 0.026–1.3 mM; for substrate **3**: $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 0.8 M, [phenol] = 0.031–0.68 mM; 3) dependence of the rate on nitrite concentration: for substrate **1** (3.1 mM): $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 0.075–1.7 M; for substrate **2** (0.5 mM): $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 0.050–1.1 M; for substrate **3** (0.68 mM): $[H_2O_2]$ = 0.36 M, $[NO_2^-]$ = 0.050–0.80 M.

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