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PEROXYNITRITE–INDUCED MODIFICATIONS OF MYOGLOBIN:

A Kinetic and Mechanistic Study

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We don't see things as they are, we see things as we are.

Anais Nin (1903-1977)

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ABSTRACT

Peroxynitrite, a biological toxin produced *in vivo* by the nearly diffusion-controlled reaction of nitrogen monoxide with superoxide, can nitrate and oxidize various biomolecules, such as thiols, lipids, carbohydrates, and nucleic acids. Modifications caused by peroxynitrite have been linked to many human diseases. In particular, increased levels of free or protein-bound 3-nitrotyrosine, a biomarker for peroxynitrite *in vivo,* have been detected in a variety of pulmonary and cardiovascular diseases as well as in neurodegenerative and chronic inflammatory disorders. These observations have led to the search of a drug that can scavenge this powerful nitrating and oxidizing agent.

Heme proteins are believed to be among the major targets for peroxynitrite *in vivo*. In particular, myoglobin and hemoglobin, present in large amounts in muscles and red blood cells, respectively, have been proposed to serve as sinks for peroxynitrite in these cells. Indeed, peroxynitrite has been shown to enter the erythrocytes and react with hemoglobin within the red blood cell. In this project, we have studied the reaction mechanism of peroxynitrite toward the oxidized forms of myoglobin and hemoglobin.

This work shows that the iron(III) forms of myoglobin and hemoglobin catalyze the isomerization of peroxynitrite to nitrate. The catalytic rate constants for the decay of peroxynitrite in the presence of metMb and metHb are $(7.7 \pm 0.1) \times 10^4$ and $(3.9 \pm 0.2) \times 10^4$ M^{-1} s^{-1}, respectively, at pH 7.0 and 20° C. The pH-dependence of the catalytic rate constants indicates that protonated form of peroxynitrite is the species that reacts with the iron(III) center of the proteins. In the presence of physiologically relevant concentrations of carbon dioxide (1.2 mM) the decay of peroxynitrite is also accelerated by these iron(III) forms of myoglobin and hemoglobin. Experiments carried out at high pH in the presence of a large excess of carbon dioxide showed that no direct reaction occurs between the iron(III) center of these proteins and 1-carboxylato-2-nitrosodioxidane, the adduct generated from the reaction of peroxynitrite anion with carbon dioxide. Analysis of the horse heart myoglobin and human hemoglobin after treatment with excess peroxynitrite showed low levels of nitration of protein-bound tyrosine residues. Moreover, these proteins efficiently protect added free tyrosine from peroxynitrite-mediated nitration. The catalytic rate constants for the decay of peroxynitrite and the extent of nitration of protein-bound and free tyrosine in the presence of metMb and metHb indicate that myoglobin is slightly a better scavenger than hemoglobin.

The isomerization of peroxynitrite to nitrate by metMb and metHb is not very efficient because of the strong hydrogen bond between the distal histidine and the aquo-ligand bound to the iron(III) center of the heme. The hydrogen bond is known to stabilize the coordinated

water molecule, thus the reactivity of peroxynitrite toward metMb and metHb is most likely regulated by the distal amino acid residues. This was demonstrated by studies with the iron(III) forms of the sperm whale myoglobin distal histidine mutants H64A, H64D, H64L, F43W/H64L, and H64Y/H93G. Also these proteins catalyzed the isomerization of peroxynitrite to nitrate. The two most efficient catalysts are H64A- and H64D-metMb, with catalytic rate constants of $(5.8 \pm 0.1) \times 10^6$ and $(4.8 \pm 0.1) \times 10^6$ M^{-1} s^{-1}, respectively, at pH 7.5 and 20 °C. Our results showed that in the absence of the distal histidine residue in myoglobin, the size of the distal ligand determines the rate of the isomerization of peroxynitrite to nitrate.

This comprehensive study of the reaction of peroxynitrite with myoglobin and hemoglobin provides new mechanistic insights for understanding the influence of single amino acid residues on heme protein-peroxynitrite interactions.

ESTRATTO

Il perossinitrito é una tossina biologica prodotta in vivo dalla reazione, a controllo diffusionale, fra il monossido d'azoto ed il superossido. Essa é in grado di nitrare ed ossidare varie biomolecole fra cui tioli, lipidi, carboidrati e acidi nucleici. Le modificazioni causate dal perossinitrito sono state collegate a molte malattie umane. In particolare, un biomarker per il perossinitrito in vivo è rappresentato dalla 3-nitro-tirosina e un aumento dei suoi livelli, sia essa libera o legata ad altri amminoacidi, è stato rivelato in una varietà di malattie polmonari e cardiovascolari così come nei disordini infiammatori neurodegenerativi e cronici. Queste osservazioni hanno condotto alla ricerca di un composto in grado di eliminare questo potente agente di nitrazione ed ossidazione.

Le eme-proteine possono rappresentare uno degli obiettivi principali per il perossinitrito in vivo. In particolare, la mioglobina e l'emoglobina, presenti in grandi quantità rispettivamente nei muscoli e nei globuli rossi, sono state proposte come scavenger per il perossinitrito in questo tipo di cellule. Inoltre, è stato indicato che il perossinitrito é in grado di entrate negli eritrociti e reagire con l'emoglobina all'interno dei globuli rossi. In questo progetto, abbiamo studiato il meccanismo di reazione del perossinitrito verso le forme ossidate di mioglobina ed emoglobina.

Questo lavoro indica che le forme del ferro (III) mioglobina ed emoglobina catalizzano l'isomerizzazione del perossinitrito a nitrato. Le costanti di velocità catalitiche per il decadimento del perossinitrito in presenza di metMb e di metHb sono rispettivamente $(7.7 \pm 0.1) \times 10^4$ e $(3.9 \pm 0.2) \times 10^4$ M^{-1} s^{-1} a pH 7.0 e 20 °C. La dipendenza dal pH delle costanti di velocità catalitiche indica che è la forma protonata del perossinitrito a reagire con il ferro (III) delle proteine. Queste proteine catalizzano il decadimento del perossinitrito anche in presenza di concentrazioni fisiologicamente rilevanti di anidride carbonica (1.2 mM). Esperimenti condotti ad alti pH ed in presenza di un largo eccesso di anidride carbonica non indicano la presenza di alcuna reazione diretta fra il ferro (III) di queste proteine e 1-carbossilato-2-nitrodiossidano, l'addotto generato dalla reazione dell'anione perossinitrito con il diossido di carbonio.

Analisi condotte utilizzando mioglobina di cuore di cavallo ed emoglobina umana con un largo eccesso di perossinitrito mostrano bassi livelli di nitrazione dei residui di tirosina presenti nella proteina. Inoltre queste proteine proteggono efficaciemente tirosine libere aggiunte in soluzione dalla nitrazione ad opera del perossinitrito e agiscono così da scavenger per il perossinitrito. L'insieme delle costanti di velocità catalitiche per il decadimento del

perossinitrito e la resa di nitrazione delle tirosine, in presenza di mioglobina ed emoglobina, indicano che la mioglobina è uno scavenger leggermente migliore dell'emoglobina.

L'isomerizzazione del perossinitrito a nitrato ad opera di metMb e o metHb non è molto efficiente a causa del forte ponte idrogeno fra l'istidina distale e la molecola d'acqua legata all'atomo di ferro dell'eme. E' noto che il legame idrogeno è in grado di stabilizzare la molecola d'acqua cosi' la reattività del perossinitrito verso la metMb e la metHb è quindi molto probabilmente regolata dai residui amminoacidici in posizione distale. Studi condotti utilizzando le forme ossidate di mioglobina di capodoglio mutate nell'istidina distale (H64A, H64D, H64L, F43W/H64L e H64Y/H93G) mostrano che anche queste proteine sono in grado di isomerizzare il perossinitrito a nitrato. I due catalizzatori piu' efficienti sono H64A- e H64D-metMb, i quali mostrano rispettivamente una costante di velocità catalitica pari a $(5.8 \pm 0.1) \times 10^6$ e $(4.8 \pm 0.2) \times 10^6$ M^{-1} s^{-1} a pH 7.5 e 20 °C. Inoltre i nostri risultati mostrano che in assenza dell'istidina distale nella mioglobina, la dimensione del ligando in posizione distale determina la velocità di isomerizzazione del perossinitrito a nitrato.

Questo studio completo del la reatione del perossinitrito con la mioglobina e l'emoglobina fornisce una nuova idea meccanicistica per la comprensione dell'influenza di un singolo ammino acido sulle interazioni proteina-perossinitrito.

ABBREVIATIONS

Mb	myoglobin
Hb	hemoglobin
$MbFe^{II}$	deoxymyoglobin
$MbFeO_2$	oxymyoglobin
$MbFe^{III}OH_2$	metmyoglobin, iron(III) myoglobin
$MbFe^{IV}=O$	ferryl myoglobin, oxoiron(IV) myoglobin
metMbCN	cyanoiron(III) myoglobin
SDS	sodium dodecyl sulfate
SDS-PAGE	SDS-polyacrylamide gel electrophoresis
SOD	superoxide dismutase
MnSOD	manganese superoxide dismutase
CuZnSOD	cupper zinc superoxide dismutase
NOS	nitric oxide synthase
NADPH	nicotinamide adenine dinucleotide phosphate
Trp	tryptophan
Tyr	tyrosine
His	histidine
TFA	trifluroacetic acid
edta	ethylenediaminetetraacetic acid
HPLC	high performance liquid chromatography
$HbFeO_2$	oxyhemooglobin
$HbFe^{III}OH_2$	methemoglobin, iron(III) hemoglobin
$HbFe^{IV}=O$	ferryl hemoglobin, oxoiron(IV) hemoglobin
ESR	electron spin resonance
H64A	histidine64alanine
H64L	histidine64leucine
H64D	histidine64aspartic acid
F43W/H64L	phenylalanine43tryptophan/histidine64leucine
H64Y/H93G	histidine64tyrosine/histidine93glycine
hhMb	horse heart myoglobin
sw-Mb	sperm whale myoglobin
DTPA	diethylenetriaminepentaacetic acid
ESI-MS	electrospray ionisation-mass spectroscopy
Mn(III)-TM-PyP	manganese(III) 5,10,15,20,-tetrakis(N-methyl-4´-pyridyl)porphyrin
Fe(III)-TMPS	iron(III) 5,10,15,20,-tetrakis(2,4,6-trimethyl-3,5-sulfonatophenyl)porphyrin

LIST OF PUBLICATIONS

The following published articles are included in part or completely in this thesis:

1. Myoglobin scavenges peroxynitrite without being significantly nitrated.
   Herold, S., Shivashankar, K., and Mehl, M. (2002) ***Biochemistry*** 41, 13460-72.

2. Metmyoglobin and methemoglobin catalyze the isomerization of peroxynitrite to nitrate.
   Herold, S., and Shivashankar, K. (2003) ***Biochemistry*** 42, 14036-46.

3. Mechanistic studies of the isomerization of peroxynitrite to nitrate catalyzed by distal histidine metmyoglobin mutants.
   Herold, S., Kalinga, S., Matsui, T., and Watanabe, Y. (2004) ***J. Am. Chem. Soc.*** 126, 6945-6955

1 INTRODUCTION AND BACKGROUND SUMMARY

1.1 OVERVIEW

Living organisms are constantly exposed to partially reduced oxygen species and partially oxidized nitrogen species with widely different reactivities. Reactive species like superoxide, hydroxyl radical and hydrogen peroxide are inevitably formed during the metabolism of dioxygen, especially from the incomplete reduction of dioxygen by the NADH oxidase of mitochondria. In addition, species like peroxynitrite and nitrogen dioxide can be formed *in vivo* and oxidize and/or nitrate various amino acids causing changes in the structure and function of proteins which may lead to many different diseases. Therefore, it is important to study the reactions of these toxic species with biomolecules and to elucidate the reaction mechanisms. However, to minimize the damage caused by these oxidizing/nitrating agents, most biological systems have developed a collection of antioxidants (eg., metalloproteins, superoxide dismutase, catalase, glutathione, vitamin C, and uric acid) that can convert toxic reactive species to less reactive derivatives.

1.2 NITROGEN MONOXIDE

In 1977, Murad et al. discovered that nitrogen monoxide has the ability to dilate blood vessels by relaxing smooth muscle tissue *(1)*. In the 1980s, it was discovered that nitrogen monoxide is produced in biological tissues by nitric oxide synthase (NOS). In 1998, three pharmacologists, Furchgott, Ignarro and Murad, were awarded the Noble Prize in Physiology and Medicine for their discovery that nitrogen monoxide acts as a signalling molecule in the cardiovascular system.

The nitric oxide synthase family of enzymes catalyzes the conversion of L-arginine to nitrogen monoxide, which acts as an effector molecule in various physiological and pathophysiological processes. Presently, three distinct isoforms of NOS have been identified: one is located exclusively in the vascular endothelium (eNOS); the second form is inducible NOS (iNOS), produced in macrophages and requires inflammatory cytokines to induce its expression (eq. 1), and the third isoenzyme is found in the brain and neuronal tissues (nNOS) *(2, 3)*. Nitrogen monoxide has various functions in the body: it regulates blood pressure, is a defensive cytotoxin of the immune system and acts as neurotransmitter.

Nitrogen monoxide is an uncharged lipophilic molecule which contains a single unpaired electron *(4)*. Since the biological half-life of nitrogen monoxide is 5–30 s, it can readily diffuse across cellular membranes and react with molecules such as dioxygen and

superoxide radicals *(2)*. Nitrogen monoxide reacts with dioxygen to give nitrogen dioxide and the rate constant for this ternary reaction in aqueous solution is $2 \times 10^6\ M^{-2}\ s^{-1}$ at 25 °C *(5)*. However, this reaction proceeds slowly under physiological conditions. Nitrogen monoxide reacts rapidly with superoxide to form peroxynitrite, which is a far more reactive molecule than its precursors (eq. 1, 2) *(6)*. Nitrogen monoxide also reacts with thiol groups in the presence of electron acceptors like dioxygen, iron(III) and copper(II) to form nitrosothiols such as *S*-nitrosocysteine, *S*-nitrosoalbumin, or *S*-nitrosohemoglobin. Nitrosothiols have been proposed to represent a stabilized form of nitrogen monoxide and thus to play important roles in the transport and release of nitrogen monoxide *(7)*.

$$\text{Cytokines} \longrightarrow \text{Cell activation} \xrightarrow{\text{NOS}} \text{NO}^{\bullet}\ \text{overproduction} \xrightarrow{O_2^{\bullet -}} \text{Peroxynitrite production} \qquad (1)$$

In red blood cells and in muscle cells, peroxynitrite is not formed because of the rapid removal of nitrogen monoxide through reaction with oxyHb and oxyMb. This reaction produces the iron(III) form of these proteins and nitrate *(8)*. Moreover, the red blood cells contain the antioxidant enzyme superoxide dismutase, which catalyzes the dismutation of superoxide radical, which is produced by the auto-oxidation of oxyHb.

1.3 PEROXYNITRITE

Peroxynitrite, a reactive transient species with a biological half life of 10–20 ms, is a potent oxidant with the ability to destroy critical cellular components *(6, 9)*. It may oxidize thiols *(10)* and/or nitrate all major types of biomolecules including DNA *(11, 12)*, proteins *(13, 14)*, and lipids *(15)*.

$$\text{NO}^{\bullet} + O_2^{\bullet -} \xrightarrow{1.6 \times 10^{10}\ M^{-1}s^{-1}} \text{ONOO}^- \underset{}{\overset{H^+}{\rightleftharpoons}} \text{ONOOH} \qquad (2)$$

$$2\ O_2^{\bullet -} + 2\ H^+ \xrightarrow[\text{SOD}]{2 \times 10^9\ M^{-1}s^{-1}} O_2 + H_2O_2 \qquad (3)$$

1.3.1 THE BIOLOGICAL CHEMISTRY OF PEROXYNITRITE

Koppenol and coworkers *(16, 17)* pointed out that the second-order rate constant for the reaction of nitrogen monoxide with superoxide is $(1.6 \pm 0.3) \times 10^{10}\ M^{-1}s^{-1}$ (eq. 2), a value which is larger than that for rate constant of the reaction of superoxide with superoxide

dismutase [2×10^9 $M^{-1}s^{-1}$, *(18)]* (eq. 3). Thus, peroxynitrite may be formed even in the presence of physiological concentrations of superoxide dismutase, if the nitrogen monoxide concentration is in the micromolar range. The normal intracellular concentration of nitrogen monoxide is ca.10 nM and that of superoxide dismutase is 10 µM *(19)*. During inflammation, the extracellular concentration of nitrogen monoxide is expected to rise to ~2 µM *(19)*. Under these conditions, nitrogen monoxide can outcompete superoxide dismutase and react with superoxide to form peroxynitrite.

The reactivity of peroxynitrite is complex and highly pH-dependent *(20)*. Kissner et al. reported that the pK_a of peroxynitrous acid is about 6.8 in 50 mM phosphate buffer but can increase up to 8, depending on the buffer composition and concentration *(16)*. The term peroxynitrite refers to the sum of peroxynitrite anion ($ONOO^-$) and its conjugate acid, peroxynitrous acid (ONOOH). Peroxynitrite can directly perform one- or two-electron oxidation reactions with electrode potentials [$E^{\circ\prime}$ (ONOOH, $H^+/NO_2^{\bullet}$, H_2O) = 1.6 ± 0.1 V] or [$E^{\circ\prime}$ (ONOOH, H^+/NO_2^-, H_2O) = 1.3 ± 0.1 V], respectively *(21)*. The peroxynitrite anion is rather stable in alkaline solutions, slowly decomposing to nitrite and dioxygen *(22)*. However, peroxynitrous acid is unstable and isomerizes to give nitrate or decomposes into a reactive intermediate which is much under debate among the scientific community.

The first proposed pathway is the decomposition of peroxynitrous acid to give nitrogen dioxide and hydroxyl radicals *(20)*. This proposed homolytic cleavage leads to a pair of radicals formed in a cage surrounded by water molecules. These caged radicals can recombine to reform peroxynitrous acid, diffuse apart to give nitrogen dioxide and hydroxyl radicals, or recombine to form nitrate (eq. 4). The yield of nitrogen dioxide and hydroxyl radicals was estimated to be 30 % *(23, 24)*. However, the rate of homolysis (0.6 s^{-1} – 10^{-4} s^{-1}) of peroxynitrous acid is slower than the rate of isomerization (1.2 s^{-1}), and thus homolysis is highly unlikely *(25)*.

$$ONOOH \underset{}{\overset{\text{Homolysis}}{\rightleftharpoons}} \underbrace{[NO_2^{\bullet}\cdots\cdots OH^{\bullet}]}_{\text{Cage}} \begin{cases} \xrightarrow{70\%} NO_3^- + H^+ \\ \xrightarrow{30\%} NO_2^{\bullet} + OH^{\bullet} \end{cases} \qquad (4)$$

Koppenol and coworkers proposed an alternative pathway for the decomposition of ONOOH, that involves an as yet undefined metastable species of peroxynitrous acid,

HOONO* *(25)*. This intermediate can rapidly isomerize to nitrate (eq. 5). As the half-life of peroxynitrous acid is less than a second, it is quite difficult to study the exact chemistry of this metastable intermediate. However, theoretical studies have shown the existence of such a metastable species *(26)*.

$$ONOOH \rightleftharpoons [ONOOH^*] \xrightarrow{\text{Isomerizes}} NO_3^- + H^+ \quad (5)$$

$$ONOOH + ONOO^- \rightleftharpoons \underset{\text{adduct}}{ONOOHONOO^-} \longrightarrow 2\,NO_2^- + O_2 + H^+ \quad (6)$$

In recent studies, no direct evidence was found for the formation of hydroxyl radical *(16, 20, 21, 25)*. In contrast, several studies reported results which are not in agreement with this hypothesis: (i) when the concentration of peroxynitrite exceeds 100 μM, its disappearance is delayed because of the formation of an adduct between peroxynitrite anion and peroxynitrous acid *(16)*. This adduct ($ONOOHONOO^-$) decomposes to give nitrite and dioxygen (eq. 6). Thus, the yields of nitrite and nitrate depend the peroxynitrite concentration and on the pH. For instance, at pH 7.0, 2.5 mM peroxynitrite gives less than 10 % nitrite, whereas at pH 10, up to 80% nitrite is formed *(27)*. (ii) The decay of peroxynitrous acid in the presence of [^{15}N]nitrite involves initial formation of an adduct between nitrite and peroxynitrite to give [^{15}N]nitrate. However, according to the homolysis hypothesis, the yields of [^{15}N]nitrate predicted for low [^{15}N]nitrite concentrations are much larger than those obtained experimentally *(28)*. (iii) The reaction of peroxynitrous acid with monohydroascorbate gives nitrate via an intermediate adduct without formation of ascorbyl radicals *(29)*. (iv) The isomerization of peroxynitrite has a small entropy of activation of 3 ± 2 eu *(23)* and a volume of activation of 6.9 ± 0.9 $cm^3\ mol^{-1}$ *(30)*, two values which are not fully compatible with homolysis. In general, activation volumes for homolysis reactions are close to 10 $cm^3\ mol^{-1}$ and values found for the isomerization of peroxynitrous acid, 6–10 $cm^3\ mol^{-1}$, do not allow to draw any conclusion regarding the mechanism of the conversion. In conclusion, the above results support the notion put forth by Koppenol et al. for the isomerization of peroxynitrite: "a radical-free mechanism rather than a free-radical mechanism" *(27)*.

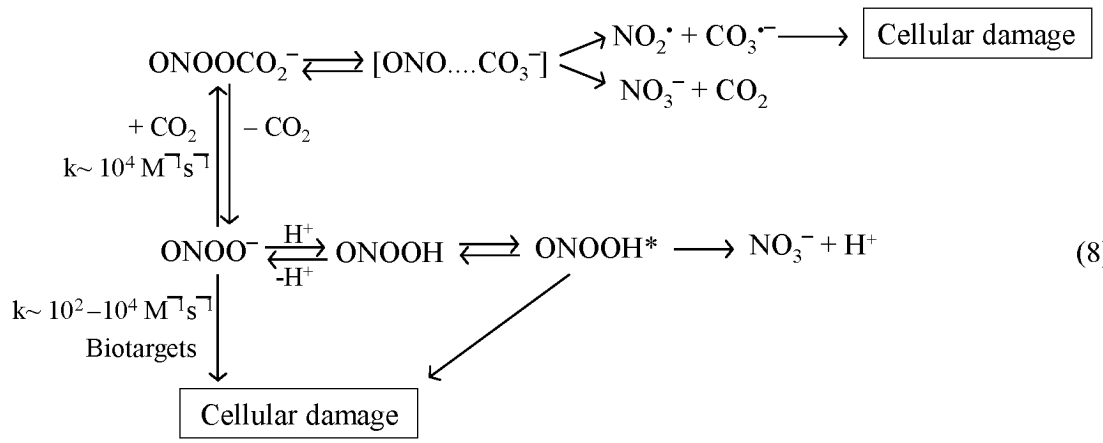
Peroxynitrite also reacts rapidly with carbon dioxide to form the adduct 1-carboxylato-2-nitrosodioxidane ($ONOOCO_2^-$), which rapidly produces carbon dioxide and nitrate, possibly via the formation of nitrogen dioxide and trioxidocarbonate (•1–). At 37 °C, the

second-order rate constant for the reaction is $5.7 \times 10^4\ M^{-1}s^{-1}$ *(31)* (eq. 7). Since the intracellular and extracellular carbon dioxide concentrations are about 1–2 mM, this reaction represents

$$ONOO^- + CO_2 \underset{}{\overset{5.7 \times 10^4\ M^{-1}s^{-1}}{\rightleftharpoons}} ONOOCO_2^- \longrightarrow \text{Products} \qquad (7)$$

one of the major routes for peroxynitrite reactivity *in vivo (31)*. Therefore, reactions of peroxynitrite *in vivo* are more likely to be mediated by $ONOOCO_2^-$ and by the products derived from its decomposition than by peroxynitrite itself *(32, 33)*. The adduct $ONOOCO_2^-$ has not yet been detected. However, the induction period observed by all contributors before a carbon dioxide/peroxynitrite mixture starts to decay represents indirect evidence *(32-36)*. The amount of radicals generated from the decay of $ONOOCO_2^-$ is still under investigation. It is assumed that 70% of the radicals formed recombine inside the solvent cage to give nitrate and carbon dioxide, while the remaining 30% is able to react with target molecules *(34, 35)*. In contrast, it has been shown that upon mixing peroxynitrite, with carbon dioxide in the presence of nitrogen monoxide, the extent of nitrogen dioxide and trioxidocarbonate (•1–) formed is less than 4% *(36)*. Nitrogen monoxide is known to react with nitrogen dioxide and trioxidocarbonate (•1–) to give carbon dioxide and nitrite, but does not react with peroxynitrite anion.

Trioxidocarbonate (•1–) is a strong oxidant, and nitrogen dioxide is both an oxidizing and a nitrating agent. The electrode potential of the couple $CO_3^{\bullet -}/CO_3^{2-}$ is 1.58 V *(37)* and that of $NO_2^{\bullet}/NO_2^-$ pair is 0.99 V *(21)*.



(8)

In conclusion, peroxynitrite can oxidize many substrates in biological systems either directly or by producing secondary oxidants (eq. 8). However, *in vivo* most of the peroxynitrite will be consumed by direct reactions, mainly with thiols, ascorbate, uric acid, transition metal centers, and carbon dioxide.

1.3.2 SYNTHESIS OF PEROXYNITRITE

Several methods have been developed to synthesize peroxynitrite. The synthesis of solid peroxynitrite salt was first reported by Bohle et al. and is considered the best method for peroxynitrite synthesis. In brief, when tetramethylammonium superoxide is mixed with a slight excess of nitrogen monoxide in liquid ammonia, a hygroscopic microcrystalline yellow solid tetramethylammonium peroxynitrite is formed. The advantage of this method is that peroxynitrite can be recrystallized to be separated from contaminating nitrite *(38)*.

In our studies we always used the method developed by Koppenol et al. *(39)*. This method has the advantage of producing peroxynitrite with no hydrogen peroxide and little contamination of nitrite. Peroxynitrite is synthesized from solid potassium superoxide mixed with purified quartz sand and gaseous nitrogen monoxide in an argon atmosphere. The solid deep yellow potassium peroxynitrite formed is then dissolved in a sodium hydroxide solution. Hydrogen peroxide formed from the excess superoxide is decomposed by addition of manganese dioxide. The concentration of resulting peroxynitrite solutions was determined by UV-spectroscopy by measuring the absorbance at 302 nm (Figure 1) [ε_{302} = 1705 M^{-1} cm^{-1} *(38)*].

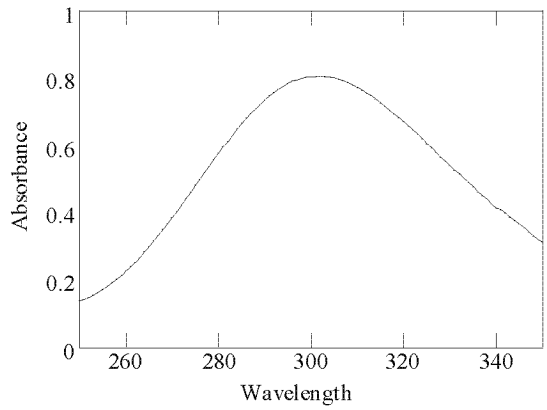


Figure 1. The Uv-vis spectrum of 0.5 mM peroxynitrite in 0.1 M NaOH with absorption maximum at 302 nm.

1.3.3 PEROXYNITRITE IN PATHOLOGY

Peroxynitrite is significantly more reactive and toxic to cells than nitrogen monoxide, superoxide or hydrogen peroxide. Peroxynitrite has been postulated to be involved in many human diseases for example, heart diseases, chronic inflammation and autoimmune diseases, cancer, Parkinson's, Alzheimer, multiple sclerosis, amyotrophic lateral sclerosis, and ischemic reperfusion injury.

1.3.3.1 THIOL OXIDATION

Peroxynitrite oxidation of thiols occurs, at least partially, through a one-electron oxidation process that may lead to sulfur-centered radicals. EPR spin-trapping studies of the peroxynitrite-mediated oxidation of cysteine, glutathione, and the thiol group of albumin demonstrated the formation of the corresponding thiyl radicals *(40, 41)*. Moreover, the yield of thiyl radicals increases in the presence of dissolved carbon dioxide and ascorbate concentrations *(42)*.

The rate constant for the reaction of peroxynitrite with thiols like cysteine and glutathione is in the order of magnitude of 10^3 $M^{-1}s^{-1}$ *(10, 21)*. Because of the high concentration of thiols *in vivo* (5-10 mM), they are considered as important targets of peroxynitrite *(14, 21)*. Although the role of glutathione as an antioxidant against peroxynitrite is disputed, this thiol has been shown to inhibit peroxynitrite-dependent formation of nitrotyrosine in the presence of carbon dioxide *(43)*.

1.3.3.2 LIPID PEROXIDATION

Peroxynitrous acid has the ability to pass through lipid bilayers and can initiate lipid peroxidation and also cause nitration of lipids *(6, 15)*. It is reported that nitrogen monoxide alone cannot cause lipid peroxidation *(44)*. In contrast, in the absence of superoxide, nitrogen monoxide inhibits lipid oxidation *(45)*. The mechanism by which nitrogen monoxide inhibits lipid peroxidation is poorly understood. It has been postulated to involve reactions between nitrogen monoxide and lipid-derived radicals *(46)*. The termination reaction between nitrogen monoxide and lipid peroxyl radicals ($LOO^{\bullet}$) yield a transient intermediate (LOONO). This species is unstable and will rearrange to give the organic nitrate ($LONO_2$) that can undergo homolysis to yield nitrogen dioxide and lipid alkoxyl radicals ($LO^{\bullet}$), or hydrolyze to give lipid alkoxide (LOH) and nitrate *(46, 47)*. However, additional reactions are possible, the lipid

alkoxyl radical can react with nitrogen monoxide to form LONO, and lipid peroxyl radicals can react with nitrogen dioxide to form $LOONO_2$.

1.3.3.3 DNA DAMAGE

Peroxynitrite causes DNA cleavage through nitration and oxidation. In addition to DNA strand breaks, peroxynitrite reacts primarily with deoxyguanine (dG) in DNA to form a variety of modifications including 8-nitro-dG and 8-oxo-dG. 8-Nitro-dG is widely considered as a specific marker for peroxynitrite-mediated DNA damage *(11)*. Recently, it has been reported that the reaction of peroxynitrite with 8-oxo-dG leads to the formation of 8-oxo-7,8-dihydroG, a compound that can be further oxidized to yield several products (for eg. 5-guanidino-4-nitroimidazole and 2, 2-diamino-4-5(2H)-oxazolone) *(48)*.

Peroxynitrite is also known to cause strand breaks in plasmid supercoiled DNA and in DNA of eukaryotic cells *(49)*. Such DNA lesions consist of single and double strand breaks, modified bases, abasic sites, and DNA-protein cross links may be responsible for a variety of biological processes such as mutagenesis, aging, and carcinogenesis.

1.3.3.4 TYROSINE/TRYPTOPHAN NITRATION

Peroxynitrite nitrates tyrosine/tryptophan residues in protein to yield nitrotyrosine (Figure 2) /nitrotryptophan which are widely considered as footprints detectable *in vivo (6, 13, 50-51)*. However, due to its short half-life, peroxynitrite cannot be detected directly *in vivo*. The mechanism of 3-nitrotyrosine formation by peroxynitrite is unclear, it has been proposed to occur through a radical mechanism which involves the reaction of tyrosine with nitrogen dioxide to form the tyrosyl radical which recombines with another nitrogen dioxide to produce 3-nitrotyrosine *(52)*. Besides the nitro derivative, several oxidation products are also formed, for instance dityrosine and 3-hydroxytyrosine. The nitration of tyrosine and tryptophan residues can cause changes in the structure and function of a protein. Tyrosine nitration has been shown to inactivate enzymes, for instance manganese superoxide dismutase *(53)*, to block tyrosine phosphorylation which disrupts the tyrosine kinase signaling pathway *(54)*, and to inactivate tyrosine hydroxylase *(55)*.

The factors determining the site of tyrosine nitration have not been completely elucidated, but some general hypotheses have been proposed. (i) Nitration often occurs when tyrosine is localized in hydrophobic domains; for instance nitration of a tyrosine located in the

hydrophobic lipid bilayer is higher than that measured for tyrosine in an aqueous solution *(56)*. (ii) In proteins, the presence of negatively charged residues near the tyrosine can increase the yield of nitration *(57)*. (iii) In some cases, the presence of a metal centre in close proximity to a tyrosine catalyzes nitration *(57)*. In conclusion, the specificity of peroxynitrite-dependent tyrosine nitration seems to depend on the secondary and tertiary structures of proteins, on the location and on the local environment of the tyrosine residue *(58)*. Although 3-nitrotyrosine is a stable product and is considered to be long lived in tissues, a report suggests the presence of an enzyme (nitrotyrosine-denitrase) that is capable of reverting protein tyrosine-nitration *(59)*.

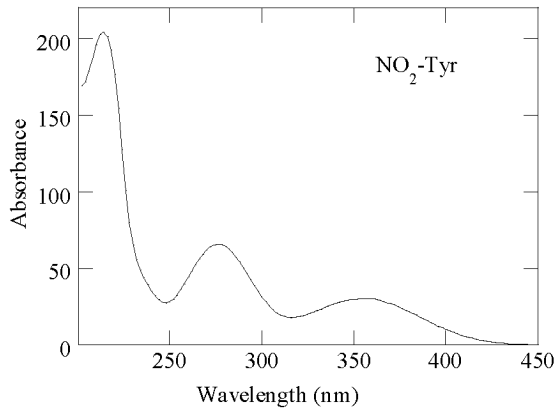


Figure 2. UV-vis spectrum of a 3-nitrotyrosine, taken from a HPLC chromatogram.

The formation of nitrotryptophans has also been detected *in vitro* after exposure of some proteins to peroxynitrite, for instance copper-zinc superoxide dismutase *(60)*, myoglobin and hemoglobin *(61)*. It has been shown that addition of carbon dioxide leads to higher yields of peroxynitrite-mediated tyrosine/tryptophan nitration. The increased nitration is due to the interaction of carbon dioxide with peroxynitrite anion to give the adduct $ONOOCO_2^-$ that nitrates tyrosine residues via its decomposition products nitrogen dioxide and trioxidocarbonate (•1–) *(34, 35, 62)*.

1.3.3.5 APOPTOSIS/NECROSIS

Peroxynitrite is also a potent inducer of apoptotic and necrotic cell death. In necrosis, cells swell and lyse releasing their cytoplasmic and nuclear contents into the intercellular milieu thus sparkling inflammation *(63)*. Apoptosis occurs when cells rapidly shrink and

exhibit fragmentations into small apoptotic bodies which are then phagocytized by macrophages *(63)*. As no cytosolic contents are released into the intercellular medium, inflammation is not triggered. The work of Salgo et al. was the first report that peroxynitrite can induce apoptosis in thymocytes *(49)*. Recently, it has been reported that low concentrations of peroxynitrite (10 μM) are sufficient to trigger apoptosis, whereas higher concentrations (200 μM) lead to necrosis in vascular smooth muscle cells *(64)*. Moreover, peroxynitrite can oxidize iron-sulfur proteins and block adenosine triphosphate synthesis in mitochondria. This may contribute to both apoptotic and necrotic cell death *(65)*.

1.3.4 DETECTION OF PEROXYNITRITE

As mentioned above, 3-nitrotyrosine formed from the reaction between peroxynitrite and free or protein bound tyrosine residues, is considered to be a biomarker for peroxynitrite production *(66)*. However, it has been demonstrated that *in vivo* nitrotyrosine is not produced exclusively by peroxynitrite. Indeed, it has been established that in the presence of hydrogen peroxide and nitrite, hemeperoxidases such as myeloperoxidase can also cause nitration, namely via nitrogen dioxide *(67)*.

There are various methods to detect and quantify 3-nitrotyrosine. HPLC analysis with UV detection is used to quantify amounts of 3-nitrotyrosine as low as ~10 pmol *(68)*. Western blotting is used to detect protein-bound 3-nitrotyrosine in pure proteins. Enzyme linked immunosorbent assay is more sensitive for the detection and quantification of 3-nitrotyrosine in biological fluids and proteins. Immunohistochemistry is widely used for detecting 3-nitrotyrosine in various tissue samples. Polyclonal and monoclonal antibodies against nitrotyrosine are commercially available for such studies. Finally, a highly specific and sensitive approach to measure 3-nitrotyrosine in proteins is the combination of gas chromatography and mass spectrometry. This method is increasingly used to quantify 3-nitrotyrosine with a detection limit below femtomol range *(69)*.

The oxidation of nonfluorescent dichlorodihydro fluorescein (DCDHF) and dihydrorhodamine 123 (DHR) to their fluorescent products is often used to detect the production of oxidants in cells *(70)*. As both DCDHF and DHR can diffuse inside the cells, they have been used to detect either intracellular or extracellular peroxynitrite. However, DCDHF and DHR oxidation products are not specific for peroxynitrite since other agents such as hydroxyl radical, nitrogen dioxide, hypochlorous acid, and hydrogen peroxide are also

able to oxidize these probes. The mechanism of DCDHF and DHR oxidation by peroxynitrite is still not completely understood. Recently, Glebska and Koppenol proposed that the reaction of peroxynitrite with DCDHF or DHR proceeds via protonation of an adduct formed between peroxynitrite and the indicator, followed by oxidation of the indicator *(71)*.

Another technique to measure the continuous formation of peroxynitrite is luminal-enhanced chemiluminescence, which shows some selectivity for peroxynitrite over other oxidants *(72)*. Peroxynitrite reacts with luminal to produce chemiluminescence, especially in the presence of carbon dioxide, by forming trioxidocarbonate (•1–) that oxidizes luminal to the luminal radical which decomposes to an electronically excited state leading to light emission *(72)*.

1.4 PEROXYNITRITE AND METAL PROTEINS

In biological systems, one of the most significant aspects of peroxynitrite chemistry is its reactivity with metal-containing proteins, in particular heme proteins *(73)*. Peroxidases such as myeloperoxidase, horseradish peroxidase, chloroperoxidase, and lactoperoxidase have been reported to react with peroxynitrite and generate the high valent oxoiron(IV) species *(74)*. Other heme-proteins such as cytochrome *c* oxidase, cytochrome *c* peroxidase and catalase also react with peroxynitrite, but the exact mechanisms of these reactions are not known *(74-77)*. Proteins like aconitase, which contains iron-sulfur centers, alcohol dehydrogenase with zinc-sulfur centers, and ceruloplasmin, which is a copper storage protein, react with peroxynitrite, which causes disruption and subsequent release of the metal: this leads to inactivation of the enzymes *(78-80)*. Peroxynitrite-mediated nitration of a single tyrosine residue in copper-zinc superoxide dismutase leads to significant loss of enzyme activity *(81)*. These observations have led to the search of a drug that can scavenge this powerful nitrating and oxidizing agent. Synthetic iron(III) and manganese(III) porphyrin derivatives have been shown to react rapidly with peroxynitrite to give nitrate in the absence of tyrosine. The catalytic rate constants for the decay of peroxynitrite in the presence of these complexes are given in Table 1. Thus, such catalysts are promising drug candidates against cytotoxicity caused by peroxynitrite.

1.4.1 MYOGLOBIN AND HEMOGLOBIN

Myoglobin and hemoglobin are proteins that act as partners in the storage and transport of dioxygen in mammals. Myoglobin is found in skeletal and heart muscles where it functions as dioxygen carrier and reservoir. Hemoglobin is found in red blood cells and is responsible for the uptake and the transport of dioxygen. In short: dioxygen is absorbed through the lungs where it binds to hemoglobin and is then transported via the blood stream to

Table 1. List of apparent rate constants for the reaction between peroxynitrite and proteins, metal complexes or amino acids. (at pH 7.0 – 7.5 and at 37 ºC).

Amino acid/Protein/Metal center	k ($M^{-1}s^{-1}$)	References
Human peroxiredoxin 5	$(7 \pm 3) \times 10^7$	*82*
Protein tyrosine phosphatase	2.2×10^7	*83*
Mn(III)-TM-2-PyP	1.85×10^7	*84*
Glutathione peroxidase (reduced)	$(8.0 \pm 0.8) \times 10^6$	*85*
Myeloperoxidase[a]	6.2×10^6	*74*
Mn(III)-TM-4-PyP	4.33×10^6	*84*
Mn(III)-TM-3-PyP	3.82×10^6	*84*
Horseradish peroxidase[b]	3.2×10^6	*74*
Fe(III)-TM-2-PyP	2.2×10^6	*86*
Ebselen	$(2.0 \pm 0.1) \times 10^6$	*87*
Peroxyredoxin alkylhydroperoxide reductase	1.5×10^6	*88*
Creatine kinase	8.85×10^5	*89*
Fe(III)-TMPS	6.45×10^5	*86*
Lactoperoxidase[a]	3.3×10^5	*74*
Zn(III)- tetrakis-(4-benzoic acid) porphyrin	$(4.9 \pm 0.1) \times 10^5$	*90*
Glyceraldehyde-3-phosphate dehydrogenase	$(2.5 \pm 0.5) \times 10^5$	*91*
E.coli MnSOD	$(1.4 \pm 0.2) \times 10^5$	*90*
Human MnSOD	$(1.0 \pm 0.2) \times 10^5$	*90*
Mn(III)- tetrakis-(4-benzoic acid) porphyrin	$(6.8 \pm 0.1) \times 10^4$	*90*
Metmyoglobin	$(8.8 \pm 0.2) \times 10^4$	*92*
Methemoglobin	$(2.1 \pm 0.1) \times 10^4$	*92*
Human CuZnSOD	$9.4 \pm 1.0) \times 10^3$	*93*
Cysteine	$(5.9 \pm 0.1) \times 10^3$	*10*
Bovine serum albumin	$(5.0 \pm 0.5) \times 10^3$	*10*
Human serum albumin	$(9.7 \pm 1.1) \times 10^3$	*94*
Tyrosine hydroxylase[b]	$(3.8 \pm 0.9) \times 10^3$	*55*
Glutathione	$(1.4 \pm 0.9) \times 10^3$	*85*
Methionine	$(1.7 \pm 0.1) \times 10^2$	*95*
Tryptophan	184 ± 11	*50*

Note: [a] determined at 12 ºC, [b] determined at 25 ºC

Human Hb Sperm Whale Mb Human Mb Horse Mb

```
Human Mb   GLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASEDLKKHGATVLTALGGILKKKGHHEAEIK
Horse Mb   GLSDGEWQQVLNVWGKVEADIAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASEDLKKHGTVVLTALGGILKKKGHHEAELK
Whale Mb   VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASEDLKKHGVTVLTALGAILKKKGHHEAELK
Human Hbα  VLSPADKTNVKAAWGKVGAHAGEYGAEALERMFLSFPTTKTYFPHF-DLS-----HGSAQVKGHGKKVADALTNAVAHVDDMPNALS
Human Hbβ  VLTPEEKSAVTALWGKVN--VDEVGGEALGRLLVVYPWTQRFFESFGDLSTPDAVMGNPKVKAHGKKVLGAFSDGLAHLDNLKGTFA

Human Mb   PLAQSHATKHKIPVKYLEFISECIIQVLQSKHPGDFGADAQGAMNKALELFRKDMASNYKELGFQG 153
Horse Mb   PLAQSHATKHKIPIKYLEFISDAIIHVLHSKHPGDFGADAQGAMTKALELFRNDIAAKYKELGFQG 153
Whale Mb   PLAQSHATKHKIPIKYLEFISEAIIHVLHSRHPGDFGADAQGAMNKALELFRKDIAAKYKELGYQG 153
Human Hbα  ALSDLHAHKLRVDPVNFKLLSHCLLVTLAAHLPAEFTPAVHASLDKFLASVSTVLTSKYR   148
Human Hbβ  TLSELHCDKLHVDPENFRLLGNVLVCVLAHHFGKEFTPPVQAAYQKVVAGVANALAHKYH   148
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Figure 3. Multiple sequence alignment of human Hb (1KD2), sperm whale Mb (1MBN), human Mb (2MM1), and horse heart Mb (1GJN). Human Mb, sperm whale Mb, horse heart Mb contain two tryptophans (7W and 14W) and two tyrosine residues (103Y and 146Y). Sperm whale Mb has an additional tyrosine at position 151Y. Human Hb contains 3 tyrosine residues per subunit.

the muscles. There, due to the difference in binding affinity, it is released from hemoglobin and taken up by myoglobin, which finally stores dioxygen until it is consumed by cytochrome *c* oxidase.

Myoglobin is a compact, predominantly alpha helical monomeric globular protein. Human, horse heart, and sperm whale myoglobins contain 153 amino acid residues (Figure 3). Eight alpha helices form an amphipathic pocket that holds the essential prosthetic group, the heme. The site at which dioxygen binds to both hemoglobin and myoglobin is the heme shown in Figure 4. The heme prosthetic group is made up by a protoporphyrin IX and an iron ion. Four of the six coordination sites of the iron are occupied by pyrrole nitrogens from the essentially planar porphyrin ring. The fifth coordination site is occupied by the nitrogen atom from the so-called proximal histidine (His93) and the sixth site is free to bind the dioxygen molecule. There is also a second histidine, the distal histidine (His64), which stabilizes the binding of dioxygen via a hydrogen bond (Figure 4) *(96)*. Myoglobin exists in three physiologically relevant forms: the deoxy-, the oxy- and the met-form. In the oxidized Fe^{III} (met) state, a water molecule or a number of various anions including azide, cyanide, or nitrite can bind Fe^{III}. In the oxy state, the iron binds dioxygen. In the Fe^{II} (deoxy) form, the 6^{th} site is empty. DeoxyMb can also bind ligands such as nitrogen monoxide or carbon monoxide. The iron in deoxyMb is 0.6 Å below the plane of the heme ring. Binding of dioxygen causes an in-plane movement of the iron ion due to change in the spin-state from high- to low-spin *(96)*.

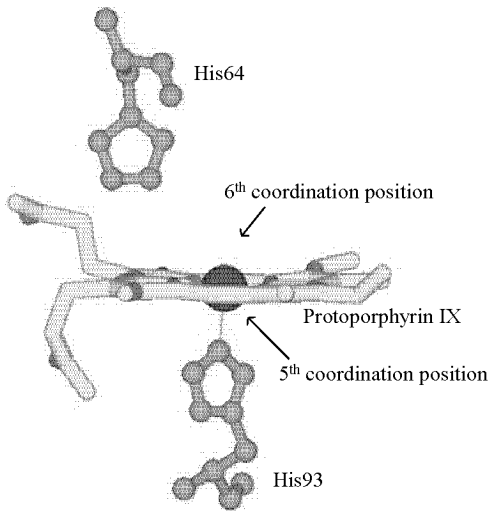

Figure 4. Diagram showing the active site of myoglobin with the proximal histidine (His93) directly bound to iron and the distal histidine (His64), which is not bound but near to the iron atom.

The hemoglobin molecule (Figure 3) is made up of four polypeptide chains; two alpha and two beta chains, with a heme bound to each chain via the proximal histidine residue, as in myoglobin (Figure 4). The alpha and beta chains have different sequences of amino acids, but they fold to form similar three dimensional structures (Figure 3). The four chains are held together by non-covalent interactions. There are four binding sites for dioxygen on the hemoglobin molecule. Oxygen binding to hemoglobin is a cooperative process: when dioxygen binds to the first subunit of deoxyHb, it increases the affinity of the remaining subunits for dioxygen. As additional dioxygen molecules are bound to the second and third subunits, dioxygen binding affinity is further increased *(96)*.

When dioxygen binds to an iron of deoxyHb, the iron ion changes from high to low spin state and moves into the plane of the heme. Since the iron is also bound to His93, this residue is also pulled towards the plane of the heme ring. The structural change near His93 is transmitted throughout the peptide backbone resulting in a significant change in the tertiary structure of the entire subunit. Conformational changes at the subunit surface lead to a new set of binding interactions between adjacent subunits. The latter changes include disruption of salt bridges and formation of new hydrogen bonds and new hydrophobic interactions, all of which contribute to the new quaternary structure. The changes in subunit interaction are transmitted from the surface to the heme binding pocket of a second deoxy subunit and result in easier access of dioxygen to the iron atom of the second heme and thus a greater affinity of the hemoglobin molecule for a second dioxygen molecule. The quarternary structure of deoxyHb is termed T (taut or tense) form, that of oxyHb, the R (relaxed) form *(96)*.

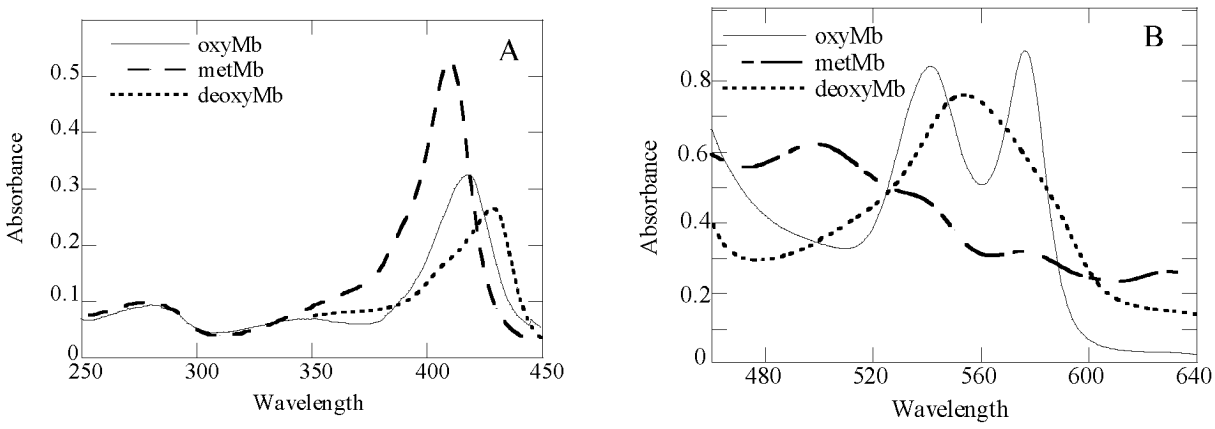

Figure 5. Uv-vis spectra of different forms of myoglobin as described in the text. The concentration of the proteins is approximately 5 μM in (A) and 50 μM in (B), in 0.1 M phosphate buffer, pH 7.0.

The Uv-visible spectrum (Figure 5) of the deoxyMb has a broad absorption maximum at ~ 560 nm while the Soret band absorbance is at 430 nm. The oxyMb has two maxima in its Uv-vis spectrum, at 542 and 580 nm. Moreover, the oxyMb has a characteristic Soret band absorbance maximum at 417 nm. The oxyMb can be oxidized (for instance, by nitrogen monoxide or peroxynitrite) to give metMb. The spectrum of metMb has absorbance maxima at 502 and 630 nm at pH 6.4, and the Soret band absorbance maximum is at 408 nM at pH 7.4.

Very recently, two additional globins have been discovered, the neuroglobin (Ngb) and the cytoglobin (Cygb) *(97)*. Neuroglobin is mainly expressed in the brain and in the retina. It is distantly related to hemoglobin and myoglobin with ~25 % amino acid identity, but shows the conserved protein fold characteristic for dioxygen binding globins. In contrast to hemoglobin and myoglobin, the iron in neuroglobin is hexacoordinate. Indeed, both in the iron(II) and the iron(III) forms, the distal and the proximal histidines are coordinated to the iron *(98)*. Although the physiological function of neuroglobin is still unknown, it has been proposed to act as a temporary dioxygen storage protein *(97)*. Recently, it has been reported that peroxynitrite reacts with the nitrosyl complex of neuroglobin ($NgbFe^{II}NO$) to form $Fe^{III}Ngb$ and release nitrogen monoxide. The reaction may be physiologically relevant as nitrosyl-Ngb has been shown to be formed *in vivo* in intact nitrogen monoxide-producing neurons. The second-order rate constant for the reaction of peroxynitrite with $NgbFe^{II}NO$ is 1.3×10^5 $M^{-1}s^{-1}$, which is larger than that for $HbFe^{II}NO$ (6×10^3 $M^{-1}s^{-1}$). This suggests that neuroglobin may scavenge peroxynitrite better than hemoglobin *(99)*. Cytoglobin is present in the cytoplasm of fibroblast-like cells in connective tissue. Although cytoglobin has been shown to bind dioxygen, the main function of cytoglobin *in vivo* is not known *(100)*. Cytoglobin and neuroglobin are highly conserved throughout the evolutionary phyla.

Myoglobin and hemoglobin are good targets for peroxynitrite as they are abundant in the human body *(73)*. The reaction of peroxynitrite with oxyHb to yield metHb has been shown to be pathophysiologically relevant as peroxynitrite is able to diffuse across the red blood cell membrane and react with oxyHb *(9)*. Minetti et al. have shown by EPR spectroscopy that tyrosyl centered radicals are partly generated from the reaction between high concentrations peroxynitrite and oxyHb. However, the yield of tyrosyl radicals is less than 1% when 1.5 mM peroxynitrite was allowed to react with oxyHb (1.5 mM) in the presence of physiological amounts of carbon dioxide *(101, 102)*. At the same time, our group reported that the peroxynitrite-mediated oxidation of oxyMb and oxyHb to the corresponding

met-forms occurs via formation of the corresponding ferryl forms of the proteins *(103)*. Ferryl myoglobin is a potent oxidant capable of damaging most biological substrates. Nitrogen monoxide is shown to reduce ferrylMb to metMb, a reaction which may represent a protection against ferryl myoglobin mediated oxidations *(104)*.

1.5 GOALS OF THIS WORK

The objective of this project was to characterize the reaction of peroxynitrite with heme-containing proteins such as myoglobin and hemoglobin. The first goal of this project was to determine the degree of peroxynitrite-mediated nitration of tyrosine residues in different forms of hemoglobin and myoglobin i.e., the oxy-, met-, apo-, deoxy- and ferryl-forms. Since peroxynitrite is known to react with carbon dioxide, present *in vivo* at a concentration of 1–1.5 mM, we decided to analyze the yields of nitration both in the presence and absence of carbon dioxide. In addition to nitration of tyrosine residues, we also planned to look for nitrated tryptophans in hemoglobin and myoglobin. A further goal was to find out whether these different forms of hemoglobin and myoglobin protect other proteins from peroxynitrite-mediated nitration. For this purpose, we planned to determine the yield of nitrotyrosine formed from the reaction between free tyrosine and peroxynitrite in the presence of different forms of hemoglobin and myoglobin, both in the absence and presence of carbon dioxide.

The second objective of this work was to determine the rate constants for the reactions of the met-forms of hemoglobin and myoglobin with peroxynitrite under physiological conditions (pH 7.0, in the absence and presence of 1.2 mM carbon dioxide). To find out whether peroxynitrite anion or peroxynitrous acid is the reactant in the reaction of peroxynitrite with the met-forms of hemoglobin and myoglobin, we planned to determine the pH dependence of the rate constants of these reaction. To better evaluate the physiological relevance of the reaction between peroxynitrite and these proteins, we planned to determine the rate constants also at 37 °C and pH 7.0 in the presence of 1.2 mM carbon dioxide. To find out whether these proteins isomerize peroxynitrite to nitrate, we planned to analyze the nitrogen containing products (nitrite and nitrate) from the reaction of met-forms of hemoglobin and myoglobin with peroxynitrite, both in the absence and presence of carbon dioxide.

To study the influence of the distal environment of metMb on the rate of decay of peroxynitrite, we used sperm whale myoglobin mutants (H64D-, H64A-, H64L-,

F43W/H64L-, and H64Y/H93G-metMb) provided by Prof. Y. Watanabe. The mutants H64A- and H64D-metMb have a water molecule which is bound to the iron, but which is not hydrogen bonded to the distal alanine or aspartic acid. The H64L-metMb mutant was choosen because the iron(III) is pentacoordinated, the coordinated water being absent. In H64Y/H93G-metMb, the Tyr64 is coordinated to heme on the distal side, and the proximal histidine is replaced by the non-coordinating glycine. The F43W/H64L-metMb mutant has a trypotphan residue (Trp43) close to the heme centre. The final goal of this project was to determine the following:

1. The rate constants for the reaction between these metMb mutants and peroxynitrite in the presence and absence of carbon dioxide.
2. The pH dependence and the influence of carbon dioxide on these rate constants.
3. The yields of the nitrogen containing products (nitrite and nitrate) from the reaction between the metMb mutants and peroxynitrite in the absence and presence of carbon dioxide.
4. The yields of 3-nitrotyrosine formed in the above metMb mutants after reaction with peroxynitrite. The efficiency of these proteins to protect added tyrosine from peroxynitrite-mediated nitration.
5. The mechanism of the reaction between peroxynitrite and the metMb mutants.

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2

MYOGLOBIN SCAVENGES PEROXYNITRITE WITHOUT BEING SIGNIFICANTLY NITRATED*

** This chapter is a part of the publication by Herold. S, Kalinga. S., and Mehl, M. Biochemistry, 2002, 41 13460-13472.*

2.1 INTRODUCTION

Despite the fact that myoglobin (Mb) is probably one of the most thoroughly studied protein, it has recently been proposed that it may display additional biological functions that remain a matter of continuing investigations. The original role attributed to Mb, that is, storage of dioxygen and facilitation of its transport through muscle tissue to mitochondria *(1)*, has recently been challenged. The discovery that a mutant mouse devoid of Mb is capable of apparently normal muscle function raised questions about the real significance of this protein *(2, 3)*. In particular, it has been proposed that Mb may be involved in the regulation of intracellular nitrogen monoxide concentration.

Overproduction of $NO^{\bullet}$ and superoxide, both generated in large quantities as a response of the immune system or in inflammatory processes, favors the production of the powerful oxidizing and nitrating agent peroxynitrite *(4)*. The peroxynitrite anion ($ONOO^-$) is stable, but the protonated form, peroxynitrous acid [HOONO, pK_a 6.8, *(5)*], isomerizes to nitrate with a rate constant of 1.2 s^{-1} at 25 °C *(5, 6)*. However, under physiological conditions peroxynitrite reacts with a number of biological targets, in particular with thiols [the second-order rate constant for the reaction of peroxynitrite with cysteine is 5.9×10^3 M^{-1} s^{-1}, at pH 7.4 and 37 °C, *(7)*], with carbon dioxide [the pH-independent second-order rate constant is 5.8×10^4 M^{-1} s^{-1} at 37 °C, *(8)*], and with metalloproteins *(9)*. Because of the relatively high concentration of CO_2 in both intra- and extracellular compartments (1-2 mM), the reaction of peroxynitrite with CO_2 is particularly relevant in biological systems. Carbon dioxide *(10, 11)*, metal complexes *(12-14)*, and metal-containing proteins *(12, 15)* have all been shown to catalyze the peroxynitrite-mediated nitration of aromatic compounds. Because of the instability of peroxynitrite under physiological conditions *(9)*, the detection of 3-nitrotyrosine (NO_2-Tyr) has become a biochemical marker for the presence of peroxynitrite in pathophysiological processes *(16)*. Extensive evidence supports the formation of NO_2-Tyr *in vivo* in a variety of different pathological conditions [for review, see ref *17*].

We have recently shown that the peroxynitrite-mediated oxidation of oxyMb and oxyHb proceeds via an intermediate ferryl complex, which, in a second step, reacts further with peroxynitrite to yield metMb and metHb, respectively *(18)*. The rate constants for the two steps of the reaction of peroxynitrite with oxyMb, at pH 7.3 and 20 °C, were determined as $(5.4 \pm 0.2) \times 10^4$ and $(2.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1}, respectively *(18)*. The corresponding rates for the reaction with oxyHb, at pH 7.0 and 20 °C, are $(8.4 \pm 0.4) \times 10^4$ and $(9.4 \pm 0.7) \times 10^4$

M^{-1} s^{-1}, respectively *(18)*. In addition, it has been shown that peroxynitrite is able to diffuse across the red blood cell membrane *(19)* and to oxidize oxyHb to metHb also within the erythrocytes. From the comparison of the values of the second-order rate constants, one can conclude that, if present in analogously high concentrations, these two proteins can efficiently compete with CO_2 and react with peroxynitrite. Thus, Hb and Mb are two of the main targets for peroxynitrite in the blood vessels and in the cardiac and skeletal muscles, where the concentrations of these two proteins are high.

Here we present detailed analyses of Hb and Mb after treatment of different forms of these proteins with variable amounts of peroxynitrite. Our results show that only very low quantities of NO_2-Tyr are formed when equivalent amounts of peroxynitrite are allowed to react with the oxy form of these proteins. Comparable amounts of nitrated amino acids are formed when metMb and metHb are treated with peroxynitrite under analogous conditions, but significantly larger yields are observed with apoMb and metMbCN. Interestingly, in addition we found that also the tryptophan residues of Mb and Hb are nitrated to a low but detectable extent. Taken together, our data suggest that the heme center of Mb may act as an efficient scavenger of peroxynitrite, protecting the globin from nitration. As peroxynitrite can irreversibly inhibit cytochrome *c* oxidase *(20)*, oxyMb may utilize an additional important pathway to maintain mitochondrial respiration, that is, rapidly react with peroxynitrite and thus prevent nitration of other cellular components.

2.2 EXPERIMENTAL PROCEDURES

2.2.1 REAGENTS

Buffer solutions were prepared from K_2HPO_4/KH_2PO_4 (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, sodium dithionite, potassium superoxide, potassium hexacyanoferrate(III), 3-nitro-L-tyrosine (NO_2-Tyr), 2-butanone, sulfanilamide, *N*-(1-naphthyl)-ethylenediaminedihydrochloride, ammonium sulfamate, and cyanogen bromide were obtained from Fluka. Sodium bicarbonate was obtained from Merck. L-tyrosine and L-tryptophan were purchased from Sigma. Pronase was obtained from Roche Molecular Biochemicals. Nitrogen monoxide was obtained from linde and passed through a NaOH solution as well as a column of NaOH pellets to remove higher nitrogen oxides before use.

2.2.2 PEROXYNITRITE AND PROTEIN SOLUTIONS

Peroxynitrite was prepared according to ref *21* from solid KO_2 and gaseous nitrogen monoxide and stored in a polyethylene bottle or in small aliquots at –20 or –80 °C. The concentration of the solutions was determined by measuring the absorbance at 302 nm [ε_{302} = 1705 M^{-1} cm^{-1}, *(22)*]. The peroxynitrite solutions contained variable amounts of nitrite (maximally 50% relative to the peroxynitrite concentration) and no hydrogen peroxide. The stock solution was diluted to the required concentration immediately before use with 0.01 M NaOH. Peroxynitrite solutions were always kept on ice.

Horse heart myoglobin was purchased from Sigma and purified, after addition of a small amount of potassium hexacyanoferrate(III), over a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. The concentration of the resulting metMb solutions was determined by measuring the absorbances at 408, 502, and/or 630 nm [ε_{408} = 188 mM^{-1} cm^{-1}, ε_{502} = 10.2 mM^{-1} cm^{-1}, and ε_{630} = 3.9 mM^{-1} cm^{-1}] between pH 6.0 and 7.5 *(23)*.

Oxymyoglobin was prepared by reducing the purchased Mb with a slight excess of sodium dithionite. The solution was purified chromatographically on a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. In some cases, the protein was purified analogously a second time to ensure complete removal of sodium dithionite. However, this procedure proved not to be necessary, as it did not influence the results of the following experiments. The concentration of the oxyMb solutions was determined by measuring the absorbance at 417, 542, and/or 580 nm [ε_{417} = 128 mM^{-1} cm^{-1}, ε_{542} = 13.9 mM^{-1} cm^{-1}, and ε_{580} = 14.4 mM^{-1} cm^{-1}, *(23)*].

ApoMb was prepared according to the method of Teale *(24)*. In brief, a concentrated HCl solution was added drop wise to a Mb solution (ca. 0.2 g in 5 mL water) until the pH reached a value of 2. Ice cooled 2-butanone was then added, and the biphasic mixture was immediately vortexed. The two phases were separated by short centrifugation, and the upper porphyrin-containing organic layer was removed with a pipet. The extraction was repeated three times until the lower protein-containing aqueous phase was completely colorless. The apoprotein solution was dialyzed against water, 2, 10, 20, and finally 50 mM phosphate buffer, pH 7.0. During the last dialysis step a precipitate formed, which was removed by centrifugation. The concentration of the supernatant apoMb solution was determined by measuring the absorbance at 280 nm [ε_{280} = 13.5 mM^{-1} cm^{-1}, *(25)*]. Contamination of the

apoprotein with unreacted Mb, assessed spectrophotometrically, was routinely below 1% (ca. 0.3%).

The cyanide-bound form of metMb (metMbCN) was prepared by adding a slight excess of KCN to metMb and was purified over a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. The concentration of the metMbCN solutions was determined by measuring the absorbance at 422 and/or 540 nm [ε_{422} = 116 mM^{-1} cm^{-1} and ε_{540} = 11.3 mM^{-1} cm^{-1}, *(23)*].

Purified human oxyHb stock solution (57 mg/mL solution of HbA_0 with approximately 1.1% metHb) was a kind gift from APEX Bioscience, Inc. (NC). The obtained solution was frozen in small aliquots (0.5-1 mL) and stored at -80 °C. Oxyhemoglobin solutions were prepared by diluting the stock solution with buffer, and concentrations (always expressed per heme) were determined by measuring the absorbance at 415, 541, and/or 577 nm [ε_{415} = 125 mM^{-1} cm^{-1}, ε_{541} = 13.8 mM^{-1} cm^{-1}, and ε_{577} = 14.6 mM^{-1} cm^{-1}, *(23)*].

MetHb solutions were prepared by oxidizing oxyHb with a slight excess of potassium hexacyanoferrate(III). The solution was purified chromatographically on a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. The concentration of the metHb solutions (always expressed per heme) was determined by measuring the absorbances at 405, 500, and/or 631 nm [ε_{405} = 179 mM^{-1} cm^{-1}, ε_{500} = 10.0 mM^{-1} cm^{-1}, and ε_{631} = 4.4 mM^{-1} cm^{-1}] between pH 6.0 and 7.5 *(23)*.

Absorption spectra were collected on a UVIKON 820, on an Analytik Jena Specord 200, or on an Analytik Jena Specord 100 spectrophotometer.

2.2.3 PREPARATION OF THE SOLUTIONS CONTAINING CARBON DIOXIDE

Experiments in the presence of 1.1 mM CO_2 were carried out by adding to the protein solutions the amount of sodium bicarbonate required (from a freshly prepared solution, 500 mM $NaHCO_3$ in H_2O) *(26)*. The values for the constant of the hydration-dehydration equilibrium $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$ at pH 7.0 are 5.15×10^{-7}, 8.41×10^{-7}, and 1.00×10^{-6} M at 0, 20, and 37 °C, respectively *(27)*. Thus, to obtain a constant CO_2 concentration of 1.1 mM, the $NaHCO_3$ concentrations present during the reactions were 6.7, 9.9, and 12.4 mM, at 0, 20, and 37 °C, respectively. After addition of $NaHCO_3$ to the protein solutions, 3-10 min elapsed (longer times when the experiments were carried out at 0 °C) before the peroxynitrite solution was added, to allow the equilibration of the various carbonated species *(26)*.

2.2.4 HPLC ANALYSIS

HPLC analysis was carried out with a Hewlett-Packard Series 1050 apparatus with a Series 1100 UV-vis detector, equipped with a VYDAC 218TP54 Protein&Peptide C18 Column (250 × 4.6 mm). Solvent A was 0.07% trifluoroacetic acid (TFA) in H_2O and solvent B 0.07% TFA in acetonitrile. Nitrotyrosine (NO_2-Tyr) was eluted (ca. 7.5 min after injection) by keeping the amount of B constant (5%) in the first 2 min and then by using an increasing linear gradient of B from 5% to 10% between 2 and 10 min and from 10% to 80% between 10 and 15 min. NO_2-Tyr was detected contemporaneously at 220, 280, 350, and 400 nm. Nitrotyrosine was quantified by measuring a calibration curve of 5-10 NO_2-Tyr standard solutions.

The nitrotryptophan products were eluted between 15 and 18 min, by following the same procedure. The different isomers were assigned by comparing the UV-vis spectra of the single peaks with those reported in the literature for 4-, 5-, 6-, and 7-nitrotryptophan *(28, 29)*.

2.2.5 ANALYSIS OF THE 3-NITROTYROSINE CONTENT IN OxyHb, metHb, oxyMb, metMb, AND apoMb AFTER REACTION WITH VARIABLE AMOUNTS OF PEROXYNITRITE

The reaction of peroxynitrite with oxyHb, metHb, oxyMb, metMb, or apoMb was carried out as follows: to 180 µL of a protein solution (112 µM in 0.1 M phosphate buffer, pH 7.0) kept at the required temperature (ice-cooled, room temperature, or in a thermostat at 37 °C) was added 20 µL of an iced-cooled peroxynitrite solution (different concentrations in 0.01 M NaOH) as a bolus and vortexed immediately. The final pH measured after mixing was always between 7.0 and 7.1. For each protein, an additional experiment was carried out with decomposed peroxynitrite as a control. For this purpose, the same peroxynitrite solution was acidified with HCl and then neutralized again with 0.1 M NaOH. The decomposition of peroxynitrite was confirmed by measuring an absorbance spectrum between 300 and 350 nm. Finally, this decomposed peroxynitrite solution was added, also in a volume ratio of 1:9, to the protein solutions. The reactions in the presence of 1.1 mM CO_2 were carried out analogously by adding the required amount of peroxynitrite to a solution containing $NaHCO_3$ (see above for the concentrations used at the different temperatures) and the protein (a total of 180 µL of a 112 µM protein solution) in a volume ratio of 1:9. After ca. 30 min, nitrite was removed by washing the solutions with a 25 mM ammonium hydrogencarbonate buffer, pH

7.8, through a 10000 MW cutoff filter (Centriplus YM-10, Amicon, Switzerland) at 3000 g until the ultrafiltrate did not show any qualitative reaction (pink coloring) with the Griess reagent *(30)*. Alternatively ca. 200 µL of an ammonium sulfamate solution (100 mM in 0.5 M HCl) was added to convert nitrite to dinitrogen. This procedure proved to be efficient at removing nitrite, as confirmed by using the Griess reagent (see above). The NO_2-Tyr yields obtained with these two nitrite-removing procedures were equivalent.

The resulting proteins were hydrolyzed by treating the solutions (200-400 µL) with an equivalent volume of 12 M HCl and heating them for 18 h at 110 °C in a closed vial. The hydrolyzed mixtures were allowed to dry by maintaining the temperature at 110 °C and by opening the vials. The residuals were redissolved in 40 µL of 0.1% trifluoroacetic acid and 20 µL was analyzed by HPLC as described above.

Alternatively the proteins were digested with Pronase as follows. After the treatment with peroxynitrite, the proteins were first dialyzed against a 25 mM ammonium hydrogencarbonate buffer, pH 7.8, to remove nitrite (see above). Lyophilized Pronase was dissolved in water (20 mg/mL) and kept on ice before use. Protein samples (100 µM) were digested by adding Pronase and $CaCl_2$ to final concentrations of 1 mg/mL and 10 mM, respectively. The samples were incubated with gentle agitation at 40 °C for 24 h. Digestion was stopped by concentrating the samples to dryness in a speed-vac apparatus. Finally, the samples were redissolved in 0.1% trifluoroacetic acid and analyzed by HPLC as described above.

2.2.6 WESTERN BLOT ANALYSIS

The protein samples were prepared in a way analogous to that described above for the HPLC analysis, by treating the protein solutions with one bolus of peroxynitrite (10% v/v). The unwashed samples were boiled for 3 min at 95 °C in an electrophoresis buffer (62.5 mM Tris-HCl, pH 6.8, 1% sodium dodecyl sulfate (SDS), 5% glycerol, 0.5 mM EDTA, and bromophenol blue) which did not contain any reducing thiols. This precaution was taken to avoid reduction of NO_2-Tyr, which has been reported to take place in the presence of hemoproteins and thiol-containing reductants *(31)*. Samples containing 10 µg of protein per lane were separated by 11% SDS polyacrylamide gel electrophoresis (SDS-PAGE) (8 × 8 cm, and 1 mm thick) at 20 mA in a Novex X cell II apparatus (Invitrogen, Groningen, Netherlands). The resolved proteins were transferred onto nitrocellulose membranes (BA 83,

Schleicher & Schüll, Dassel, Germany) by the application of 25 V for 90 min in a wet blot apparatus (transfer buffer: 12 mM Tris, 96 mM glycine, and 20% methanol). Transfer of proteins was checked by reversible Ponceau-S staining (0.1% Ponceau-S in 5% acetic acid). The same protein aliquots where run in parallel on a separate gel and were stained with Coomassie blue. The membranes were blocked by incubation with 5% (w/v) nonfat dry milk powder in phosphate buffered saline (PBS: 137 mM NaCl, 2.7 mM KCl, 10 mM Na_2HPO_4, and 2 mM KH_2PO_4, pH 7.4) for 1 h. After extensive washing with PBST (phosphate buffered saline with 0.05% Tween 20), the blots were incubated overnight at 4 °C either with polyclonal antibodies at 1:2000 dilution *(16)* or with the monoclonal 1A6 nitrotyrosine antibodies (UpstateBiotechnology, Lake Placid, USA) at 1:1000 dilution (in PBST with 0.05% (w/v) nonfat milk powder). The membranes were then washed five times for 5 min in PBST and incubated for ca. 45 min at room temperature with horseradish peroxidase-conjugated goat anti-rabbit secondary antibodies (Jackson Immunoresearch, West Grove, PA) at 1:5000 dilution (in PBST with 0.05% (w/v) nonfat milk powder). The blots were again washed 5 times and the immunoreactive bands were detected by enhanced chemiluminescence (ECL) (Apbiotech, Dübendorf, Switzerland) according to the suppliers instructions (incubation with ca. 0.125 mL/cm^2 for 1 min). Labeled proteins were visualized by exposing medical X-ray films (Fuji RX) to the blot for 1-5 min.

2.2.7 STATISTICS

Most experiments reported in this article were carried out by different people at least in triplicate on independent days. The results are given as mean values of at least three experiments plus or minus the corresponding standard error.

2.3 RESULTS

2.3.1 IMMUNOHISTOCHEMICAL ANALYSIS OF THE GLOBIN AFTER REACTION OF OXYHEMOGLOBIN, Oxy-, Met-, AND apo MYOGLOBIN WITH PEROXYNITRITE

Western blot analysis with anti-3-nitrotyrosine antibody showed that exposure of oxyMb to an excess of peroxynitrite (25 equiv) at pH 7.0 and 0 °C did not yield to significant nitration (lane 8 in Figure 1A). In contrast, under our experimental conditions, that is, when an about 100 μM protein solution was treated with one bolus of peroxynitrite (10% v/v) at pH 7.0 and 0 °C, nitration of oxyHb was detectable when at least 5 equiv of peroxynitrite was

added (lane 5 in Figure 1B), in agreement to previous reports *(19, 32-35)*. As expected *(32)*, in the presence of 1.1 mM CO_2, Hb was already nitrated by only 2 equiv of peroxynitrite (lane 9 in Figure 1B), and nitration of oxyMb could also be detected (lane 7 in Figure 1A).

To identify whether the oxidation state of the heme influences significantly the yield of nitration, we treated metMb with peroxynitrite under analogous conditions. As depicted in Figure 1A, similar amounts of nitrated protein were detected when metMb was mixed with 25 equiv of peroxynitrite both in the absence and presence of CO_2 (lanes 5 and 4, respectively). Finally, to elucidate the role of the heme center in the reaction of Mb with peroxynitrite, we determined the degree of nitration obtained by mixing peroxynitrite with apoMb.

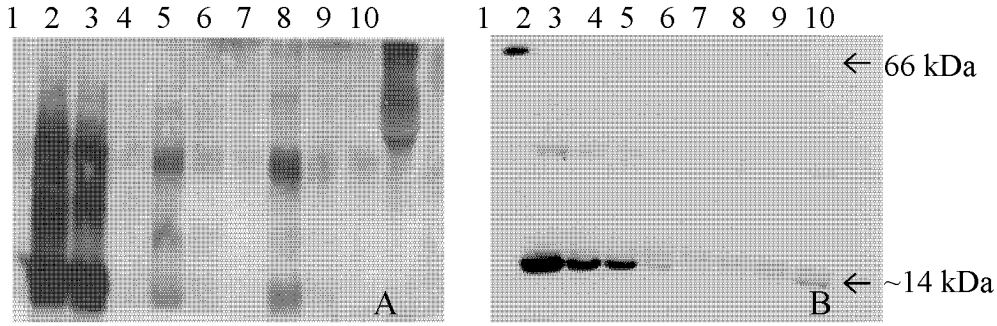

Figure 1. Anti-nitrotyrosine immunoblotting of Mb (A) and Hb (B). Samples were separated on a 11% SDS polyacrylamide gel and examined by western blot analysis with a polyclonal antibody against NO_2-Tyr as described in the experimental section. (Gel A) The proteins (100 μM) were incubated In 0.1M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the absence (lanes 2, 3, 4, 5, 6, 8, 9 and 10) and presence of 1.1 mM CO_2 (lanes 1, 4, and 7). Lane 1, apoMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 2 and 3, apoMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite in the presence of CO_2; lane 4, metMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 5 and 6, metMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lane 7, oxyMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 8 and 9, oxyMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lane 10, BSA treated with 25 equiv of peroxynitrite. Gel (B) The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the absence (lanes 1-8 and 10) and presence of CO_2 (lane 9). Lane 1, bovine serum albumine (BSA) treated with 10 equiv of peroxynitrite; lanes 2-8, oxyHb mixed with 25, 10, 7.5, 5, 2, 1, and 0.5 equiv of peroxynitrite, respectively; lane 9, oxyHb mixed with 2 equiv of peroxynitrite in the presence of 1.1 mM CO_2; lane 10, oxyHb treated with decomposed peroxynitrite.

In contrast to oxy- and metMb, when apoMb was mixed with 25 equiv of peroxynitrite under the same conditions, the protein was nitrated to a significant degree (lane 2 in Figure

1A). The amount of nitration was even more pronounced in the presence of 1.1 mM CO_2 (lane 1 in Figure 1A).

From the Western blot of Mb (Figure 1A), it appears that peroxynitrite treatment of oxy- and metMb causes the formation of dimers which are also nitrated. Visualization of the proteins by staining with Coomassie blue (Figure 2) showed that the amount of dimerized protein was very small, thus suggesting that the dimer is preferentially nitrated. Work is in progress to characterize this dimeric product and to understand the mechanism of its formation.

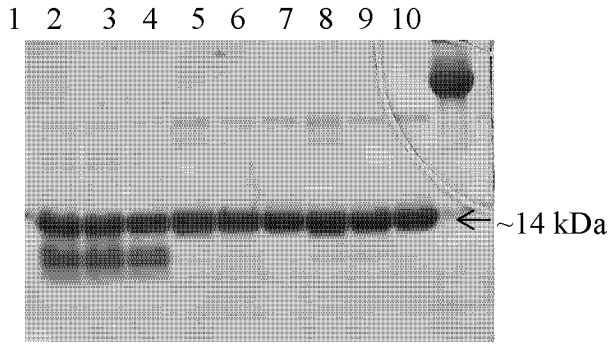

Figure 2. Coomassie blue staining of peroxynitrite-treated Mb (compare with figure 1A). The proteins (10 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the absence (lanes 2, 3, 5, 6, 8, 9 and 10) and presence of 1.1 mM CO_2 (lanes 1, 4 and 7). Samples were separated on 11% SDS-PAGE and visualized by staining the gel with coomassie blue. Lane 1: apoMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 2-3: apoMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lane4: metMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 5-6: metMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lane 7: oxyMb mixed with 25 equiv of peroxynitrite in the presence of CO_2; lanes 8-9: oxyMb mixed with 25 equiv of peroxynitrite and with decomposed peroxynitrite; lane 10 BSA treated with 25 equiv of peroxynitrite.

2.3.2 QUANTIFICATION OF THE AMOUNT OF 3-NITROTYROSINE GENERATED

To quantify the yields of nitration, we hydrolyzed the proteins treated with peroxynitrite with hydrochloric acid and then analyzed them by HPLC. To avoid artifacts due to tyrosine nitration by nitrite (always present as a contaminant in our peroxynitrite solutions, up to 50% relative to the peroxynitrite concentration) under acidic conditions, we thoroughly washed the samples by using size exclusion membranes. Alternatively, nitrite was removed by addition of a concentrated acidic ammonium sulfamate solution. Control experiments showed that NO_2-Tyr does not react with ammonium sulfamate and is stable under our hydrolysis conditions *(see Table A in Appendices)*. Exposure of oxy-, met-, and apoMb to a

range of peroxynitrite concentrations (at pH 7.0 and 0 °C) induced dose-dependent increase in the nitration of

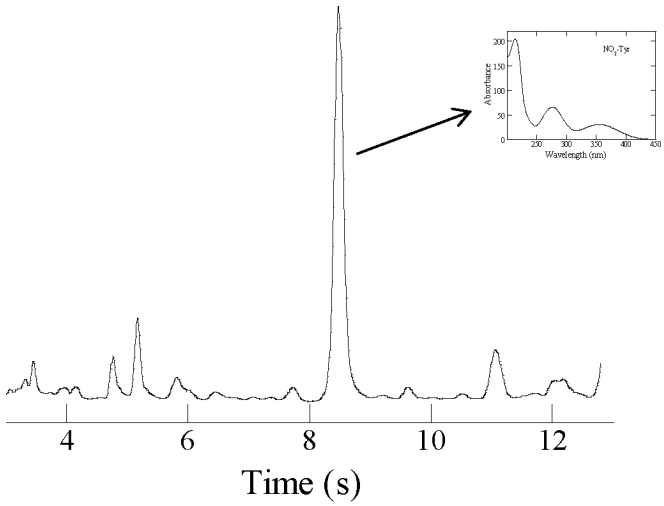

Figure 3. HPLC chromatograms (measured at 350 nm) of the products of the reactions of apoMb (~100 μM) with 5 equiv of peroxynitrite, in 0.1 M phosphate buffer pH 7.0, after complete acid hydrolysis as described in the experimental section. In the inset, the UV-vis spectra of the peak corresponding to NO_2-Tyr.

the proteins. Indeed, NO_2-Tyr could clearly be identified in the HPLC chromatogram of the hydrolyzed samples measured at 350 nm (Figure 3). The data, shown in Figure 4 *(and summarized in Appendices Table B)*, confirm the results of the immunohistochemical analyses presented above. In the absence of CO_2, less than 5% of the two available tyrosine residues of oxyMb and about 7% of those in metMb are nitrated by a 20-fold excess of peroxynitrite, whereas apoMb is nitrated up to about 20%. Interestingly, addition of 5 or 10 equiv of peroxynitrite led to very similar nitration yields of the available tyrosine residues in the two different forms of the holoproteins, with the NO_2-Tyr yields in oxyMb slightly larger than those in metMb. In the presence of 1.1 mM CO_2 (Figure 4B), the same trend was observed at all peroxynitrite concentrations studied, and the absolute NO_2-Tyr yields were significantly higher. Addition of 20 equiv of peroxynitrite led to 30.5%, 14.7%, and 8.3% nitration of the available tyrosine residues in apoMb, oxyMb, and metMb, respectively. Addition of 1 equiv of peroxynitrite, still a large excess compared to the relative concentrations likely to be found *in vivo*, resulted only in the nitration of 2.7%, 1.3%, and 0.5% of the available tyrosine residues in apoMb, oxyMb, and metMb, respectively.

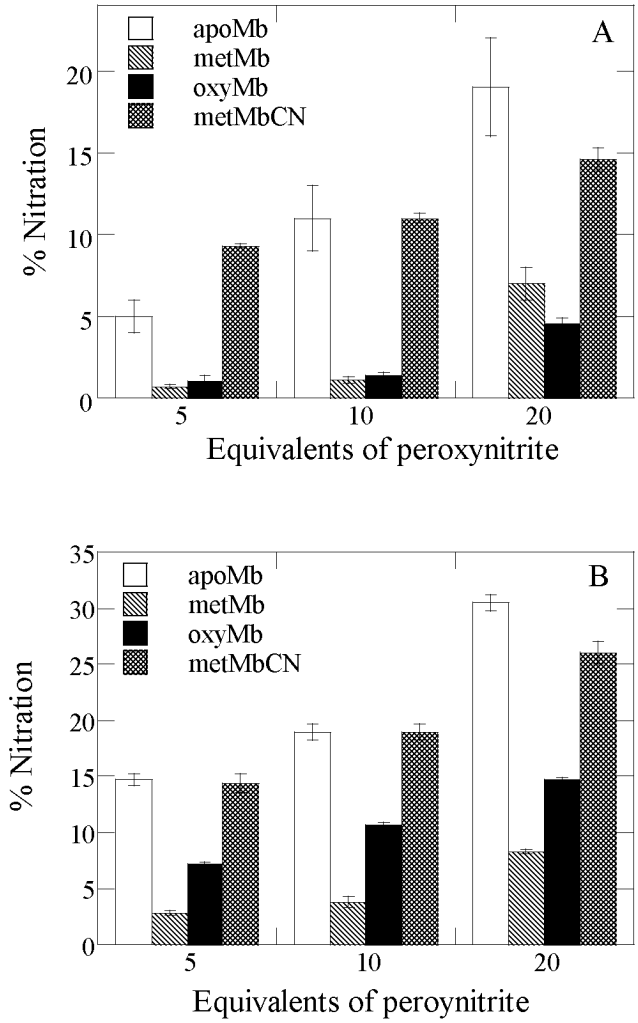

Figure 4. NO_2-Tyr yields (% relative to the total content of the protein, that is, 2 tyrosine residue per heme), determined by HPLC after acid hydrolysis, from the reaction of apo-, met-, oxyMb, and metMbCN (100 μM) with different amounts of peroxynitrite (5, 10, and 20 equiv) at 0 °C and pH 7.0 (A) in the absence and (B) presence of 1.1 mM CO_2. Note the different scale of the y axis in the two plots.

To confirm our hypothesis that in oxy- and metMb the heme center scavenges peroxynitrite and thus prevents nitration of the two tyrosine residues of the globin, we determined the degree of NO_2-Tyr formation in metMbCN treated with peroxynitrite under analogous conditions. The cyanide-bound Mb is extremely stable and its UV-vis spectrum is only slightly modified even after treatment with 20 equiv of peroxynitrite (Figure 5). Preliminary results showed that when oxyMb is treated with a large excess of peroxynitrite (≥ 20 equiv), a species is partly formed in which the heme is cross-linked to the globin, possibly via Tyr103. This species has a characteristic broad absorbance maximum around 590 nm and

seems to be partly generated also by treatment of metMbCN with 20 equiv of peroxynitrite. As depicted in Figure 4A *(and summarized in Appendices Table B)*, metMbCN was nitrated to an extent comparable to that of apoMb. In the presence of 1.1 mM CO_2 (Figure 4B) the same pattern was observed: nitration yields of peroxynitrite-treated metMbCN were comparable to those found for apoMb and significantly larger than those determined for oxy- and metMb. Peroxynitrite cannot react directly with the iron center of metMbCN, as the iron is coordinatively saturated and cyanide is not displaced during the reaction. As a consequence, peroxynitrite cannot be scavenged and thus leads to significant nitration of the tyrosine residues of the globin.

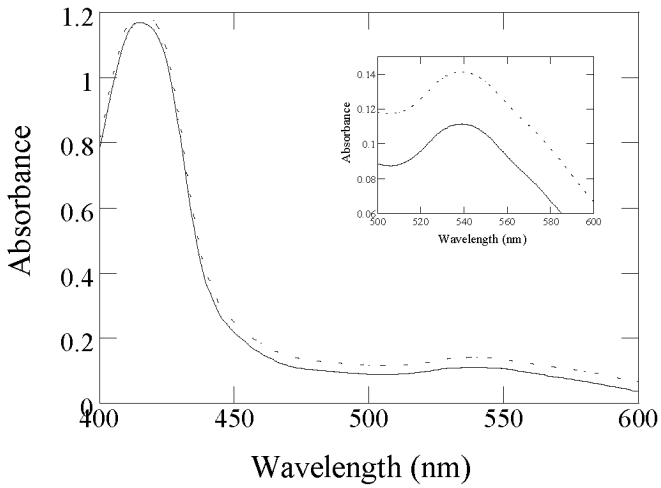

Figure 5. Uv-vis spectrum of the metMbCN (10 μM) in 0.1 M phosphate buffer pH 7.0 (A) and treated with 20 equiv of peroxynitrite (B) at room temperature, pH 7.0 *(see also Figure B in Appendices)*.

Analogous studies were carried out with oxyHb and metHb. As shown in Figure 6 *(and summarized in Appendices Table B)*, treatment of these two forms of Hb (100 μM) with increasing concentrations of peroxynitrite (100-2000 μM), at pH 7.0 and 0 °C, both in the absence and presence of CO_2 (1.1 mM), induced a dose-dependent nitration of the tyrosine residues of the globins. As suggested by the immunohistochemical analyses presented above, the nitration yields were significantly higher than those obtained for the corresponding experiments with Mb. For instance, addition of 20 equiv of peroxynitrite in the presence of 1.1 mM CO_2 led to 22% and 17.6% nitration of the available tyrosine residues in oxyHb and metHb, respectively. Both in the absence and presence of 1.1 mM CO_2, under comparable conditions, the nitration yields were always higher for oxyHb than for metHb. Our data with

1 equiv of peroxynitrite are comparable to those reported recently for the nitration, at pH 7.4, of 1 mM oxyHb and metHb with 1 mM peroxynitrite *(32, 35)*.

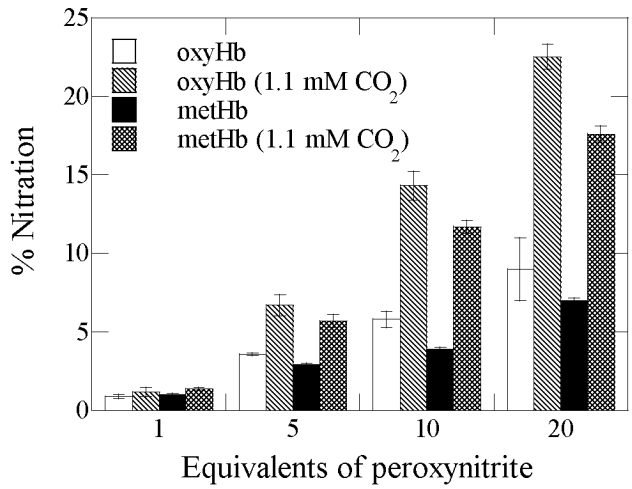

Figure 6. NO_2-Tyr yields (% relative to the total content of the protein, that is, 3 tyrosine residue per heme), determined by HPLC after acid hydrolysis, from the reaction of met- and oxyHb (100 μM) with different amounts of peroxynitrite (1, 5, 10, and 20 equiv) at 0 °C and pH 7.0 in the absence and presence of 1.1 mM CO_2.

As during the course of these studies Groves and co-workers reported significantly higher nitration yields of metMb by peroxynitrite *(36)*, to validate our results we determined the yields of nitration by hydrolyzing the proteins with Pronase, a milder method. In an independent set of experiments, we treated the proteins with different amounts of peroxynitrite at pH 7.0 and 0 °C as described above. Each peroxynitrite-treated protein solution was then divided in two samples, one of which was hydrolyzed with HCl and the other one digested with Pronase. Nitrotyrosine yields in both samples were then determined by HPLC analysis. Comparison of the results obtained with the two different methods indicates that the nitration yields measured are reproducible (within 10% error) and are only slightly larger when determined after digestion with pronase *(see Figure A in Appendices)*.

As mentioned above, the peroxynitrite solutions used for the experiments described in this paper contained variable amounts of nitrite (maximally 50%, expressed relative to the peroxynitrite concentration). Thus, we studied the influence of nitrite on the yield of the peroxynitrite-mediated nitration of the tyrosine residues in apo-, met-, and oxyMb, both in the absence and presence of 1.1 mM CO_2. For this purpose, we treated the proteins (at 0 °C and pH 7.0) with 20 equiv of peroxynitrite (final concentration 2 mM, contaminated with

approximately 1 mM nitrite) premixed with different amounts of NO_2^-. The total final concentrations of NO_2^- were 1, 2, 3, and 5 mM (considering also the amount derived from the contamination of the peroxynitrite solution). Analysis of the globin indicated that the NO_2-Tyr yields apparently slightly decreased with increasing concentration of added nitrite *(see Table C in Appendices)*. However, the changes were higher than 10% only in the case of the reaction of peroxynitrite with apoMb in the presence of CO_2 (NO_2-Tyr yields: 30.5% ± 0.7%, 25.5% ± 0.2%, 24% ± 1%, and 20.8% ± 0.8% for 1, 2, 3, and 5 mM total NO_2^-, respectively). Taken together, these results suggest that variable amounts of contaminating nitrite in the peroxynitrite solutions may be one of the factors that cause slight discrepancies in the nitration yields obtained with different peroxynitrite preparations. However, as already stated above, our data were always reproducible within an error margin of ~10%.

Finally, we carried out one further experiment in the presence of the metal chelator diethylenetriaminepentaacetic acid (DTPA), to rule out any involvement of traces of free transition metal ions in the nitration reactions studied. For this purpose, we added 0.1 mM DTPA to our 0.1 M phosphate buffer pH 7.0 and determined the yield of nitration of oxyMb treated with 20 equiv of peroxynitrite, both in the absence and presence of 1.1 mM CO_2 (at 0 °C). Interestingly, under these conditions the NO_2-Tyr yields were slightly higher than those obtained in the absence of DTPA (no added CO_2, 4.6% ± 0.3% and 8.9% ± 0.7%; 1.1 mM CO_2, 14.7% ± 0.2% and 16.0% ± 0.5%, in the absence and presence of 0.1 mM DTPA, respectively). This result suggests that in our experiments free metal ions are not involved in the nitration of the proteins. In contrast, as already reported by Goldstein et al. *(37)*, DTPA may interact with peroxynitrite and thus addition of this metal chelator may influence the reactivity of peroxynitrite.

Last, we studied the influence of the temperature on the yields of nitration, both in the absence and presence of 1.1 mM CO_2. When apo-, met-, oxyMb, met-and oxyHb were treated with peroxynitrite at room temperature (at pH 7.0), the yields of nitration were significantly larger for the three forms of Mb, but were almost equivalent for the two Hb forms, compared to the results obtained at 0 °C *(see Table B in Appendices)*. For instance, addition of 20 equiv of peroxynitrite in the presence of 1.1 mM CO_2 led to 43.4%, 19%, and 15% nitration of the available tyrosine residues in apoMb, oxyMb, and metMb, respectively. In contrast, addition of 20 equiv of peroxynitrite in the presence of 1.1 mM CO_2 nitrated 23.6% and 17.8% of the available tyrosine residues in oxyHb and metHb, respectively.

2.3.3 IDENTIFICATION OF NITRATED TRYPTOPHAN RESIDUES

HPLC analyses of pronase-digested peroxynitrite-treated oxy-, met-, and apoMb suggested that, in addition to NO_2-Tyr, other nitrated species were formed, as additional peaks were present in the chromatograms measured at 350 nm. As shown for the reaction of apoMb with 10 equiv of peroxynitrite (Figure 7), four main peaks with elution times between 15 and 18 min displayed spectra characteristic for nitrotryptophan derivatives (insets of Figure 7) *(28, 29)*. In the presence of 1.1 mM CO_2, the total yields of nitrated products were higher but the same four species were generated at similar relative yields. Attempts were made to identify the nature of the four nitrated products. By direct comparison with the known absorbance spectra of the different nitrotryptophan derivatives *(28, 29)*, we could unambiguously assign the peaks at 15.6, 16.1, and 17.5 min to the 6-, 4-, and 5-nitrotryptophan, respectively.

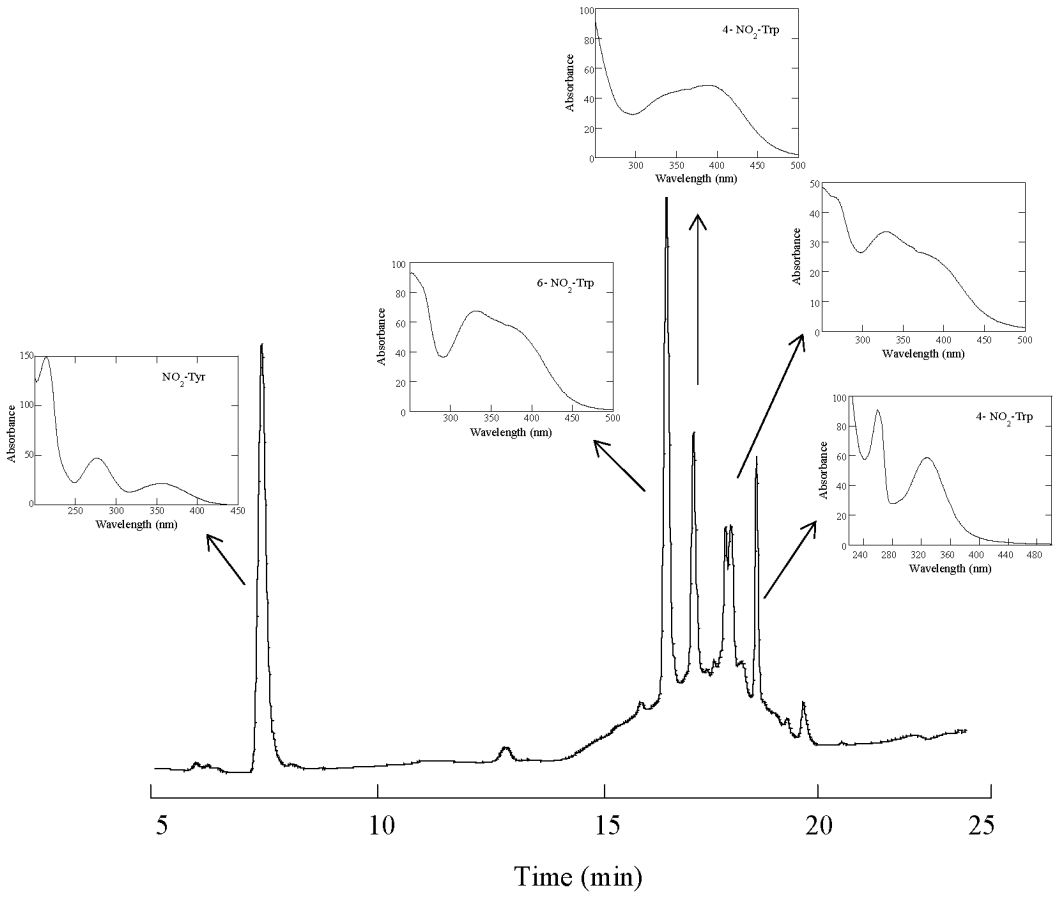

Figure 7. HPLC chromatograms (measured at 350 nm) of the products of the reactions of apoMb (~100 μM) with 10 equiv of peroxynitrite, in 0.1 M phosphate buffer pH 7.0 at 0 °C, after complete digestion with pronase as described in the experimental section. In the insets, the UV-vis spectra of the peaks corresponding NO_2-Tyr (ca. 7.5 min), 6-NO_2-Trp (ca. 15.5 min), 4-NO_2-Trp (ca. 16 min) and 5-NO_2-Trp (ca.17.5 min), and that of the peak with an elution time of ca 16.8 min.

HPLC analyses of the pronase-digested peroxynitrite-treated metHb and oxyHb samples also indicated the formation of nitrated tryptophan residues. As shown in Figure 8, the two peaks of the chromatogram eluting at 15.5 and 16 min could be assigned to 6- and 4-nitrotryptophan, respectively. However, additional nitrated species and/or decomposition products also elute at similar retention times and are very likely to be responsible for the slightly modified absorption spectra obtained for these two nitrotryptophan products (Figure 8 insets). Preliminary results suggested that the yields of nitrated tryptophan residues of the two forms of Hb were lower than those of the corresponding Mb species.

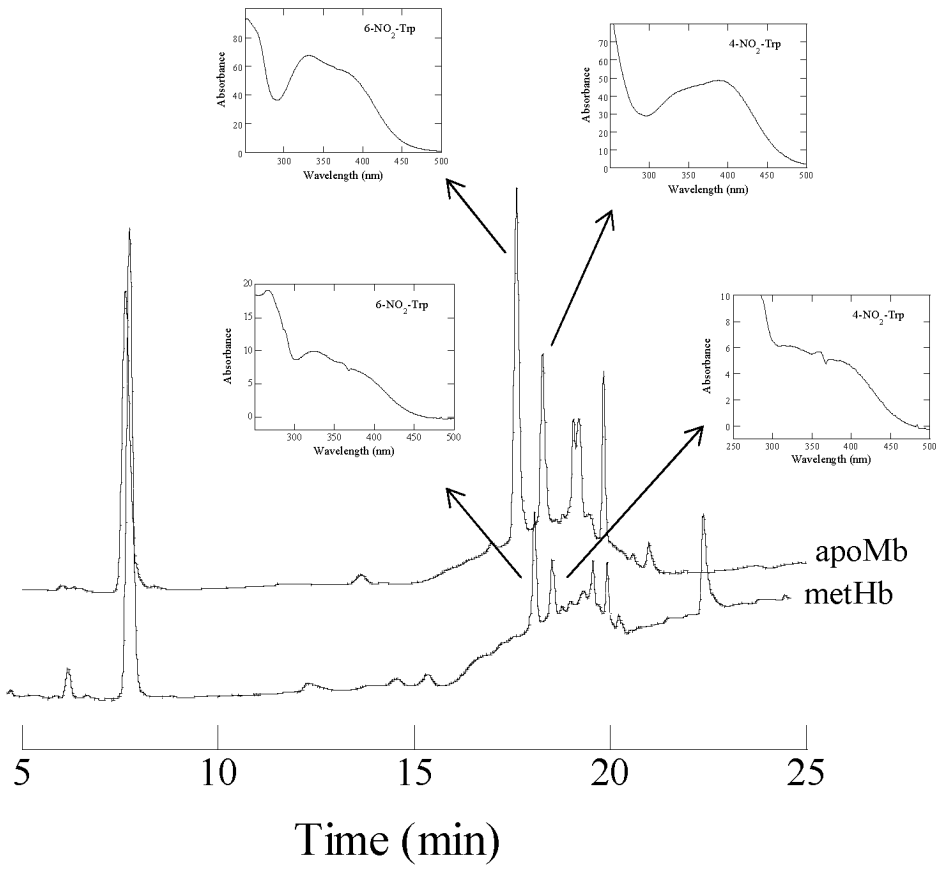

Figure 8. HPLC chromatograms (measured at 350 nm) of the products of the reactions of ~100 μM apoMb (top) and metHb (bottom) with 10 equiv of peroxynitrite, in 0.1 M phosphate buffer pH 7.0 at 0 °C, after complete digestion with pronase as described in the experimental section. In the insets, the UV-vis spectra of the peaks corresponding NO_2-Tyr , 6-NO_2-Trp and 4-NO_2-Trp

2.4 DISCUSSION

The kinetic studies reported in our previous work *(29)* suggest that oxyMb may play an important role *in vivo* for the detoxification of peroxynitrite. Indeed, the second-order rate constant for the reaction of peroxynitrite with oxyMb is $(5.4 \pm 0.2) \times 10^4$ M^{-1} s^{-1} (at pH 7.3 and 20 °C), a value slightly larger than that for the reaction of peroxynitrite with CO_2 [2.3×10^4 M^{-1} s^{-1}, at pH 7.3 and 24 °C, *(40)*]. To be an efficient scavenger, Mb should not be damaged in the course of its reaction with peroxynitrite. In this work, we have analyzed extensively the globins after the reaction of different forms of Mb and Hb with variable amounts of peroxynitrite.

2.4.1 NITRATION OF TYROSINE RESIDUES

Several qualitative studies with immunohistochemical analyses have shown that oxy- and metHb are nitrated by peroxynitrite to a dose-dependent extent *(19, 32-35)*. In agreement with previous studies *(32, 35)*, treatment of oxyHb and metHb with 1 equiv of peroxynitrite yields about 1% of NO_2-Tyr (expressed relative to the total amount of tyrosine in the protein, that is, 3 Tyr residues per heme). Furthermore, addition of 1 equiv of peroxynitrite to oxy- and metMb also led to approximately 1% nitration. In contrast, when the two proteins are treated with a larger excess of peroxynitrite our results show that oxy- and metMb are nitrated to a significantly lower extent than the corresponding Hb forms. Clear differences can already be detected with 5 equiv of peroxynitrite. As the rate constant for the reaction of peroxynitrite with oxyHb is larger than that for its reaction with oxyMb [$(8.4 \pm 0.4) \times 10^4$ M^{-1} s^{-1} and $(5.4 \pm 0.2) \times 10^4$ M^{-1} s^{-1}, respectively *(18)*], these data suggest that other factors, in addition to the rate of reaction with peroxynitrite, must influence the yields of nitration. Horse heart Mb has two Tyr residues (Tyr103 and Tyr146), whereas the two chains of human Hb have each three Tyr residues (Tyr24, Tyr42, and Tyr140 in the α- and Tyr35, Tyr130, and Tyr145 in the β-chain, respectively). The observation that Hb and Mb are nitrated to a different extent suggests that the exact position of the tyrosine residue within the protein structure strongly influences the nitration yields.

In most of our experiments, the nitration yields of the oxy forms of the proteins were slightly larger than those of the corresponding met-forms. The exact mechanism of the reaction of metMb and metHb with peroxynitrite is not known. However, in analogy to the mechanism of the reaction between His64-mutated metMb and peroxynitrite *(41)*, it is

conceivable that metMb and metHb simply catalyze the isomerization of peroxynitrite to nitrate. In contrast, we have previously shown that the reaction of oxyMb and oxyHb with peroxynitrite proceeds via the ferryl form of these proteins *(18)*. Thus, the higher nitration yields obtained for the oxy forms of the proteins may derive from reactions with additional oxidizing and nitrating species generated from the interaction of peroxynitrite with oxyMb or oxyHb.

In the presence of 1.1 mM CO_2 the absolute yields of nitration are all significantly larger, in particular for the oxy forms of the proteins. In the presence of CO_2, the reaction between oxyMb and peroxynitrite proceeds at a faster rate [$(4.6 \pm 0.3) \times 10^5$ M^{-1} s^{-1}, at pH 7.5, 20 °C, and 1.2 mM CO_2], but the yield of the reaction is significantly lower *(Herold S, Exner M, Boccini F., Chem. Res. Toxicol. 2003, 16, 390-402)*. Thus, only a small amount of peroxynitrite is scavenged by the protein. The nitration yields of Mb are always lower than those of the corresponding forms of Hb. This result suggests that Mb can scavenge peroxynitrite better than Hb also in the presence of CO_2.

As we chose to keep the amount of CO_2 added constant throughout our experiments (1.1 mM), at the highest peroxynitrite concentrations used (20 equiv, 2 mM), CO_2 was present at a substoichiometric level relative to peroxynitrite. However, as the reaction of CO_2 with peroxynitrite is catalytic *(42)*, a large amount of CO_2 is rapidly regenerated from this reaction. Nevertheless, the relative yields of nitration of all Mb and Hb forms studied are almost linearly dependent on the amount of added peroxynitrite in the absence of CO_2 but show a saturation effect in the presence of 1.1 mM CO_2. This result suggests that at higher peroxynitrite concentrations the direct, that is, not CO_2-mediated, oxidation of the protein by peroxynitrite may represent a significant reaction pathway. As the NO_2-Tyr yields are lower in the absence of added CO_2, this may lead to the reduced NO_2-Tyr yields obtained in the experiments with 20 equiv peroxynitrite.

To confirm our hypothesis that the heme center of Mb protects the protein from peroxynitrite-mediated modifications, we have also analyzed the NO_2-Tyr yields obtained from the reaction of apoMb and metMbCN with different amounts of peroxynitrite under the same conditions. Interestingly, our results show that apoMb and metMbCN are nitrated to a significantly larger extent than metMb and oxyMb, both in the absence and presence of 1.1 mM CO_2. ApoMb has been shown to retain the main features of the secondary and tertiary structure of holoMb *(43, 44)*. However, because of the observed loss of about 15-20% of its

α-helicity *(43, 44)*, it cannot be excluded that small conformational changes in apoMb cause larger exposition of the Tyr residues to the surface and thus leads to higher nitration yields. Nevertheless, the observation that metMbCN is nitrated to an degree comparable to that of apoMb rather suggests that the main factor that determines the extent of nitration of the globin is the availability of the heme center to react directly with peroxynitrite or with species derived from the decay of HOONO and/or $ONOOCO_2^-$. Thus, our results demonstrate that the heme centers of oxy- and metMb efficiently scavenge peroxynitrite, which would otherwise nitrate the tyrosine residues of the globin.

It has recently been reported that reaction of metMb (100 μM) with 5, 10, and 20 equiv of peroxynitrite, carried out in the absence of added CO_2, yields 20, 36, and 50% nitration of selectively one of the two tyrosine residues (Tyr103) in the protein *(36)*. The large discrepancies between our and these yields of nitration are difficult to rationalize (note that our results are expressed as percentage nitration of both tyrosine residues). However, as the experimental procedure used by Groves and co-workers *(36)* has not been described in detail, one can speculate that the reaction has been carried out at a different temperature and/or with different volumetric ratios of the two reagents. Our results show that the NO_2-Tyr yields were significantly larger at higher temperatures (20 vs 0 °C). However, preliminary results of the reactions carried out at 37 °C suggest that nitration yields are not significantly larger than those at 20 °C (see Chapter 3). Different reactions that can take place in the presence of peroxynitrite: isomerization to nitrate, decomposition to dioxygen and nitrite via an adduct between the peroxynitrite anion and its conjugate acid *(45, 46)*, and finally reaction with the protein. Evidently, which of these reactions takes place depends on experimental conditions such as local peroxynitrite concentration, pH, and temperature. Thus, the technique used to carry out the experiments may greatly influence the results. In all our experiments, care was taken to rapidly mix the peroxynitrite solution with that containing the proteins and to always use the same volumetric ratio of 1:9.

In contrast to the preferential nitration of Tyr103 in metMb *(46)*, we found that when apoMb is mixed with an excess of peroxynitrite, Tyr146 is nitrated first. Tyr103 is (possibly) nitrated only when Mb is treated with a very large molar excess of peroxynitrite (more than 20 equiv) *(refer our published article for details)*. The same preferential nitration of Tyr146 over Tyr103 is obtained when apoMb is treated with tetranitromethane *(47)*, a reagent known to nitrate tyrosine residues *(48)*. However, it has also been reported that electrooxidation of

apo- and metMb at alkaline pH in the presence of sodium nitrite yields selectively nitrated Tyr103, together with small amounts of nitrated Phe106 and His97 *(49)*. Analysis of the three-dimensional structure of Mb *(50)* suggests that nitration of Tyr103 and not Tyr146 would rather be expected. Indeed, Tyr103 is on the outside of the protein with the phenolic hydroxyl group facing the solvent and the closest ring carbon atom 3.3 Å from the heme iron center. In contrast, Tyr146 is significantly less accessible and its closest tyrosine ring carbon atom is 9.7 Å from the heme iron atom.

As mentioned above, despite the fact that apoMb retains the main features of the secondary and tertiary structure of holoMb *(43, 44)*, it has been shown that the accessibility of protein groups for chemical reagents changes significantly, thus pointing to an altered conformation and increased dynamics of the molecule *(51)*. Consequently, the difference in the sites and yields of nitration between apo- and metMb may be due to these structural changes. The observation that electronitration of apoMb leads to a different selectivity may be explained by the significantly different conditions under which the nitration reaction was carried out, in particular the high pH (9.4) and the large nitrite concentration (50 mM) *(49)*. Indeed, under these conditions conformational changes may have taken place and different kinds of reactive intermediates may be responsible for nitration.

The modifications of Hb by peroxynitrite have been investigated thoroughly by Minetti and co-workers *(32, 35)*. Treatment of oxyHb with 1 equiv of peroxynitrite in the presence of 1.3 mM CO_2 leads to the following approximate nitration yields (relative to the unmodified subunits): Tyr42 4.5%, Tyr140 1%, and Tyr24 0.05% in the α-chain plus Tyr130 3% in the β-chain *(32)*. Interestingly, analogous studies with metHb lead to a somewhat different nitration pattern with the following approximate nitration yields (relative to the unmodified subunits): Tyr42 2.5%, Tyr140 2.5% and Tyr24 0.1% in the α-chain and Tyr130 3% in the β-chain *(35)*. For both forms of Hb, in the absence of CO_2 the total yield of nitration was about 30% lower without significantly affecting the nitration pattern *(32, 35)*. These data also support our hypothesis that removal of the heme from metMb may lead to a different nitration pattern of the globin.

2.4.2 NITRATION OF TRYPTOPHAN RESIDUES

Tryptophan is another amino acid that is known to be nitrated by peroxynitrite. In contrast to the reaction with tyrosine, the reaction between peroxynitrite and tryptophan is

bimolecular and proceeds with a rate of 38 ± 3 M^{-1} s^{-1} [at pH 7.1 and 25 °C] *(38)*. Previous reports agree that 6-NO_2-Trp is the main reaction product *(38, 39)*, but the formations of 5-NO_2-Trp *(39)* and oxidized products *(52)* have also been observed. Under our experimental conditions, that is, when tryptophan is allowed to react with an excess of peroxynitrite, we could also unambiguously identify 4-nitrotryptophan as an additional nitrated product *(data not shown, refer our published article of details)*.

Most mammalian myoglobins contain two tryptophan residues at invariant positions 7 and 14 of the amino acid sequence *(23)*. Fluorescence data show that in holoMb both tryptophan residues are located in a hydrophobic environment and are equally exposed to the solvent (about 50%) *(53)*. In contrast, in apoMb Trp7 is in a more polar environment and fully accessible to solvent while surroundings of Trp14 are hydrophobic, and it is inaccessible to external quenchers *(54)*. This observation points to an altered conformation and increased dynamics of the N-terminal region in apoMb *(54)*. Human Hb contains three tryptophan residues each αβ-dimer: one in the α-chain [Trpα14] and two in the β-chain [$Trp^{\beta}15$ and $Trp^{\beta}37$] *(23)*.

Our studies with the different forms of Mb and Hb clearly show that in all species investigated, that is, apo-, met-, oxyMb, and met- and oxyHb, some of the tryptophan residues are nitrated by addition of peroxynitrite. All three derivatives, 6-, 5-, and 4-nitrotryptophan were definitely identified when apo-, oxy-, and metMb were treated with an excess of peroxynitrite, whereas only 6- and 4-nitrotryptophan were detected in peroxynitrite-treated oxy- and metHb. However, for all the proteins studied, 6-nitrotryptophan seems to be the isomer generated to the largest extent. It has recently been shown that when sperm whale Mb is treated with hydrogen peroxide, the ferryl form of the protein and, among other transient radical species, a peroxyl radical centered on tryptophan are formed *(55)*. Site-directed mutations of all three tyrosine residues or Trp7 did not prevent formation of this radical, which in contrast was not formed when Trp14 was mutated, implicating Trp14 as the specific site of the peroxidation *(55)*. Analogously, the two tryptophan residues of the Hb β-chain (Trp15 and Trp37) are also oxidized in the course of the reaction between hydrogen peroxide and oxyHb *(56)*. With the results obtained up to now, we cannot assert whether Trp7 and/or Trp14 is modified by reaction of peroxynitrite with different forms of Mb. However, digestion of Mb with chymotrypsin should allow for the distinction of eventual modifications of each single tryptophan residues. These experiments are in progress in our laboratory.

Interestingly, we found only one other report, that appeared while this work was in progress *(57)*, in which nitrated tryptophan residues have unambiguously been identified in a protein treated with peroxynitrite. Yakamura and co-workers showed that treatment of human recombinant Cu,Zn-superoxide dismutase with an excess of peroxynitrite leads to a dose-dependent decrease in the tryptophan fluorescence, which arises from the only tryptophan residue (Trp32) of the protein (human Cu,Zn-superoxide dismutase does not have any tyrosine residues) *(57)*. The decrease in fluorescence was enhanced 20-25% in the presence of 1.3 mM CO_2 relative to the CO_2-free reaction. The difference absorption spectrum between the modified and the control enzymes has been mentioned to resemble that of 6-NO_2-Trp, even though the data have not been shown *(57)*. Other authors have reported reduction of fluorescence in peroxynitrite-treated bovine serum albumin (BSA), attributed to oxidation and/or nitration of tryptophan residues *(58)*, oxidation of Trp residues in BSA *(59)*, oxidation of Trp residues in BSA and collagen IV *(52)*, and formation of protein radicals centered on tryptophan residues in blood plasma *(60)*.

In conclusion, our results suggest that tryptophan is an additional amino acid that can easily be nitrated by peroxynitrite in proteins, possibly even to a larger extent than tyrosine residues. Thus, the different nitrotryptophan derivatives may represent additional, not yet fully recognized footprints for peroxynitrite and may also be used as biomarkers for oxidative stress-related diseases *in vivo*.

2.5 SUMMARY AND POSSIBLE BIOLOGICAL IMPLICATIONS

Peroxynitrite has been proposed to be formed when ischemic tissues are reperfused and to be one of the species responsible for oxidative lesions found in these tissues *(61)*. However, our previous work showed that deoxyMb, which is also present in significant concentrations in ischemic tissues, is among the species which react at the highest rate with peroxynitrite [k = ~10^6 M^{-1} s^{-1}, at pH 7.4 and 20 °C *(18)*]. This reaction, which is thus likely to take place *in vivo*, yields nitrite and another oxidizing species, $MbFe^{IV}$=O. Ascorbate, present in tissues in mM concentration, can reduce $MbFe^{IV}$=O, though at a rather slow rate [2.7 ± 0.8 M^{-1} s^{-1} at pH 7.0 and 25 °C, *(62)*]. In addition, we have shown that $MbFe^{IV}$=O is rapidly reduced among others by peroxynitrite and nitrogen monoxide *(18)*. Taken together, these data suggest that the overall reaction of deoxy- or oxyMb with peroxynitrite may represent a detoxification pathway for this strong oxidant. Our current results indicate that the

heme center of Mb protects against peroxynitrite-mediated oxidation and/or nitration of the globin. An excess of peroxynitrite nitrates Tyr146 and Trp7 and/or Trp14 in apoMb, but significantly lower yields, are obtained for oxyMb and metMb. Under physiological conditions, with Mb concentrations around 200 μM and considerably lower amounts of peroxynitrite, its half-life is reduced from about 4 s (in the absence of any other substrate) to less than 100 ms, and nitration yields are irrelevant. In addition, as the rate of the reaction between peroxynitrite and oxyMb *(18)* is in the same order of magnitude than that for the formation of $ONOOCO_2^-$ from peroxynitrite and CO_2 *(8, 42)*, Mb can partly scavenge peroxynitrite also in the presence of CO_2. This is supported also by the observation that the second-order rate constant for the reaction of oxyMb and peroxynitrite is larger in the presence of CO_2. Nitration yields in the presence of CO_2 are higher, but are still insignificant when physiological relevant concentrations of peroxynitrite are allowed to react with Mb. It has recently been suggested that Mb may play an important role in the regulation of cellular respiration, by scavenging $NO^{\bullet}$, an inhibitor of cytochrome *c* oxidase. Peroxynitrite has also been shown to irreversibly inhibit this enzyme *(20)*. Thus, our results show that scavenging this strong oxidizing and nitrating agent may represent an additional important physiological function of Mb. In this contest, preliminary results showed that oxyMb can prevent peroxynitrite-mediated nitration of free tyrosine (see Chapter 3). Under physiological conditions, this reaction may be relevant as the Mb concentration is mostly larger than that of free tyrosine, normally present in the range 20-80 μM *(63)*. In addition, it is likely that Mb can protect also other proteins from oxidative modification by peroxynitrite.

Finally, we have identified two of the first examples of tryptophan nitration by peroxynitrite within proteins. Even though we have not quantified the results, 6-NO_2-Trp seems to be the preferred isomer, but 5- and 4-NO_2-Trp were also unambiguously identified in Mb and 4-NO_2-Trp in Hb. As we have found nitrated tryptophan residues when both apo- and holoMb were treated with peroxynitrite, our results suggest that this modification may not be found only in hemoproteins and may thus represent, in addition to NO_2-Tyr, a biomarker for peroxynitrite *in vivo*.

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3 METMYOGLOBIN AND METHEMOGLOBIN CATALYZE THE ISOMERIZATION OF PEROXYNITRITE TO NITRATE*

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3.1 ABSTRACT

Hemoproteins, in particular, myoglobin and hemoglobin, are among the major targets of peroxynitrite *in vivo*. The oxygenated forms of these proteins are oxidized by peroxynitrite to their corresponding iron(III) forms (metMb and metHb). This reaction has previously been shown to proceed via the corresponding oxoiron(IV) forms of the proteins. In this paper, we have conclusively shown that metMb and metHb catalyze the isomerization of peroxynitrite to nitrate. The catalytic rate constants were determined by stopped-flow spectroscopy in the presence and absence of 1.2 mM CO_2 at 20 and 37 °C. The values obtained for metMb and metHb, with no added CO_2 at pH 7.0 and 20 °C, are $(7.7 \pm 0.1) \times 10^4$ and $(3.9 \pm 0.2) \times 10^4$ M^{-1} s^{-1}, respectively. The pH-dependence of the catalytic rate constants indicates that HOONO is the species that reacts with the iron(III) center of the proteins. In the presence of 1.2 mM CO_2, metMb and metHb also accelerate the decay of peroxynitrite in a concentration-dependent way. However, experiments carried out at pH 8.3 in the presence of 10 mM CO_2 suggest that $ONOOCO_2^-$, the species generated from the reaction of $ONOO^-$ with CO_2, does not react with the iron(III) center of Mb and Hb. Finally, we showed that different forms of Mb and Hb protect free tyrosine from peroxynitrite-mediated nitration. The order of efficiency is metMbCN < apoMb < metHb < metMb < ferrylMb < oxyHb < deoxyHb < oxyMb. Taken together, our data show that myoglobin is always a better scavenger than hemoglobin. Moreover, the globin offers very little protection, as the heme-free (apoMb) and heme-blocked (metMbCN) forms only partly prevent nitration of free tyrosine.

3.2 INTRODUCTION

Peroxynitrite the strong oxidizing and nitrating agent generated from the diffusion-limited reaction of $NO^{\bullet}$ with $O_2^{\bullet -}$ *(1)*, is a molecule of considerable biological interest *(2)*. A variety of compounds present in biological systems have been shown to be modified by peroxynitrite (for a recent review, see ref *3*). Targets for peroxynitrite-mediated oxidation and/or nitration are amino acids such as tyrosine *(4)*, tryptophan *(5, 6)*, cysteine *(7)*, and methionine *(8)*; nucleic acids *(9)*; and membrane lipids *(10)*. Moreover, the selenocysteine-containing glutathione peroxidase has been reported to catalytically reduce peroxynitrite to nitrite by using glutathione (*11*).

Metal-containing proteins, in particular, hemoproteins, can also be modified by peroxynitrite. Among others, peroxynitrite inhibits the cytosolic iron-thiolate protein

aconitase *(12, 13)*, Mn-superoxide dismutase *(14)*, and induces the release of zinc and the oxidation of the thiol moiety of zinc-finger-containing proteins *(15)*. Amid hemoproteins that have been reported to react with peroxynitrite are peroxidases *(16)*, catalase *(16)*, cytochrome-*c* *(17)*, cytochrome P450 *(18)*, nitric oxide synthase *(19)*, and cytochrome *c* oxidase *(20)*. Moreover, synthetic iron(III)- and manganese(III)-metalloporphyrins have been shown to catalyze the isomerization of peroxynitrite to nitrate *in vitro (21, 22)* and to be cytoprotective against peroxynitrite *in vivo (23)*.

Under physiological conditions, one of the main targets of peroxynitrite is CO_2, present in millimolar concentration in most tissues. Reaction of $ONOO^-$ with CO_2 leads to $ONOOCO_2^-$, a stronger nitrating agent than peroxynitrous acid.

Hemoglobin (Hb) and myoglobin (Mb), by large the hemoproteins present in the highest amount in the human body, also react with peroxynitrite. We have shown that, in the presence of an excess of peroxynitrite, oxyMb and oxyHb are oxidized to their corresponding iron(III) forms (metMb and metHb) via the formation of ferrylMb and ferrylHb, respectively *(24, 25)*. In addition, we have recently proposed that, since the proteins are nitrated only to a very small extent by an excess of peroxynitrite, this reaction represents an efficient scavenging pathway for peroxynitrite *(26)*. Here, we report kinetic studies of the reaction of peroxynitrite with metMb and metHb in the presence and absence of added CO_2. Analysis of the nitrogen-containing products suggests that the iron(III) forms of these proteins catalyze the isomerization of peroxynitrite to nitrate. Comparison with the heme-free (apoMb) and the heme-blocked (metMbCN) forms of Mb indicate that the reaction is mediated by the heme center, despite no absorbance changes being detected in the UV-vis spectrum of metMb upon mixing with peroxynitrite. In the presence of physiological amounts of CO_2 (1.1–1.2 mM), metMb and metHb catalyze the decay of peroxynitrite by competing with CO_2 for peroxynitrite. No direct reaction seems to take place between $ONOOCO_2^-$ and the iron(III) center of the proteins.

3.3 EXPERIMENTAL PROCEDURES

3.3.1 CHEMICALS

Potassium phosphate buffers were prepared from KH_2PO_4/K_2HPO_4 (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, sodium dithionite, potassium hexacyanoferrate(III), 2-butanone, sulfanilamide, *N*-(1-naphthyl)-

ethylenediaminedihydrochloride, ammonium sulfamate, trifluoroacetic acid, hydrogen peroxide, and acetonitrile (99.8%) were obtained from Fluka. Sodium bicarbonate was purchased from Merck. Bovine serum albumin (fraction V) and L-tyrosine were obtained from Fluka. Catalase (bovine liver, 17 000 units/mg of protein) was purchased from Sigma. Nitrogen monoxide was obtained from Linde and passed through a column of NaOH pellets to remove higher nitrogen oxides before use.

3.3.2 PEROXYNITRITE, CARBON DIOXIDE, AND PROTEIN SOLUTIONS

Peroxynitrite was synthesized from KO_2 and nitrogen monoxide according to ref *27* and stored in small aliquots at –80 °C. The peroxynitrite solutions contained variable amounts of nitrite (maximally 50% relative to the peroxynitrite concentration) and no hydrogen peroxide. The stock solution was diluted with 0.01 M NaOH, and the concentration of peroxynitrite was determined spectrophotometrically prior to each experiment by measuring the absorbance at 302 nm (ε_{302} = 1705 M^{-1} cm^{-1}, ref *28*).

Experiments in the presence of CO_2 were carried out by adding to the protein solutions the required amount of a freshly prepared 0.5 or 1 M sodium bicarbonate solution. The values for the constant of the hydration-dehydration equilibrium $CO_2 + H_2O \leftrightarrows H^+ + HCO_3^-$ were derived from ref *29* by taking into consideration the ionic strength of the solutions. After the addition of bicarbonate, the protein solutions were allowed to equilibrate for at least 5 or 3 min at 20 or 37 °C, respectively. For the experiments carried out in the absence of CO_2, the buffers and the 0.01 M NaOH solutions were thoroughly degassed.

Horse heart myoglobin was purchased from Sigma. Solutions of oxyMb, metMb, ferrylMb, and the cyanide-bound form of metMb (metMbCN) were prepared as described previously *(26, 30)*. ApoMb was prepared according to the method of Teale *(26, 31)*. Purified human oxyHb stock solution (57 mg/mL solution of HbA_0 with approximately 1.1% metHb) was a kind gift from APEX Bioscience, Inc. (Research Triangle Park, NC). MetHb and deoxyHb were prepared as reported earlier *(32)*. The concentration of Hb was always expressed per heme. Absorption spectra were collected on a UVIKON 820 spectrophotometer.

3.3.3 KINETIC STUDIES

The decay rates of peroxynitrite in the presence of different forms of Mb and Hb were studied with an Applied Photophysics SX18MV-R single-wavelength stopped-flow instrument. The width of the cell in the spectrophotometer is 1 cm, and the mixing time of the instrument is about 2 ms. The kinetic traces were collected at 302 nm, and the data were analyzed with the SX18MV-R operating software or with Kaleidagraph, version 3.52. The results of the fits of the traces (averages of at least 10 single traces) from at least three experiments were averaged to obtain each observed rate constant, given with the corresponding standard deviation.

For the experiments at pH 7.0, the protein solutions of the required concentrations were prepared under aerobic conditions in 0.1 M phosphate buffer pH 6.8 (in the absence of CO_2) or pH 6.6 (in the presence of CO_2). Peroxynitrite solutions were prepared by diluting the stock solution immediately before use with 0.01 M NaOH to achieve the required concentration. The experiments were carried out either at 20 or at 37 °C. The pH was always measured at the end of the reactions. The protein solutions for the experiments at pH 6.5 and 8.0 were prepared analogously in 0.1 M phosphate buffer pH 6.3 and 7.5, respectively. The protein solutions for the experiments in the presence of 10 mM CO_2 at pH 7.3 and 8.3 were both prepared in 0.1 M phosphate buffer pH 6.5 containing 71.5 mM sodium bicarbonate. The two different pH values were obtained by varying the concentration of the NaOH solution with which the peroxynitrite stock solution was diluted. Specifically, 0.01 M NaOH was used to reach a final pH of 7.3 and 0.05 M NaOH to obtain a final pH of 8.3. In some cases, the protein/bicarbonate solution was prepared and kept in a gastight SampleLock Hamilton syringe to avoid loss of CO_2. This additional precaution proved not to be necessary.

3.3.4 NITRITE AND NITRATE ANALYSIS

Product analysis was carried out as described previously *(33)* by anion chromatography with conductivity detection with a Metrohm instrument (IC Separation Center 733, IC Detector 732 and IC pump 709) equipped with an Anion SUPER-SEP (6.1009.000) column and an Anion SUPER-SEP (6.1009.010) precolumn. Calibration curves were obtained by measuring 5-10 standard sodium nitrite and sodium nitrate solutions in 5 mM phosphate buffer. The samples were prepared by mixing 500 µL of the apoMb, metMb, and metHb solutions (50-1500 µM in 0.1 M phosphate buffer pH 6.8 or in 0.1 M phosphate

buffer pH 6.6 containing 22 mM sodium bicarbonate) at room temperature with 500 µL of an ice-cooled peroxynitrite solution (200 µM in 0.01 M NaOH) while vortexing. The reaction mixture was diluted 1:10 with water and analyzed within ca. 5 min. At least two analyses of three separate experiments were carried out for each protein. Nitrite and nitrate contamination in peroxynitrite was determined by adding to 500 µL of an ice-cooled 50 mM phosphoric acid solution 500 µL of the same peroxynitrite solution used for the reactions. Under these conditions, peroxynitrite exclusively isomerizes to nitrate. Thus, ion chromatographic analysis after dilution of the samples with water (1:10) gives directly the amount of nitrite present in the peroxynitrite solutions. Nitrate contamination was determined by subtracting from the amount of nitrate found in this experiment the peroxynitrite concentration measured spectrophotometrically. Usual nitrite and nitrate contaminations were in the range of 20-50 and 0-10% of the peroxynitrite concentration, respectively.

3.3.5 REVERSE PHASE HPLC ANALYSIS

HPLC analysis was carried out with a Hewlett-Packard Series 1050 apparatus with a Series 1100 UV-vis detector, equipped with a VYDAC 218TP54 Protein&Peptide C18-Column (250 × 4.6 mm). Solvent A was 0.07% TFA in H_2O and solvent B was 0.07% TFA in acetonitrile. Nitrotyrosine was eluted (ca. 7.5 min after injection) by keeping the amount of B constant (5%) in the first 2 min and then by using an increasing linear gradient of B from 5 to 10% between 2 and 10 min and from 10 to 80% between 10 and 15 min. NO_2-Tyr was detected contemporaneously at 220, 280, 350, and 400 nm. NO_2-Tyr was quantified by measuring a calibration curve of five to 10 nitrotyrosine standard solutions.

3.3.6 ANALYSIS OF THE FREE NITROTYROSINE CONTENT GENERATED BY THE REACTION OF PEROXYNITRITE WITH oxyMb, metMb, apoMb, metMbCN, FerrylMb, oxyHb, deoxyHb, AND metHb IN THE PRESENCE OF ADDED FREE TYROSINE

The reactions were carried out at room temperature by adding 100 µL of an ice-cooled peroxynitrite solution (2 mM in 0.01 M NaOH) to 900 µL of a solution containing tyrosine (112 µM) and different concentrations of oxyMb, metMb, apoMb, metMbCN, metHb, and oxyHb (0-55.5 µM in 0.05 M phosphate buffer pH 7.5), in the absence and presence of 1.1 mM CO_2. For the reactions with deoxyHb, 1.8 mL of an oxyHb solution (16.6, 27.8, or 55.5

μM in 0.05 M phosphate buffer pH 7.5) containing tyrosine (112 μM) was placed in a sealable cell for anaerobic applications and thoroughly degassed until the recorded UV-vis spectrum indicated the complete formation of deoxyHb. Then, 200 μL of an ice-cooled degassed peroxynitrite solution (2 mM in 0.01 M NaOH) was added directly in the cell by using a gastight SampleLock Hamilton syringe. For the reactions with ferrylMb, 1.765 mL of a metMb solution (5.6-56.6 μM in 0.05 M phosphate buffer pH 7.5) was allowed to react with 10 equiv of H_2O_2 (10 μL of a 1-10 mM solution) for 6 min. Then, excess H_2O_2 was destroyed by the addition of 5 μL of a solution of catalase in water (approximately 1 mg/mL). After 1 min, 20 μL of a 10 mM tyrosine solution (basic) was added, followed by the addition of 200 μL of an ice-cooled peroxynitrite solution (2 mM in 0.01 M NaOH). All the samples were analyzed by HPLC as described above.

3.3.7 ANALYSIS OF THE NITROTYROSINE CONTENT IN BSA, TYROSINE, apoMb, oxyMb, metMb, oxyHb, AND metHb AFTER REACTION WITH VARIABLE AMOUNTS OF PEROXYNITRITE

The reaction of peroxynitrite with BSA, apoMb, oxyMb, metMb, oxyHb, and metHb was carried out at 37 °C as described previously *(26)*. In brief, 20 μL of an ice-cooled peroxynitrite solution (different concentrations in 0.01 M NaOH) was added as a bolus while vortexing to 180 μL of a protein solution (112 μM in 0.1 M phosphate buffer, pH 7.0) kept in a thermostat at 37 °C. After ca. 30 min, nitrite was removed by adding ca. 200 μL of an ammonium sulfamate solution (100 mM in 0.5 M HCl) and subjected to acid hydrolysis as described previously *(26)*. Finally, the samples were analyzed by HPLC as described above. For comparison, an analogous experiment was carried out by adding a peroxynitrite solution to a tyrosine solution under identical experimental conditions.

3.3.8 STATISTICS

The experiments reported in this article were carried out at least in triplicate on independent days. The results are given as mean values of at least three experiments plus or minus the corresponding standard deviation.

3.4 RESULTS

3.4.1 KINETIC STUDIES AT 20 AND 37 °C

The decay rate of peroxynitrite (100 μM) in the presence of the iron(III) forms of Mb and Hb was determined by stopped-flow spectroscopy at 20 °C and pH 7.0. The reactions were studied by following the absorbance changes at 302 nm, the characteristic absorbance maximum for peroxynitrite. All the measured traces could be fitted well to a single-exponential expression. As shown in Figure 1A, the observed peroxynitrite decay rates (k_{obs}) increased linearly with increasing metMb and metHb concentration. The values of k_{cat} (Table 1), obtained from the linear fits of the two plots, are in the order of magnitude of 10^4 $M^{-1}s^{-1}$, but metMb is a slightly more efficient catalyst than metHb.

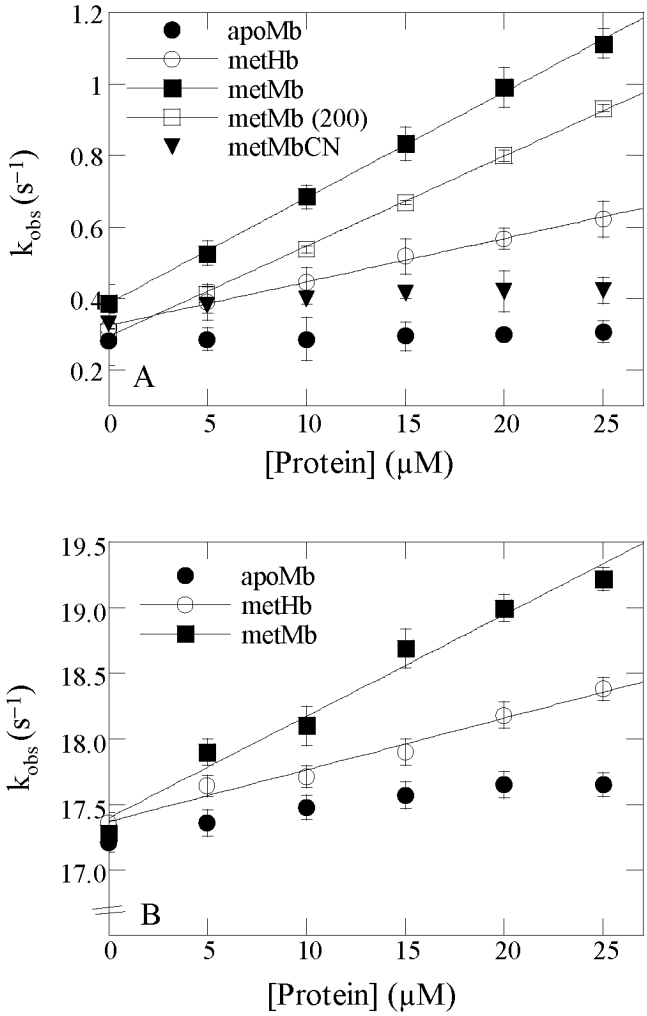


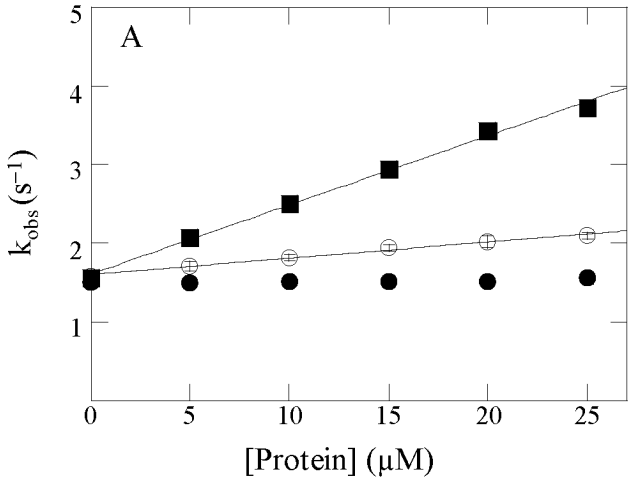
Figure 1. Plots of k_{obs} vs protein concentration (apoMb, metMb, CN, metMb, and metHb) for the protein-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer pH 7.0 and 20 °C. metMb (200): metMb-catalyzed decay of 200 μM peroxynitrite. Data in the absence (A) and presence (B) of 1.2 mM CO_2. The second-order rate constants resulting from the linear fits depicted are given in Table 1.

For comparison, we determined the decay rates of peroxynitrite also in the presence of different amounts of the heme-free protein (apoMb) and a heme-blocked form (metMbCN). As depicted in Figure 1A, the values of the observed rate constants for the decay of peroxynitrite (100 μM) were nearly unchanged when the concentration of these two forms of Mb was varied in the range of 0-25 μM. Because of the physiological relevance attributed to the reaction between carbon dioxide and peroxynitrite, we studied the protein-mediated decay of peroxynitrite also in the presence of 1.2 mM CO_2. As shown in Figure 1B and summarized in Table 1, metMb and metHb accelerated the decay of peroxynitrite (100 μM) in a concentration-dependent extent. In general, the values of k_{cat} were all approximately 2-3 times larger than those obtained in the absence of CO_2. Thus, also in the presence of CO_2, metMb was more efficient than metHb. The analogous experiment with apoMb showed that this protein form only slightly accelerated the decay of peroxynitrite.

Table 1. Summary of the Catalytic Rate Constants ($\times 10^4$ M^{-1} s^{-1}) for the Protein-Mediated Decay of Peroxynitrite (100 μM) in 0.05 M Phosphate Buffer pH 7.0, at 20 °C and 37 °C.

Protein	No CO_2		1.2 mM CO_2	
	20 °C	37 °C	20 °C	37 °C
metMb	2.9 ± 0.1	8.8 ± 0.2	7.7 ± 0.1	26.5 ± 0.2
metHb[a]	1.2 ± 0.1	2.1 ± 0.1	3.9 ± 0.2	18.2 ± 0.1

[a] The rate constants for metHb are expressed per heme.



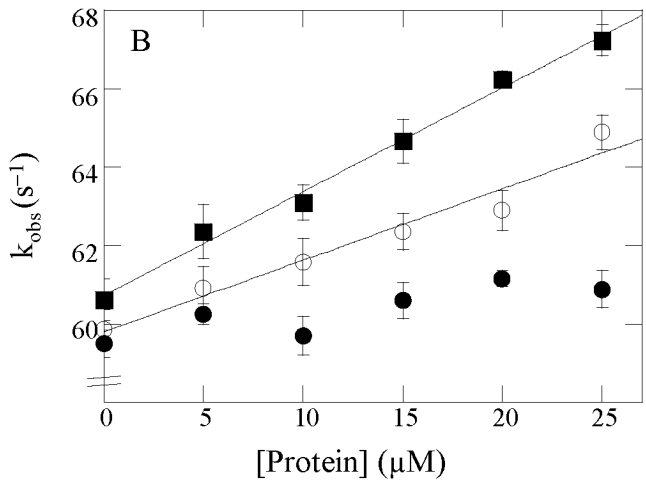


Figure 2. Plots of k_{obs} versus protein concentrations apoMb (●), metMb (■) and metHb (○) for the protein catalyzed decay of peroxynitrite in 0.05 M phosphate buffer, pH 7.0, and 37 °C. Data in the absence (A) and presence (B) of 1.2 mM CO_2. The second-order rate constants resulting from the linear fits depicted are given in Table 1.

The kinetics of the protein-catalyzed decay of peroxynitrite in the absence and presence of 1.2 mM CO_2 were studied also at 37 °C (Figure 2), to better evaluate the physiological relevance of these reactions. As expected, at a higher temperature, the values for k_{cat} were all 2-4 times larger (Table 1), and metMb was still the most efficient catalyst, both in the absence and in the presence of CO_2.

To confirm the catalytic nature of the protein-mediated decay of peroxynitrite, we determined the observed rate constants for the disappearance of peroxynitrite in the presence of metMb and metHb over the peroxynitrite concentration range of 50-250 μM (at 20 °C and pH 7.0). The protein concentration was always kept constant (10 μM), and the reactions were carried out both in the absence and in the presence of CO_2. As shown in Figure 3, in both cases the values of the observed rate constants were nearly constant and only slightly decreased with increasing peroxynitrite concentration. In agreement with previous reports *(34)*, a similar trend was observed also in the absence of the proteins. Indeed, at peroxynitrite concentrations higher than 50 μM and under neutral conditions, it has been proposed that an adduct is formed between the peroxynitrous acid and its deprotonated form *(34, 35)*. The decrease in the observed rate constant at higher peroxynitrite concentrations may suggest that either this adduct reacts at a lower rate with the heme or that it has to dissociate before it can react with the heme.

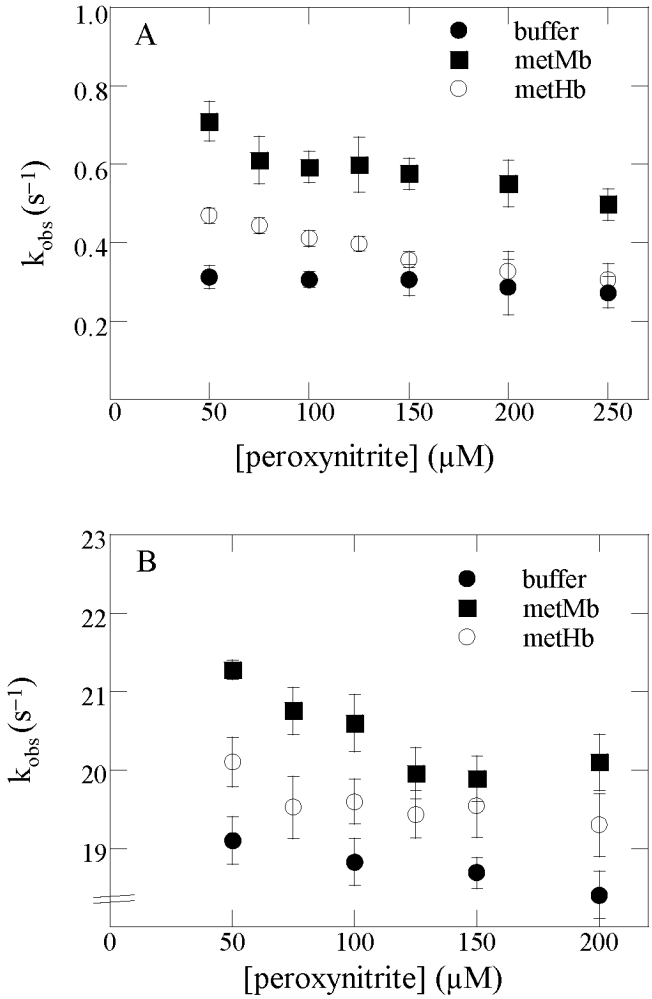


Figure 3. Plots of k_{obs} vs peroxynitrite concentration for the decay of peroxynitrite (at pH 7.0 and 20 °C) in the absence of added protein (buffer) and in the presence of 10 μM metMb or 10 μM metHb. Data in the absence (A) and presence (B) of 1.3 mM CO_2.

To find out whether the peroxynitrite concentration influences the value of k_{cat}, we measured the decay of 200 μM peroxynitrite in the presence of various amounts of metMb (0-50 μM) under similar conditions. As shown in Figure 1A (metMb 200), at each metMb concentration the observed rate constants (k_{obs}) were slightly lower than those obtained with 100 μM peroxynitrite (metMb). However, the values of k_{cat}, obtained from the linear fits of the two plots, were nearly identical: $(2.9 \pm 0.1) \times 10^4$ and $(2.5 \pm 0.1) \times 10^4$ M^{-1} s^{-1} for the reactions with 100 and 200 μM peroxynitrite, respectively.

3.4.2 pH-DEPENDENCE OF THE CATALYTIC RATE CONSTANT IN THE ABSENCE OF CO_2

The decay rate of peroxynitrite (100 μM) in the presence of apoMb, metMb, and metHb was determined also at pH 6.5 and 8.0 (at 20 ºC). As shown in Figure 4, in both cases apoMb did not influence the rate of peroxynitrite decay. At pH 6.5, the catalytic rate constants for the metMb- and metHb-mediated decay of peroxynitrite were significantly

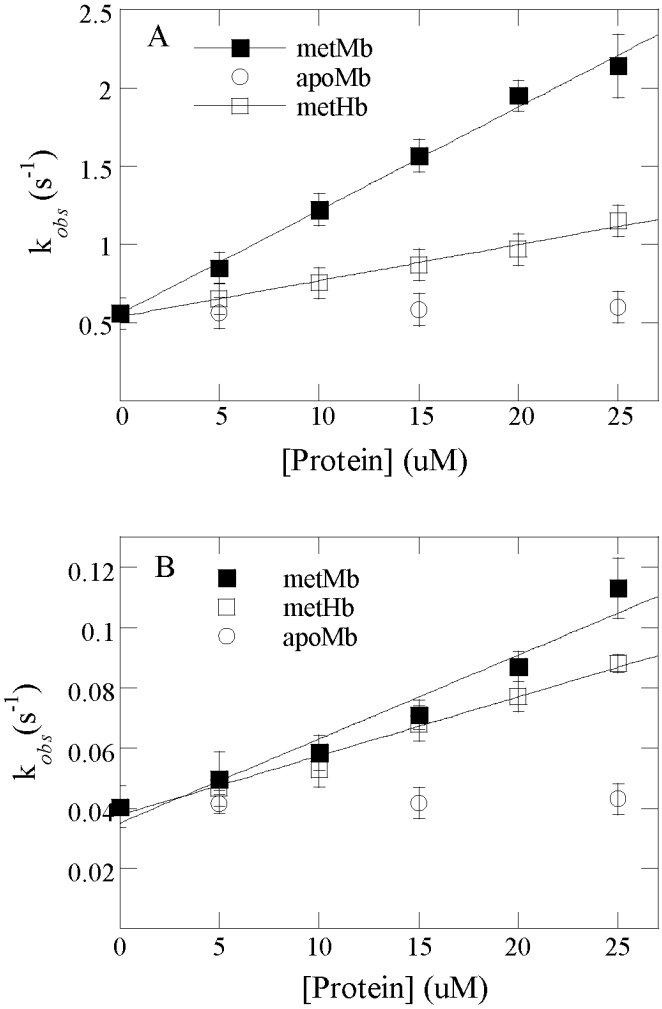


Figure 4. Plots of k_{obs} vs protein concentration (apoMb, metMb, and metHb) for the protein-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer (A) pH 6.5 and (B) pH 8.0 (20 ºC). The second-order rate constants resulting from the linear fits depicted are given in the text.

larger than those obtained at pH 7.0, that is $(6.6 \pm 0.3) \times 10^4$ and $(2.3 \pm 0.1) \times 10^4$ M^{-1} s^{-1}, respectively (Figure 4A). In contrast, at pH 8.0, metMb and metHb were significantly less effective (Figure 4B). Indeed, the values of the catalytic rate constants for the metMb- and

metHb-mediated decay of peroxynitrite measured at pH 8.0 were (0.27 ± 0.03) × 10^4 and (0.19 ± 0.01) × 10^4 M^{-1} s^{-1}, respectively.

3.4.3 PEROXYNITRITE DECAY IN THE PRESENCE OF A LARGE EXCESS OF CO_2 (10 mM) AND apoMb, metMb, OR metHb

The influence of apoMb, metMb, and metHb on the decay rate of peroxynitrite (100 μM) was determined also in the presence of a large excess of CO_2. Interestingly, with 10 mM CO_2, both at pH 7.3 and at pH 8.3, the rate of decay of peroxynitrite was not affected by the addition of increasing amounts of apoMb, metMb, or metHb (Figure 5).

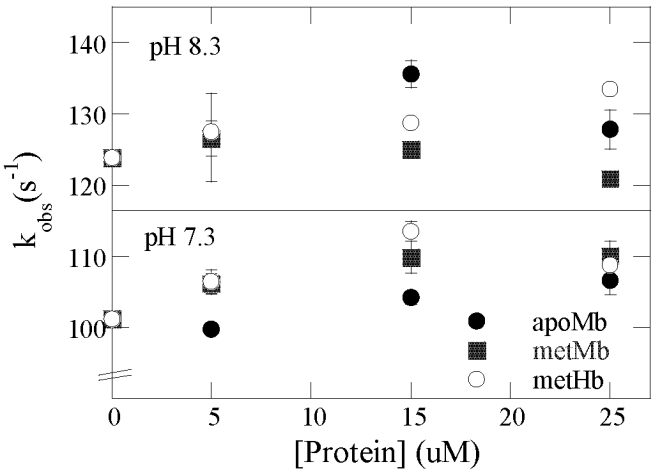


Figure 5. Plots of k_{obs} vs protein concentration (apoMb, metMb, and metHb) for the decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer in the presence of 10 mM CO_2 (bottom) at pH 7.3 or (top) at pH 8.3 (20 °C). The second-order rate constants resulting from the linear fits depicted are given in the text.

3.4.4 ANALYSIS OF THE NITROGEN CONTAINING PRODUCTS

The products of the protein-catalyzed decay of peroxynitrite were analyzed by ion chromatography. In agreement with previous reports *(35)*, spontaneous decay of 100 μM peroxynitrite at pH 7.0 and 20 °C yielded 67 ± 2% nitrate and 34 ± 3% nitrite. In the presence of the iron(III) forms of Mb and Hb, the nitrite yield continuously decreased with increasing protein concentration (25-750 μM), and the nitrate yields increased to a comparable extent (Table 2). In the presence of 750 μM metMb or metHb, peroxynitrite isomerized nearly quantitatively to nitrate, and the amount of nitrite found corresponded to that already present in the peroxynitrite solution. Increased nitrate yields have recently been reported for the

metHb-catalyzed decay of peroxynitrite (100 μM) at pH 7.4 *(36)* and for the metMb-mediated isomerization of peroxynitrite (500 μM) at pH 7.6 *(37)*. ApoMb did not influence significantly the nitrate yields, but lowered the nitrite yields; thus, the total amount of nitrogen-containing products decreased with increasing protein concentration (Table 2). As previously shown *(26)*, this observation suggests that under these conditions the protein is nitrated to a significant extent.

Table 2. Product distribution of peroxynitrite (100 μM) decay in the presence of different concentrations of apoMb, metMb and metHb (in 0.05 M phosphate buffer pH 7.0, and 20 °C), in the absence and presence of 1.1 mM CO_2. The data are expressed as percentage yields.

	No added CO_2			1.1 mM CO_2		
[Proteins] (μM)	NO_2^-	NO_3^-	Total	NO_2^-	NO_3^-	Total
No protein	34 ± 3	67 ± 2	101	17 ± 2	85 ± 3	102
apoMb 25	29 ± 3	65 ± 1	94	17 ± 3	81 ± 4	99
apoMb 50	26 ± 5	66 ± 2	92	16 ± 3	83 ± 4	99
apoMb 100	24 ± 3	68 ± 4	92	18 ± 1	83 ± 2	101
apoMb 200	20 ± 1	66 ± 3	86	12 ± 2	84 ± 2	96
metMb 25	20 ± 3	78 ± 4	98	17 ± 3	84 ± 3	101
metMb 50	16 ± 4	81 ± 2	97	14 ± 2	85 ± 2	99
metMb 100	13 ± 5	85 ± 3	98	9 ± 2	85 ± 2	94
metMb 200	8 ± 3	86 ± 2	94	7 ± 4	91 ± 3	98
metMb 500	4 ± 2	96 ± 3	100	nd	nd	nd
metMb 750	< 1	99 ± 2	99	nd	nd	nd
metHb 25	24 ± 4	76 ± 4	100	16 ± 4	81 ± 4	97
metHb 50	19 ± 5	81 ± 4	100	12 ± 3	85 ± 2	97
metHb 100	15 ± 4	87 ± 4	103	6 ± 2	91 ± 3	97
metHb 200	8 ± 3	85 ± 4	93	3 ± 2	95 ± 2	98
metHb 500	5 ± 2	94 ± 2	99	nd	nd	nd
metHb 750	< 1	97 ± 2	97	nd	nd	nd

nd: not determined

As expected, in the presence of 1.1 mM CO_2, the yield of nitrate generated from the decay of 100 μM peroxynitrite (at pH 7.0 and 20 °C) was significantly larger. In agreement with previous reports *(35)*, we obtained 17 ± 2% nitrite and 85 ± 3% nitrate. Also in the presence of 1.1 mM CO_2, the addition of metMb and metHb led to a concentration-dependent increase of the nitrate yields (Table 2). In contrast, apoMb caused a slight decrease of the nitrite yields without influencing significantly the nitrate yields (Table 2). As pointed out

above, this result is in agreement with our previous observation that apoMb is significantly nitrated under these conditions.

3.4.5 PROTECTION AGAINST PEROXYNITRITE-MEDIATED NITRATION OF FREE TYROSINE

To investigate whether different forms of myoglobin can protect against peroxynitrite-mediated nitration, we determined the yield of 3-nitrotyrosine (NO_2-Tyr) formed from the reaction of peroxynitrite with free tyrosine in the presence of increasing quantities of the protein. For this purpose, we mixed 200 μM peroxynitrite with 100 μM tyrosine in the presence of increasing amounts of apoMb, oxyMb, metMb, ferrylMb, and metMbCN (in 0.05 M phosphate buffer, pH 7.4 and 20 °C). As shown in Figure 6A *(and summarized in Table D in Appendices)*, oxyMb, ferrylMb, and metMb are rather efficient scavengers of peroxynitrite. In the presence of 50 μM of these proteins, the relative NO_2-Tyr yields (relative to the amount found in the absence of the proteins) were 0, 14 ± 2, and 21 ± 2%, respectively. In contrast, the addition of 50 μM heme-free apoMb and heme-blocked metMbCN lowered the relative NO_2-Tyr yields only to 63 ± 3 and 74 ± 1%, respectively. To study the efficiency of the reduced form of the proteins under the same conditions, we chose to use deoxyHb as it can be prepared in a much simpler way than deoxyMb, by thoroughly degassing an oxyHb solution. DeoxyHb was slightly more effective than oxyHb. For instance, the addition of 50 μM deoxyHb or oxyHb led to relative NO_2-Tyr yields of 0 and 8 ± 3%, respectively *(see Table D in Appendices)*. In agreement with our kinetic studies, oxyHb and metHb were found to be less efficient scavengers than the corresponding Mb forms. The addition of 50 μM metHb led to the formation of 27 ± 1% NO_2-Tyr, whereas only 21 ± 2% was formed in the presence of 50 μM metMb *(see Table D in Appendices)*.

Analogous studies were carried out in the presence of CO_2. As previously reported *(38)*, the addition of CO_2 leads to an increase of the absolute NO_2-Tyr yields produced from the reaction of peroxynitrite with tyrosine. As observed in the absence of CO_2, the addition of increasing amounts of oxyMb and metMb led to a significant, concentration-dependent decrease of the NO_2-Tyr yields. However, in the presence of 1.1 mM CO_2, these two forms of Mb were less efficient to prevent the peroxynitrite-mediated nitration of added tyrosine (Figure 6B *and see Table D in Appendices)*. The order of efficiency of the four Mb forms studied was identical to that obtained in the absence of CO_2. In the presence of 50 μM

oxyMb, the best scavenger, the NO_2-Tyr yield was 26 ± 2% of the amount obtained in the absence of the protein. Interestingly, small quantities of metMbCN reduce the nitration of free tyrosine to a larger extent in the presence than in the absence of CO_2.

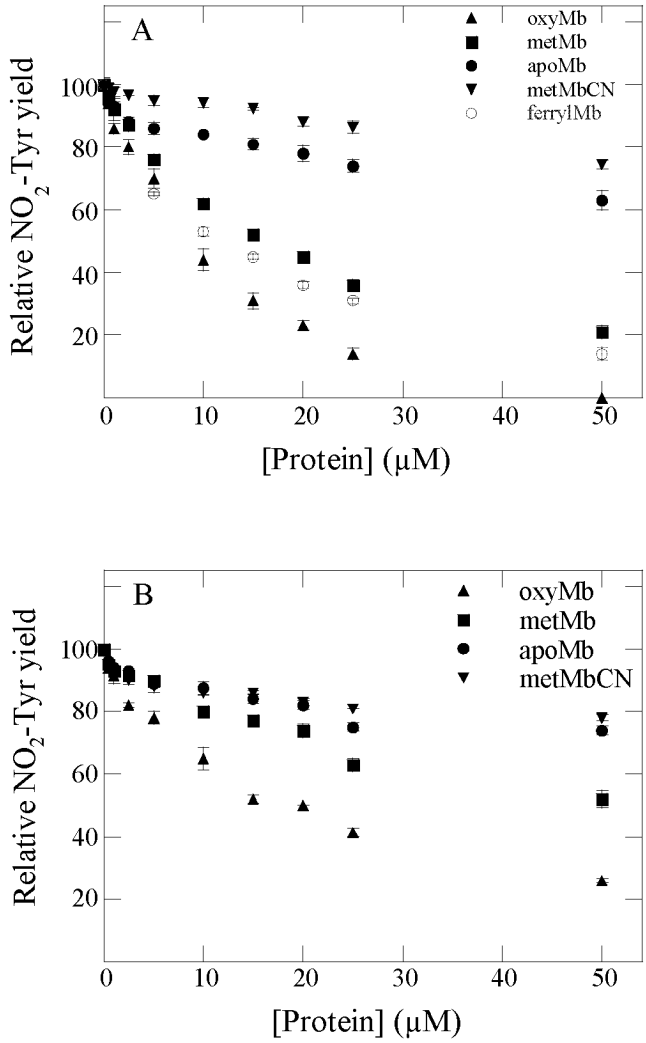
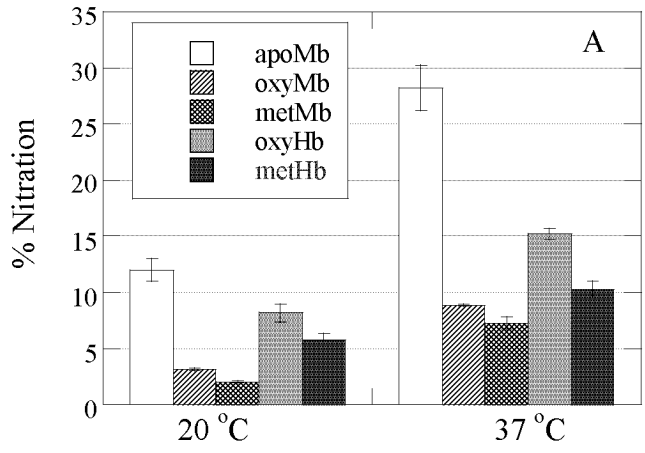


Figure 6. Relative yield of NO_2-Tyr formed from the reaction of peroxynitrite (200 μM) and tyrosine (100 μM), in the presence of different concentrations of oxyMb, metMb, apoMb, ferrylMb, and metMbCN in 0.05 M phosphate buffer pH 7.4, 20 ºC. Experiments in the absence (A) and presence (B) of 1.1 mM CO_2. Relative yield = (yield with added protein/yield with no protein) × 100%.

3.4.6 NITRATION OF THE GLOBIN AFTER TREATMENT OF Mb AND Hb WITH PEROXYNITRITE AT 37 °C

We have previously determined the extent of nitration of the tyrosine residues upon reaction of different forms of Mb and Hb with an excess of peroxynitrite at 0 and 20 °C *(26)*. Our preliminary results showed that the NO_2-Tyr yields were only slightly larger when the reactions were carried out at 37 °C instead of 20 °C *(26)*. Thus, we completed these studies by exposing at 37 °C apoMb, oxyMb, metMb, oxyHb, and metHb (100 μM) to an excess of peroxynitrite (1000 μM) in phosphate buffer pH 7.0. For comparison, we treated also tyrosine and BSA with peroxynitrite under identical conditions. As shown in Figure 7A, at 37 °C the percentages of nitration of the two available tyrosine residues of all forms of Mb and of the three tyrosine residues of oxyHb and metHb were 2-3 times larger than those obtained at 20 °C *(see Table E in Appendices)*. In contrast, in the presence of 1.1 mM CO_2, the yield of nitrotyrosine produced when the oxygenated forms of Mb and Hb were allowed to react with peroxynitrite at 37 °C were nearly identical to those obtained at 20 °C (Figure 7B *and see Table E in Appendices)*.

The percentages of nitration of apoMb, metMb, and metHb were larger at 37 °C also in the presence of CO_2. As observed previously at lower temperatures *(26)*, also at 37 °C apoMb was always nitrated to the largest extent, and the tyrosine residues of the iron(III) forms of Mb and Hb were slightly less nitrated than those of the corresponding oxygenated forms, both in the absence and in the presence of CO_2. As anticipated, the addition of CO_2 always led to higher relative NO_2-Tyr yields.



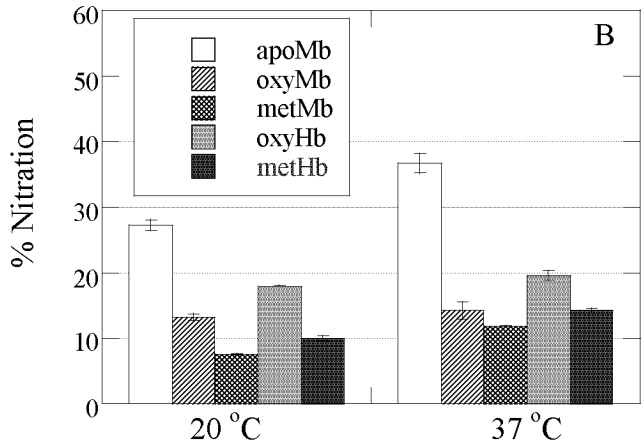


Figure 7. NO_2-Tyr yields, determined by HPLC after acid hydrolysis of the proteins, from the reaction of apoMb, oxyMb, metMb, oxyHb, and metHb (100 μM) with 10 equiv of peroxynitrite in the absence (A) and presence (B) of 1.1 mM CO_2 at 20 and 37 °C, pH 7.0.

As expected, the exposure of L-tyrosine and BSA to a range of peroxynitrite concentrations under similar conditions (at 20 and 37 °C) induced a dose-dependent increase in the nitration yields (Figures 8 and 9). For both tyrosine and BSA, the nitration yields were

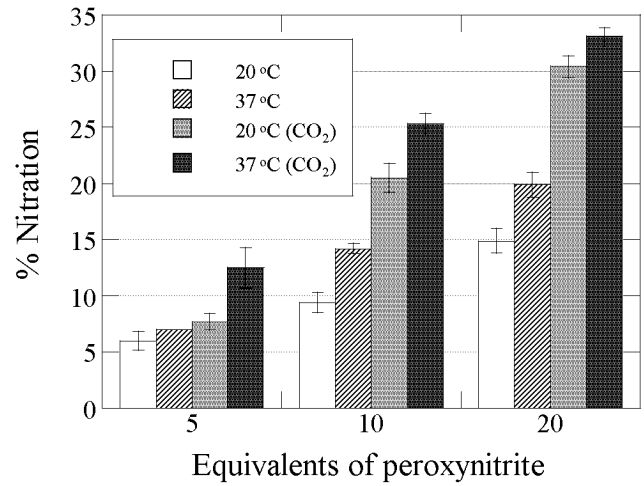


Figure 8. NO_2-Tyr yields, determined by HPLC, from the reaction of tyrosine with different amounts of peroxynitrite in the absence and presence of 1.1 mM CO_2, at 20 and 37 °C, pH 7.0.

mostly larger at higher temperatures *(Table F in Appendices)*. Interestingly, in the absence of CO_2, this temperature-mediated increase in the nitration yields was more pronounced at

higher concentrations of peroxynitrite, in particular for tyrosine. In contrast, in the presence of CO_2, larger differences between the NO_2-Tyr yields obtained at 20 and 37 °C were observed when smaller amounts of peroxynitrite were used.

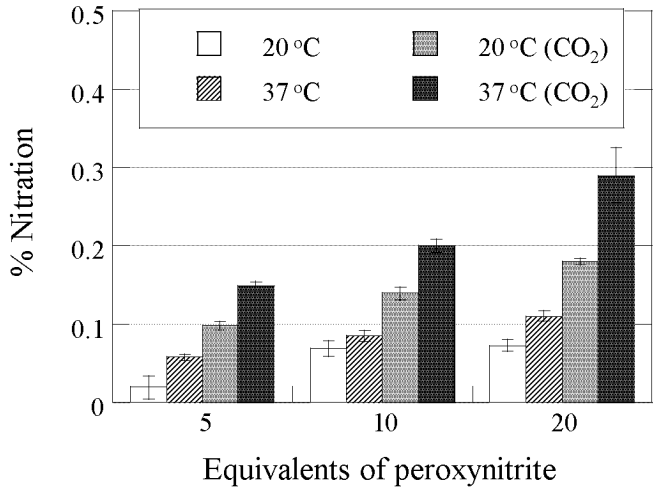


Figure 9. NO_2-Tyr yields determined by HPLC after acid hydrolysis of the protein, from the reaction of BSA with different amounts of peroxynitrite in the absence and presence of 1.1 mM CO_2, at 20 °C and 37 °C, pH 7.0.

3.5 DISCUSSION

The reaction of peroxynitrite with oxyMb and oxyHb has been reported to yield the corresponding iron(III) forms *(39-41)*. We have shown that these two reactions proceed in two steps via the formation of the corresponding ferryl species *(24)*. The second-order rate constants for the two steps of the reaction between peroxynitrite and oxyHb are $(3.3 \pm 0.1) \times 10^4$ and $(3.3 \pm 0.4) \times 10^4$ M^{-1} s^{-1} (at pH 7.4 and 20 °C), respectively *(Boccini, F., Domazou, A. S and Herold. S. (2004) J. Phys. Chem. A 108, 5800-5805)*. The values for the rate constants of the reaction with oxyMb are slightly larger, $(5.4 \pm 0.2) \times 10^4$ and $(2.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1} (at pH 7.3 and 20 °C), respectively. It is conceivable that the reaction between peroxynitrite and oxyHb takes place *in vivo*, as peroxynitrite has been shown to be able to diffuse across the erythrocyte membrane and oxidize oxyHb within the red blood cells *(39, 42)*.

In contrast, we and others have shown that upon mixing peroxynitrite with the iron(III) forms of Hb and Mb, no changes are detected in the UV-vis absorbance spectra of the proteins *(ref 43 and references therein, see also Figure B in Appendices)*. This

observation may suggest that no reaction takes place between peroxynitrite and the heme centers of metMb or metHb. However, despite the lack of absorbance changes in the UV-vis spectra of the proteins, the iron(III) forms of sperm whale myoglobin mutants, in which the distal histidine residue had been replaced with either an aspartic acid or an alanine, have been shown to be efficient catalysts for the isomerization of peroxynitrite to nitrate *(44)*. In addition, we have recently reported that the tyrosine residues of metMb and metHb are nitrated by peroxynitrite to a significantly smaller extent than those of apoMb *(26)*. Taken together, these results suggest that a reaction must take place between peroxynitrite and heme centers of metMb and metHb.

3.5.1 KINETIC STUDIES OF THE REACTION OF PEROXYNITRITE WITH THE IRON(III) FORMS OF Mb AND Hb

In this paper, we have shown that there is a linear dependence between the observed rate of decay of an excess peroxynitrite and the concentration of both metMb and metHb. This result suggests that the iron(III) forms of these proteins catalyze the decay of peroxynitrite. This assumption is supported by the further observation that a variation of the peroxynitrite concentration does not affect significantly the value of the observed rate of peroxynitrite decay. Finally, analysis of the nitrogen-containing products showed that metMb and metHb increase the production of nitrate from the decay of peroxynitrite in a concentration-dependent way. Taken together, our results indicate that metMb and metHb catalyze the isomerization of peroxynitrite. The measured values of the catalytic rate constants are $(2.9 \pm 0.1) \times 10^4$ and $(1.2 \pm 0.1) \times 10^4$ M^{-1} s^{-1} (at pH 7.0 and 20 °C), respectively. Thus, as for the reaction with the oxygenated forms of these proteins, the rate of the reaction between peroxynitrite and myoglobin is larger than that of its reaction with hemoglobin. The value for the reaction with metMb is consistent with those recently reported: $(1.4 \pm 0.1) \times 10^4$ M^{-1} s^{-1} (at pH 7.4 and 20 °C) *(44)* and 1.03×10^4 M^{-1} s^{-1} (at pH 7.6) *(37)*. For comparison, the sperm whale metMb mutant H64A has a k_{cat} value of $(6.0 \pm 0.1) \times 10^6$ M^{-1} s^{-1} (at pH 7.4 and 20 °C) *(44)*. This significantly larger rate is reflected in the extremely small quantities (1.6 μM) of this mutant needed to completely convert 100 μM peroxynitrite to nitrate under conditions analogous to those used in the present work *(44)*.

The pH-dependence of the catalytic rate constant for metMb and metHb suggests that HOONO is the species that reacts with the iron(III) center of these proteins. Alternatively, the

decrease of the value of k_{cat} with increasing pH may be a consequence of the deprotonation of the water coordinated to the iron(III) center, which leads to a stronger Fe^{III}–O bond. However, as the pK_a value for the acid-alkaline transition of metMb is 8.05 and that for metHb is 8.93 *(45)*, no significant difference would be expected between the rate constants at pH 6.5 and 7.0. In contrast, at pH 6.5, the values of k_{cat} are approximately twice as large as those at pH 7.0. The acid-alkaline transition could be at least in part responsible for the significant decrease of k_{cat} at pH 8.0. A further possible explanation for the observed pH-dependence is that protonation of the distal histidine at low pH and its consequent shift away from the active site pocket *(46)* facilitate the entrance of peroxynitrite and its binding to the iron. Nevertheless, if this was true, no significant difference would be expected among the k_{cat} values at pH 7.0 and 8.0, as the pK_a of the distal histidine is lower than 6. In contrast, at pH 8.0, the values of k_{cat} are approximately 10 times smaller than those at pH 7.0. Taken together, these results strongly indicate than HOONO is the species that reacts at a larger rate with metMb and metHb. This conclusion is in agreement with the results obtained with the H64A-metMb mutant. Also for this protein, the values of k_{cat} increase with decreasing pH *(44)*.

To further probe the role of the iron center for the catalysis of the decay of peroxynitrite, we also investigated the reaction of peroxynitrite with increasing amounts of apoMb and metMbCN. As expected, the heme-free apoMb and the heme-blocked metMbCN do not affect the decay rate of peroxynitrite. Moreover, analysis of the nitrogen-containing products showed that apoMb does not induce an increase of the nitrate yield produced by the decay of peroxynitrite. Taken together, these results confirm that the iron(III) centers of metMb and metHb play a critical role in the catalysis of the isomerization of peroxynitrite.

The rapid reaction between the peroxynitrite anion and the ubiquitous CO_2 has been shown to be one of the principal pathways for peroxynitrite consumption in biological systems *(38)*. Despite the high CO_2 concentration present in the plasma (ca. 1.3 mM), it has been established that significant amounts of peroxynitrite can diffuse inside the red blood cells, via the anion channels or by simple diffusion and oxidize oxyHb *(42)*. We have previously shown that the peroxynitrite-mediated oxidation of oxyMb proceeds via the ferrylMb also in the presence of added CO_2 *(25)*. The second-order rate constants for the two reaction steps are $(41 \pm 7) \times 10^4$ and $(3.2 \pm 0.2) \times 10^4$ M^{-1} s^{-1} (at pH 7.5, 20 °C, and in the presence of 1.2 mM CO_2), respectively *(25)*. The corresponding values of the rate constants

for the reaction with oxyHb are $(35 \pm 3) \times 10^4$ and $(10.6 \pm 0.6) \times 10^4$ M^{-1} s^{-1} (at pH 7.4, 20 °C, and in the presence of 1.2 mM CO_2) *(Boccini, F., Domazou, A. S. and Herold. S. (2004) J. Phys. Chem. A 108, 5800-5805).* Thus, for both proteins the first reaction step, the formation of the ferryl form from the oxy form, is significantly accelerated by the addition of CO_2, and once more Mb reacts faster than Hb.

Also in the presence of 1.1 mM CO_2, the reaction of peroxynitrite with metMb and metHb does not induce any changes in the UV-vis spectra of the proteins (data not shown). However, our stopped-flow and ion chromatography studies show that, also in the presence of physiological concentrations of CO_2 (1.1-1.2 mM), metMb and metHb catalyze the isomerization of peroxynitrite to nitrate, whereas apoMb has no effect on its decay rate. The values of the catalytic rate constants are approximately 3 times larger than those determined in the absence of CO_2 (Table 1).

Interestingly, in the presence of 10 mM CO_2 both at pH 7.3 and 8.3, metMb and metHb did not influence the decay rate of peroxynitrite. After the 4 ms approximately needed for mixing, under the conditions chosen for the experiment at pH 8.3, a large fraction of peroxynitrite is already converted to $ONOOCO_2^-$. Because of the alkaline pH, most of the remaining peroxynitrite is in its deprotonated form. Thus, nearly no HOONO will be available for reaction with metMb or metHb. As no effect is observed by the addition of increasing amounts of metMb or metHb, our data indicate that no fast reaction takes place between $ONOOCO_2^-$ and the iron(III) center of these two proteins. Taken together, these results suggest that the catalytic effect observed in the presence of 1.2 mM CO_2 is due to the competing reaction between HOONO, still present in significant amounts after mixing the reagents at pH 7.0, and the iron(III) center of the proteins. However, according to this mechanism, the value of k_{cat} should not be influenced by the addition of CO_2. The larger values of k_{cat} obtained in the presence of 1.2 mM CO_2 could reflect the increased rate observed with lower amounts of peroxynitrite (Figure 3). Indeed, despite the fact that 100 μM peroxynitrite is mixed with the proteins, a large amount of peroxynitrite is rapidly consumed by its reaction with CO_2; thus, lower amounts of peroxynitrite are available for reaction with the protein. Alternatively, large concentrations of HCO_3^-/CO_2 could cause conformational changes of the proteins, which could lead to a faster reaction with HOONO.

The hypothesis that $ONOOCO_2^-$ does not react with metMb or metHb also explains why in the presence of 1.2 mM CO_2 larger amounts of metMb and metHb were needed

to get a significant increase in the nitrate yields. Under the conditions of our experiment, the very fast reaction between peroxynitrite and CO_2 has a half-life of ca. 40 ms, whereas in the presence of 50 μM metMb, the half-life of the protein-catalyzed process is 180 ms. Thus, only about 20% of peroxynitrite will react with metMb, and the nitrate yields will be only slightly larger than those obtained in the absence of the protein.

3.5.2 PROTECTION OF metMb AND metHb AGAINST PEROXYNITRITE-MEDIATED NITRATION

The ability of metMb and metHb to protect against peroxynitrite-mediated nitration of free tyrosine is another feature that confirms that these proteins are active catalysts for the isomerization of peroxynitrite. Pietraforte et al. *(36)* have shown that metHb prevents nitration of the dipeptide Ala-Tyr (1 mM) by 1 mM peroxynitrite in a concentration-dependent way. We carried out a similar experiment with different forms of Mb and showed that oxyMb is the species that most efficiently inhibits the nitration of free tyrosine by peroxynitrite. Higher concentrations of metMb were needed to achieve a similar extent of protection, and significant nitration of free tyrosine was obtained also in the presence of large amounts of apoMb and metMbCN. These data further confirm that the heme center of Mb plays a key role to scavenge peroxynitrite. The protection provided by high concentrations of apoMb and metMbCN is likely to be due to the concurrent reaction of peroxynitrite with the two tyrosine, two tryptophan, and/or three methionine residues of the globin. The observation that metMbCN is slightly more efficient than apoMb in protecting free tyrosine from peroxynitrite-mediated nitration suggests that in apoMb, because of partial unfolding of the globin *(47, 48)*, some of these residues may be less accessible. The protection efficiency of metMb can be rationalized by comparing the rate of the protein-catalyzed isomerization of peroxynitrite with that of its spontaneous decay. For instance, under the conditions of our experiment, the rate of the reaction between 25 μM metMb and 200 μM peroxynitrite is 0.72 s^{-1} ($k_{obs} = (2.9 \times 10^4\ M^{-1}\ s^{-1}) \times (25 \times 10^{-6}\ M) = 0.72\ s^{-1}$) and that of the decay of peroxynitrite is 0.3 s^{-1} *(34)*. Thus, in the presence of 25 μM metMb, approximately 70% of peroxynitrite will be converted to nitrate by its reaction with metMb, and 30% will be available for reaction with free tyrosine. In this experiment, we have expressed the amount of nitrotyrosine produced in relative yields, that is, we have arbitrarily set the nitration yield obtained from the reaction with 200 μM peroxynitrite (in the absence of protein) equal to 100%. Thus, if 30%

of peroxynitrite is available for reaction with tyrosine, we expect a relative nitration yield of 30%. This calculated value is in good agreement with the experimental yield obtained, that is, 36 ± 2%.

As the reaction between oxyMb and peroxynitrite is not catalytic, the higher protection provided by this form of Mb in the presence of a large excess of peroxynitrite over the protein is probably due to a combination of the reactions of peroxynitrite with oxyMb and with metMb. Indeed, the half-life of the reaction of 200 μM peroxynitrite with 2.5 μM oxyMb is approximately 64 ms *(25)*, whereas that of the spontaneous peroxynitrite decay is ca. 2.3 s. Thus, the rapid oxidation of oxyMb generates metMb, which catalyzes the decay of excess peroxynitrite, still present in large quantities when all oxyMb is oxidized.

FerrylMb is slightly less efficient than oxyMb as fewer equivalents of peroxynitrite are consumed to generate metMb *(25)*, which also in this case catalyzes the isomerization of the rest of peroxynitrite present after complete reduction of ferrylMb. As expected by comparing the kinetic parameters, metHb and oxyHb were a little less effective than the corresponding Mb forms. The observation that deoxyHb is more efficient than oxyHb is probably also due to the larger value of the rate constant for the reaction between peroxynitrite and deoxyHb *(Boccini, F., Domazou, A. S and Herold. S. (2004) J. Phys. Chem. A 108, 5800-5805)*. In the presence of CO_2, higher concentrations of the proteins, in particular of metMb and oxyMb, are required to achieve similar protection. This observation can also be explained by comparing the rates of all the reactions in the system. In the presence of 1.1 mM CO_2, the observed rate of peroxynitrite decay is 17.4 s^{-1}, whereas the isomerization catalyzed by 25 μM metMb proceeds at a rate of 1.9 s^{-1}. Thus, in the presence of 25 μM metMb, most peroxynitrite (90%) will be available for reaction with free tyrosine, and only 10% will be converted to nitrate by its reaction with the iron center of metMb. This calculated NO_2-Tyr yield (90%) is significantly larger than that obtained experimentally (63 ± 2%). This difference is due to the protection imparted by the globin. Indeed, the addition of 25 μM apoMb or metMbCN reduces the amount of nitration of free tyrosine by 20-25%. Moreover, amino acid analysis of metMb after treatment with an excess of peroxynitrite showed significant nitration of the tyrosine residues *(26)*. In summary, by considering the protection provided both by the globin (20-25%) and by the iron(III) center (10%), the expected NO_2-Tyr yield is 65-70%, a value close to the experimentally obtained value of 63 ± 2%. As discussed above for the reaction in the absence of CO_2, the higher protection imparted

by oxyMb probably derives from the combination of the reactions of peroxynitrite/CO_2 with oxyMb and with metMb.

Under physiological conditions, the protein concentration will always be significantly larger than that of peroxynitrite. Mb and Hb are mostly found in the oxygenated form, with only a small percentage present in the oxidized iron(III) form. Consequently, since the second-order rate constants of the reactions of peroxynitrite with oxyMb and oxyHb are in the same order of magnitude as the catalytic rate constants for its metMb- and metHb-mediated isomerization, oxyHb and oxyMb will be the species primarily involved in the scavenging of peroxynitrite *in vivo*. The deoxygenated forms of Mb and Hb have been shown to react with peroxynitrite at a rate 1-2 order of magnitude larger than that for the reaction with the oxygenated proteins *(24)*. Thus, the reaction with deoxyHb may play a role in venous blood, whereas deoxyMb will scavenge peroxynitrite only under hypoxic conditions, for instance, such as found in ischemia.

3.5.3 COMPARISON OF THE EXTENT OF NITRATION AT 20 AND 37 ºC

We have previously shown that the extent of nitration of the tyrosine residues upon mixing, at 0 or 20 ºC, different forms of Mb and Hb with an excess of peroxynitrite are larger when the reactions are carried out at 20 ºC *(26)*. The results described in this paper show that, in the absence of added CO_2, the NO_2-Tyr yields are even larger at 37 ºC. As already observed at lower temperatures, metMb is always nitrated to a significantly smaller extent than metHb. The catalytic rate constants determined in this work for the metMb- and metHb-mediated isomerization of peroxynitrite at 20 and 37 ºC provide a key to interpret this result. Indeed, both at 20 and at 37 ºC the values of k_{cat} for metMb are 2.5-4 larger than those for metHb. Thus, in the presence of metMb, a larger amount of peroxynitrite is isomerized to NO_3^- and cannot nitrate the tyrosine residues of the protein.

At higher temperatures, all the proteins are nitrated to a larger extent despite the fact that the catalytic rate constants are 2-3 times larger. This result is due to the accelerated decay of peroxynitrite, the process that leads to the reactive species responsible for nitration and that proceeds significantly faster at higher temperatures. For instance, at 20 ºC, the rate of the reaction between 100 μM metMb and 1000 μM peroxynitrite is ca. 3 s^{-1} and that of the decay of peroxynitrite is 0.3 s^{-1}. At 37 ºC, the rate of the protein-catalyzed (100 μM metMb) reaction is 3 times larger (8.8 s^{-1}), whereas that of the decay of peroxynitrite is 5 times larger

($1.5\ s^{-1}$). Interestingly, nitration yields of tyrosine and BSA are only slightly larger at 37 °C. Thus, the significant increase in the nitration of metMb and metHb arises from a decrease in the potency of these proteins to scavenge peroxynitrite rather than from an intrinsic higher nitration efficiency of peroxynitrite at higher temperatures.

At both temperatures studied, oxyMb and oxyHb are nitrated to a larger extent than metMb and metHb, despite the fact that the oxy forms prevent more effectively the nitration of free tyrosine by peroxynitrite (Figure 6). This result suggests that, as has recently been shown by EPR spectroscopy *(41, 49)*, tyrosine radicals are generated in some extent from the reaction of peroxynitrite with oxyHb, and most likely, with oxyMb. A similar EPR spectrum has been observed upon mixing of metHb with peroxynitrite, but its intensity was 52% lower than that obtained with oxyHb *(36)*. As the nitration yields of the two tyrosine residues of oxyHb and metHb are very low, the pathways leading to the tyrosine radicals must be secondary. Nevertheless, the higher radical yields obtained for oxyHb may be responsible for the larger nitration yields of oxyHb (and oxyMb).

A further noteworthy result is the significant increase of nitration of apoMb at 37 °C (almost twice as much as at 20 °C). This result suggests that apoMb may be significantly unfolded at higher temperatures; thus, the tyrosine residues may be more exposed and nitrated more easily.

As expected, since it has been shown that carbon dioxide increases the peroxynitrite-mediated yields of NO_2-Tyr *(38, 50)*, the addition of carbon dioxide in all the systems discussed above led to increased nitration. Moreover, also in the presence of added CO_2, the yields of nitration were mostly larger at a higher temperature. Two exceptions are represented by metMb and metHb. Interestingly, treatment of the iron(III) forms of these proteins with peroxynitrite at 20 or 37 °C led to comparable yields of nitration of their tyrosine residues. This observation can be explained by comparing the rates of all the reactions in the system at the two temperatures. At 20 °C, in the presence of 1.1 mM CO_2, the rate of the reaction between 100 μM metMb and 1000 μM peroxynitrite is ca. $7.7\ s^{-1}$ and that of the decay of peroxynitrite is $17.4\ s^{-1}$. At 37 °C, the rate of the protein-catalyzed (100 μM metMb) reaction is ca. 3.5 times larger ($26.5\ s^{-1}$) and that of the decay of peroxynitrite is also ca. 3.5 times larger ($60\ s^{-1}$). Thus, as both reactions are accelerated to the same extent, no significant changes are observed in the nitration yields.

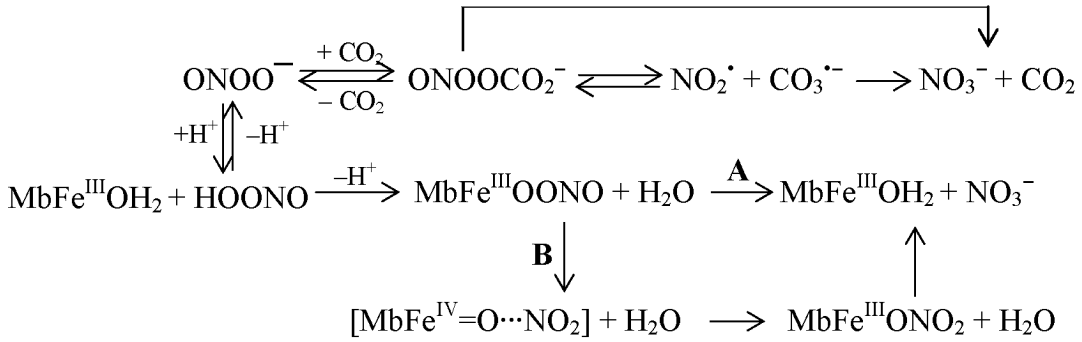
3.6 MECHANISTIC CONSIDERATIONS AND CONCLUSIONS

The work presented here shows conclusively that metMb and metHb catalyze the isomerization of peroxynitrite to nitrate, despite the fact that no changes are observed in the UV-vis spectrum of the proteins upon treatment with peroxynitrite. As shown in Scheme 1, two mechanisms may be formulated to interpret these results. It is conceivable that the first step of the reaction is represented by a ligand substitution reaction. The coordinated water is replaced by peroxynitrite, and the peroxynitrito-complex $MbFe^{III}OONO$ is generated. Unexpectedly, our results suggest that the protonated form of peroxynitrite, HOONO, is the species that reacts with the iron(III) center. Because of the strong hydrogen bond between the distal histidine and the aquo-ligand in metMb, this reaction is likely to proceed at a slow rate. Indeed, it has previously been established that ligand binding to metMb is significantly accelerated in mutants in which the coordinated water is absent or in which the distal histidine has been replaced by an amino acid residue that cannot form hydrogen bonds *(51)*. The metMb peroxynitrito complex has been shown to be unstable at neutral pH and to rapidly decay to metMb and nitrate *(52)*. Thus, if the binding of peroxynitrite is the rate-limiting step, one would expect that $MbFe^{III}OONO$ does not accumulate in concentrations large enough to be detected. Moreover, the UV-vis spectrum of $MbFe^{III}OONO$ is very similar to that of metMb under neutral or acidic conditions *(52)*, a feature that makes its detection even less likely. The corresponding hemoglobin complex $HbFe^{III}OONO$ is more stable, and under alkaline conditions, its increased stability and UV-vis spectrum significantly different from that of the basic form of metHb ($HbFe^{III}OH$) may allow for its detection *(53)*. Nevertheless, ligand substitution reactions of metHb and metMb are significantly slower under alkaline conditions, as the coordinated hydroxide ion is a stronger ligand for iron(III) than the water molecule *(45)*. Thus, if both the rate of formation and the rate of decay are slowed, also in this case $HbFe^{III}OONO$ will not accumulate in concentrations large enough to be detected.

Our previous mechanistic studies of the decay of $MbFe^{III}OONO$ and $HbFe^{III}OONO$ to their corresponding iron(III) forms and nitrate suggest that this reaction proceeds in a concerted way (pathway A in Scheme 1) *(52)*. However, Groves and co-workers proposed an alternative mechanism *(37)*. According to their hypothesis, the reaction of the peroxynitrito complex to the final products proceeds in three steps: homolytic cleavage of the O-O bond of the peroxynitrito ligand with generation of nitrogen dioxide and the ferryl form of the protein,

partial recombination to the nitrate complex, and finally, dissociation of nitrate (pathway B in Scheme 1). Groves and co-workers based their mechanism on the

Scheme 1: Possible pathways for the reaction between metMb and peroxynitrite, in the absence and presence of CO_2.



observation that in the presence of 100-200 μM metMb the nitrate yields generated from the decay of 500 μM peroxynitrite leveled off around 11%. Thus, they suggested that part of $NO_2^•$ eludes the reaction with ferrylMb and undergoes hydrolysis to nitrite and nitrate. However, our data in the presence of a large excess of metMb or metHb (750 μM) show that, if the experimental conditions are chosen so that the decay of peroxynitrite proceeds exclusively via the protein-catalyzed pathway, no nitrite is generated. Thus, no $NO_2^•$ is produced from the peroxynitrite complex, which yields quantitatively nitrate *(52)*. This conclusion is supported by our previous observation that no nitrite is formed when peroxynitrite is allowed to react with 1.6 μM of the effective isomerization catalyst H64A-metMb. Moreover, Pietraforte et al. also obtained nearly quantitative nitrate yields (94-97%) from the decay of 100 μM peroxynitrite in the presence of 500 μM metHb (at pH 7.4) *(36)*.

The values of the catalytic rate constants for the metMb- and metHb-mediated isomerization of peroxynitrite are in the same order of magnitude as that of the reaction between CO_2 and peroxynitrite. Thus, in the presence of physiological amounts of CO_2, metMb and metHb can compete with CO_2 for peroxynitrite, and at least in part, catalyze its isomerization to nitrate (Scheme 1).

Our data at high pH in the presence of 10 mM CO_2 clearly indicate that no direct reaction takes place between $ONOOCO_2^-$ and the iron(III) center of the proteins. Interestingly, Pietraforte et al. arrived to the same conclusion, partly based on experiments

that in our hands gave different results *(36)*. Specifically, they showed that the product distribution of the decay of 100 μM peroxynitrite in the presence of 1.2 mM CO_2 is not influenced by the addition of increasing amounts of metHb (5-500 μM) *(36)*. The only difference between these two experiments is the temperature: from the bicarbonate concentration used to reach a CO_2 concentration of 1.2 mM, it looks as if these experiments were carried out at 37 °C, whereas our data were obtained at 20 °C.

In conclusion, our results confirm and extend previous studies demonstrating that the oxy-, deoxy-, and met-forms of Mb and Hb are efficient scavengers for peroxynitrite *(24-26, 36, 54)*. The catalytic rate constants obtained in this work for the metMb- and metHb-catalyzed isomerization of peroxynitrite are essential to appraise the role of the different protein forms in this scavenging process.

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4 MECHANISTIC STUDIES OF THE ISOMERIZATION OF PEROXYNITRITE TO NITRATE CATALYZED BY DISTAL HISTIDINE METMYOGLOBIN MUTANTS*

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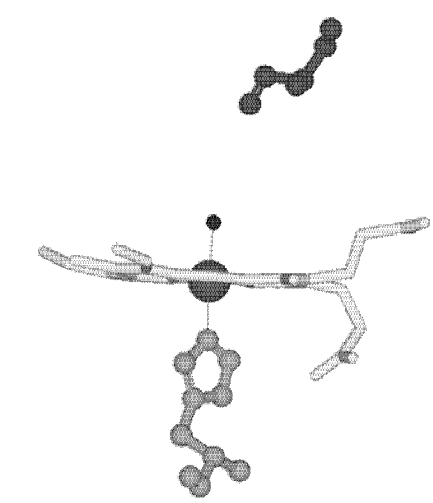
4.1 ABSTRACT

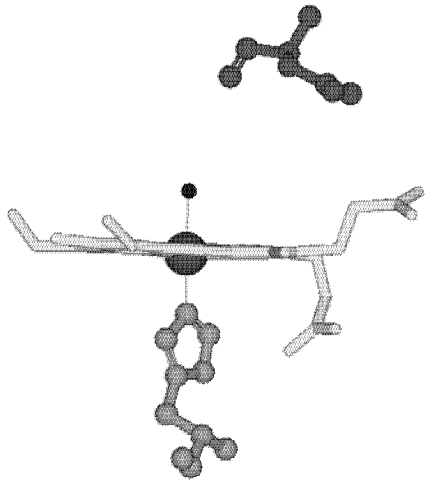
Hemoproteins are known to react with the strong nitrating and oxidizing agent peroxynitrite according to different mechanisms. In this article, we show that the iron(III) forms of the sperm whale myoglobin (swMb) mutants H64A, H64D, H64L, F43W/H64L, and H64Y/H93G catalyze the isomerization of peroxynitrite to nitrate. The two most efficient catalysts are H64A (k_{cat} = (5.8 ± 0.1) × 10^6 M^{-1} s^{-1}, at pH 7.5 and 20 °C) and H64D metMb (k_{cat} = (4.8 ± 0.1) × 10^6 M^{-1} s^{-1}, at pH 7.5 and 20 °C). The pH dependence of the values of k_{cat} shows that HOONO is the species which reacts with the heme. In the presence of physiologically relevant concentrations of CO_2 (1.2 mM), the decay of peroxynitrite is accelerated by these metMb mutants via the concurring reaction of HOONO with their iron(III) centers. Studies in the presence of free added tyrosine show that the metMb mutants prevent peroxynitrite-mediated nitration. The efficiency of the different sw metMb mutants correlates with the value of k_{cat}. Finally, we show that sw WT-metMb is nitrated to a larger extent than horse heart metMb, a result that suggests that the additional Tyr151 is a site of preferential nitration. Again, the extent of nitration of the tyrosine residues of the metMb mutants correlates with the values of k_{cat}.

4.2 INTRODUCTION

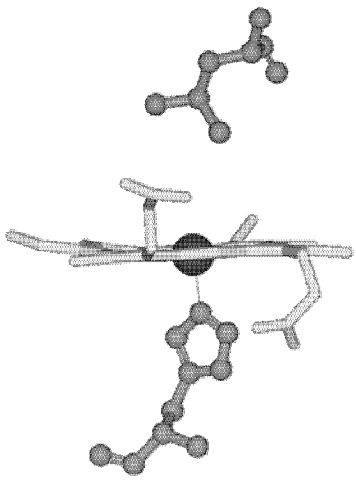
Peroxynitrite *(1)*, a strong oxidizing and nitrating agent generated *in vivo* from the diffusion-controlled reaction between $NO^{\bullet}$ and $O_2^{\bullet -}$ *(2)*, reacts with hemoproteins according to radically different pathways. In general, the rate constants for the reactions of peroxynitrite with hemoproteins are rather large. Thus, when present in large concentrations these proteins can conceivably compete with CO_2, the major target of peroxynitrite *in vivo*. The reaction between the peroxynitrite anion and CO_2 is quite fast (3 × 10^4 M^{-1} s^{-1} at 24 °C) (*3, 4)* and yields the adduct 1-carboxylato-2-nitrosodioxidane ($ONOOCO_2^-$) *(3, 5)*, a nitrating agent stronger than peroxynitrous acid. The iron(III) forms of peroxidases such as horseradish peroxidase, lactoperoxidase, myeloperoxidase, and chloroperoxidase have been reported to catalyze the decay of peroxynitrite by a complex mechanism which involves the high valent oxoiron(IV) (ferryl) species *(6, 8)*. In the presence of phenolic compounds, these peroxidases have been shown to significantly increase the yield of the peroxynitrite-mediated nitration *(7)*. A similar mechanism was observed for the reactions of peroxynitrite with the iron(III) forms



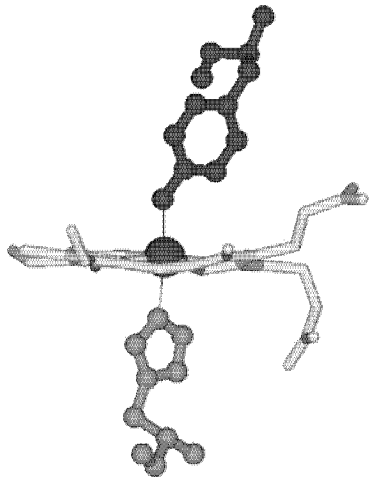
H64A



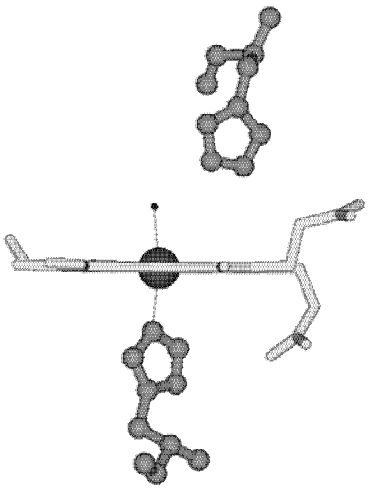
H64D



H64L



H64Y



WT

Figure 1. Heme ligation of, H64A-metMb (PDB No.102M) *(47)*, H64D-metMb (H64D/V68A, PDB No.1LUE) *(16)*, H64L-metMb (in the crystallized double mutant H64L/F43H, PDB No.1OFK, a water molecule is bound to the heme) *(48)*, H64Y-metMb (PDB No.1YMA) *(49)* and wild-type metMb (His64, PDB no. 1YMA) *(46)*.

of cytochrome P450 enzymes such as $P450_{BM-3}$ and $P450_{NOR}$ *(9)*. Oxyhemoglobin (oxyHb) and oxymyoglobin (oxyMb) are oxidized by an excess of peroxynitrite to their corresponding iron(III) forms (metHb and metMb, respectively) in a two-step mechanism: first the proteins are oxidized to their oxoiron(IV) forms, which in the second reaction step are reduced by peroxynitrite *(10)*. Cytochrome *c* oxidase reacts with peroxynitrite to generate $NO^{\bullet}$ (in its reduced iron(II) form) or nitrite (in its iron(III) form) *(11, 12)*. However, high peroxynitrite concentrations irreversibly inhibit this enzyme *(11, 12)*.

We have recently shown that human metHb and horse heart metMb (hh metMb) catalyze, albeit not very efficiently, the isomerization of peroxynitrite to nitrate *(13)*. The reduced reactivity of the iron(III) form of these proteins contrasts the intrinsic high reactivity of iron(III)-porphyrin complexes toward peroxynitrite and is due to the strong hydrogen bond present in metMb and metHb between the distal histidine (His64) and the water molecule coordinated to the iron(III) center (Figure 1) *(13)*. Indeed, synthetic water-soluble iron(III)-porphyrin complexes efficiently catalyze the isomerization of peroxynitrite to nitrate, the best catalyst being $[Fe(III)(TMPyP)]^{5+}$ ($k_{cat} = 1.6 \times 10^6\ M^{-1}\ s^{-1}$, at pH 7.4 and 37 °C) *(14)*.

In this article, we extended our preliminary studies *(15)* and investigated the reactivity toward peroxynitrite of the iron(III) forms of the sperm whale Mb mutants, H64A, H64D, H64L, F43W/H64L, and H64Y/H93G. In all these mutants, the distal histidine is replaced by an amino acid which cannot undergo a hydrogen bond with the water molecule bound to the heme (Figure 1). Asp64 could theoretically undergo a hydrogen bond, but it has been shown by X-ray crystallography that it does not point to the heme but toward Arg45 (Figure 1) *(16)*. In H64D-metMb, the water molecule bound to the iron is stabilized by hydrogen bonds to other water molecules present in the distal pocket *(16)*. Moreover, the H64L Mb mutant was chosen because the iron(III) center is pentacoordinated, the coordinated water being absent *(17)*. We have recently shown that, upon mixing with different forms of hh Mb, peroxynitrite induces not only formation of nitrotyrosine but also of different nitrotryptophan isomers *(18)*. Thus, the double mutant F43W/H64L Mb was investigated to determine the extent of nitration of a tryptophan residue close to the heme. Finally, the last Mb mutant studied, H64Y/H93G, exhibits a completely different axial ligation pattern. In its iron(III) form, only the tyrosine residue Tyr64 is coordinated to the heme on the distal side (compare structure of H64Y-metMb in Figure 1). The originally coordinated proximal histidine (His93) is replaced by the non coordinating glycine. Thus, reaction of peroxynitrite with H64Y/H93G metMb will

necessarily take place on the proximal side of the heme, and peroxynitrite will have to follow a different pathway to reach the iron center. We have carried out kinetic studies of the decay of peroxynitrite catalyzed by sw metMb and by these five metMb mutants at different pH values and CO_2 concentrations. In addition, we have analyzed the nitrogen-containing products of these reactions, the efficiency of these proteins to protect peroxynitrite-mediated nitration of free tyrosine, and the extent of tyrosine (and tryptophan) nitration upon addition of an excess peroxynitrite.

4.3 EXPERIMENTAL PROCEDURES

4.3.1 CHEMICALS

Buffers were prepared from KH_2PO_4/K_2HPO_4 (Fluka) with deionized Milli-Q water. Sodium nitrite, sodium nitrate, 3-nitrotyrosine, L-tyrosine, ammonium sulfamate, trifluoroacetic acid, hydrogen peroxide, calcium chloride, and acetonitrile (99.8%) were obtained from Fluka. Sodium bicarbonate was purchased from Merck. Pronase was obtained from Roche Molecular Biochemicals. The Enhanced Chemiluminescence kit and the nitrocellulose membrane were purchased from Amersham Biosciences and Schleicher & Shuell, respectively. Nitrogen monoxide was obtained from Linde and passed through a column of NaOH pellets to remove higher nitrogen oxides before use.

4.3.2 PEROXYNITRITE, CARBON DIOXIDE, AND PROTEIN SOLUTIONS

Peroxynitrite was synthesized from KO_2 and nitrogen monoxide according to ref 19 and stored in small aliquots at –80 °C. The peroxynitrite solutions contained variable amounts of nitrite (maximally 50% relative to the peroxynitrite concentration) and no hydrogen peroxide. The stock solution was diluted with 0.01 M NaOH, and the concentration of peroxynitrite was determined spectrophotometrically before each experiment by measuring the absorbance at 302 nm [$\varepsilon_{302} = 1705\ M^{-1}\ cm^{-1}$] *(20)*.

Experiments in the presence of CO_2 were carried out by adding the required amount of a freshly prepared 0.5 or 1 M sodium bicarbonate solution to the protein solutions as described in detail in ref 13. The values for the constant of the hydration-dehydration equilibrium $CO_2 + H_2O \leftrightarrow H^+ + HCO_3^-$ were derived from ref 21 by taking into consideration the ionic strength of the solutions. After addition of bicarbonate, the protein solutions were

allowed to equilibrate for at least 5 min at 20 °C. For the experiments carried out in the absence of CO_2, the buffers and the 0.01 M NaOH solutions were thoroughly degassed.

Over-expressed and purified recombinant sperm whale Mb (WT-Mb) was purchased from Sigma. MetMb was prepared by adding a small amount of potassium hexacyanoferrate(III) and purified over a Sephadex G25 column by using a 0.1 M phosphate buffer solution (pH 7.0) as the eluent. H64A, H64L, and H64D sperm whale Mb mutants were prepared according to refs 22 and 23. The Phe43→Trp mutation was introduced by the polymerase chain reaction (PCR)-based method as reported before *(22, 23)*. The His64→Tyr mutation on H93G sperm whale Mb was introduced by the cassette mutagenesis *(Roach, M. P., Ozaki, S.I., Watanabe, Y. (2000) Biochemistry 39, 1446-1454)*. The concentrations of WT-metMb and the iron(III) form of the sperm whale Mb mutants were determined by measuring (at pH 7.0) the absorbance at 409 nm for WT-metMb [ε_{409} = 157 mM^{-1} cm^{-1}] *(24)*, at 407 nm for H64A and H64D metMb [ε_{407} = 136 mM^{-1} cm^{-1} and ε_{407} = 154 mM^{-1} cm^{-1}, respectively], at 393 nm for H64L metMb [ε_{393} = 103 mM^{-1} cm^{-1}], at 394 nm for F43W/H64L metMb [ε_{394} = 103 mM^{-1} cm^{-1}], and at 406 nm for H64Y/H93G metMb [ε_{406} = 115 mM^{-1} cm^{-1}]. All the absorption spectra were collected on a UVIKON 820 spectrophotometer.

4.3.3 STOPPED-FLOW EXPERIMENTS

The kinetics of peroxynitrite decay in the presence of WT-metMb and its mutants were studied with an Applied Photophysics SX18MV-R single-wavelength stopped-flow instrument. The length of the cell in the spectrophotometer is 1 cm, and the mixing time of the instrument is about 2 ms. The kinetic traces were collected at 302 nm, and the data were analyzed with Kaleidagraph, version 3.52. The results of the fits of the traces (averages of at least 5-8 single traces) from at least three experiments were averaged to obtain each observed rate constant, given the corresponding standard deviation. The protein solutions of the required concentrations were prepared under aerobic conditions in 0.1 M phosphate buffer. In general, the pH of the buffer was slightly lower than the desired final pH, since upon mixing with the alkaline peroxynitrite solution the pH always rose between 0.3 and 0.6 units. For the experiments carried out in the presence of CO_2, the pH of the buffer had to be even lower, because addition of sodium bicarbonate also raised the pH of the protein solutions. Obviously, the concentration of bicarbonate needed to obtain a final (after mixing) concentration of 1.2 mM CO_2 depended on the pH and varied from 2 to 65 mM as the pH was

raised from 6.0 to 8.5. Thus, the required initial pH of the protein solution had to be determined separately for each experiment. Specifically, for the experiments at pH 6.0, the protein solutions had a pH of 5.7 (in the absence of CO_2) or of 5.5 (in the presence of CO_2); for the experiments at pH 6.5, the protein solutions were prepared in phosphate buffer pH 6.2 (in the absence of CO_2) or pH 6.0 (in the presence of CO_2); for the experiments at pH 7.0, the protein solutions had a pH of 6.7 (in the absence of CO_2) or of 6.5 (in the presence of CO_2); and for the experiments at pH 7.5, the protein solutions were prepared in phosphate buffer pH 7.1 (in the absence of CO_2) or pH 6.8 (in the presence of CO_2). Finally, for the experiments under alkaline conditions at pH 8.0, the protein solutions were prepared in phosphate buffer pH 7.4 (in the absence of CO_2) or pH 7.0 (in the presence of CO_2); for those at pH 8.5, the protein solutions had a pH of 7.8 (in the absence of CO_2) or of 7.5 (in the presence of CO_2). If not specified, all the measurements were carried out at 20 °C and the pH was always measured at the end of the reactions for control.

Rapid-scan UV-vis spectra were measured by using an OLIS RSM 1000 rapid scanning monochromator stopped-flow apparatus (On-Line Instrument System, Inc). The solutions were prepared as described above for the single-wavelength stopped-flow experiments.

4.3.4 DETERMINATION OF NITRITE AND NITRATE

Product analysis was carried out as described previously *(25)* by anion chromatography with conductivity detection with a Metrohm instrument (IC Separation Center 733, IC Detector 732, and IC pump 709) equipped with an Anion SUPER-SEP (6.1009.000) column and an Anion SUPER-SEP (6.1009.010) precolumn. Calibration curves were obtained by measuring 5-10 standard sodium nitrite and sodium nitrate solutions in 5 mM phosphate buffer. The samples were prepared by mixing 100 µL of WT-metMb and metMb mutants (1-25 µM in 0.1 M phosphate buffer pH 6.9 or in 0.1 M phosphate buffer pH 6.8 containing 22 mM sodium bicarbonate) at room temperature with 100 µL of an ice-cooled peroxynitrite solution (200 µM in 0.01 M NaOH) while vortexing. The reaction mixture was diluted 1:10 with water and analyzed within ca. 5 min. At least two analyses of three separate experiments were carried out for each protein. The contamination of nitrite and nitrate in peroxynitrite was determined as reported recently *(13)*. Usual nitrite and nitrate

contaminations were in the range 20-50% and 0-10% of the peroxynitrite concentration, respectively.

4.3.5 ANALYSIS OF THE FREE 3-NITROTYROSINE CONTENT

The reaction of peroxynitrite with sperm whale WT-metMb and metMb mutants in the presence of L-tyrosine was carried out at room temperature by adding 100 µL of an ice-cooled peroxynitrite solution (2 mM in 0.01 M NaOH) to 900 µL of a solution containing tyrosine (112 µM) and different protein concentrations (0-10 µM in 0.05 M phosphate buffer pH 7.4), in the absence and presence of 1.2 mM CO_2. All the samples were analyzed by reverse-phase HPLC on a Hewlett-Packard Series 1050 apparatus with a series 1100 UV-vis detector, equipped with a VYDAC 218TP54 Protein & Peptide C18-Column (250 × 4.6 mm^2), and fluorescence detection (222 and 400 nm, excitation and emission wavelength, respectively) as described previously *(18)*. Nitrotyrosine was eluted at ca. 7.5 min after injection and was detected contemporaneously at 220, 280, 350, and 400 nm. NO_2-Tyr was quantified by measuring a calibration curve of 5-10 nitrotyrosine standard solutions.

4.3.6 ANALYSIS OF THE PROTEIN-BOUND 3-NITROTYROSINE CONTENT

The reaction of peroxynitrite with WT-metMb and its mutants was carried out at 20 °C as described previously *(18)*. In brief, 20 µL of an ice-cooled peroxynitrite solution (different concentrations in 0.01 M NaOH) was added as a bolus while vortexing to 180 µL of a protein solution (112 µM in 0.1 M phosphate buffer, pH 7.0) kept at room temperature. After ca. 30 min, nitrite was removed by adding ca. 200 µL of an ammonium sulfamate solution (100 mM in 0.5 M HCl) and subjected to acid hydrolysis as described previously *(18)*. Finally, the samples were analyzed by HPLC as described above.

4.3.7 WESTERN BLOT ANALYSIS

After treatment with peroxynitrite as described above for the HPLC samples, the proteins were boiled for 3 min at 95 °C in Laemmli sample buffer containing no reducing agents. Samples (5 µg of per lane) were subjected to an 11% SDS-polyacrylamide gel, and were electrophoretically transferred to a pure nitrocellulose membrane in 25 mM Tris, 192 mM glycine, and 20% methanol. Membranes were blocked overnight at 4 °C with 5% dry milk in phosphate-buffered saline containing 0.2% Tween 20. Then the membranes were

probed with either monoclonal or a polyclonal antibody to 3-nitrotyrosine as described previously *(18)*. Complexes were detected by using peroxidase conjugated goat anti-mouse or goat anti-rabbit (1:5000) IgG and then visualized by using the enhanced chemiluminescence detection kit.

4.3.8 PRONASE DIGESTION OF NITRATED PROTEINS

Proteins for pronase digestion were treated with 20 equiv of peroxynitrite at 20 °C and pH 7.0, as described in detail (see section *3.35 and 3.36*) above for the HPLC samples. Nitrated proteins (200 µL) were mixed with 200 µL of ammonium sulfamate (50 mM) to remove excess nitrite prior to digestion. After ca. 30 min, $CaCl_2$ was added to the samples (100 µM) to a final concentration of 10 mM. After addition of Pronase (1 mg/mL), the samples were incubated for 12 h at 40 °C, and then another 1 mg/mL Pronase was added and incubated again for 12 h at 40 °C. Before HPLC separation the samples were dried by speed-vac (Eppendorf concentrator 5301 connected to a vacuum pump) at 45 °C, dissolved in 0.1% TFA, and analyzed by HPLC as described above.

4.3.9 STATISTICS

The experiments reported in this article were carried out at least in triplicate on independent days. The results are given as mean values of at least three experiments plus or minus the corresponding standard deviation.

4.4 RESULTS AND DISCUSSION

4.4.1 KINETIC STUDIES AT PH 7.5

Kinetic studies of the decay of peroxynitrite (100 µM) in the presence of the sperm whale WT-metMb, the distal histidine mutants (H64L, H64A, and H64D metMb), and the double mutants (F43W/H64L and H64Y/H93G metMb) were performed by single-wavelength stopped-flow spectroscopy at 20 °C. The traces were collected at 302 nm, the characteristic absorbance maximum for peroxynitrite *(see Figure C in Appendices)*. All the measured traces could be fitted well to a single-exponential expression. For all proteins studied, the observed peroxynitrite decay rates increased linearly with increasing protein concentration (Figure 2 and 3). The values of k_{cat} obtained from the linear fits are summarized in Table 1. In similar studies with horse heart metMb, we have previously

shown that the acceleration of the rate of decay of peroxynitrite is due to its reaction with the heme center of the protein *(13)*. Indeed, we showed that the heme-free apoMb or the heme-blocked MbFe(III)CN do not catalyze the decay of peroxynitrite *(13)*.

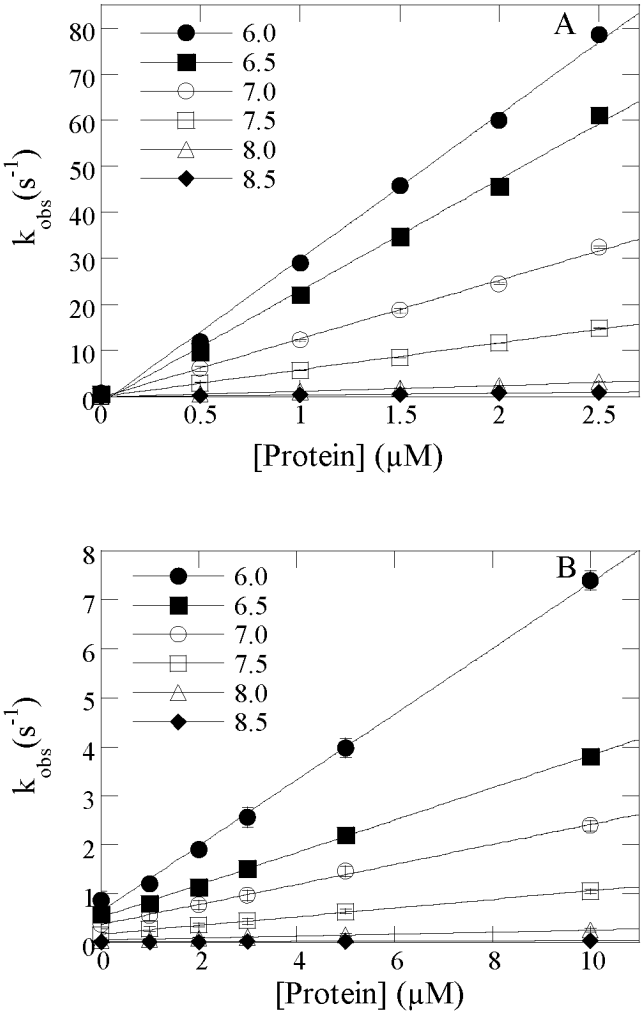


Figure 2. Plots of k_{obs} versus different concentrations of (A) H64A metMb and (B) H64L metMb for the protein-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer in the pH range 6.0-8.5 at 20 °C.

The values of the rate constants of the reactions of peroxynitrite with H64A and H64D metMb are among the largest measured for reactions with peroxynitrite *(26)* and are comparable to those of the most efficient iron(III) porphyrin catalysts such as $[Fe(III)TPPS]^{3-}$ ($k_{cat} = 8.6 \times 10^5$ M^{-1} s^{-1}, at pH 7.4 and 37 °C) and $[Fe(III)TMPyP]^{5+}$ ($k_{cat} = 1.6 \times 10^6$ M^{-1} s^{-1},

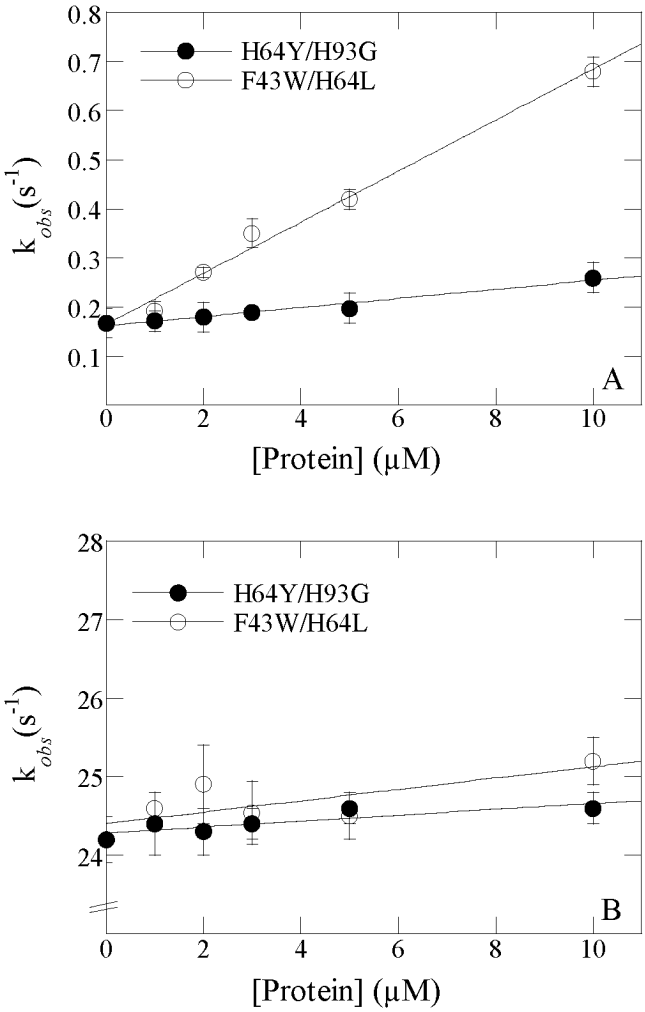


Figure 3. Plots of k_{obs} versus protein concentrations for the protein-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer pH 7.5 and 20 °C. Data in the absence (A) and presence (B) of 1.2 mM CO_2.

at pH 7.4 and 37 °C) *(14)*. Interestingly, the only protein containing a pentacoordinated heme (H64L metMb) was not the most efficient catalyst: the two most active mutants were H64A and H64D metMb, which both have a water molecule as the sixth ligand of the iron center. As leucine is larger than alanine and aspartic acid, our results indicate that, when the hydrogen bond that stabilizes the coordinated water is not present, the reactivity toward peroxynitrite is regulated mostly by steric factors. Indeed, substitution of Phe43, a residue close to the heme on its distal side, with the larger Trp residue also slightly reduced the reactivity of the heme toward peroxynitrite (compare the k_{cat} value of F43W/H64L metMb with that of H64L metMb). The H64Y/H93G metMb mutant was the least efficient catalyst (Table 1): its k_{cat} value was even lower than that of sw WT-metMb, which is somewhat lower

than that recently reported for hh metMb under the same conditions (k_{cat} = 2.9 × 10^4 M^{-1} s^{-1}) *(13)*.

Table 1. Summary of the catalytic rate constants for the protein-catalyzed decay of peroxynitrite (100 μM) measured at pH 7.5 and 20 °C in the presence of different amounts of CO_2

Proteins	No CO_2	0.6 mM CO_2[c]	1.2 mM CO_2
H64A metMb	(5.8 ± 0.1) × 10^6	(5.0 ± 0.2) × 10^6	(5.4 ± 0.1) × 10^6
H64D metMb	(4.8 ± 0.1) × 10^6	(3.8 ± 0.1) × 10^6	(4.9 ± 0.1) × 10^6
H64L metMb	(5.7 ± 0.1) × 10^4	(5.2 ± 0.4) × 10^4	(6.1 ± 1.8) × 10^4
F43W/H64L metMb	(5.2 ± 0.2) × 10^4	*nd*[a]	(7.2 ± 0.3) × 10^4
H64Y/H93G metMb	(0.9 ± 0.1) × 10^4	*nd*[a]	(3.7 ± 0.1) × 10^4
swWT- metMb	(1.6 ± 0.1) × 10^4	*nd*[a]	*nd*[a]
hh metMb[b]	(1.2 ± 0.1) × 10^4	*nd*[a]	(3.9 ± 0.2) × 10^4

[a] Not determined. [b] Reference 13. [c] *see Figure D in Appendices*.

This observation suggests that the proximal side of the heme is less accessible to peroxynitrite. Alternatively, the lower catalytic activity may be attributed to the reduced Lewis acidity of the iron center in H64Y/H93G metMb, a consequence of the tyrosinate ligand bound to the heme. Interestingly, a correlation seems to be present between the second-order rate constants of azide binding to H64A-, H64L-, and WT-metMb and the catalytic rate constant for the protein-catalyzed decay of peroxynitrite. At pH 7.0 and 20-25 °C, the values of the azide binding constants also follow the order H64A-metMb >> H64L-metMb > WT-metMb (2000, 34, and 2.9 mM^{-1} s^{-1}, respectively) *(17)*. Indeed, it has been shown that azide binding is also strongly influenced by the size of the distal ligands *(17)*.

Analogous kinetic studies were carried out in the presence of different amounts of CO_2. For all proteins studied, the measured traces could be fitted well to a single-exponential expression. As expected, the values of the observed rate constants were significantly larger than those measured in the absence of CO_2 (Figure 3) because of the rapid reaction of peroxynitrite and CO_2 *(4)*.

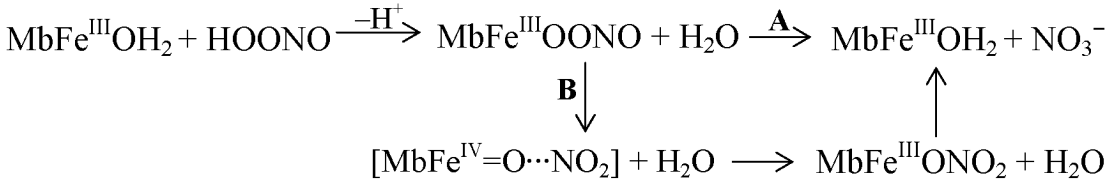
However, as summarized in Table 1, no significant changes were found between the catalytic rate constants (obtained from the linear fit of the plot of the observed rate constants

versus protein concentrations) determined in the absence or in the presence of either 0.6 or 1.2 mM CO_2 *(27)*. As recently observed for the reaction of peroxynitrite with metMb and metHb *(13)*, the results obtained here suggest that also in the presence of CO_2 the metMb mutants accelerate the decay of peroxynitrite via the fast concurring reaction between peroxynitrite and the iron(III) center of the proteins. For all Mb mutants studied, we followed their reactions with peroxynitrite, both in the absence and presence of CO_2, also by rapid-scan spectroscopy in the range 480-650 nm (each millisecond, up to 50 s). In all cases, no changes were observed in the UV-vis spectra of the proteins upon addition of an excess of peroxynitrite *(see Figure E in Appendices)*, in analogy to the parallel reaction with hh metMb and human metHb *(13)*. We have previously reported that the Fe(III)OONO complex, generated as an intermediate during the reaction of $MbFeO_2$ or $HbFeO_2$ with $NO^{\bullet}$, can be detected only under alkaline conditions *(28, 29)*. Moreover, at pH 9.5, the metMb and metHb-peroxynitrite complexes decay to nitrate and the corresponding iron(III) form of the proteins without formation of observable high valent intermediates *(28, 29)*. At pH 7.0, the $NO^{\bullet}$ – mediated oxidation of oxyMb and oxyHb yields metMb and metHb, respectively, without formation of any detectable intermediates *(28, 29)*. Taken together, these previous results are in excellent agreement with the data presented here. Binding of peroxynitrite to the iron(III) center of the metMb mutants is likely to be the rate-determining step, followed by isomerization and dissociation of nitrate. Thus, no intermediates can be observed during these reactions.

The formation of nitrate and metMb from the intermediate MbFe(III)OONO can proceed according to two possible mechanisms: the coordinated peroxynitrite either isomerizes to free nitrate in a concerted mechanism (pathway A in Scheme 1) or generates an oxoiron(IV)/nitrogen dioxide intermediate which rapidly recombines to the metMb-nitrato complex from which nitrate dissociates (pathway B in Scheme 1). We have previously shown that, under alkaline conditions the formation of metMb and nitrate from MbFe(III)OONO (generated from the reaction between oxyMb and $NO^{\bullet}$) must proceed in a concerted way according to pathway A (in Scheme 1) *(29)*. Indeed, the rate of dissociation of nitrate from $MbFe(III)NO_3$ is significantly slower than that of MbFe(III)OONO decay *(29)* and would thus have to be the rate-determining step of the reaction between oxyMb and $NO^{\bullet}$. In pathway B, the oxoiron(IV) intermediate can be detected only if its reaction with $NO_2^{\bullet}$ is the rate-determining step. Interestingly, Groves and co-workers recently reported that the reaction of

peroxynitrite with the H64D/V68L sperm whale metMb mutant leads to the formation of detectable amounts of the high valent oxoiron(IV) form *(30)*.

Scheme 1. Possible Mechanisms for the MetMb-Catalyzed Isomerization of Peroxynitrite



The intermediate produced from the reaction of CO_2 with $ONOO^-$, $ONOOCO_2^-$, has been shown to partly generate $CO_3^{\bullet-}$ upon its decomposition *(5, 31)*. The yield of radicals formed from this decay reaction is still a matter of discussion. Indeed, it has been proposed that 30% *(32, 33)* or less than 5% *(34)* of $ONOOCO_2^-$ decays to form the radicals $NO_2^\bullet$ and $CO_3^{\bullet-}$. Our data suggest that the heme center of the iron(III) form of the Mb mutants does not react with these radicals. Indeed, preliminary pulse radiolysis studies of the reaction between hh metMb and $CO_3^{\bullet-}$ show that the iron center is not oxidized and that $CO_3^{\bullet-}$ reacts only with amino acids of the globin *(35)*.

4.4.2 pH DEPENDENCE OF THE CATALYTIC RATE CONSTANTS

Kinetic studies of the decay of peroxynitrite in the presence of different concentrations of H64A and H64L metMb were carried out in the pH range 6.0-8.5 (at 20 °C). As shown in Figure 2 and summarized in Table 2, for both proteins the values of k_{cat} increased with decreasing pH. At pH 6.0 the values of the catalytic rate constants for H64A and H64L metMb were nearly 2 orders of magnitude larger than those obtained at pH 8.5. A possible explanation for this observation is that peroxynitrous acid (ONOOH) is the species that reacts with the iron center of these proteins. If one assumes that the pH dependence originates from a single ionizable group, the relationship given in eq 1 exists between k_{cat} and the proton concentration.

$$k_{cat} = (k_{cat'} \times [H^+])/(K + [H^+]) \qquad (1)$$

Table 2. Summary of the catalytic rate constants for the decay of peroxynitrite (100 μM) measured at different pH and 20 °C in the absence and presence of CO_2.

pH	H64A (10^6 $M^{-1}s^{-1}$)		H64L (10^4 $M^{-1}s^{-1}$)	
	No CO_2	1.2 mM CO_2	No CO_2	1.2 mM CO_2
6.0	31 ± 1	*nd*	67 ± 1	*nd*
6.5	24 ± 1	23 ± 1	33 ± 1	66 ± 1
7.0	13 ± 1	*nd*	20 ± 1	*nd*
7.5	5.8 ± 0.5	5.4 ± 0.5	5.7 ± 0.1	5.4 ± 0.2
8.0	1.2 ± 0.1	*nd*	2.0 ± 0.1	*nd*
8.5	0.40 ± 0.01	0.10 ± 0.03	0.24 ± 0.01	0.42 ± 0.01

nd: not determined

In eq 1, $k_{cat'}$ stays for the pH-independent catalytic rate constant and K for the dissociation constant of the protonable group. Despite the narrow pH range studied, 6.0–8.5, the experimental data could be fitted rather well to eq 1. The best fits for the two reactions gave values of pK = 6.7 ± 0.5 and $k_{cat'}$ = (3.7 ± 0.1) × 10^7 M^{-1} s^{-1} for H64A metMb and values of pK = 6 ± 1 and $k_{cat'}$ = (1.1 ± 0.1) × 10^5 M^{-1} s^{-1} for H64L metMb *(see Figure F in Appendices)*. Although a large error was associated with the value of the pK obtained for the reaction with H64L metMb, these results support the hypothesis that HOONO (pK = 6.8) *(36)* is the species that reacts with the iron(III) center of these proteins.

This result was unexpected, as it might rather have been anticipated that the positively charged [(porphyrin)Fe(III)OH_2]$^+$ complex reacts preferentially with the deprotonated form $ONOO^-$. Interestingly, previous studies of the reaction between oxyMb and peroxynitrite showed that HOONO is the reactive species also in this system *(10)*. Taken together, these results may suggest that the diffusion of HOONO toward the heme is facilitated compared to that of $ONOO^-$.

The identical pH dependence of k_{cat} for H64A and H64L metMb suggests that the water molecule bound to the heme in H64A metMb (and absent in H64L metMb) is not responsible for the decrease of the value of k_{cat} at higher pH (deprotonation of the coordinated H_2O can generate a hydroxide, which is likely to be more difficult to displace). Finally, a last explanation for the observed pH dependence of the k_{cat} values may be that conformational changes take place in the protein under acidic conditions and cause the observed acceleration.

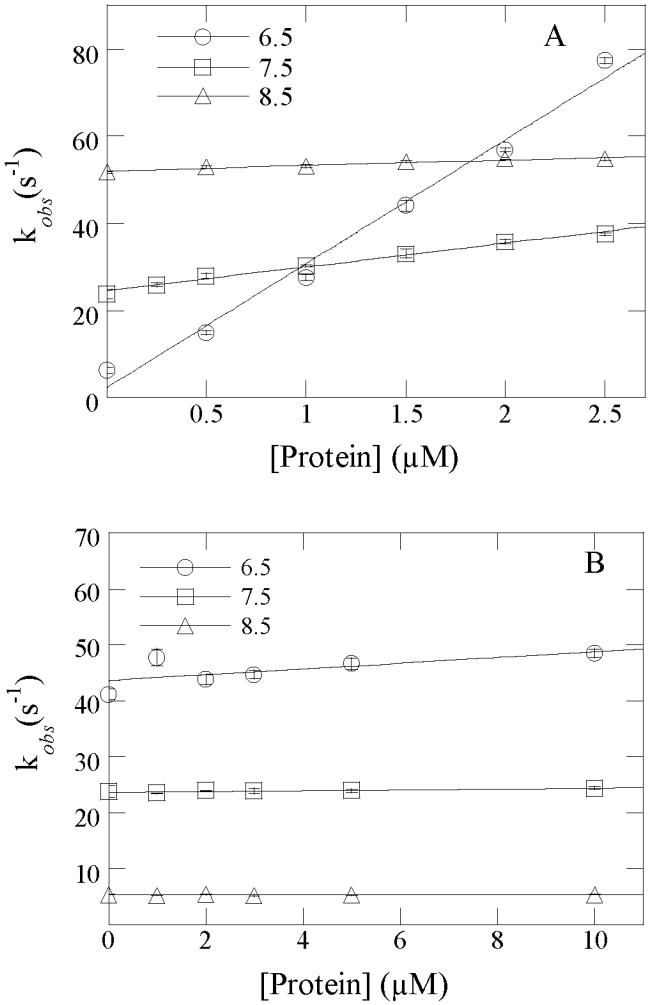


Figure 4. Plots of k_{obs} versus different concentration of H64A (A) and H64L (B) for the decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer in the pH range 6.5–8.5 at 20 °C. Data in the presence of 1.2 mM CO_2.

Analogous kinetic studies at different pH values were carried out with H64A and H64L metMb also in the presence of 1.2 mM CO_2. As summarized in Table 2, the catalytic rate constants increased with decreasing pH. Moreover, in most cases the values obtained in the presence of added CO_2 were nearly identical to those measured in its absence (Figure 4). This result supports the hypothesis that the acceleration observed is independent of CO_2 and always arises from the reaction of HOONO with the iron(III) center of the protein. To further confirm this hypothesis, we studied the reactions of H64A and H64L metMb with peroxynitrite under alkaline conditions (pH 8.0) and in the presence of a large excess of CO_2 (5 mM). Under these conditions, essentially no HOONO is left after the mixing time. As

shown in Figure 5, the decay rate of peroxynitrite was not affected by addition of increasing amounts of either of the proteins. The large errors associated with the values of k_{obs} and the scattering of the data are due to the experimental problems linked to the preparation of different solution with exactly the same CO_2 concentration and pH. Taken together, our results show that the acceleration of the decay of peroxynitrite observed in the presence of physiologically relevant CO_2 concentrations (1.2 mM) arise from the concurring reaction of peroxynitrite with the iron(III) center of the proteins. However, with the results obtained from these kinetic studies it is not possible to find out whether the heme center of Mb mutants

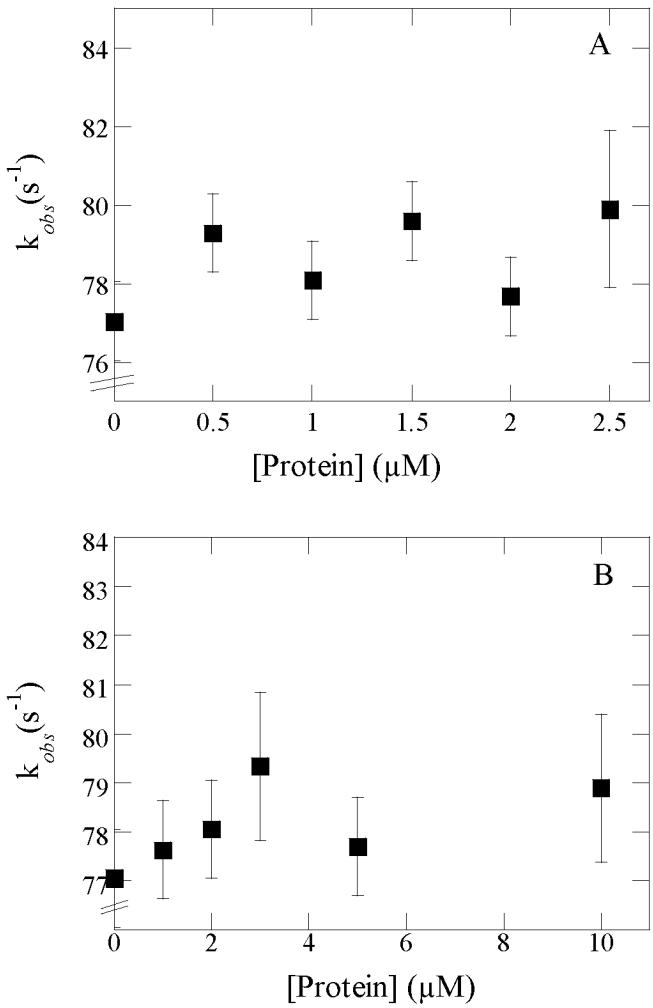


Figure 5. Plots of k_{obs} versus different concentration of H64A (A) and H64L (B) for the decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer in the presence of 10 mM CO_2, pH 8.0 (20 °C).

reacts with $ONOOCO_2^-$, the adduct generated from the reaction of $ONOO^-$ with CO_2, or with the products of its decomposition. Nevertheless, since during the reaction of the metMb mutants with peroxynitrite no changes were observed in the UV-vis spectra, and since $CO_3^{\bullet-}$ does not oxidize metMb (see above), reactions may take place only with the amino acids residues of the globin.

4.4.3 ANALYSIS OF THE NITROGEN-CONTAINING PRODUCTS

The products of the protein-catalyzed decay of peroxynitrite were analyzed by ion chromatography with conductivity detection. As expected *(37)*, in the absence of the proteins, the decomposition of peroxynitrite (100 μM) yielded 67 ± 3% nitrate and 32 ± 3% nitrite (at pH 7.0). As summarized in Table 3, addition of different amounts of the proteins led to a concentration-dependent increase of the amount of nitrate formed, accompanied by a corresponding decrease of the nitrite yields. The efficiency of the proteins to catalyze the

Table 3. Distribution of the products of peroxynitrite (100 μM) decay in the presence of WT-Mb and Mb mutants (in 0.05 M phosphate buffer pH 7.0, and 20 °C), in the absence and presence of 1.1 mM CO_2. The data are expressed as percentage yields.

[Mb] (μM)	No added CO_2			1.2 mM CO_2		
	NO_2^-	NO_3^-	Total	NO_2^-	NO_3^-	Total
No protein	32 ± 3	67 ± 3	99	15 ± 3	87 ± 2	102
H64A (1μM)	3 ± 1	95 ± 1	98	2 ± 3	99 ± 4	101
H64A (5 μM)	0	100 ± 2	100	0	100 ± 3	99
H64D (1 μM)	5 ± 2	96 ± 3	101	3 ± 1	97 ± 2	100
H64D (5 μM)	0	100 ± 3	100	0	100 ± 2	100
H64L (1 μM)	29 ± 3	73 ± 4	102	20 ± 3	81 ± 3	101
H64L (5 μM)	21 ± 2	81 ± 2	102	18 ± 2	81 ± 2	99
H64L (25 μM)	10 ± 2	91 ± 3	101	8 ± 2	93 ± 2	101
F43W/H64L (25)	15 ± 2	86 ± 2	101	13 ± 4	87 ± 3	100
H64Y/H93G (25M)	20 ± 2	80 ± 3	100	18 ± 3	84 ± 3	102
WT-Mb (25μM)	24 ± 3	77 ± 2	101	20 ± 2	82 ± 3	102

isomerization of peroxynitrite to nitrate correlated with the value of k_{cat}. Thus, addition of 5 μM of either of the two best catalysts, H64A or H64D metMb, led to complete isomerization

of peroxynitrite (100 μM) to nitrate, and no nitrite could be detected. In contrast, larger amounts of all other proteins were required for complete conversion of peroxynitrite to nitrate. These results are in agreement with our previous observation that addition of a large amount (750 μM) of hh metMb or human metHb also leads to the quantitative isomerization of peroxynitrite to nitrate *(13)*. Taken together, our data indicate that when the decomposition of peroxynitrite occurs exclusively via the heme, either in the presence of a very efficient catalyst such as H64A metMb or by addition of very large amounts of the ineffective catalyst hh metMb, no nitrite is generated. This conclusion is in contrast to the results of Groves and co-workers *(38)* who observed that a constant level of approximately 10% nitrite was produced from the decay of peroxynitrite even in the presence of large concentrations of metMb. According to the mechanism proposed by these authors (pathway B in Scheme 1) *(38)*, nitrite is generated from $NO_2^{\bullet}$, which partly eludes the reaction with ferrylMb and undergoes hydrolysis to nitrite and nitrate.

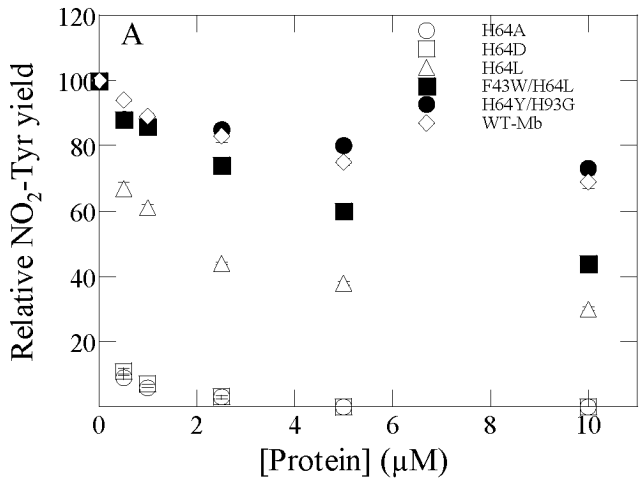
As expected, in the presence of 1.2 mM CO_2 the yield of nitrate generated from the decay of 100 μM peroxynitrite (in the absence of the proteins, at pH 7.0 and 20 °C) was significantly larger: we obtained 15 ± 3% nitrite and 87 ± 2% nitrate. As summarized in Table 3, also under these conditions addition of small amounts (1-5 μM) of the active catalysts H64A and H64D metMb led to the complete conversion of peroxynitrite to nitrate. In contrast, in the presence of 25 μM of the less effective catalysts (H64L, H64L/F43W, H64Y/H63G, and WT-metMb), the products distribution remained approximately unchanged. These results are in agreement with the hypothesis that the best Mb catalysts can efficiently isomerize the peroxynitrite that eludes reaction with CO_2.

4.4.4 PROTECTION AGAINST PEROXYNITRITE-MEDIATED NITRATION OF FREE TYROSINE

To find out whether the metMb mutants investigated protect against peroxynitrite-mediated nitration or catalyze this reaction, we determined the yield of 3-nitrotyrosine (NO_2-Tyr) formed from the reaction of peroxynitrite with free tyrosine in the presence of different concentrations of the proteins. For this purpose, we mixed 200 μM peroxynitrite with 100 μM tyrosine in the presence of increasing amounts (0-10 μM) of H64A, H64D, H64L, F43W/H64L, H64Y/H93G, and WT-metMb (in 0.05 M phosphate buffer, pH 7.4 and 20 °C). As shown in Figure 6A *(and summarized in Table G in Appendices)*, H64A and H64D metMb

are very efficient scavengers of peroxynitrite: in the presence of 5 μM of these proteins no NO_2-Tyr was generated. As expected from the values of k_{cat}, the other proteins studied were significantly less efficient. However, the NO_2-Tyr yields always decreased with increasing amounts of proteins added (Figure 6A).

The different extent of protection exhibited by the metMb mutants can be explained by comparing the rate of the protein-catalyzed isomerization of peroxynitrite with that of the proton-mediated process. For instance, under the conditions of our experiment the rate of the reaction between 5 μM H64A metMb and 200 μM peroxynitrite is 29 s^{-1} (k_{obs} = (5.8 × 10^6 M^{-1} s^{-1}) × (5 × 10^{-6} M) = 29 s^{-1}) and the decay of peroxynitrite is 0.3 s^{-1} at pH 7.4. Thus, in agreement with our experimental results, in the presence of 5 μM of H64A metMb less than 1% of peroxynitrite is expected to react with tyrosine and over 99% will be isomerized through its reaction with H64A metMb. In contrast, the reaction of peroxynitrite (200 μM) with 10 μM of less efficient mutant H64L proceeds at a rate of 0.57 s^{-1} (k_{obs} = (5.7 × 10^4 M^{-1} s^{-1}) × (10 × 10^{-6} M) = 0.57 s^{-1}). Thus, under these conditions only approximately 34% of peroxynitrite is expected to be available for reaction with free tyrosine and should lead to a relative NO_2-Tyr yield of 34% (a relative yield of 100% corresponds to the amount obtained in the absence of the protein, with 100% peroxynitrite), in excellent agreement with our experimental value of 30 ± 1%.



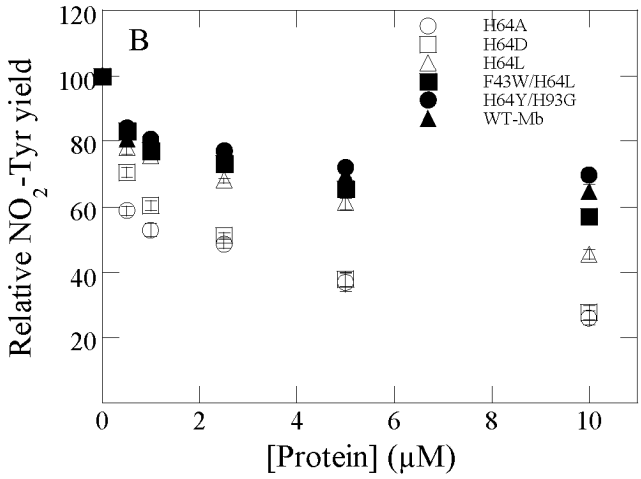


Figure 6: Relative yield of NO_2-Tyr formed from the reaction of peroxynitrite (200 μM) and tyrosine (100 μM), in the presence of WT-Mb and Mb mutants in 0.05 M phosphate buffer pH 7.4, 20 °C. Experiments in the absence (A) and presence (B) of 1.2 mM CO_2. Relative yield = (yield with added protein/yield with no protein) × 100%.

Analogous studies were carried out in the presence of 1.2 mM CO_2. As expected *(4)*, addition of CO_2 led to an increase of the absolute NO_2-Tyr yields generated from the reaction of peroxynitrite with tyrosine. As observed in the absence of CO_2, addition of increasing amounts of all metMb mutants studied induced a concentration-dependent decrease of the NO_2-Tyr yields (Figure 6B *and as summarized in Table G in Appendices)*. The order of efficiency of the different mutants was not influenced by addition of CO_2, and once more mirrored the values of k_{cat}. However, in the presence of 1.2 mM CO_2, the metMb mutants were all less efficient to prevent the peroxynitrite-mediated nitration of added tyrosine. For instance, in the presence of 5 μM H64A metMb the relative NO_2-Tyr yield was 37 ± 3%. This result can be explained by the significant increase of the rate of decay of peroxynitrite in the presence of 1.2 mM CO_2, the process that leads to NO_2-Tyr formation. Indeed, under the conditions of our experiments the decay rate of peroxynitrite is approximately 24.5 s^{-1}. In the presence of 5 μM H64A metMb peroxynitrite reacts with the protein at a rate of 27 s^{-1}, and thus a relative nitration yield of 47% would be expected. The observation that the expected yield is larger than that measured experimentally suggests that the protein reacts with $ONOOCO_2^-$ and/or the products derived from its decomposition and thus partly prevents the formation of free NO_2-Tyr. This hypothesis is supported by the yields obtained in the

presence of the less efficient catalysts which were all significantly lower than the calculated yields. For instance, the expected NO_2-Tyr yield in the presence of 10 μM H64L metMb is 96%, whereas only 45 ± 1% NO_2-Tyr was obtained. As discussed above, the heme is not involved in this scavenging process, which must thus take place through reaction with the amino acid residues of the globin.

To further support the hypothesis that the nitrating agent derived from the reaction of peroxynitrite with CO_2 can be scavenged by the globin, we carried out a similar experiment at high pH (8.0) and in the presence of a very large CO_2 concentration (5 mM). As already mentioned above, under these conditions essentially no HOONO is left after the mixing time. Under these conditions, addition of 200 μM peroxynitrite to 100 μM Tyr in the presence of 2.5-10 μM H64A or H64L reduced the amount of NO_2-Tyr formed up to 20% *(see Table H in Appendices)*. Taken together, our data show that in the presence of physiological concentrations of CO_2 the peroxynitrite that eludes reaction with CO_2 is scavenged by the iron(III) center, whereas the amino acid residues of the globin scavenge, albeit not very efficiently, $ONOOCO_2^-$ and/or the products derived from its decomposition.

4.4.5 NITRATION OF THE GLOBINS AFTER TREATMENT OF THE metMb MUTANTS WITH EXCESS PEROXYNITRITE

Sperm whale Mb contains three tyrosine residues (Tyr103, Tyr146, and Tyr151), one more (Tyr151) than hh Mb. The extent of nitration of these residues after treatment of the mutants with an excess of peroxynitrite was first assessed by Western blot analysis with anti-NO_2-Tyr antibody. As shown in Figure 7, exposure of H64A and H64D metMb to 10 or 20 equiv of peroxynitrite induced nitration in a dose-dependent way. In comparison, the less efficient scavenger H64L metMb was nitrated to a larger extent. Moreover, the amount of nitration of F43W/H64L, H64Y/H93G metMb was comparable to that of sw WT-metMb, but was significantly higher than that of hh metMb *(18)*. Taken together, these blots suggest that the metMb mutants were nitrated to an extent inversely correlated to their efficiency as peroxynitrite scavengers, that is, H64A ≈ H64D >> H64L ~ F43W/H64L < H64Y/H93G ~ WT-metMb. Interestingly, after treatment with 20 equiv of peroxynitrite a second nitrated band of approximately 29 kDa was present in the blots, in particular H64L, H64Y/H93G, and F43W/H64L metMb. A much smaller amount of nitrated dimer was formed from the reaction

with hh metMb *(18)*. Thus, our results suggest that Tyr151, which is not present in hh metMb, is responsible for the formation of the dimer.

A similar observation was made upon reaction of hydrogen peroxide with sw metMb and hh metMb *(39)*. Indeed, Ortiz de Montellano and co-workers demonstrated that sw metMb (but not hh metMb) forms a dityrosine cross-link between Tyr151 on one molecule and Tyr103 on the other *(39)*. Moreover, site-directed mutagenesis of Tyr103 and/or Tyr146 to a phenyalanine still led to the formation of dimers, whereas mutation of Tyr151 completely blocked dimerization *(40)*. Thus, Tyr151-Tyr151 dimers must also be formed.

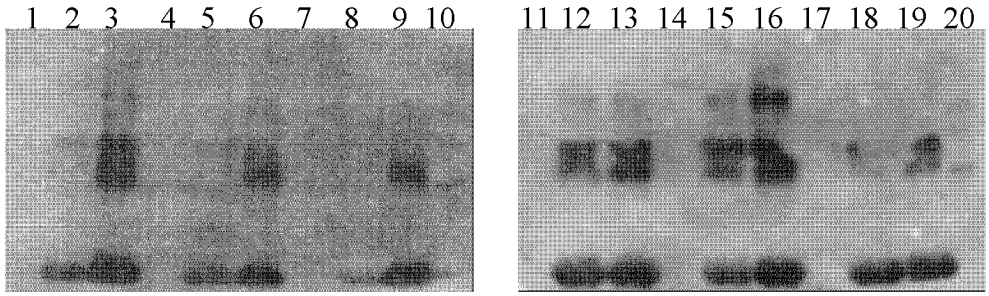


Figure 7. Anti-nitrotyrosine immunoblotting of WT-metMb and metMb mutants. Samples were separated on an 11% SDS polyacrylamide gel and examined by Western blot analysis with a polyclonal antibody against nitrotyrosine as described in the Experimental Procedures. The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the absence of added CO_2. Lanes 1-3, H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 4-6, H64D metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 7-9, H64A metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 10, molecular weight marker; lanes 11-13, H64Y/H93G metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 14-16, F43W/H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 17-19, WT-metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 20, molecular weight marker.

As shown in Figure 8, in the presence of 1.2 mM CO_2, the blots of all the proteins treated analogously with peroxynitrite showed much more intense bands, suggesting a larger extent of nitration. Moreover, the amount of dimeric and trimeric nitrated proteins was considerable, in particular F43W/H64L, H64L, and H64D metMb. Interestingly, visualization of the proteins by staining with coomassie Blue (Figure 9) indicated that the largest fraction of the proteins was in the monomeric form, thus suggesting that the oligomers are preferentially nitrated. It has been reported that under neutral conditions Trp radicals generated within a protein can transfer their electron deficiency to tyrosine residues *(41)*.

This process takes place at rates in the range 10^2-10^4 s^{-1} and leads to the formation of Tyr radicals *(41)*. Our results suggest that Trp43, located close to the heme, may be oxidized by peroxynitrite in the presence of CO_2, lead to the formation of additional Tyr radicals, and consequently generate larger amounts of nitrotyrosine as well as of dimeric and/or oligomeric species. Interestingly, recent reinvestigation of the reaction of sw metMb with H_2O_2 also showed the formation of trimeric as well as tetrameric products *(42)*.

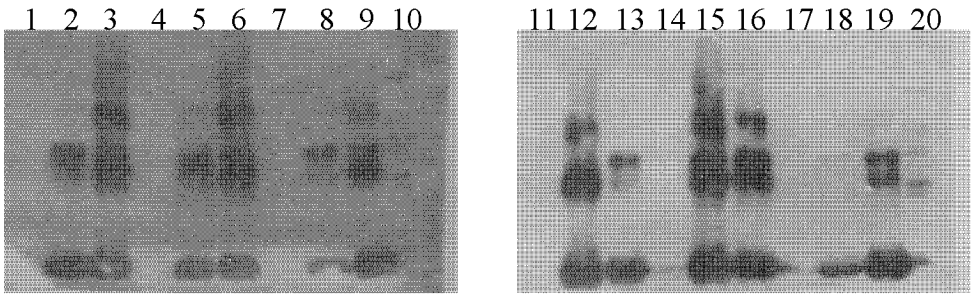


Figure 8. Anti-nitrotyrosine immunoblotting of WT-metMb and metMb mutants. Samples were separated on an 11% SDS poly acrylamide gel and examined by Western blot analysis with a polyclonal antibody against nitrotyrosine as described in the Experimental Procedures. The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the absence of added CO_2. Lanes 1-3, H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 4-6, H64D metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 7-9, H64A metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 10, molecular weight marker; lanes 11-13, H64Y/H93G metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 14-16, F43W/H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 17-19, WT-metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 20, molecular weight marker.

To quantify the amount of nitration of the tyrosine residues in H64A, H64D, H64L, F43W/H64L, H64Y/H93G, and WT-metMb, we mixed these proteins with an excess of peroxynitrite (at 20 °C and pH 7.0), subjected them to acid hydrolysis, and analyzed the products by HPLC. To avoid artifactual nitration by nitrite present in the peroxynitrite solution (up to 50% relative to the peroxynitrite concentration), we treated the samples with a concentrated acidic ammonium sulfamate solution (before hydrolysis with HCl). The results, shown in Figure 10 *(and summarized in Table I in Appendices)* confirm the qualitative data of the immunohistochemical analyses discussed above. The three tyrosine residues of the two efficient scavengers, H64A and H64D metMb, were nitrated less than 1%, even upon addition of 20 equiv of peroxynitrite. Interestingly, despite the similar values of k_{cat}, F43W/H64L

metMb was always nitrated to a significantly larger extent than H64L metMb. As discussed above, Trp43 may be responsible for the formation of larger amounts of NO_2-Tyr, upon reaction of F43W/H64L metMb with peroxynitrite. Indeed, Trp43 radicals, generated either from the direct reaction of Trp43 with peroxynitrite or by a mechanism mediated by the heme, may form additional Tyr radicals, which may recombine with $NO_2^{\bullet}$ to yield NO_2-Tyr.

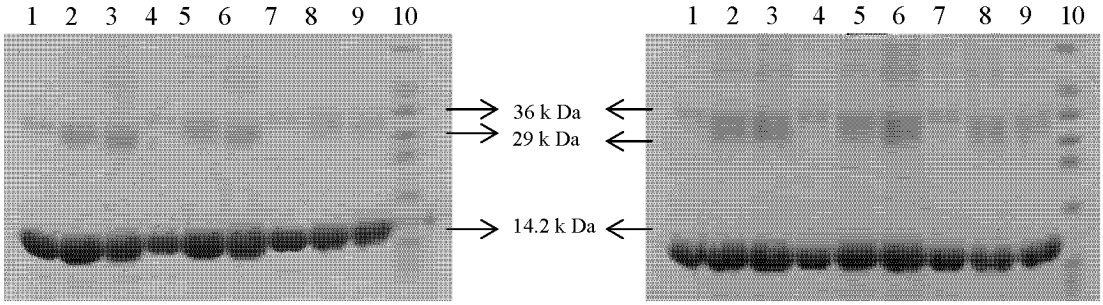


Figure 9. Coomassie blue staining of WT-metMb and met Mb mutants (compare with Figure 5). The proteins (100 μM) were incubated in 0.1 M phosphate buffer pH 7.0 at 0 °C with decomposed peroxynitrite (negative control) and with increasing amounts of peroxynitrite in the presence of 1.2 mM CO_2. Lanes 1–3, H64L metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 4–6, H64D metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lanes 7–9, H64A metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 10, molecular weight marker; lanes 11–13, H64Y/H93G metMb mixed with decomposed peroxynitrite, 20, or 10 equiv of peroxynitrite; lanes 14–16, F43W/H64L metMb mixed with decomposed peroxynitrite, 20, or 10 equiv of peroxynitrite; lanes 17–19, WT-metMb mixed with decomposed peroxynitrite, 10, or 20 equiv of peroxynitrite; lane 20, molecular weight marker.

As shown in Figure 10, HPLC analyses confirmed that sw-metMb is nitrated to a significantly larger extent than hh-metMb, confirming the hypothesis that Tyr151 is nitrated more readily than Tyr103 and Tyr146. Finally, the most nitrated Mb mutant was H64Y/H93G metMb. Because the UV-vis spectrum of the protein remained unchanged even after treatment with a large excess of peroxynitrite *(see Figure I in Appendices)*, the increased extent of nitration does not arise from nitration of the additional Tyr64. Indeed, such a modification would be expected to cause discernible changes in the absorbance spectrum of the protein. Taken together, these results rather indicate that the higher degree of nitration is due to the poor ability of H64Y/H93G metMb to scavenge peroxynitrite.

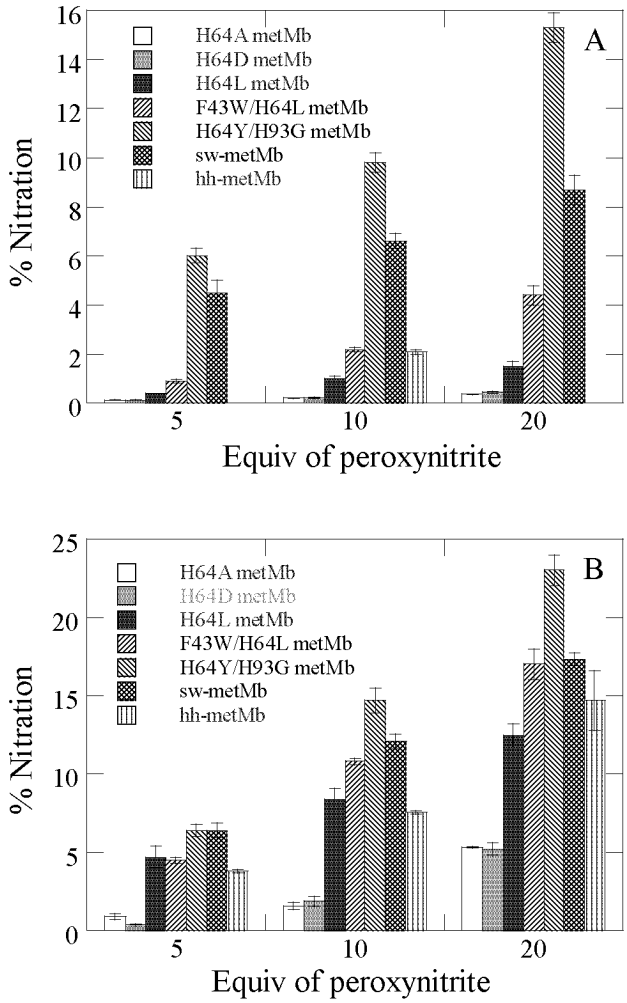


Figure 10. NO_2-Tyr yields (% relative to the total content of tyrosine residues available for nitration, that is, three residues per heme for sw Mb and two for hh Mb), determined by HPLC after acid hydrolysis of the proteins, from the reaction of WT-metMb and metMb mutants (100 μM) with 5, 10, and 20 equiv of peroxynitrite (A) in the absence and (B) in the presence of 1.2 mM CO_2 at 20 °C and pH 7.0. The data are summarized in Table H in Appendices.

In the presence of 1.2 mM CO_2, the yields of NO_2-Tyr produced after treatment of all proteins with an excess of peroxynitrite were generally higher but followed a trend analogous to that observed in the absence of CO_2 *(see Table I in Appendices* and Figure 10B); note the different scale of the *y*-axis in the two plots of Figure 10). Because of the large values of k_{cat}, H64A and H64D metMb can still compete with CO_2 for peroxynitrite and significantly reduce the extent of nitration of the tyrosine residues in the protein. All other metMb mutants studied display a value of k_{cat} close to that of the second-order rate constant of the reaction between peroxynitrite and CO_2 (3×10^4 M^{-1} s^{-1} at 24 °C) *(4)*. Thus, in the presence of 1.2

mM, only a small amount of peroxynitrite can be detoxified by the heme center of these mutants. It is important to note that in the experiments with 10-20 equiv of peroxynitrite (1-2 mM) the concentration of CO_2 is comparable to that of peroxynitrite. However, it has been shown that the reaction between CO_2 and $ONOO^-$ is catalytic and regenerates CO_2 *(43)*, and thus these reaction conditions may simply lead to a slower reaction (since the pseudo-first-order conditions are not maintained). Finally, as clearly depicted in Figure 10B, the yields of nitration of the tyrosine residues of sw-metMb were significantly larger than those of hh-metMb, again indicating that Tyr151 is a preferential site of nitration also in the presence of CO_2.

To confirm the NO_2-Tyr yields obtained after acid hydrolysis of the treated proteins, we hydrolyzed the nitrated proteins also by a milder method that is, with pronase, and then analyzed the samples by HPLC. As shown in Figure 11 *(and summarized in Table J in Appendices)*, both in the absence and in the presence of 1.2 mM CO_2, the yields of NO_2-Tyr were slightly larger (5-25%) than those quantified after acid hydrolysis. As recently reported *(18)*, this small difference may be due to partial decomposition of NO_2-Tyr during acid hydrolysis.

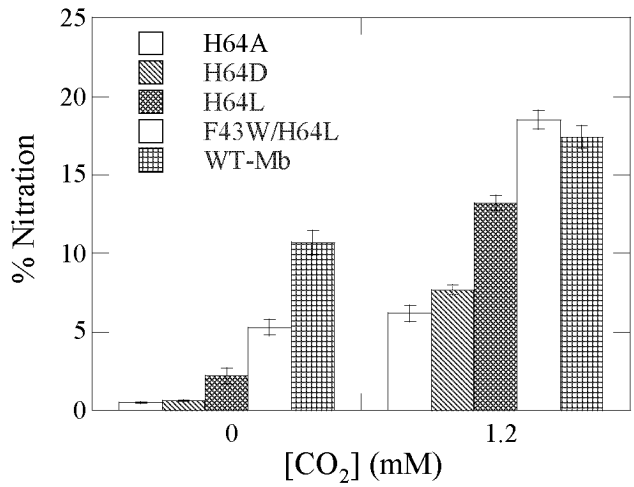


Figure 11. NO_2-Tyr yields (% relative to the total content of the proteins, that is, three tyrosine residues per heme), determined by HPLC after Pronase digestion after treatment of WT-Mb and Mb mutants (100 μM) with 20 equiv of peroxynitrite, in the presence of 0 or 1.2 mM added CO_2 (at 20 °C and pH 7.0).

Interestingly, HPLC analyses of pronase-digested protein samples showed that, in addition to NO_2-Tyr, other nitrated species were formed, as additional peaks were present in the chromatograms measured at 350 nm (Figure 12). For H64L, F43W/H64L, and WT-

metMb, four peaks of the chromatogram measured between 17 and 20 min displayed absorbance spectra characteristic for nitrotryptophan derivatives.

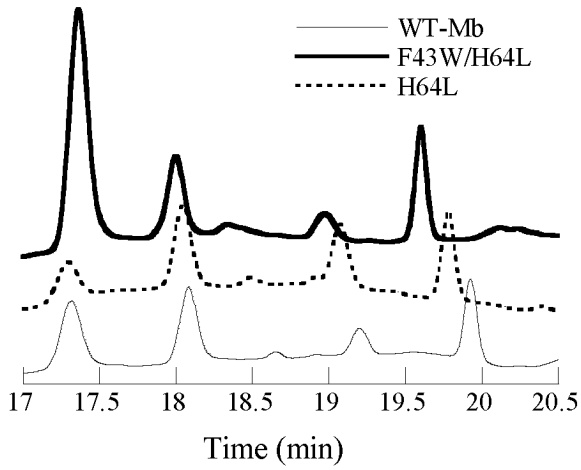


Figure 12. Sections corresponding to the nitrotryptophan species of the HPLC chromatograms (measured at 350 nm) of the products of the reactions of 100 μM WT-metMb, F43W/H64L metMb, and H64L metMb with 20 equiv of peroxynitrite, in 0.1 M phosphate buffer pH 7.0 at 0 °C, after complete digestion with pronase as described in the Experimental Procedures.

The assignment of the peaks to the corresponding NO_2-Trp isomers was rather difficult because of the presence of an overlapping absorbance probably derived from heme impurities. However, comparison of the spectra with those known for the different NO_2-Trp isomers *(18, 44, 45)*, allowed for the assignment of the peaks at 17.3 (Peak 1), 18.0 (Peak 2), and 19.8 min (Peak 4) to the 6-, 4-, and 5-nitrotryptophan, respectively. We have recently shown that 6-, 5-, and 4-NO_2-Trp are generated also when apo-, oxy- or metMb (horse heart) are treated with excess of peroxynitrite *(18)*. Additional studies are required to identify which of the two tryptophan residues, Trp7 and/or Trp14, is modified upon reaction with peroxynitrite. As shown in Figure 12, comparison of the intensity of the NO_2-Trp peaks suggests that the additional Trp residue present in F43W/H64L metMb may also be nitrated. Indeed, especially the peak at 17.3 min assigned to 6-NO_2-Trp was significantly larger for peroxynitrite-treated F43W/H64L metMb.

4.5 CONCLUSIONS

We have shown that the reactivity of peroxynitrite toward metMb is regulated by the distal amino acid residues. The strong hydrogen bond between the distal histidine and the water molecule bound to the iron(III) center in WT-metMb considerably reduces the efficiency of the protein to catalyze the isomerization of peroxynitrite to nitrate. In the absence of a residue which can undergo hydrogen bonds, the size of the distal ligands determines the effectiveness of the catalysis. In the presence of CO_2, only the fraction of peroxynitrite that eludes reaction with CO_2 reacts with the iron(III) center of the protein, and this reaction is responsible for the observed catalytic effect. The adduct $ONOOCO_2^-$ and/or the products derived from its decay are partly scavenged by the globin. However, this reaction is not catalytic and consequently significantly less effective.

In contrast to peroxidases, the iron(III) forms of WT-metMb and its mutants protect free tyrosine from peroxynitrite-mediated nitration. The extent of protection is directly correlated to the value of k_{cat}. In the absence of free tyrosine, sperm whale metMb is significantly more nitrated by an excess of peroxynitrite than hh metMb. This result suggests that, analogously to the reaction with H_2O_2, Tyr151 is more easily modified, and thus larger amounts of nitrotyrosine and oligomerization products are detected upon reaction with an excess of peroxynitrite. The double mutant F43W/H64L metMb, which contains an additional Trp residue close to the distal pocket of the heme, is the mutant nitrated to the largest extent. This result suggests that facilitated generation of Trp radicals followed by H-atom transfer produces Tyr radicals, which can then lead to nitrotyrosine or dityrosine modifications. In conclusion, our results show that myoglobin represents an ideal platform to investigate the influence of single amino acid residues on the reactivity of hemoproteins with peroxynitrite

4.5 REFERENCES AND NOTES

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5 GENERAL DISCUSSION

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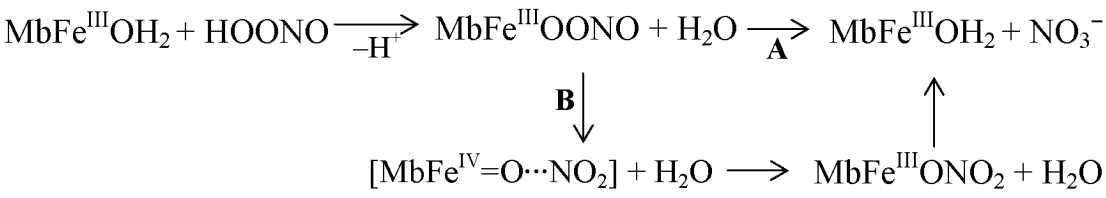
Nitrogen monoxide reacts rapidly with superoxide to produce peroxynitrite which has been postulated to be involved in many human diseases *(1-4)*. It has been reported that peroxynitrite formation in arteries leads to hypertension and/or atherosclerosis *(5)*. Thus, many researchers are currently searching for a drug/catalyst that can scavenge peroxynitrite. Myoglobin has been shown to efficiently scavenge nitrogen monoxide, leading to an attenuation of the heart diseases, especially cardiac arrest *(6)*. Thus, we propose that myoglobin and hemoglobin present in high concentration in the cardiac and skeletal muscle and in the blood vessels, respectively, may act as scavengers of nitrogen monoxide and/or peroxynitrite.

Our group has previously shown that the oxygenated forms of myoglobin and hemoglobin are good scavengers for peroxynitrite *(7, 8)*. The reaction of peroxynitrite with oxyhemoglobin (and oxymyoglobin) leads to methemoglobin (and metmyoglobin). This reaction proceeds in two steps via a ferryl intermediate. The second-order rate constants for the peroxynitrite-mediated oxidation of oxymyoglobin and oxyhemoglobin are on the order of 10^4 M^{-1} s^{-1} *(7, 8)*. In this project, the main objective was to study the reaction of peroxynitrite with the iron(III) forms of myoglobin and hemoglobin.

Our kinetic studies showed that the iron(III) forms of myoglobin and hemoglobin are good catalysts for peroxynitrite. In contrast to the reaction of peroxynitrite with oxyhemoglobin and with oxymyoglobin, no intermediate or absorbance changes were observed upon addition of an excess of peroxynitrite to methemoglobin or metmyoglobin solutions. However, to explain the catalytic isomerization of peroxynitrite to nitrate, we have proposed that this reaction proceeds as shown in Scheme 1. In the first step, the coordinated

Scheme 1. Possible Mechanisms for the MetMb-Catalyzed Isomerization of Peroxynitrite.



water is replaced by peroxynitrite, and thus we suggest that an intermediate iron(III) peroxynitrite complex ($MbFe^{III}OONO$) must be generated. Since this complex could not be

detected, it probably decays to nitrate and metmyoglobin at a very fast rate. Hence, binding of peroxynitrite to metmyoglobin must be the rate-determining step. The decay of the intermediate $MbFe^{III}OONO$ can proceed according to two possible mechanisms (Pathway A and B) as shown in Scheme 1. The same Mb- or Hb-$Fe^{III}OONO$ complexes have previously been characterized by UV-vis and EPR spectroscopy as intermediates of the reaction of nitrogen monoxide with oxymyoglobin or with oxyhemoglobin at alkaline pH *(9-11)*. The $MbFe^{III}OONO$ decays to give nitrate and metmyoglobin, and the rate constant reported is 205 ± 5 s^{-1} at pH 9.5 and 20 °C *(10)*. In another experiment, $MbFe^{III}ONO_2$, generated by pulse radiolysis from the reaction between ferryl myoglobin and nitrogen dioxide, has been reported to decay to give nitrate and metmyoglobin at a rate of 35 s^{-1} *(10)*. Recent re-investigation of this reaction showed that this value is probably incorrect *(S. Herold, personal communication)*. Indeed, the values of the nitrate dissociation rate constants measured in our group in a series of new preliminary experiments were not very reproducible but they were always significantly lower than 205 s^{-1} *(S. Herold, personal communication)*. Taken together, these dissociation constants suggest that the decay of $MbFe^{III}OONO$ to nitrate and metmyoglobin cannot occur via the formation of the corresponding nitrato-complex (pathway B in Scheme 1). Thus, based on these observations, we propose that the intermediate formed from the reaction between peroxynitrite and metmyoglobin decays to give nitrate and metmyoglobin via pathway A (in Scheme 1).

Very recently, Goldstein et al. re-measured the rate of dissociation of nitrate from $MbFe^{III}ONO_2$, also generated by pulse radiolysis from the reaction between ferryl myoglobin and nitrogen dioxide, and obtained a value of 190 ± 20 s^{-1} (at pH 9.7 and 20 °C) *(12)*. Under their slightly different experimental conditions, the value obtained for the observed rate constant was reproducible. Additionally, by considering the calculated lifetime of alkyl peroxynitrites to nearly 1 μs, they suggested that the $MbFe^{III}OONO$ complex should rapidly convert to $MbFe^{III}ONO_2$ via ferryl myoglobin and nitrogen dioxide *(12)*. Thus, they concluded that $MbFe^{III}OONO$ cannot be detected and that the intermediate characterized under alkaline conditions is $MbFe^{III}ONO_2$. However, the UV-vis spectrum of the hemoglobin peroxynitrite complex ($HbFe^{III}OONO$) is different from that of the nitrato complex ($HbFe^{III}ONO_2$) *(10)* and thus their conclusion cannot be correct. In conclusion, based on the new value reported for the rate constant of the decay of $MbFe^{III}ONO_2$, which is essentially identical to that of the decay of $MbFe^{III}OONO$, we suggest that both pathways (A and B in Scheme 1) are possible for the metmyoglobin-catalyzed isomerization of peroxynitrite.

While our work was in progress, Groves and co-workers also studied the metmyoglobin-catalyzed isomerization of peroxynitrite and proposed a mechanism similar to pathway B in Scheme 1 *(13)*. They suggested that part of the nitrogen dioxide eludes the reaction with ferryl myoglobin and undergoes hydrolysis to nitrite and nitrate. Their conclusion is based on the observation that a constant level of approximately 10% nitrite was produced from the decay of peroxynitrite in the presence of 100-200 μM of metmyoglobin *(13)*. However, we showed that in the presence of higher concentrations of metmyoglobin (750 μM) peroxynitrite is converted quantitatively to nitrate. If large amounts of nitrogen dioxide escape recombination, one might expect ferryl myoglobin to be observed, but no intermediate was reported to be detected *(13)*. Thus, our results indicate that if the decomposition of peroxynitrite to nitrate proceeds via pathway B (in Scheme 1) nitrogen dioxide and ferryl myoglobin react quantitatively to $MbFe^{III}ONO_2$.

Furthermore, we have shown that when His64 is replaced with alanine or aspartic acid, very low concentrations (5 μM) of these proteins were needed to achieve 100% isomerization of peroxynitrite to nitrate. The efficiency of these proteins to catalyze the isomerization of peroxynitrite is consistent with the larger value of k_{cat} (see below). These data indicate that, in contrast to the hypothesis of Groves and co-workers *(13)*, in the presence of efficient catalysts (H64A or H64D), the decomposition of peroxynitrite does not lead to nitrite formation. Thus, this result supports the above discussed mechanism for the decay of $MbFe^{III}OONO$ complex: if the reaction proceeds via pathway B (in Scheme 1), the reaction between ferryl myoglobin and nitrogen dioxide must be quantitative.

The catalytic rate constants for the decay of peroxynitrite in the presence of the iron(III) forms of myoglobin and hemoglobin is lower compared to that of water-soluble iron(III)-porphyrin complexes such as iron(III) 5, 10, 15, 20-tetrakis- [*N*-methyl-4'-pyridyl] porphyrin (Fe(III)-TM-2-PyP) and iron(III) 5, 10, 15, 20-tetrakis-[2, 4, 6-trimethyl-3. 5-sulfonatophenyl] porphyrin (Fe(III)-TMPS) *(14)*. These complexes react rapidly with peroxynitrite, therefore, are proposed to be promising drug candidates against peroxynitrite toxicity. However, to explain the difference in catalytic rate constants, we hypothesized that the reactivity of peroxynitrite with the iron(III) center of the proteins is most likely regulated by the distal histidine which is strongly hydrogen bonded to the water molecule coordinated to the iron(III) center. Indeed, our studies showed that mutants of sperm whale metmyoglobin in which the distal histidine was substituted with amino acid residues that cannot undergo a hydrogen bond with the coordinated water, react faster with peroxynitrite and catalyze its

isomerization to nitrate more efficiently. The catalytic rate constants for the decay of peroxynitrite are (5.8 ± 0.1) × 10^6 and (4.8 ± 0.1) × 10^6 M^{-1} s^{-1}, (at pH 7.5 and 20 °C) for H64A- and H64D-metMb, respectively. These values are comparable to those of the most efficient iron(III)-porphyrin catalysts such as Fe(III)-TM-2-PyP (k_{cat} = 2.2 × 10^6 M^{-1} s^{-1}, at pH 7.4 and 37 °C) and Fe(III)-TMPS (k_{cat} = 6.4 × 10^5 M^{-1} s^{-1}, at pH 7.4 and 37 °C) *(14)*.

Apart from its reaction with the heme center, in principle peroxynitrite could induce nitration of aromatic residues in these proteins *(1, 2)*. However, to be an efficient scavenger, myoglobin or hemoglobin should not be damaged in the course of its reaction with peroxynitrite. Our studies showed that the tyrosine residues of the oxy- and the met-forms of hemoglobin and myoglobin are nitrated to less than 1% upon addition of 1 equiv of peroxynitrite. These nitration yields are physiologically irrelevant since the concentration of peroxynitrite *in vivo* will never be as high as that of myoglobin and hemoglobin. Indeed, no nitrated tyrosine residues were found in hemoglobin of aged red blood cells *(15)*. Our mechanistic studies showed relatively larger nitration yields of tyrosine and tryptophan residues in apomyoglobin or in the cyanide-bound form of iron(III) myoglobin (metMbCN) treated with peroxynitrite. This result suggests that the iron center in the oxy- and met-forms of the proteins plays a role in inhibiting peroxynitrite-mediated nitrotyrosine formation.

During the course of this study, Groves and co-workers reported very large nitration yields (50%) of metmyoglobin after addition of 20 equiv of peroxynitrite *(13)*. The amount of nitration was determined with mass spectrometry. However, no experimental details were reported *(13)*. In our hands, the same reaction gave nitration yields lower than 10%, quantified by HPLC analysis after hydrolysis of the protein. Unfortunately, first attempts to use the ESI-mass spectrometer of our laboratory to measure the nitrotyrosine yields did not give consistent results. Therefore, this argument is still unresolved.

Furthermore, experiments with different forms of myoglobin and hemoglobin showed that oxymyoglobin is the species that most efficiently prevents peroxynitrite-mediated nitration of added free tyrosine. Significantly larger nitration yields of free tyrosine were observed by addition of peroxynitrite in the presence of apomyoglobin or of metMbCN. Taken together, these results strongly indicate that the heme center in the oxy- and met-forms of myoglobin and hemoglobin acts as an efficient scavenger of peroxynitrite and thus may prevent peroxynitrite-mediated nitration not only of the tyrosine and tryptophan residues of the globins but also those of other proteins.

Interestingly, the iron(III) forms of cytochrome P450 enzymes such as $P450_{NOR}$, and $P450_{BM}$ have been shown to catalyze the peroxynitrite-mediated nitration of tyrosine residues, rather than to protect nitration of target molecules *(16, 17)*. However, in these proteins the reaction of the iron(III) form with peroxynitrite generates the ferryl species and nitrogen dioxide, which induce nitration of tyrosine residues located close to the heme *(16, 17)*.

Under physiological conditions, hemoglobin and myoglobin are mostly found in the oxygenated form with only 1-5% present in the oxidized iron(III) form of the corresponding proteins. Since the second-order rate constants of the reactions of peroxynitrite with oxymyoglobin and oxyhemoglobin (10^4 M^{-1} s^{-1}) are on the same order of magnitude as the catalytic rate constants for the metmyoglobin- and methemoglobin-catalyzed isomerization of peroxynitrite, oxyhemoglobin and oxymyoglobin will be the species primarily involved in the scavenging of peroxynitrite *in vivo (7)*. Moreover, the deoxygenated forms of myoglobin and hemoglobin have been shown to react with peroxynitrite at a rate of 2 orders of magnitude larger (10^6 M^{-1} s^{-1}) than that for the reaction with the corresponding oxygenated proteins *(8)*. Thus, under hypoxic conditions, the reaction of the deoxygenated forms of the protein with peroxynitrite may be of biological relevance.

Under ischemic conditions such as low dioxygen tension, one can argue if there is formation of nitrogen monoxide and/or peroxynitrite in venous blood. Two different enzymes may produce nitrogen monoxide or superoxide under different conditions *(18)*. When there is sufficient supply of L-arginine and dioxygen, nitric oxide synthase makes nitrogen monoxide *(1, 2)*, whereas the same enzyme may generate considerable amounts of superoxide when the concentrations of arginine and cofactors are limited *(18)*. Xanthine oxidase generates superoxide, for example, during reperfusion after ischemia *(19)*, whereas nitrite reduction to nitrogen monoxide occurs preferentially during hypoxia *(20)*. Moreover, nitrite can react with deoxyhemoglobin to generate nitrosylated hemoglobin ($HbFe^{II}NO$) *(21-23)*. Cosby et al. have shown that intra-arterial infusion of nitrite at physiological levels caused vasodilation in healthy humans, thus, demonstrating that nitrogen monoxide can be liberated from nitrosylated hemoglobin *vivo (24)*. Thus, there is an efficient pathway to make nitrogen monoxide and peroxynitrite also under hypoxic conditions. Deoxymyoglobin and deoxyhemoglobin may play a role as peroxynitrite scavengers. Indeed, these proteins are present in high concentrations and are among the species, which react with the highest rate constants with peroxynitrite *(7, 8)*.

In conclusion, the concentrations of myoglobin and hemoglobin in cardiac and skeletal muscle and in the blood vessels, respectively, are significantly larger than that of peroxynitrite (which is often generated continuously in nM range), and our results indicate that under physiological conditions, these proteins may trap most peroxynitrite by its reaction with the heme center and prevent damage to the surrounding biomolecules.

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6 ADDITIONAL STUDIES*

In the present chapter, additional studies on the reaction of myoglobin with peroxynitrite are summarized. In particular, the following three projects have been investigated in part to support the results of the preceding chapters. However, these studies are not complete and further work is necessary.

1. Influence of tryptophan residues in sperm whale myoglobin on the rate of decay of peroxynitrite. Construction and purification of W7F/W14A-swMb.
2. Quantification of the extent of nitration of Mb by peroxynitrite with ESI-MS.
3. Nitration of apoMb with a large excess of peroxynitrite and heme reinsertion.

** Part of the work described in this Chapter was carried out in the laboratory of Prof. Y.Watanabe, Department of Bioinorganic Chemistry, Nagoya University, JAPAN.*

6.1 INFLUENCE OF TRYPTOPHAN RESIDUES IN sw-Mb ON THE RATE OF DECAY OF PEROXYNITRITE. CONSTRUCTION AND PURIFICATION OF W7F/W14A-swMb.

6.1.1 INTRODUCTION

Sperm whale myoglobin (sw-Mb) contains three tyrosine residues (Tyr103, Tyr146, and Tyr151) and two tryptophans (Trp7 and Trp14). The reaction of the iron(III) form of sw WT-Mb with hydrogen peroxide yields ferryl myoglobin and protein-centered radicals *(1)*. During the reaction of sw-metMb with hydrogen peroxide, either Tyr151 or Tyr103 are oxidized to the corresponding tyrosine radicals, which may lead to intermolecular cross-links (Tyr-Tyr) *(1)*. In addition to tyrosines, at least one of the two tryptophan residues has been proposed to be involved in the formation of a transient tryptophan radical *(2)*. EPR studies by spin trapping have shown the formation of a tryptophanyl radical at a low hydrogen peroxide to metMb ratio (1:1), whereas a higher ratio of hydrogen peroxide to protein (5:1) led to generation of tyrosyl radicals *(3)*.

Peroxynitrite is known to react directly with Trp or via the secondary radicals (trioxocarbonate (•1–), nitrogen dioxide) formed from the decay of peroxynitrite in the presence of carbon dioxide. The direct reaction has an apparent rate constant of 130 M^{-1} s^{-1} at pH 7.0 and 25 ºC *(4, 5)*. We have previously shown that apart from nitrotyrosine, nitro-tryptophans are formed in the apoMb, swWT-, F43W/H64L- and H64L-metMb mutants upon reaction with peroxynitrite *(6)*. It has been reported that the Trp radicals generated within a protein can transfer their electron deficiency to tyrosine residues *(7, 8)*. This process takes place at rates in the range 10^2-10^4 s^{-1} and leads to the formation of tyrosine radicals *(7)*. Since the amount of peroxynitrite-mediated nitration in F43W/H64L-metMb is larger compared to that of H64L-metMb, we proposed that the Trp43 (located close to the heme in F43W/H64L-metMb) may be oxidized by peroxynitrite, lead to the formation of Trp and consequently of Tyr radicals. These radicals may recombine with nitrogen dioxide to yield nitrotyrosine and nitro-tryptophan *(6)*. Interestingly, EPR studies with spin traps have shown that tryptophan-centered protein radicals are formed in human plasma after reaction with excess peroxynitrite *(9)*.

The double mutant W7F/W14A sw-Mb would be an interesting protein to study the extent of tyrosine nitration without the influence of Trp radicals. However, the simultaneous substitution of both Trp residues has been reported to cause incorrect protein folding with a

subsequent loss of the heme *(10-12)*. Investigations with W7F- and W14F-swMb mutants might allow identification of which of the two tryptophan residues influence peroxynitrite-mediated Trp/Tyr nitration. These two mutants fold correctly, since their absorption spectra are identical to that of native sw WT-metMb. Here we report the initial results of the investigation of the reaction of W7F- and W14F-metMb with peroxynitrite.

6.1.2 EXPERIMENTAL PROCEDURES

6.1.2.1 CONSTRUCTION OF W7F/W14A-swMb

Construction of the mutants was carried out in the laboratory of Prof. Y. Watanabe (Nagoya University, JAPAN). The double mutant W7F/W14A-swMb was constructed by using QuikChange™ PCR-mutagenesis and the DNA sequence was confirmed by Big Dye Terminator Sequence. The mutants W7F- and W14F-swMb had already been constructed in the Group. The QuikChange™ site-directed mutagenesis system allows rapid introduction of mutations into the sequence of interest by using a pair of complementary mutagenesis primers. The primers used for making W7F/W14A-swMb: Sense; G GTT CTG CAT GTT **GCT** GCT AAA GTT GAA GC, Antisense; GC TTC AAC TTT AGC **AGC** AAC ATG CAG AAC *(bold letters correspond to the site of mutagenesis)*.

6.1.2.2 PROTEIN EXPRESSION AND PURIFICATION OF W7F- AND W14F-swMb

The expression and the purification of W7F- and W14F-swMb mutants were performed according to the method described by Springer *(13)*. In brief, the protein expression was achieved by using the pUC19 expression vector containing a *lacZ* promoter in the *E. coli* TB-1 strain. Prior to protein purification, the yield of protein expression was examined by SDS-polyacrylamide gel (SDS-PAGE) (Figure 1), as described by Laemmli *(14)*.

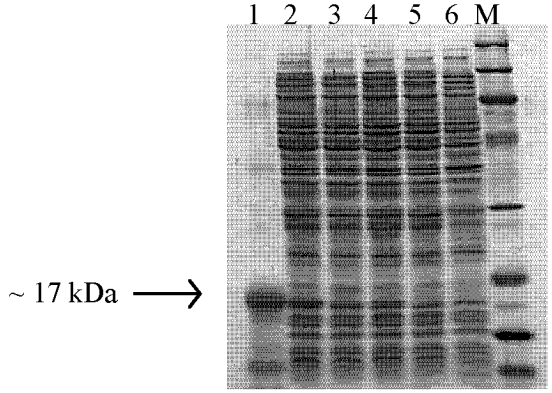


Figure 1. Protein expression profile of W7F/W14A-, W7F- and W14F-swMb. lane 1; Purified WT-swMb, lane 2; WT-swMb, lane 3; W7F, lane 4; W14F, lane 5; W7F/W14A (in Top 10 *E.coli* cells), lane 6; lane 5; W7F/W14A (in JM109 *E.coli* cells) and M; SDS-PAGE marker.

For myoglobin purification, LB (Luria-Bertini) medium containing 50 µg/mL ampicillin was inoculated with an overnight starter culture of TB-1 *E. coli* cells. The culture was allowed to grow at 37 °C with vigorous shaking (100 rpm) for 12 hr. Bacterial cells were collected by spinning at 8000 rpm for 10 min. Cell pellets were resuspended in the following lysis buffer: 50 mM Tris, 0.5 mM dithiothreitol (DTT), 1 mM edta, and 1 mM phenylmethylsulphonyl fluoride (PMSF), pH 8.0. Approximately 50 ml of lysis buffer was used to resuspend the cells originating from ~ 1 L culture. The solution was sonicated for 25 s on ice (10 times). After centrifugation at 17,500 rpm for 20 min, ammonium sulfate (55% w/v) was added to the collected supernatant and the Mb solution was stirred for 1 h at 4 °C. After another centrifugation, the supernatant was collected and ammonium sulfate was added up to a 90% w/v concentration. The solution was stirred for 2 h at 4 °C and after centrifugation the supernatant was dialysed overnight at 4 °C in 15 mM potassium phosphate buffer, pH 6.0 containing 1 mM edta. After dialysis, the protein solution was centrifuged for 10 min at 17,500 rpm and the supernatant was loaded on a CM52 column equilibrated with 25 mM potassium phosphate buffer of pH 6.0. The protein elution was carried out with a linear gradient of 0-50 mM phosphate buffer of pH 9.0 (Figure 2).

The fractions collected were concentrated with an Amicon ultra-filtration unit with a 10 kDa cut off membrane filter. The concentrated protein was subjected to gel filtration (Sephadex G100) with FPLC (Amersham), and the single peak eluted was collected. After 10% SDS-PAGE in reducing conditions, the collected proteins showed a single band with apparent molecular mass of 17 kDa *(data not shown)*. The purified W7F- and W14F-swMb proteins were used for preliminary tyrosine nitration and peroxynitrite decay kinetics studies in our laboratory.

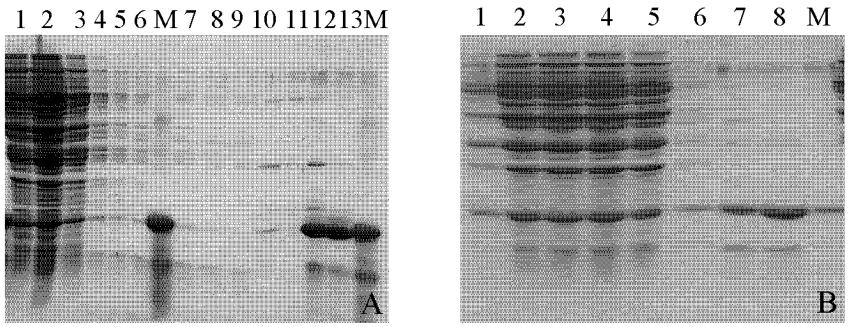


Figure 2. SDS-PAGE (10%) of protein purification profile of the W7F- (A) and W14F-swMb (B) mutants. Fractions eluted from CM52 column; fraction 12, 13 of A and the fractions 7 and 8 of B corresponds to myoglobin at 90% initial purity. M is purifed wild type swMb as marker.

6.1.2.3 STOPPED-FLOW EXPERIMENTS

The kinetics of peroxynitrite decay in the presence of W7F-and W14F-swMb mutants were studied with an Applied Photophysics SX18MV-R single-wavelength stopped-flow instrument as described in previous chapters. The kinetic traces were collected at 302 nm, and the data were analyzed with Kaleidagraph, version 3.52. The protein solutions of the required concentrations were prepared under aerobic conditions in 0.1 M potassium phosphate buffer. The protein solutions had a pH of 6.7 so that after mixing with peroxynitrite, the final pH was around 7.0. Peroxynitrite solutions were prepared by diluting the stock solution immediately before use with 0.01 M NaOH to achieve the required concentration. The measurements were carried out at 20 °C and the pH was always measured at the end of the reactions.

6.1.2.4 REVERSE PHASE HPLC ANALYSIS

HPLC analysis was carried out with a Hewlett-Packard Series 1050 apparatus with a Series 1100 UV-vis detector, equipped with a VYDAC 218TP54 Protein & Peptide C18-Column (250 × 4.6 mm). Solvent A was 0.07% TFA in H_2O and solvent B was 0.07% TFA in acetonitrile. The reaction of peroxynitrite with W7F- and W14F-swMb mutants was carried out at 20 °C similar to as described in Chapter 4. In brief, 20 µL of an ice-cooled peroxynitrite solution (different concentrations in 0.01 M NaOH) was added as a bolus while vortexing to 180 µL of a protein solution (112 µM in 0.1 M phosphate buffer, pH 7.0). After ca. 30 min, nitrite was removed by adding ca. 200 µL of an ammonium sulfamate solution (100 mM in 0.5 M HCl) and subjected to acid hydrolysis. Finally, the samples were analyzed by HPLC (*for details see previous Chapters).*

6.1.3 RESULTS AND DISCUSSION

6.1.3.1 KINETIC STUDIES

The decay of peroxynitrite in the presence of the iron(III) form of the W7F-swMb or of W14F-swMb mutants was measured by following the absorbance changes at 302 nm in the stopped flow spectrometer. As shown in Figure 3, the observed rates of decay of peroxynitrite (100 µM) increased linearly with increasing proteins concentration (0-25 µM). The catalytic rate constants measured for the protein-mediated decay of peroxynitrite are (1.9 ± 0.1) × 10^4 and (1.9 ± 0.3) × 10^4 $M^{-1}s^{-1}$ for W7F- and W14F-metMb, respectively. These values are nearly identical to the recently reported k_{cat} value of (1.6 ± 0.1) × 10^4 $M^{-1}s^{-1}$

measured for sw WT-metMb under similar conditions *(5)*. It is known that the reaction between peroxynitrite and tryptophan is slow and the rate constant measured is 130 $M^{-1}s^{-1}$ *(4)*. Since the mutated tryptophan residues do not change the structure of the active pocket in sperm whale myoglobin, there is no acceleration of the decay of peroxynitrite in the presence of the mutants. Therefore, replacement of either tryptophan residue in sperm whale myoglobin has little or no effect on the rate of decay of peroxynitrite.

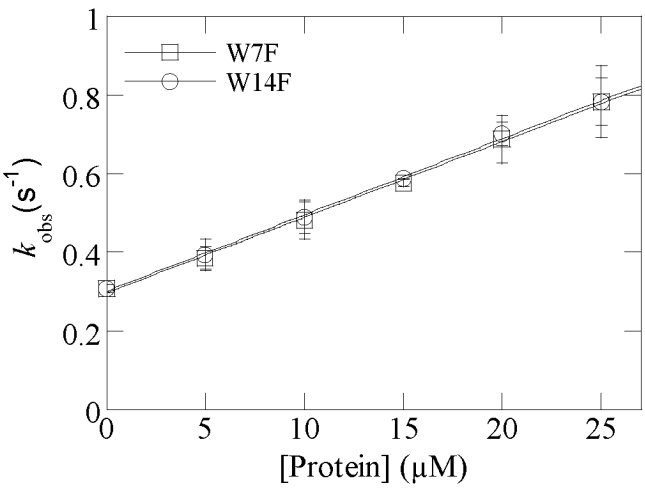


Figure 3. Plots of k_{obs} versus protein concentrations for the W7F- and W14F- metMb mutant-catalyzed decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer pH 7.0 and 20 °C.

Interestingly, the iron(III) forms of cytochrome P450 enzymes such as $P450_{NOR}$ and $P450_{BM3}$ have been reported to catalyze the decay of peroxynitrite via an intermediate ferryl species *(15)*. However, the results obtained from our stopped-flow studies show no detectable high valent oxoiron(IV) intermediates in the course of the reaction between peroxynitrite and Trp mutants.

6.1.3.2 NITROTYROSINE ANALYSIS

We analyzed the 3-nitrotyrosine content in W7F- and W14F-metMb after addition of excess of peroxynitrite. As shown in Figure 4, exposure of the mutants to 5, 10 or 20 equiv of peroxynitrite induced nitration in a dose-dependant way. Treatment with 20 equiv of peroxynitrite led to 7.3 ± 0.7 and 7.2 ± 0.2 % of nitration of the tyrosine residues in W7F- and W14F-metMb, respectively. In comparison, the sw WT-metMb was nitrated to 8.7 ± 0.6 % (Figure 4).

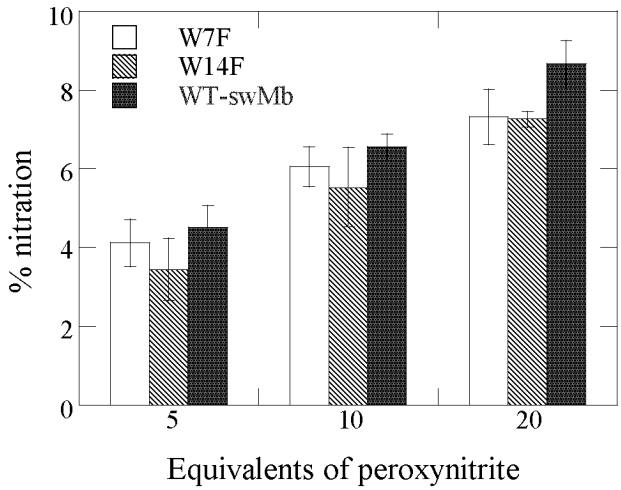


Figure 4. NO_2-Tyr yields (% relative to the total content of tyrosine residues available for nitration, that is, three residues per heme), determined by HPLC after acid hydrolysis of the proteins, from the reaction of sw WT-metMb, W7F- and W14F-metMb (100 μM) with 5, 10, and 20 equiv of peroxynitrite at 20 ºC and pH 7.0.

In myoglobin, Trp7 is located at the protein surface pointing away from the heme, while Trp14 is buried inside the protein. The distances from the carbon (C5) of Trp7 and Trp14 to the heme iron in sw-Mb are ~19 and ~12.8 Å, respectively. If one considers the location and distance of Trp14 to the heme centre, it would be expected that peroxynitrite generates Trp14 radicals by a mechanism mediated by the heme. However, the direct reaction between peroxynitrite and Trp7 can generate Trp7 radicals. These radicals can abstract hydrogen atom from Tyr to give Tyr radicals which may recombine with nitrogen dioxide to yield nitrotyrosine. Since the extent of tyrosine nitration in these two mutants was identical to that of sw WT-metMb, we suggest that the tryptophan radicals generated by direct reaction between peroxynitrite and tryptophans might not be involved in nitrotyrosine formation. These results confirm that the two tryptophans, W7 and W14 do not influence the yields of 3-nitrotyrosine in sw-Mb.

In other hemeproteins, the presence of a tyrosine residue in close proximity to the metal centre has been shown to result in higher nitration yields, due to a metal-catalyzed reaction via formation of ferryl intermediates *(15-17)*. We have reported higher nitrotyrosine yields in the F43W/H64L-metMb mutant in which a tryptophan residue is localized near the heme centre. However, no ferryl intermediate was detected in the course of its reaction with peroxynitrite *(6)*.

6.2 QUANTIFICATION OF THE EXTENT OF NITRATION OF MYOGLOBIN BY PEROXYNITRITE WITH ESI-MS.

6.2.1 INTRODUCTION

Groves and co-workers reported that the percentage nitration of tyrosine residues in horse heart metMb is about 50% upon treatment with 20 equiv of peroxynitrite *(18)*. They quantified the yield of 3-nitrotyrosine by using mass spectrometry (ESI-MS). In contrast, we have found that under analogous conditions metMb is nitrated to less than 10%, by HPLC analysis. Comparable results were obtained when nitrotyrosine was quantified after HCl hydrolysis or pronase digestion of the protein *(19)*. This discrepancy prompted us to reconfirm the amount of nitration in peroxynitrite-treated myoglobin by using mass spectroscopy.

6.2.2. EXPERIMENTAL CONDITIONS

6.2.2.1 ESI-MS SPECTROSCOPY

ESI-MS data were acquired with a single quadruopole mass spectrometer (Thermo Finnigan MSQ). The sample flow rate into the electrospray source was 5 μL/min, and data were typically acquired for 5-10 min while scanning over the *m/z* range 300-1500 at 4 scan/s. The cone voltage (sample orifice to skimmer potential) ramp was employed to optimize sensitivity over the *m/z* range scanned. The temperature of the spray capillary was held at 100 to 200 °C. The pressure of sheath gas was ~500 kPa of nitrogen. The samples were prepared by mixing 90 μL of the metMb and F43W/H64L-metMb solutions (25 μM in 0.1 M phosphate buffer pH 7.2) at room temperature with 10 μL of an ice-cooled peroxynitrite solution (400 μM in 0.01 M NaOH) while vortexing. Before injection into the ESI-MS instrument, the reaction mixture was always diluted 10 times with aqueous acetonitrile (50%) containing 0.1% TFA. All the above reactions were carried out in Eppendorf tubes (Micro-centrifuge tubes). In one experiment, a 10 μL peroxynitrite solution (100 μM in 0.01 M NaOH) taken in an Eppendorf tube was directly injected into the ESI-MS instrument.

6.2.2.2 FLUORESCENCE EXPERIMENTS

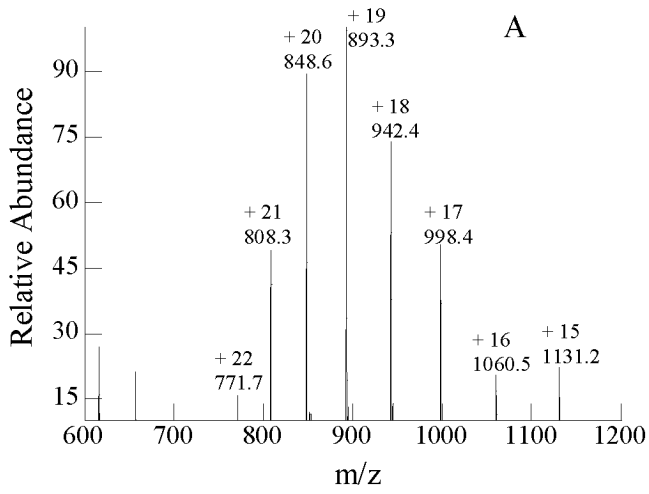
Fluorescence spectra were recorded on a Perkin Elmer LS 55 flurorescence spectrophotometer supplied with DM-3000 software (data interval 0.5 nm, scan speed 50 nm/min). Fluorescence emission was measured in the ratio mode and corrected for the

appropriate solvent-blanks, as well as for wavelength dependent bias of the optics and detection system. The excitation of fluorescence was set at 290 nm. The emission was scanned from 290 to 450 nm and integrated within this range for further analysis. We measured a fluorescence spectrum of apoMb (2 μM in 0.01 M phosphate buffer) and after reaction with 20 equiv of peroxynitrite (40 μM in 0.01 M NaOH) at pH 7.0 at 25 °C.

6.3 RESULTS AND DISCUSSION

6.3.1 NITRATION ANALYSIS BY USING ESI-MS

Figure 5A shows the control ESI-MS spectrum of metMb. After deconvolution, the mass of the protein was calculated to be 16955.7 Da, in good agreement with the calculated mass of 16956.0 Da. From the spectrum of metMb treated with 20 equiv of peroxynitrite (Figure 5B), we calculated that the mass of the modified metMb appears to be same as that of untreated protein i.e. 16955.7 Da. However, the spectra of the protein solutions treated peroxynitrite always contained new peak groups in the *m/z* range 300 to 700 *(data not shown)*. This observation may suggest degradation or cleavage of the protein. Moreover, the intensity of the peaks corresponding to the intact protein of the peroxynitrite-treated protein was lower than that of the control sample (compare y-axis of Figure 5A and 5B). Since the relative abundance is related to the number of times an ion of that *m/z* ratio strikes the detector, the observed low abundance may be due to protein degradation. From these results, we are unable to determine the extent of nitration in peroxynitrite-treated metMb.



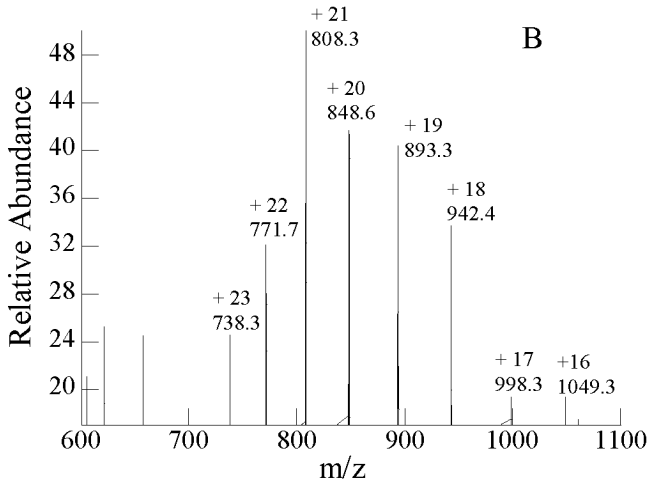


Figure 5. ESI-MS spectrum of metMb (A) and with 20 equiv peroxynitrite (B). *(Note that the scales of the relative abundance are different)*

We have shown that treatment of F43W/H64L-metMb with an excess of peroxynitrite leads to nitration of not only tyrosine but also tryptophan residues *(6, 19)*. Moreover, the yield of tyrosine nitration was larger than that observed for H64L-metMb. We proposed that the additional tryptophan (Trp43) localized near the heme may be oxidized and contribute to the formation of tyrosine radicals which can combine with nitrogen dioxide to yield 3-nitrotyrosine. In this work, we tried to quantify the yield of nitrotyrosine/nitrotryptophan in F43W/H64L-metMb by using ESI-MS. Figure 6A shows the ESI-MS spectrum of F43W/H64L-metMb. The calculated mass of the protein after deconvolution is 17331.6 Da in agreement with the expected mass of 17333.0 Da. As shown in Figure 6B, additional peaks appeared after reaction with 20 equiv of peroxynitrite. These peaks show high intensity and are shifted by ~ 2 units in the same region of *m/z* ratio of untreated protein. After deconvolution, the calculated mass of peroxynitrite-treated F43W/H64L-metMb was 17390.0 Da, a difference of plus ~ 60 Da.

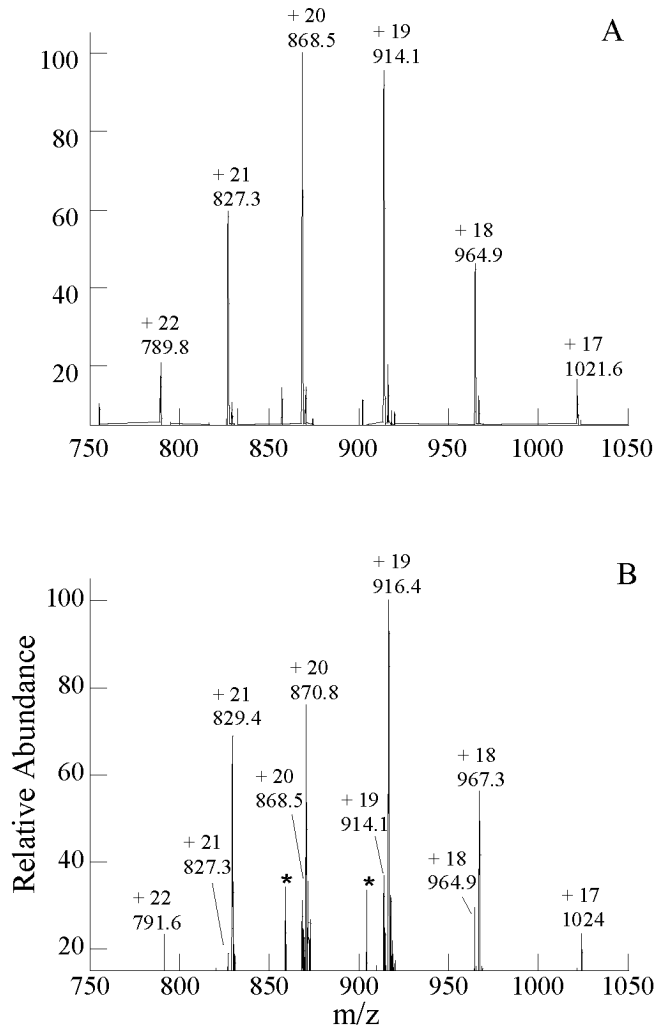


Figure 6. ESI mass spectrum obtained for F43W/H64L-metMb (A) and after reaction with 20 equiv of peroxynitrite (B).

Insertion of an oxygen molecule and a NO_2 group to the residues in a protein results in the increase of mass of plus ~ 16 and ~ 46 Da, respectively. We suggest that the increase in mass observed in peroxynitrite-treated F43W/H64L-metMb is probably due to oxidation and/or nitration of amino acid residues in the protein. However, because this result was obtained from a single experiment, further work has to be carried out.

Since all the above reactions were carried out in Eppendorf tubes and addition of peroxynitrite always led to some additional peaks in the protein, we tried to analyze the peroxynitrite solution taken in an Eppendorf tube. Direct injection of 100 μM peroxynitrite (in 0.01 M NaOH) into the ESI-MS instrument showed appearance of peaks similar to the protein peaks (Figure 7). The formation of these peaks suggests that peroxynitrite can react

with the polymer or coatings of the Eppendorf tube and may mislead in interpreting the protein peaks. Therefore, for further experiments, care should be taken to carry out reactions with peroxynitrite in glass vials.

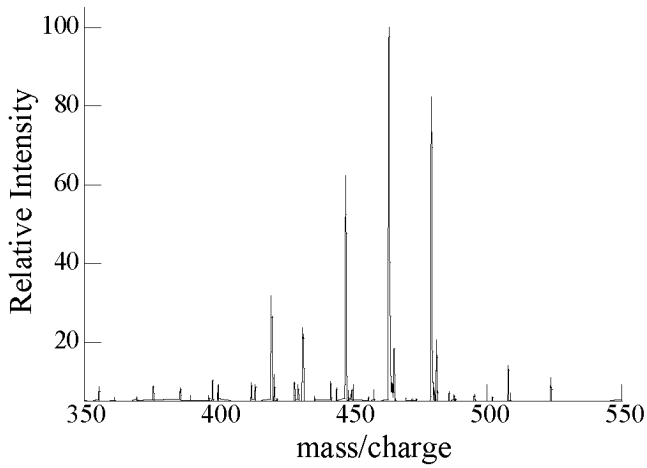


Figure 7. ESI-MS spectrum obtained after injecting a peroxynitrite solution.

6.3.2 FLUORESCENCE MEASUREMENT

Horse heart myoglobin contains two Trp residues at position 7 and 14 in the amino acid sequence. It has been shown that the replacement of Trp with phenylalanine at this position does not affect the correct protein fold whereas the simultaneous substitution of both Trp causes incorrect folding *(11, 12)*. These observations suggest that the tryptophan residues play an important role in driving the folding process and in determining the structure of apoMb.

We have shown that addition of 20 equiv of peroxynitrite leads to the formation of nitro-tryptophans in apoMb (horse heart) *(19)*, sw WT-, F43W/H64L- and H64L-metMb mutants (sperm whale) *(6)*. Here, we investigate the reaction of peroxynitrite with apoMb by using fluorescence spectroscopy. To avoid contamination from the tyrosine emission, protein samples were excited at 290 nm, a wavelength at which the absorption of this residue is negligible. The fluorescence spectrum of apoMb had an emission maximum at 350 nm (Figure 8). As shown in Figure 8, the fluorescence of apoMb decreased after treatment with 20 equiv of peroxynitrite. These observations suggest that the tryptophans in apoMb are readily modified at the aromatic ring of Trp by addition of peroxynitrite (Figure 8).

In the apoMb it was observed that Trp7 is more accessible to solvent than Trp14 *(20)*, we propose that addition of peroxynitrite may lead to the decay of fluorescence of Trp7 more readily than Trp14. However, studies with the apo form of the Trp substituted phenylalanine mutants, W7F- and W14F-swMb might help to identify which of the two tryptophans are modified upon addition of peroxynitrite.

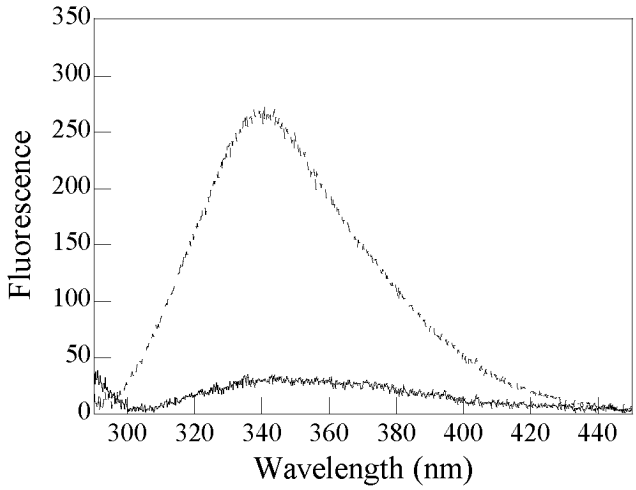


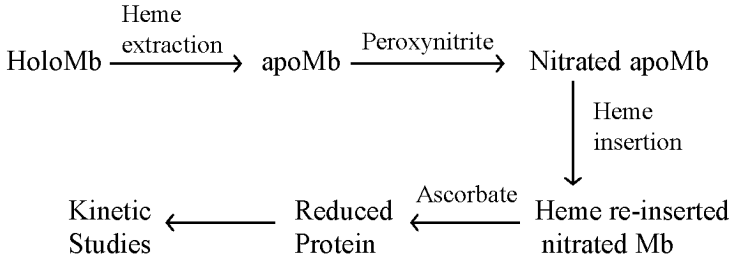
Figure 8. Fluorescence spectra of apoMb (dotted line) and with 20 equiv of peroxynitrite (straight line).

6.3 NITRATION OF apoMb WITH LARGE EXCESS PEROXYNITRITE AND HEME REINSERTION STUDY

6.3.1 INTRODUCTION

We have shown that the tyrosine residues of oxy- and the met-forms of myoglobin or hemoglobin are nitrated to very low levels (less than 10%) after addition of 20 equiv of peroxynitrite (see Chapter 2 for details). Addition of carbon dioxide, at physiological relevant concentrations, increased the level of tyrosine nitration to ~14% *(19)*. However, addition of 1 equiv of peroxynitrite in the presence of carbon dioxide resulted in ~1% nitration of tyrosine residues in oxyMb and metMb *(19)*. Thus, under physiological conditions the nitration yields are not relevant, since the formation of peroxynitrite *in vivo* is in the nanomolar range *(21)*.

We were interested to find out whether nitration of myoglobin or hemoglobin alters the function of these proteins, for instance, dioxygen binding or catalysis of the isomerization of peroxynitrite. Scheme 1 shows the general steps involved in this study; the apoMb is nitrated with large excess of peroxynitrite in the presence of carbon dioxide. After heme insertion, the nitrated protein is reduced by ascrobate, since strong reducing agent like sodium dithionite are known to reduce 3-nitrotyrosine to aminotyrosine *(22)*.



Scheme 1.

6.3.2 EXPERIMENTAL PROCEDURES

6.3.2.1 PREPARATION OF apoMb AND QUANTIFICATION OF 3-NITROTYROSINE

ApoMb was prepared according to the method of Teale *(see Chapter 2 for brief description, and ref 23)*. The reaction of peroxynitrite with apoMb was carried out as follows: to 0.8 mL of a protein solution (1 mM in 0.1 M phosphate buffer, pH 6.5 containing 22 mM bicarbonate) kept at room temperature we added 0.2 mL of an ice-cooled peroxynitrite

solution (50 or 75 equiv. in 0.01 M NaOH) as a bolus and vortexed immediately. The final pH measured after mixing was between 7.0 and 7.1. A part of the nitrated protein (200 μL) was subjected to HCl hydrolysis and HPLC analysis for quantification of 3-nitrotyrosine as described in Chapter 2.

6.3.2.2 HEME INSERTION IN NITRATED apoMb

The nitrated apoMb was suspended in 0.1 M potassium phosphate buffer pH 9.0 containing 0.1 M KCl and 10 % glycerol. The solution was concentrated to ~10 mL with an Amicon concentrator with a 10 kDa cutoff membrane filter. A hemin (Fe-protoporphyrin IX) solution (2 mL and 1.2 equiv) was prepared in 0.1 M NaOH and added drop-wise to the apoMb solution while stirring at 4 °C. The mixture was allowed to stir for 10 h at 4 °C and then loaded on a Sephadex G25 gel-filtration column equilibrated with 0.1 M phosphate buffer pH 9.0 containing 1 mM edta. The purified sample was concentrated (~10 mL) and subjected to CM52 column equilibrated with 0.1 M phosphate buffer pH 7.0. The eluted protein was collected and concentrated to ~5 mL.

6.3.3 RESULTS AND DISCUSSION

6.3.3.1 QUANTIFICATION OF 3- NITROTYROSINE

The HPLC chromatogram of apoMb after reaction with 75 equiv of peroxynitrite in the presence of carbon dioxide and acid hydrolysis showed two peaks with high intensity, eluted at ~ 8 min and 13 min. The hydrolyzed protein samples were measured at 350 nm. As shown in Figure 9, the spectrum of the peak at 8 min corresponds to the 3-nitrotyrosine and that at 13 min appears similar to that of nitrotyrosine. The chromatogram of apoMb after reaction with 50 equiv of peroxynitrite also showed similar additional peak at 13 min *(data not shown)*. We suggest that the additional peak eluted at ~13 min is di-nitrotyrosine. The formation of 3, 5-dinitrotyrosine has been reported from the reaction of free tyrosine and small peptides with peroxynitrite in the presence of carbon dioxide *(24, 25)*. However, these reports did not show the spectrum of 3, 5-dinitrotyrosine.

Nitrotyrosine was quantified by measuring a calibration curve of 5-10 nitrotyrosine standard solutions. Exposure of apoMb to 50 and 75 equiv of peroxynitrite in the presence of 1.2 mM carbon dioxide led to 48 ± 4 and 61 ± 2 % nitration of the available tyrosine residues (Tyr103 and Tyr146), respectively. The relative yield of nitrotyrosine was calculated from the area of both peaks in the chromatogram.

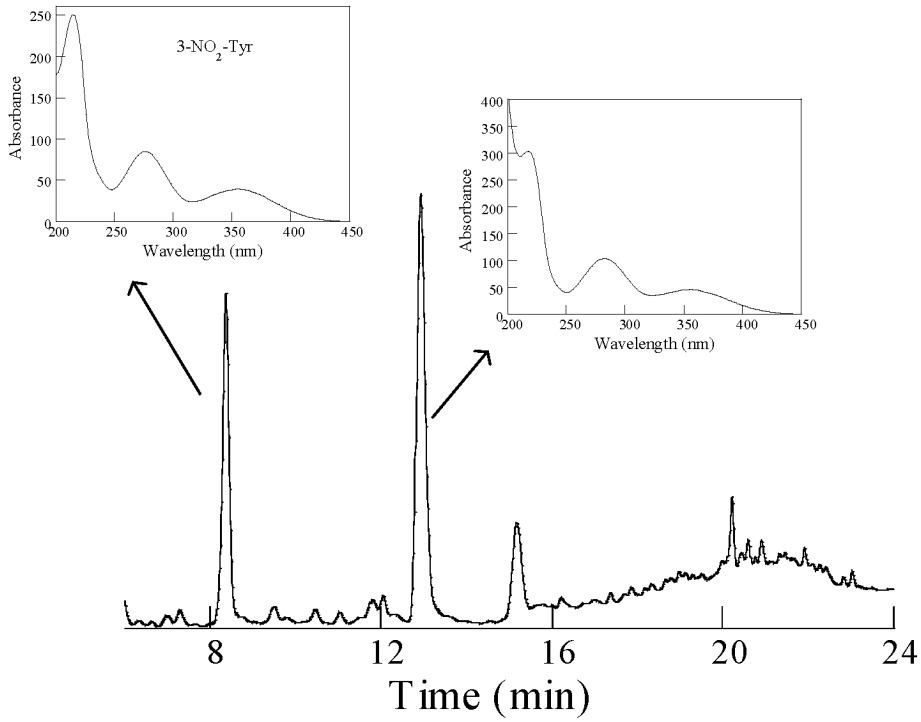


Figure 9. HPLC chromatogram (measured at 350 nm) of the products of the reactions of apoMb (~100 μM) with 75 equiv of peroxynitrite, in 0.1 M phosphate buffer pH 7.0, after complete acid hydrolysis as described in the experimental section. In the insets, the UV-vis spectra of the peak corresponding to NO_2-Tyr (~8 min) and possible di-nitrotyosine (~13 min).

6.3.3.2 REDUCTION OF THE NITRATED HEME-REINSERTED metMb

The Soret band absorbance maximum for metMb is at 408 nm *(26)*. As shown in Figure 10, the nitrated apoMb after heme insertion (unpurified) has an absorbance maximum at 410 nm where the unbound hemin is known to absorb. The purified nitrated metMb (after Sephadex G25) showed a shift in absorbance maximum to 396 nm. We tried to reduce the nitrated protein with ascorbic acid or with sodium dithionite. DeoxyMb is known to have an absorbance maximum at 435 nm *(26)*. As shown in Figure 10, we were unable to reduce the nitrated metMb.

The UV visible spectrum of metMb has absorbance maxima at 502 and 630 nm *(26)*. The nitrated purified metMb shows maxima at 542, 591 and 640 nm (Figure 11). The reaction of the metMb with hydrogen peroxide is known to lead to the formation of $MbFe^{IV}$=O *(1)*, which has absorption maxima at 549 and 588 nm *(27)*. However, the reaction of nitrated metMb with hydrogen peroxide does not lead to changes in the UV spectra (Figure 11). In metMb, cyanide binds strongly to heme iron and forms characteristic visible band at

540 nm *(26)*. However, addition of potassium cyanide did not lead to changes in the UV visible spectra of nitrated metMb (Figure 11). Similarly, no changes were observed for the Soret band *(data not shown)*.

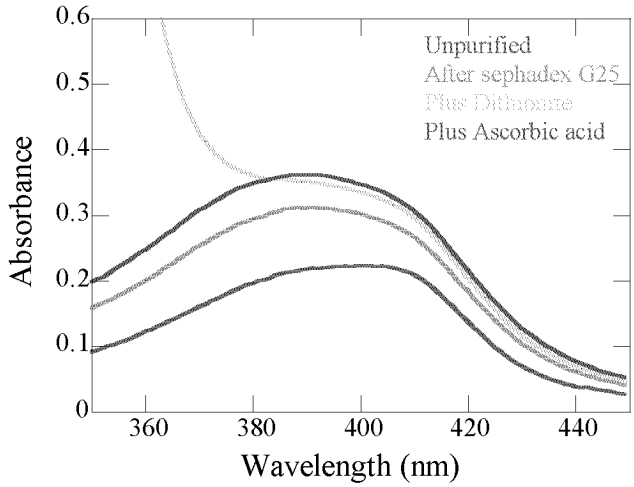


Figure 10. The UV absorption spectrum of the unpurified apoMb (blue), after purification with Sephadex G25 (green), reduced with sodium dithionite (yellow) and with ascorbic acid (red).

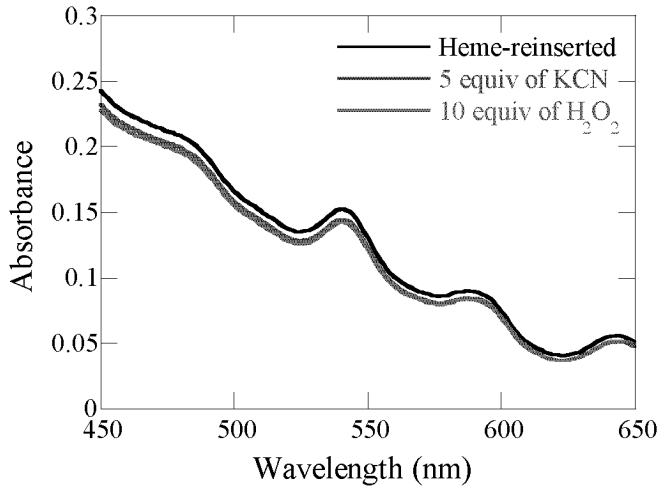


Figure 11. The UV visible spectra of purified metMb (black), after reaction with 10 equiv of hydrogen peroxide (blue) and with 5 equiv of potassium cyanide (red).

The spectral differences between metMb and in the modified metMb may be due to several factors. One of the possible explanations could be that nitration of apoMb can lead to conformational changes in the protein. Thus, the heme group may bind to different amino

acid residues in the protein and loose contact with the proximal and the distal histidine. These changes may make the protein resistant to the reduction by agents like ascorbic acid or sodium dithionite.

Alternatively, the proximal and the distal histidine could both coordinate to the heme iron to give a hexacoordinate complex. However, studies have shown that these hexacoordinated heme proteins can be reduced readily by sodium dithionite *(28)*. Since in our experiment the heme-inserted nitrated apoMb could not be reduced by sodium dithionite, a hexacoordinated heme is not very likely (Figure 10). Moreover, if the nitrated metMb would be hexacoordinate with His64 bound to the iron, addition of potassium cyanide is expected to lead to displacement of the coordinated His.

Since the nitrotyrosine yields of peroxynitrite-treated metMbCN and apoMb are similar (see Chapter 2), we also carried out experiments with metMbCN. However, treatment of metMbCN with a large excess of peroxynitrite (50–100 equiv) caused the formation of a cross-link between the porphyrin and the protein backbone (most likely Tyr). Indeed, the reaction of metMbCN with peroxynitrite caused a dose-dependent decrease of the amount of heme that can be extracted by acidic heme extraction with 2-butanone *(data not shown)*. Heme/protein cross-linking has been shown to take place analogously when metMb was treated with hydrogen peroxide *(29)*.

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APPENDICES

Table A. Area measured at 350 nm for NO_2-Tyr (200 μM) mixed with sodium nitrate (10 and 20 equiv) and ammonium sulfamate (100 mM) and HCl hydrolysis for 16 hr at 110 °C. *(see page 42)*

No.	Samples	Area at 350 nm
1	NO_2-Tyr	1958
2.	NO_2-Tyr + HCl hydrolysis	1901
3.	NO_2-Tyr + Ammonium sulfamate	1924
4.	NO_2-Tyr + Ammonium sulfamate + HCl hydrolysis	1926
5.	NO_2-Tyr + 10 equiv $NaNO_3^-$ + Ammonium sulfamate + HCl hydrolysis	1890
6.	NO_2-Tyr + 20 equiv $NaNO_3^-$ + Ammonium sulfamate + HCl hydrolysis	1932

Table C. NO_2-Tyr yields (% relative to the total content of the protein, that is, 2 tyrosine residue per heme), determined by HPLC after acid hydrolysis, from the reaction of apo-, met-, and oxyMb (100 μM) with 20 equiv of peroxynitrite (final concentration 2 mM) containing 1, 2, 3, and 5 mM sodium nitrite. The experiments were carried out at 0 °C, pH 7.0 and in the absence or presence of 1.1 mM CO_2. *(see page 47)*

Protein	Equivalents of peroxynitrite/nitrite							
	No added CO_2				1.1 mM CO_2			
	20/10	20/20	20/30	20/50	20/10	20/20	20/30	20/50
apoMb	19 ± 3	23 ± 2	30 ± 1	17.0 ± 0.6	30.5±0.7	25.5±0.2	24 ± 1	20.8 ±0.8
metMb	7 ± 1	7.1	nd	nd	8.3 ± 0.2	10 ± 3	10.0 ±0.2	7.3 ±0.8
oxyMb	4.6±0.3	4.2±0.9	3.8±0.4	3.5±0.3	14.7±0.2	13.4 ±0.1	15 ± 1	15 ± 2

nd: not determined

Table B. NO_2-Tyr yields (% relative to the total content of the protein, that is 2 tyrosine residues per heme for myoglobin and 3 tyrosine residues per heme for hemoglobin), determined by HPLC after acid hydrolysis, from the reaction of apo-, met-, oxyMb, metMbCN, met-, oxyHb (100 μM) with different amounts of peroxynitrite (1, 5, 10, and 20 equiv) at 0 or 20 ºC and pH 7.0 in the absence and presence of 1.1 mM CO_2. *(see page 43, 45, 47)*

Protein	Temperature	Equivalents of peroxynitrite							
		No added CO_2				1.1 mM CO_2			
		1	5	10	20	1	5	10	20
apoMb	0 ºC	nd	5 ± 1	11 ± 2	19 ± 3	2.7 ± 0.1	14.7±0.5	19.0 ± 0.7	30.5±0.7
	20 ºC	nd	Nd	12 ± 1	nd	2.8 ± 0.1	21 ± 1	27.3 ± 0.8	43 ± 2
metMb	0 ºC	nd	0.7 ± 0.1	1.1 ± 0.2	7 ± 1	0.52 ±0.05	2.8 ± 0.2	3.8 ± 0.5	8.3±0.2
	20 ºC	nd	nd	2.1 ± 0.1	nd	0.7 ± 0.1	3.8 ± 0.1	7.6 ± 0.1	15 ± 2
oxyMb	0 ºC	nd	1.0 ± 0.4	1.4 ± 0.2	4.6 ± 0.3	1.3 ± 0.1	7.2 ± 0.2	10.7 ± 0.2	14.7±0.2
	20 ºC	nd	nd	3.2 ± 0.1	nd	1.8 ± 0.5	9 ± 1	13.3 ± 0.5	19.0±0.1
metMbCN	0 ºC	nd	9.3 ± 0.1	11.0±0.3	14.6±0.7	nd	14.4±0.8	19.0 ± 0.7	26 ± 1
metHb	0 ºC	1.0±0.1	2.9 ± 0.1	3.9 ± 0.1	7.0 ± 0.2	1.4 ± 0.1	5.7 ± 0.4	11.7 ± 0.4	17.6±0.5
	20 ºC	nd	nd	nd	nd	2.0 ± 0.1	7.0 ± 0.1	10.0 ± 0.4	17.8±0.2
oxyHb	0 ºC	0.9±0.1	3.6 ± 0.1	5.8 ± 0.5	9 ± 2	1.2 ± 0.3	6.7 ± 0.7	14.3 ± 0.9	22.5±0.8
	20 ºC	nd	nd	nd	nd	1.5 ± 0.1	8.8 ± 0.1	18.0 ± 0.1	23.6±0.4

nd: not determined

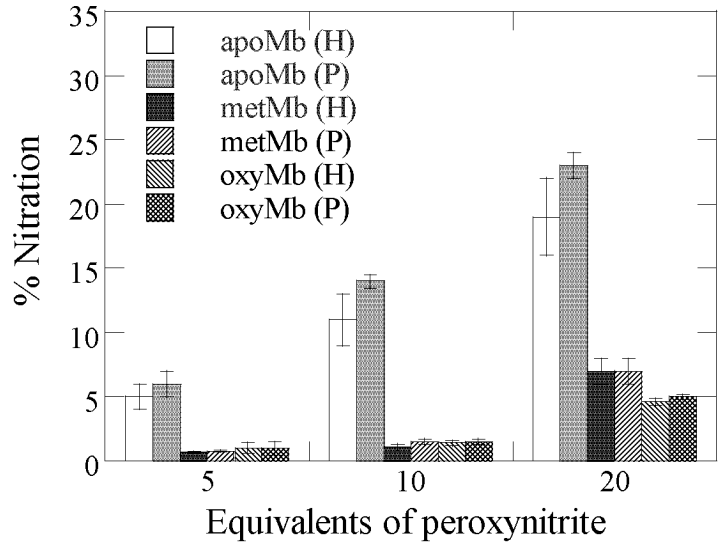

Figure A. Comparison of the NO_2-Tyr yields (% relative to the total content of the protein, that is 2 tyrosine residues per heme), determined by HPLC, from the reaction of apo-, met-, and oxyMb (100 μM) with different amounts of peroxynitrite (5, 10, and 20 equiv) at 0 °C and pH 7.0 after acid hydrolysis (HCl) and pronase digestion (P) of the proteins. *(see page 46)*

Table D. Relative yield of NO_2-Tyr formed from the reaction of peroxynitrite (200 μM) and tyrosine (100 μM), in the presence of different concentrations of oxyMb, metMb, apoMb, and metMbCN in 0.05 M phosphate buffer pH 7.4, 20 °C. The table shows the data in the absence and presence of 1.1 mM CO_2. Relative yield = (yield with added protein/yield with no protein) ×100%. *(see page 75)*

[protein] (μM)	Relative NO_2-Tyr yield											
	No CO_2								1.1 mM CO_2			
	oxyMb	ferrylMb	metMb	apoMb	metMbCN	oxyHb	deoxyHb	metHb	oxyMb	metMb	apoMb	metMbCN
No protein	100 ± 2	100 ± 2	100 ± 1	100 ± 1	100.0 ± 0.3	100 ± 1	100 ± 1	100.0 ± 0.3	100 ± 1	100 ± 1	100 ± 1	100 ± 1
0.5	94 ± 2	n.d	96 ± 1	94.1 ± 0.5	99 ± 1	n.d	n.d	n.d	94 ± 1	95 ± 1	96.0 ± 0.5	95 ± 1
1	86 ± 2	n.d	92 ± 4	93 ± 1	98 ± 1	n.d	n.d	n.d	92 ± 3	93 ± 1	94 ± 1	93 ± 1
2.5	80 ± 2	n.d	87 ± 2	88 ± 1	97.2 ± 0.5	n.d	n.d	n.d	82 ± 1	92 ± 1	93 ± 1	90 ± 1
5	70 ± 3	65.0 ± 0.6	76 ± 2	86 ± 2	95 ± 1	n.d	n.d	n.d	78 ± 2	90 ± 1	89 ± 1	88 ± 2
10	44 ± 4	53 ± 2	62 ± 1	84.0 ± 0.5	94 ± 1	n.d	n.d	n.d	65 ± 4	80 ± 1	88 ± 2	86 ± 1
15	31 ± 1	45.0 ± 0.8	52 ± 2	81 ± 2	92.2 ± 0.4	33 ± 2	n.d	62 ± 2	52 ± 1	77.3 ± 0.5	84 ± 1	86 ± 1
20	23 ± 2	36 ± 1	45 ± 1	78 ± 2	88 ± 1	n.d	n.d	n.d	50.1 ± 0.5	74 ± 2	82 ± 1	83 ± 1
25	14 ± 1	31 ± 1	36 ± 2	74 ± 2	86 ± 1	n.d	20 ± 2	n.d	42 ± 1	63 ± 2	75 ± 1	81.1 ± 0.5
50	0	14 ± 2	21 ± 2	63 ± 3	75 ± 1	8 ± 3	0	27 ± 1	26 ± 1	52 ± 3	74 ± 1	78 ± 1

n.d: not determined

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Table E. NO_2-Tyr yields (% relative to the total content of the protein, that is 2 tyrosine residues per heme for myoglobin and 3 tyrosine residues per heme for hemoglobin), determined by HPLC after acid hydrolysis, from the reaction of apoMb, metMb, oxyMb, metHb and oxyHb (100 μM) with 10 equivalents of peroxynitrite at 20 ºC or 37 ºC and pH 7.0 in the absence and presence of 1.1mM CO_2. *(see page 77)*

Protein	Temperature	10 equiv of peroxynitrite	
		No CO_2	1.1 mM CO_2
apoMb	20 ºC	12 ± 1	27.3 ± 0.8
	37 ºC	28 ± 2	36.7 ± 1.5
metMb	20 ºC	2.1 ± 0.1	7.6 ± 0.1
	37 ºC	7.3 ± 0.5	11.9 ± 0.3
oxyMb	20 ºC	3.2 ± 0.1	13.3 ± 0.5
	37 ºC	8.9 ± 0.7	14.3 ± 1.3
metHb	20 ºC	5.8 ± 0.6	10.0 ± 0.4
	37 ºC	10.3 ± 0.7	14.3 ± 0.3
oxyHb	20 ºC	8.2 ± 0.8	18.0 ± 0.1
	37 ºC	15.2 ± 0.5	19.6 ± 0.8

Table F. NO_2-Tyr yields (% relative to the total content of the protein) determined by HPLC after acid hydrolysis, from the reaction of BSA and Tyrosine (100 μM) with 5, 10 and 20 equivalents of peroxynitrite at 20 ºC or 37 ºC and pH 7.0 in the absence and presence of 1.1 mM CO_2. *(see page 78)*

Protein	Equiv. of $ONOO^-$	Equivalents of peroxynitrite			
		No CO_2		1.1 mM CO_2	
		20 ºC	37 ºC	20 ºC	37 ºC
BSA	5	0.02 ± 0.015	0.058 ± 0.004	0.098 ± 0.005	0.15 ± 0.004
	10	0.07 ± 0.01	0.085 ± 0.008	0.14 ± 0.01	0.20 ± 0.01
	20	0.07 ± 0.01	0.11 ±. 006	0.18 ± 0.004	0.29 ± 0.035
Tyrosine	5	6 ± 1	7 ± 1	7.7 ± 0.7	12.5 ± 1.8
	10	9 ± 1	14.2 ± 0.5	20.5 ± 1.3	25 ± 1
	20	15 ± 1	20 ± 1	30 ± 1	33 ± 1

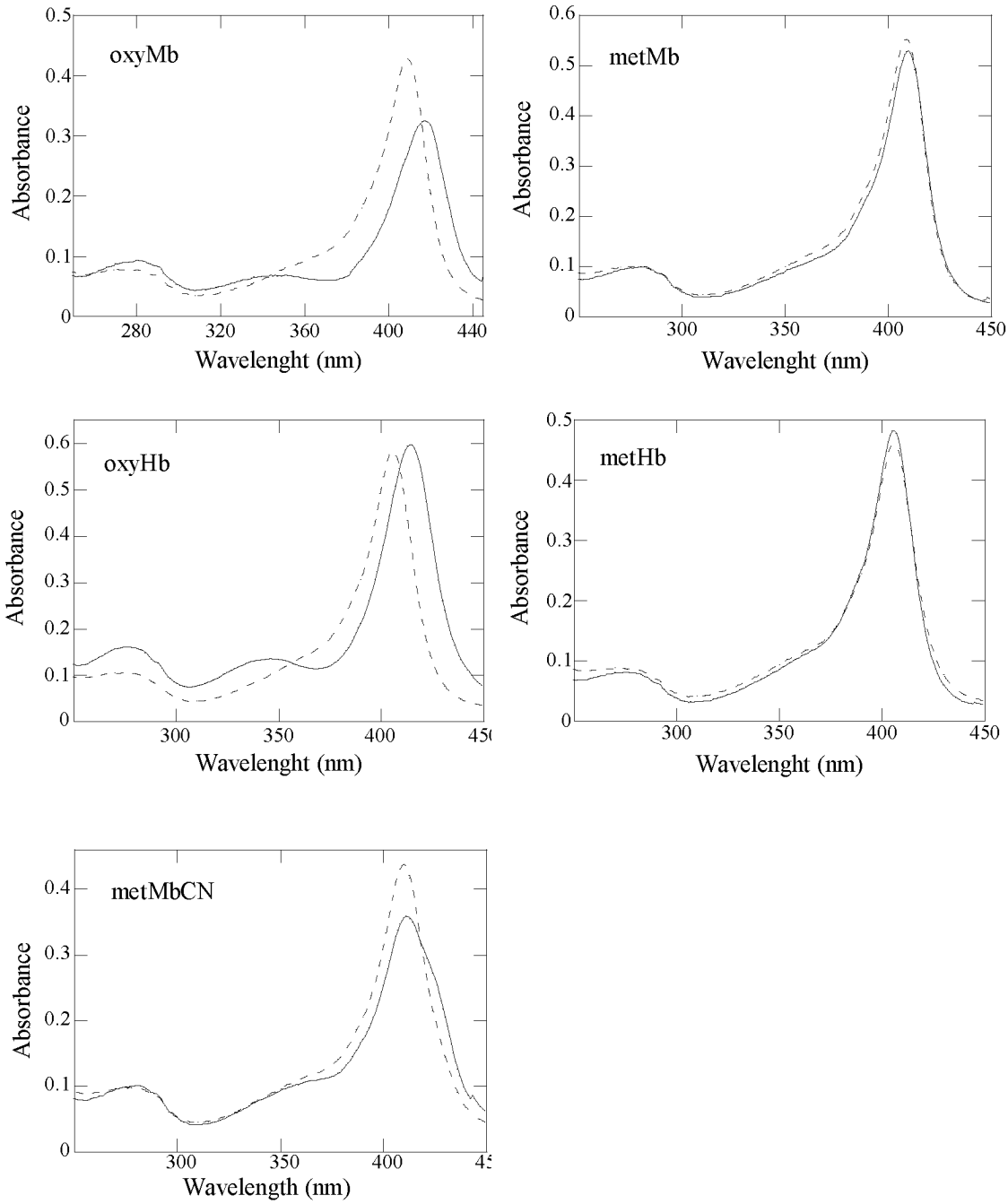

Figure B. Uv-vis spectra of oxyMb, metMb,oxyHb, metHb and metMbCN (5 µM) in 0.1 M phosphate buffer pH 7.0 (straight lines) and treated with 25 equiv of peroxynitrite (dotted lines). *(see page 45, 79)*

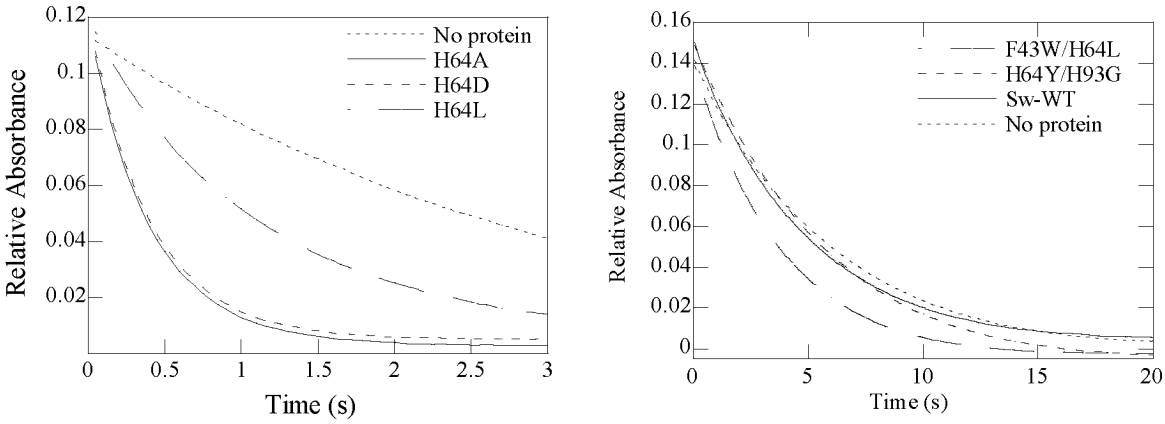

Figure C. Time courses measured by stopped-flow spectroscopy at 302 nm for the decomposition of 100 μM peroxynitrite in 0.05 M phosphate buffer at pH 7.0, 20 ºC: (A) in the absence of added proteins and with added mutants H64A, H64D, H64L-metMb; (B) with added F43W/H64L, H64Y/H93G and Sw-WT-metMb. Note the difference in time scale. *(see page 101)*

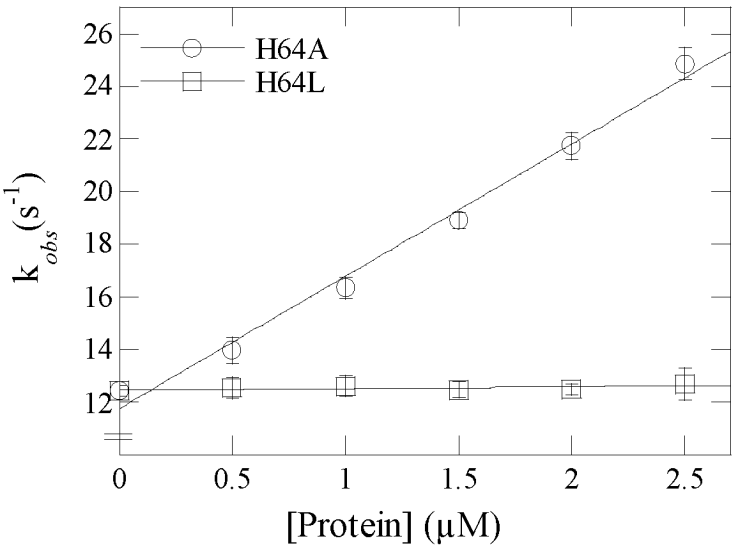

Figure D. Plots of k_{obs} versus protein concentration for the decay of peroxynitrite (100 μM) in 0.05 M phosphate buffer in the presence of 0.6 mM CO_2, pH 7.5 and 20 °C. *(see page 104)*

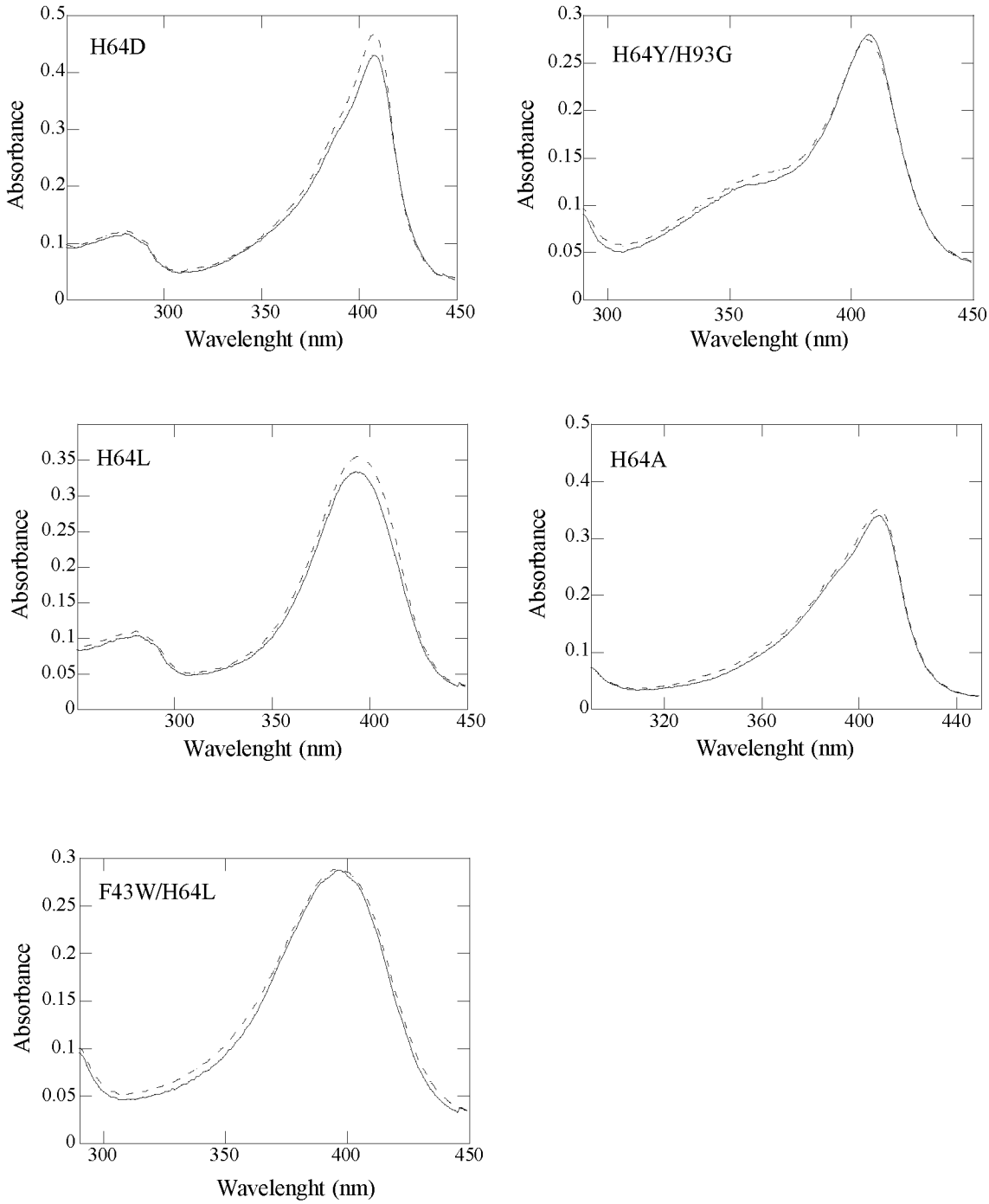

Figure E. Uv-vis spectra of H64D, H64Y/H93G, H64L, H64A and F43W/H64L (5 μM) in 0.1 M phosphate buffer pH 7.0 (straight lines) and treated with 25 equiv of peroxynitrite (dotted lines). *(see page 105)*

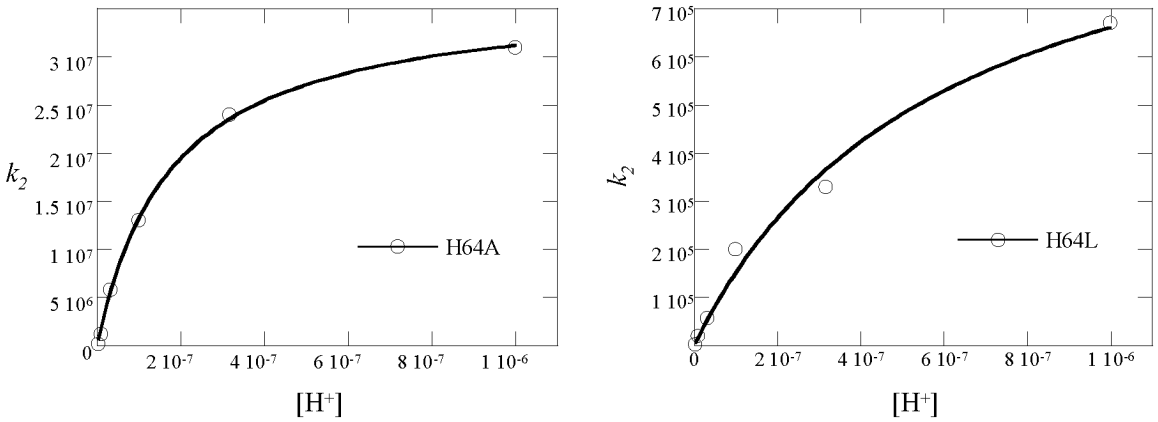

Figure F. Plot showing the k_{cat} value for H64A and H64L metMb. The values of pK = 6.7 ± 0.5 and $k_{cat'}$ = (3.7 ± 0.1) × 10^7 M^{-1} s^{-1} for H64A metMb and values of pK = 6 ± 1 and $k_{cat'}$ = (1.1 ± 0.1) × 10^5 M^{-1} s^{-1} for H64L metMb. *(see page 107)*

Table H. Relative yield of NO_2-Tyr formed from the reaction of peroxynitrite (200 μM) and tyrosine (100 μM), in the presence of different concentrations of H64A and H64L in 0.05 M phosphate buffer pH 8.0, 20 °C. The table shows the data in the presence of 5 mM CO_2. Relative yield = (yield with added protein/yield with no protein) × 100%. *(see page 114)*

Protein (μM)	Relative NO_2-Tyr yield	
	H64A	H64L
No protein	100 ± 3	100 ± 3
2.5	111 ± 6	93 ± 9
5	94 ± 4	88 ± 4
7.5	99 ± 5	80 ± 8
10	77 ± 4	94 ± 1

Table G. Relative yield of NO_2-Tyr formed from the reaction of peroxynitrite (200 μM) and tyrosine (100 μM), in the presence of different concentrations of H64A, H64D, H64L, F43W/H64L, H64Y/H93G and Sw-WT in 0.05 M phosphate buffer pH 7.4, 20 °C. The table shows the data in the absence and presence of 1.1 mM CO_2. Relative yield = (yield with added protein/yield with no protein) × 100%. *(see page 111, 113)*

[protein] (μM)	Relative NO_2-Tyr yield											
	No CO_2						1.2 mM CO_2					
	H64A	H64D	H64L	F43W/H64L	H64Y/H93G	Sw-WT	H64A	H64D	H64L	F43W/H64L	H64Y/H93G	Sw-WT
No protein	100	100±1	100 ± 1	100 ± 1	100 ± 1	100 ± 1	100±1	100±1	100±1	100 ± 1	100 ± 1	100 ± 1
0.5	9	11	67	87.0± 0.5	87 ± 1	94 ± 1	59 ± 2	70 ± 1	78 ± 2	83 ± 2	84 ± 0.5	81 ± 1
1	6	7	61	85±1.5	86 ± 1	89 ± 3	53 ± 2	60 ± 1	75 ± 1	77 ± 1	81 ± 1	79 ± 1
2.5	3	3	44	73±1	85 ± 0.5	83 ± 2	49 ± 1	51 ± 1	68 ± 1	73 ± 2	77 ± 1	77 ± 1
5	0	0	38 ± 1	60±2	79 ± 1	75 ± 2	37 ± 3	38 ± 2	61 ± 2	65 ± 3	72 ± 1	67 ± 2
10	0	0	30 ± 1	44±2	72 ± 1	68 ± 1	26 ± 2	28 ± 2	45 ± 1	57 ± 1	70 ± 2	65 ± 1

Table I. NO_2-Tyr yields (% relative to the total content of the protein) determined by HPLC after acid hydrolysis, from the reaction of H64A, H64D, H64L, F43W/H64L, H64Y/H93G and Sw-WT (100 μM) with 5, 10 and 20 equivalents of peroxynitrite at 20 °C and pH 7.0 in the absence and presence of 1.2 mM CO_2. *(see page 116, 117, 118)*

Protein	Equivalents of peroxynitrite					
	No CO_2			1.2 mM CO_2		
	5	10	20	5	10	20
H64A	0.12 ± 0.01	0.21 ± 0.02	0.37 ± 0.02	0.94 ± 0.2	1.6 ± 0.2	5.32 ± 0.05
H64D	0.12	0.21 ± 0.03	0.45 ± 0.04	0.37 ± 0.07	1.9 ± 0.3	5.2 ± 0.4
H64L	0.4 ± 0.02	1.0 ± 0.1	1.5 ± 0.2	4.7 ± 0.7	8.4 ± 0.7	12.5 ± 0.7
F43W/H64L	0.9 ± 0.08	2.2 ± 0.1	4.4 ± 0.4	4.5 ± 0.2	10.8 ± 0.2	17.4 ± 1.2
H64Y/H93G*	4.5 ± 0.3	7.3 ± 0.4	11.5 ± 0.6	4.8 ± 0.4	11.0 ± 0.8	17.4 ± 1.5
Sw-WT	4.5 ± 0.5	6.6 ± 0.3	8.7 ± 0.6	6.4 ± 0.5	12.1 ± 0.5	17.3 ± 0.4

* data shows H64Y/H93G with respect to 4 tyrosine residues, and the rest with 3.

Table J. NO_2-Tyr yields (% relative to the total content of the protein) determined by HPLC after Pronase digestion, from the reaction of H64A, H64D, H64L, F43W/H64L, H64Y/H93G and Sw-WT (100 μM) with 20 equivalents of peroxynitrite at 20 °C and pH 7.0 in the absence and presence of 1.2 mM CO_2. *(see page 119)*

Protein	% Nitration	
	No CO_2	1.2 mM CO_2
H64A	0.50 ± 0.03	6.2 ± 0.5
H64D	0.63 ± 0.05	7.7 ± 0.3
H64L	2.2 ± 0.5	13.2 ± 0.5
F43W/H64L	5.3 ± 0.5	18.5 ± 0.6
Sw-WT	10.7 ± 0.8	17.4 ± 0.7

CURRICULUM VITAE

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RESEARCH EXPERIENCE

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PUBLICATIONS

1. Herold, S., Kalinga, S., Matsui, T. and Watanabe, Y., *J. Am. Chem. Soc. 2004, 126,* 6945-6955
2. Herold, S. and Shivashankar, K. *Biochemistry 2003, 42,* 14036-14046.
3. Herold, S., Shivashankar, K. and Mehl, M. *Biochemistry 2002, 41,* 13460-13472.
4. Shivashankar, K., Subbayya, I. N. and Balaram, H., *J. Mol. Microbiol. Biotechnol. 2001, 3,* 557-562.
5. Sujay Subbayya, I. N., Sukumaran, S., Shivashankar, K. and Balaram, H., *Biochem. Biophys. Res. Commun. 2000, 272,* 596-602.

ACHIEVEMENTS

1. ETH PhD qualifying examination grades: 6.0/6.0 from Prof. C. Richter (ETHZ, Biochemistry) and 4.5/6.0 from Prof. D. Hilvert (ETHZ, Bioorganic Chemistry).
2. Received student travel award from the University of Singapore to attend the 2nd International Conference on Structural Biology and Functional Genomics 2002, December 2-4, SINGAPORE.
3. Received student travel grant from Swiss Chemical Society to attend the 2nd International Conference on Biomedical Spectroscopy 2003, July 5-8, UNITED KINGDOM.
4. Received best poster presentation award at the 2nd International Conference on Biomedical Spectroscopy 2003, July 5-8, UNITED KINGDOM.
5. Received student travel grant from 3rd International Conference, Biology, Chemistry, and Therapeutic Applications of Nitric Oxide 2004, May 24-28, JAPAN.
6. Received student travel allowance for summer internship 2004 in Japan from Boehringer Ingelheim Fonds, GERMANY.
7. Received COE Fellowship for summer internship, July-September 2004, Nagoya University, JAPAN.

PARTICIPATION IN CONFERENCES

1. Oral Presentation on the 3rd International Conference, Biology, Chemistry, and Therapeutic Applications of Nitric Oxide 2004, May 24-28, Nara, JAPAN.
2. A poster presentation on the 2nd International Conference on Biomedical Spectroscopy 2003, July 5-8, London, UNITED KINGDOM.
3. A poster presentation on the 2nd International Conference on Structural Biology and Functional Genomics 2002, December 2-4, NUS, SINGAPORE.
4. A poster presentation on the 5th Swiss Micro-symposium on Radicals in Health and Disease 2002, June 28, Gottlieben, SWITZERLAND.
5. 2nd Intl. Conference on Biology, Chemistry and Therapeutic Applications of Nitric Oxide 2002, June 16-20, Prague, CZECH REPUBLIC. Herold. S., Shivashankar. K., and Röck. G. *Nitric Oxide. (2002)* 6, 401.
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7. Herold. S., Boccini. F., and Kalinga. S. Interaction of peroxynitrite with hemoglobin and myoglobin. *Free. Rad. Biol. Med. (2004)* 36, S3-S4.
8. Shivashankar. K., and Herold. S. Myoglobin catalyze the isomerization of peroxynitrite to nitrate. *Nitric oxide. (2004)* 11, 54.