Scavenging of Peroxynitrite by Oxyhemoglobin and Identification of Modified Globin Residues†

Maurizio Minetti,*,‡ Donatella Pietraforte,‡ Virginia Carbone,§ Anna Maria Salzano,§,|| Giuseppe Scorza,‡ and Gennaro Marino§,||

Laboratorio di Biologia Cellulare, Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161 Roma, Italy, CNR, Centro Internazionale di Servizi di Spettrometria di Massa, Via Pansini 5, I-80131, Napoli, Italy, and Dipartimento di Chimica Organica e Biologica, Università di Napoli Federico II, Via Mezzocannone 16, I-80134 Napoli, Italy

Received December 6, 1999; Revised Manuscript Received February 28, 2000

ABSTRACT: Peroxynitrite is a strong oxidant involved in cell injury. In tissues, most of peroxynitrite reacts preferentially with CO_2 or hemoproteins, and these reactions affect its fate and toxicity. CO_2 promotes tyrosine nitration but reduces the lifetime of peroxynitrite, preventing, at least in part, membrane crossing. The role of hemoproteins is not easily predictable, because the heme intercepts peroxynitrite, but its oxidation to ferryl species and tyrosyl radical(s) may catalyze tyrosine nitration. The modifications induced by peroxynitrite/CO_2 on oxyhemoglobin were determined by mass spectrometry, and we found that αTyr42, βTyr130, and, to a lesser extent, αTyr24 were nitrated. The suggested nitration mechanism is tyrosyl radical formation by long-range electron transfer to ferrylhemoglobin followed by a reaction with $^{\bullet}NO_2$. Dityrosine (α24−α42) and disulfides (β93−β93 and α104−α104) were also detected, but these cross-linkings were largely due to modifications occurring under the denaturing conditions employed for mass spectrometry. Moreover, immunoelectrophoretic techniques showed that the 3-nitrotyrosine content of oxyhemoglobin sharply increased only in molar excess of peroxynitrite, thus suggesting that this hemoprotein is not a catalyst of nitration. The noncatalytic role may be due to the formation of the nitrating species $^{\bullet}NO_2$ mainly in molar excess of peroxynitrite. In agreement with this hypothesis, oxyhemoglobin strongly inhibited tyrosine nitration of a target dipeptide (Ala−Tyr) and of membrane proteins from ghosts resealed with oxyhemoglobin. Erythrocytes were poor inhibitors of Ala−Tyr nitration on account of the membrane barrier. However, at the physiologic hematocrit, Ala−Tyr nitration was reduced by 65%. This "sink" function was facilitated by the huge amount of band 3 anion exchanger on the cell membrane. We conclude that in blood oxyhemoglobin is a peroxynitrite scavenger of physiologic relevance.

Tyrosine nitration appears to represent in vivo a prominent pathway of protein modification in several different pathological conditions (*1*−*4*). The detection of stable 3-nitrotyrosine residues in pathologic tissues was suggestive of the existence of nitrating pathways and considered a possible marker for the production of peroxynitrite[1] in vivo. It should be mentioned, however, that probably peroxynitrite is not the only tyrosine nitrating agent, as this modification can also occur through mechanisms involving peroxidases, nitrite, and hydrogen peroxide (*5*).

Peroxynitrite is a powerful oxidant that is formed by the diffusion-limited reaction between nitrogen monoxide ($^{\bullet}NO$, more frequently referred to as nitric oxide) and superoxide anion ($O_2^{\bullet-}$) at a faster rate (*6*, *7*) than that of the reaction of superoxide with superoxide dismutase (*8*). In tissues, however, most of peroxynitrite does not react directly with several biological targets because of its fast reaction with CO_2 and proteins containing metal centers (*9*−*12*). These compounds are highly concentrated in tissues (from micromolar to millimolar), and their reactions with peroxynitrite, thus, precede those with other biological targets, including low molecular weight antioxidants (*10*). The $ONOO^-/CO_2$ reaction is of particular relevance because it significantly decreases the lifetime of peroxynitrite (*10*) and leads to the formation of a postulated secondary oxidant, the nitrosoperoxycarboxylate[2] anion adduct, $ONOOCO_2^-$ (*9*, *13*). The lifetime of $ONOOCO_2^-$ is too short to allow it to cross a biological membrane (*10*), but it has been suggested that the radicals produced in the decay process, $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ (*14*−*16*), increase the nitration of tyrosine residues and one-electron oxidations (*10*, *12*, *13*, *17*−*20*).

The reaction of peroxynitrite with some hemoproteins ($k = 10^4-10^7$ M^{-1} s^{-1}) leads to redox modification of metal centers and tyrosyl radical formation, but it is unclear whether these reactions protect the tissue or contribute to peroxynitrite-mediated damage (*11*, *12*, *21*−*23*). Since tyrosine

† This work was supported in part by Istituto Superiore di Sanità research project: "Natural antioxidants and effects on chronic-degenerative diseases".

* Corresponding author: Dr. M. Minetti, Department of Cell Biology, Istituto Superiore di Sanità, Viale Regina Elena 299, I-00161 Roma, Italy. Telephone: +39 06 49902907. Fax: +39 06 49387143. E-mail: mminetti@iss.it.

‡ Istituto Superiore di Sanità.

§ Centro Internazionale di Servizi di Spettrometria di Massa.

|| Università di Napoli Federico II.

[1] This term refers to both the anion oxoperoxynitrate (1−), $ONOO^-$, and its conjugate acid hydrogen oxoperoxynitrate, ONOOH.

[2] IUPAC-recommended name is 1-carboxylato-2-nitrosodioxidane.

10.1021/bi9927991 CCC: $19.00
Published on Web 05/10/2000

nitration by peroxynitrite is strongly dependent on the simultaneous availability of tyrosyl and $^{\bullet}NO_2$ radicals (*9, 16, 21*), hemoproteins are in principle expected to increase tyrosine nitration if they can promote the formation of these radicals. Recently, it has been demonstrated that the heme-thiolate protein $P450_{nor}$ can catalyze peroxynitrite-mediated formation of 3-nitrotyrosines in target molecules (*21*). It has been suggested that the ferrylheme oxidizes target tyrosines to tyrosyl radicals, which in turn react with $^{\bullet}NO_2$ to yield 3-nitrotyrosines (*21*). However, the action of hemoproteins is determined by several factors including (i) the solvent exposure of tyrosine residues, (ii) the existence in the vicinity of tyrosine residues of metal centers or polar amino acid side chains (*21, 24*), and (iii) the ability to convey oxidizing equivalents from the heme to the tyrosine residue(s) and from tyrosine residue(s) to other targets (*25, 26*), processes involving long-range electron transfer or electron tunneling (ET).

Recent studies suggested, that in the vasculature, oxyhemoglobin (oxyHb)[3] is one of the most important targets for peroxynitrite (*12, 27, 28*). This belief is based on (i) the high concentration of oxyHb in blood, (ii) a second-order rate constant for peroxynitrite comparable to that of CO_2 (*12*), and (iii) the fast diffusion of peroxynitrite across the erythrocyte membrane (*12*). We reported in a previous work that peroxynitrite reacts as a peroxide with oxyHb, oxidizing the heme to a ferryl species (in this work, we use the term ferryl to denote generically high valent oxo-iron species such as ferryl or perferryl heme) and forming a long-lived tyrosyl radical(s) similar to that (those) produced by hydrogen peroxide (*28*). This oxidative pathway involves a bimolecular reaction between peroxynitrite and oxygenated heme and is thus mechanistically different from the one-electron oxidations mediated by $^{\bullet}NO_2$ and/or $CO_3^{\bullet-}$ radicals formed during $ONOOCO_2^-$ decay.

The experimental design of our study was focused on the properties of oxyHb in peroxynitrite-mediated tyrosine nitration measured either in target molecules or in globin residues by mass spectrometry (MS). The picture that emerges is oxyHb acting as a scavenger of peroxynitrite without catalyzing tyrosine nitration.

MATERIALS AND METHODS

Materials. Anti-3-nitrotyrosine monoclonal antibodies were obtained from Upstate Biotechnology Incorp. (Lake Placid, NY). Acetonitrile was of the highest purity grade from J. T. Baker (Milan, Italy). Iodoacetamide was from Fluka (Buchs, Switzerland), and endoproteinase AspN was from Boehringer Mannheim (Monza, Italy). All other reagents were from Sigma (St. Louis, MO).

DIDS Treatment, Preparation of Ghosts, and Hemoglobin Purification. Heparinized fresh human blood was obtained from normal donors following informed consent. An aliquot of erythrocytes washed in isotonic phosphate buffer (0.15 M NaCl, 5 mM sodium phosphate, pH 7.4) was suspended at 20% hematocrit and incubated with 10 mM 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS). After 15 min at room temperature, cells were extensively washed in isotonic phosphate buffer to remove excess DIDS. The oxyHb content of control and DIDS-treated erythrocytes was 99.8 ± 0.2%. A concentrated hemolysate and oxyHb purification by ionic exchange column was obtained as previously described (*28*). MetHb was prepared from oxyHb by the addition of potassium ferricyanide in a 1:1.2 molar ratio. Excess ferricyanide was removed by a Sephadex G-25 column. The concentration of oxyHb and metHb expressed per heme group was determined spectrophotometrically (*29*). CO-Hb was obtained from oxyHb by bubbling with CO gas for 3 min. Hemoproteins were concentrated by centrifugation on Microcon (cutoff 10 kDa, Amicon, Beverly, MA). Hb-free ghosts were resealed in 0.13 M KCl, 5 mM diethylenetriaminepentaacetic acid (DTPA), 0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 5 mM phosphate buffer, pH 7.4, at 37 °C for 10 min. In some experiments, ghosts were resealed in the presence of different concentrations of purified oxyHb to allow oxyHb trapping. After resealed ghosts were extensively washed, the yield of incorporated oxyHb ranged from 10 to 30%. To avoid metal-catalyzed nitration by peroxynitrite (*30*), buffers were treated extensively with Chelex 100 (Bio-Rad, Richmond, CA), and all samples contained 0.1 mM DTPA.

Treatments with Peroxynitrite. Peroxynitrite was synthesized from nitrite and H_2O_2 as described by Radi et al. (*31*) and treated with MnO_2 (2 mg/mL, 30 min, 4 °C) to eliminate excess H_2O_2. The peroxynitrite concentration was determined at 302 nm ($\epsilon = 1670\ M^{-1}\ cm^{-1}$). Peroxynitrite was added as a bolus to samples, buffered with 150 mM phosphate, 0.1 mM DTPA, and 25 mM sodium bicarbonate, pH 7.4 (phosphate/DTPA/bicarbonate) and submitted to vigorous vortexing. Bicarbonate was added to the phosphate buffer from a 1 M stock solution, and the dissolved CO_2 was 1.3 mM with 25 mM bicarbonate, as measured by a blood gas analyzer (ABL 330 Radiometer, Copenhagen, Denmark). Decomposed peroxynitrite was obtained by inactivation at acidic pH. A slight excess of HCl was added to the alkaline solution of peroxynitrite before its addition to biological targets. NO_2^- is able to induce oxyHb oxidations (*32*) and is present as a contaminant of peroxynitrite preparations. However, we observed that oxyHb induced the nitration of Ala−Tyr only in the presence of $NO_2^- > 1$ mM and with the simultaneous addition of $H_2O_2 \geq 0.5$ mM (unpublished results).

Gel Electrophoresis and Western Blot. After being exposed to peroxynitrite, erythrocytes, ghosts, or oxyHb were dissolved in electrophoresis loading buffer (1% sodium dodecyl sulfate, 7.5% glycerol, 0.1% β-mercaptoethanol, 0.01% Bromophenol blue, 0.055 M Tris-HCl, pH 6.7, final concentrations) in which the concentration of β-mercaptoethanol was lowered to 0.1% (14 mM) to avoid heme- and thiol-dependent reduction of 3-nitrotyrosines (*33*). Proteins were separated by sodium dodecyl sulfate−polyacrylamide gel electrophoresis (SDS−PAGE) and for Western blot analysis were transferred to nitrocellulose paper (Sleicher & Schuell, Dassel, Germany) at 35 V overnight and incubated with monoclonal anti-3-nitrotyrosine antibodies as previously described (*34*). Specificity of the anti-3-nitrotyrosine anti-

[3] Abbreviations: oxyHb, oxyhemoglobin; metHb, methemoglobin; ferrylHb, ferrylhemoglobin; CO-Hb, carbon monoxide hemoglobin; phosphate/DTPA/bicarbonate, 150 mM phosphate−0.1 mM DTPA−25 mM sodium bicarbonate, pH 7.4, buffer; MS, mass spectrometry; LC/ES, liquid chromatography/electrospray; MALDI/MS, matrix laser desorption ionization; IS, ionspray; SDS−PAGE, sodium dodecyl sulfate−polyacrylamide gel electrophoresis; TFA, trifluoroacetic acid; DTPA, diethylenetriaminepentaacetic acid; DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; ET, electron transfer or electron tunneling.

bodies was established by the absence of reactivity after reduction to 3-aminotyrosine before SDS−PAGE analysis (*33*). Band intensity of stained proteins (Red Ponceau S) or band intensity of X-ray films was quantified by densitometric analysis (GS-700 Imaging densitometer, Bio-Rad, Hercules, CA).

Liquid Chromatography−Electrospray Mass Spectrometry (LC/ES/MS). Peroxynitrite-treated oxyHb was analyzed by LC/ES/MS "on-line" using an LCQ ion trap instrument (Finnigan Corp., San José, CA). Individual globin chains were fractionated using a Phenomenex Jupiter C4 column (250 × 4.6 mm, 300 Å) (Torrance, CA) using 0.25% trifluoroacetic acid (TFA) in H_2O/CH_3CN 4:1 (solvent A) and 0.1% TFA in H_2O/CH_3CN 2:3 (solvent B) by means of a linear gradient from 57 to 70% solvent B for 35 min. One-third of the eluate was injected into the electrospray ion source, and the spectra were acquired and elaborated using the software provided by the manufacturer. Modified globin chains were purified by preparative HPLC on an HP 1100 apparatus (Hewlett-Packard, Palo Alto, CA) using a Phenomenex Jupiter C4 column (250 × 10 mm, 300 Å). Fractions were collected manually and dried down in a Speedy-Vac centrifuge (Savant, Farmingdale, NY). Modified globin chains were carboxyamidomethylated by incubation with a 10-fold molar excess of iodoacetamide (relative to SH groups) in 0.1 M Tris-HCl and 1 mM EDTA, pH 8.0, for 30 min at room temperature under nitrogen atmosphere in the dark. Alkylated globins were desalted and then analyzed by ionspray mass spectrometry (IS/MS) on an API/100 quadrupole mass spectrometer (Perkin-Elmer, Norwalk, CT). Relative yields were calculated on the area value related to the extract ions [m/z = 1225.0 (MH_{13}^{+13}), m/z = 1327.0 (MH_{12}^{+12}), m/z = 1447.5 (MH_{11}^{+11})] for nitrated β-chain; [m/z = 1443.4 (MH_{22}^{+22}), m/z = 1512.0 (MH_{21}^{+21}), m/z = 1587.5 (MH_{20}^{+20})] for β-chain dimer; [m/z = 1168.1 (MH_{13}^{+13}), m/z = 1265.2 (MH_{12}^{+12}), m/z = 1379.9 (MH_{11}^{+11})] for nitrated α-chains; [m/z = 1211.5 (MH_{22}^{+22}), m/z = 1261.9 (MH_{21}^{+21}), m/z = 1316.6 (MH_{20}^{+20})] for α-chain dimers calculated as percent of the area value related to the extract ions; [m/z = 1221.6 (MH_{13}^{+13}), m/z = 1323.3 (MH_{12}^{+12}), m/z = 1443.5 (MH_{11}^{+11})] for unmodified β-chain; and [m/z = 1164.7 (MH_{13}^{+13}), m/z = 1261.6 (MH_{12}^{+12}), m/z = 1376.1 (MH_{11}^{+11})] for unmodified α-chains.

Chemical and Enzymatic Hydrolysis. Carboxyamidomethylated samples were digested with CNBr in 70% TFA at room temperature for 18 h in the dark. Samples were then diluted 10 times with water and lyophilized. CNBr fragments were purified by HPLC on a narrowbore Phenomenex Jupiter C4 column (250 × 2.0 mm, 300 Å) using 0.1% TFA (solvent A) and 0.07% TFA in 95% CH_3CN (solvent B) by means of a two-step gradient; the column was equilibrated at 30% of solvent B, and after 5 min of isocratic elution, the acetonitrile concentration was raised from 30 to 45% in 15 min and to 60% in 38 min; the elution was monitored both at 220 and 360 nm (absorbance of 3-nitrotyrosine) at a flow rate of 0.2 mL/min. Purified CNBr peptides were digested either with chymotrypsin or with pepsin. Hydrolysis with chymotrypsin was performed in 0.4% ammonium bicarbonate, pH 7.5, at 37 °C for 2 h using an enzyme-to-substrate ratio of 1:50 (w/w). Hydrolysis with pepsin was carried out in 5% formic acid at 37 °C for 30 min using an enzyme-to-substrate ratio of 1:150 (w/w). Resulting peptide mixtures were analyzed directly by LC/ES/MS using the LCQ instrument described above. Proteolytic digest was fractionated on a narrowbore C18 column using a linear gradient from 5 to 60% of 0.07% TFA in 95% acetonitrile over 60 min at a flow rate of 0.2 mL/min. Effluent was directly inserted into the ion source through the electrospray probe, and ES/MS spectra were acquired through the entire analysis by using the software provided by the manufacturer. Hydrolysis with endoproteinase AspN was carried out in 0.4% ammonium bicarbonate containing 10% acetonitrile, pH 8.5, at 37 °C overnight using an enzyme-to-substrate ratio of 1:50 (w/w). An aliquot of peptide mixture was submitted to MALDI/MS analysis.

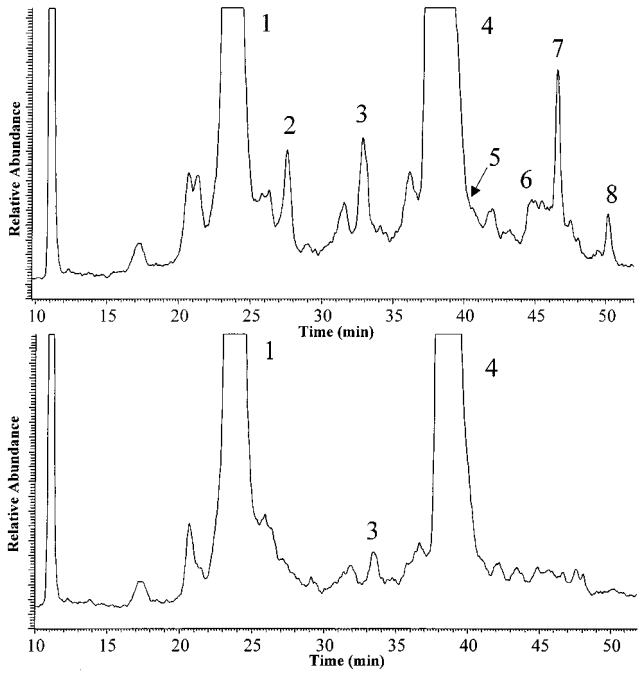
Matrix Laser Desorption Ionization Mass Spectrometry (MALDI/MS) Analysis. MALDI mass spectra were recorded using a Voyager DE MALDI-TOF mass spectrometer (PerSeptive Biosystem, Boston, MA). A mixture of analyte solution, α-cyano-4-hydroxycinnamic acid, and bovine insulin was applied to the metallic sample plate and dried under vacuum. Mass calibration was performed using the molecular ions from the bovine insulin (5734.2 Da) and the matrix (379.1 Da) as internal standards. Raw data were analyzed using computer software provided by the manufacturer and are reported as average masses.

Spectrophotometric Determination of 3-Nitrotyrosine. Treatment of Ala−Tyr with peroxynitrite was performed in phosphate/DTPA/bicarbonate. Samples were centrifuged at 4 °C on Microcon (cutoff 3 kDa), and 3-nitrotyrosine was detected in the eluate. 3-Nitrotyrosine content was measured using differential absorbance at 430 nm under acidic and alkaline conditions (*35*). It should be noted that nitration of tyrosine by peroxynitrite is zero order for the substrate, and thus results are not affected by small changes in dipeptide concentration due to its diffusion or transport inside the erythrocyte. This was confirmed experimentally by obtaining comparable results using 0.5−2 mM Ala−Tyr.

Analysis of the Tridimensional Model of Crystallized OxyHbA. Distances of residues from the heme and their relative orientations were obtained using the program Swiss-Pdb Viewer v3.51 that is available through the Internet (http://www.expasy.ch/spdbv/mainpage.html). Since there is no single value to assign the distances between a residue and a heme, we chose the shortest distance to the porphyrin group expressed in angström (1 Å = 10^{-10} m).

RESULTS

Characterization of Globin Residues Modified by Peroxynitrite. Previous studies (*12, 23*) have shown that oxidative modifications by peroxynitrite occur primarily at the β-chain with the formation of Hb dimers and 3-nitrotyrosine. We analyzed in more detail the modifications induced by peroxynitrite/CO_2 on oxyHb (1 mM) in a 1:1 molar ratio by MS in combination with chemical and enzymatic hydrolysis. The reaction mixture was analyzed by LC/ES/MS immediately or within 24 h after sample freezing without appreciable changes. Mass spectra of the different Hb species, separated by HPLC, immediately revealed the nature of the modifications (Figure 1). The mass data suggested that reaction of peroxynitrite resulted in a single nitration on both chains and cross-linking formation between two α- and two β-chains, respectively. It should be stressed that the yield of



Fraction	Measured MW (Da)	Interpretation	Target Residues
1	15866.4± 0.7	β	-
2	15910.7±0.4	Mono-nitrated β chain	Tyr 130
3	31730.3±1.3	β-β	Cys 93
4	15126.1±0.7	α	-
5	15171.0±0.2	Mono-nitrated α chain	Tyr 42
6	15170.3±0.6	Mono-nitrated α chain	Tyr 24
7	30250.1±1.3	α-α	Tyr 24-Tyr42
8	30249.0±0.2	α-α	Cys 104

FIGURE 1: Identification of structural modifications induced by peroxynitrite on oxyHb. LC/ES/MS analysis of reaction products of 1 mM oxyHb in phosphate/DTPA/bicarbonate treated with 1 mM peroxynitrite (upper) or 1 mM decomposed peroxynitrite (lower). See Results for details.

Table 1: Effects of the Post-Addition of Ascorbate on OxyHb Residues Modified by Peroxynitrite/CO_2[a]

target	yield (%)[b] without ascorbate	yield (%)[b] with ascorbate	% inhibition with ascorbate
NO_2−Tyr130	2.85 ± 0.2	2.50 ± 0.2	12[c]
Cys93−Cys93	2.40 ± 0.1	1.05 ± 0.2	56
NO_2−Tyr42	2.35 ± 0.2	2.35 ± 0.2	0
NO_2−Tyr24	0.30 ± 0.1	0.30 ± 0.1	0
Tyr24−Tyr42	0.70 ± 0.1	0.25 ± 0.1	64
Cys104−Cys104	0.20 ± 0.1	0.03 ± 0.02	85

[a] Peroxynitrite treatment was as described in the legend to Figure 1. [b] Yields were calculated as percent of the relative unmodified subunit (see Materials and Methods). [c] Not statistically significant, $p = 0.2$.

nitrotyrosine was 2.6−2.8% of the respective unmodified subunit (Table 1).

To identify the modified residues, peroxynitrite-treated species were purified by HPLC and carboxyamidomethylated to prevent undesired reactions of free SH groups. In all cases, CNBr hydrolysis was revealed to be instrumental in locating the targeted residues (unpublished results). Modified CNBr peptides were separated by HPLC and submitted to proteolytic digestion. The fraction corresponding to nitrated β-chains showed the presence of a nitrated peptide 56−146. Only Tyr130 was shown to be nitrated by peroxynitrite out of the two tyrosines that this peptide contains. Analysis of fractions corresponding to the α-nitrated chain revealed that Tyr residues in positions 24 and 42 were involved. A semiquantitative analysis showed that nitration of Tyr42 represents about 87% of the total nitration on the α-chain (Table 1). One of the cross-linked species of the α-chain was identified as a dimer formed by two Tyr residues involving residues 24 and 42. Both nitrotyrosine and dityrosine are indicative of the initial formation of tyrosyl radicals. Dityrosines are stable end products of the radical−radical reaction between two tyrosyl radicals, whereas tyrosine nitration mostly derives from the tyrosyl and $^{\bullet}NO_2$ radicals reaction (*10*, *21*).

Peroxynitrite-treated Hb also showed the presence of a disulfide bridge between two Cys residues in position 104 of two different α-chains as demonstrated by digestion with pepsin. Identification of amino acidic residues involved in the formation of a cross-link between two β-chains was obtained by means of hydrolysis with endoprotease AspN of the dimer of the C-terminal peptide 56−146. This analysis showed that the Cys residue in position 93 of the β-chain formed a disulfide bridge. The formation of a disulfide bond from two thiols may be due to thiyl radical−thiyl radical or thiyl radical−thiolate reactions (*36*).

OxyHb treated with previously decomposed peroxynitrite did not show an HPLC profile indicative of modified globin residues, excluding a small formation of β93−β93 disulfide cross-linkings (Figure 1), thus ruling out a major contribution of peroxynitrite decomposition or contamination products in the observed globin modifications.

Some protein radical species, such as cysteinyl and tryptophanyl radicals, are relatively short-lived species (decay during sample mixing or within a few seconds), while tyrosyl radical(s) and ferrylHb are exceptionally long-lived species (≥10−20 min) (*19*, *28*, *37*). The possibility that some of the observed modifications may occur during denaturing conditions introduced for LC/ES/MS analysis was therefore considered. Before MS analysis, we added 500 μM ascorbic acid at room temperature 60 s after the addition of peroxynitrite. Under these conditions, ascorbic acid is not expected to reduce preformed nitrotyrosines (*33*), disulfides, or dityrosines but reduces tyrosyl radicals with formation of the relatively unreactive ascorbyl radical (*38*). The post-addition of ascorbate did not significantly affect the intensity of nitrotyrosines in the α- or β-chains, while it did reduce the intensity of dityrosine and disulfides (Table 1). We deduce that cross-linking of peroxynitrite-treated oxyHb occurs to a large extent (>50%) during denaturing conditions and that long-lived oxidants (e.g., ferrylHb and tyrosyl radicals) are probably involved.

Effect of Peroxynitrite/OxyHb Ratio on Globin Oxidative Modifications. There is evidence that peroxynitrite can form a complex with heme compounds or hemoproteins (*21*, *27*, *39*), and it was therefore of interest to determine oxidative modifications induced on globin by different oxyHb/peroxynitrite molar ratios. A convenient way to summarily investigate the extent of cross-linking and tyrosine nitration is by immunoelectrophoretic methods.

Figure 2A shows the SDS−PAGE pattern of purified oxyHb (0.25−3 mM) treated with 1 mM $ONOO^-$. Peroxynitrite 1:1 or in excess to oxyHb induced dose-dependently

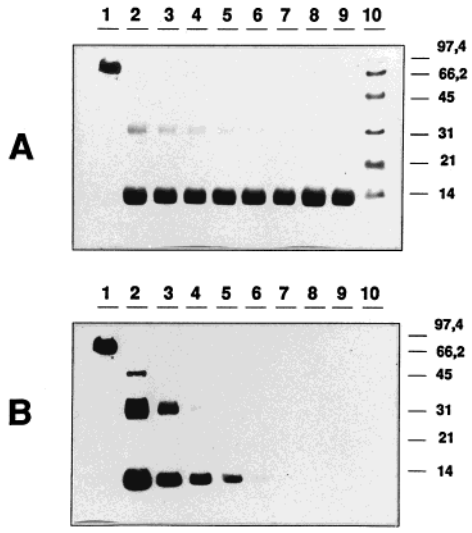


FIGURE 2: Modifications induced by peroxynitrite on oxyHb detected by immunoelectrophoretic methods. (A) Electrophoretic pattern of purified oxyHb (0.25−3 mM) treated with 1 mM peroxynitrite in phosphate/DTPA/bicarbonate and visualized by Red Ponceau S staining. Lanes 2−7, oxyHb/peroxynitrite molar ratios of 0.25:1, 0.5:1, 0.7:1, 1:1, 2:1, and 3:1, respectively. Lanes 8 and 9, oxyHb treated with decomposed peroxynitrite at oxyHb/peroxynitrite molar ratios of 0.25:1 and 3:1, respectively. Lane 1, BSA (1 mM) treated with 1 mM peroxynitrite in phosphate/DTPA/bicarbonate. Lane 10, standards with molecular mass in kDa. (B) Western blot analysis of gel A with anti-3-nitrotyrosine antibodies. Each lane was loaded with 10 μg of oxyHb. Proteins were separated by 15% SDS−PAGE.

the formation of a new band at 30−35 kDa compatible with cross-linked Hb dimers (Figure 2A, lanes 2−5). This band was not due to S−S bridges as the gels were run under reducing conditions. The formation of dimers was not observed in oxyHb treated with decomposed peroxynitrite (Figure 2A, lanes 8−9). The same gel was submitted to Western blot analysis with anti-3-nitrotyrosine antibodies, and the results are shown in Figure 2B. Interestingly, nitrated Hb bands were clearly evident at a 1:1 molar ratio or in excess of peroxynitrite (Figure 2B, lanes 2−5), whereas they were virtually absent with oxyHb in excess (Figure 2B, lanes 6−7) or with oxyHb treated with decomposed peroxynitrite (Figure 2B, lanes 8−9). The lack of nitration in samples with oxyHb in molar excess to peroxynitrite was not an artifact due to the "dilution" of the modified globins in excess of oxyHb, because nitration was not detected even overloading the gel with 30 μg of Hb (results not shown). In molar excess of peroxynitrite, nitrated bands with apparent molecular weights compatible with the formation of nitrated Hb dimers (30−35 kDa) and trimers (45−50 kDa) were also detected (Figure 2B, lanes 2−3). In agreement with MS results, the addition of 500 μM ascorbic acid 60 s after peroxynitrite and before exposing the sample to denaturing conditions reduced the formation of Hb dimers. OxyHb treated with peroxynitrite at a 1:1 molar ratio and receiving ascorbate showed an intensity of Hb dimers reduced by 45 ± 8%.

Owing to the comparable rate constant of peroxynitrite for oxyHb and for CO_2 ($k \sim 1$ and 3×10^4 M^{-1} s^{-1} at 25 °C, respectively) (*9*, *12*), it is important to stress that at low oxyHb concentrations most of the peroxynitrite interacts with CO_2, thereby prompting the reaction of oxyHb with $ONOOCO_2^-$, whereas at high oxyHb concentrations, more

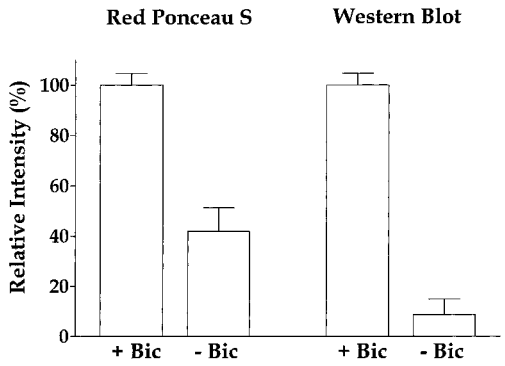


FIGURE 3: Comparison of Hb dimer intensity and nitration in the presence or in the absence of bicarbonate. Purified oxyHb was treated with peroxynitrite at a oxyHb/peroxynitrite molar ratio of 0.25:1 and submitted to SDS−PAGE and Western blot analysis as in the legend to Figure 1. Hb−Hb dimer intensity and nitration were estimated by densitometric analysis performed on dimer band stained with Red Ponceau S (left) or after Western blot with anti-3-nitrotyrosine antibodies (right). Band intensity was expressed as a percentage of the value in the sample containing 25 mM bicarbonate. Data are the mean values ± SD, $n = 3$.

peroxynitrite interacts directly with oxyHb. This last scenario is closer to the situation inside red cells (17−22 mM oxyHb). In a previous work, we suggested that $ONOOCO_2^-$ was the peroxide involved in the formation of tyrosyl radical(s) by peroxynitrite (*28*). Further studies of tyrosyl radical yield at different pCO_2 revealed that the radical, although significantly enhanced by CO_2, was induced also by $ONOO^-$ (unpublished results). We therefore performed some experiments in the absence of added bicarbonate. The omission of bicarbonate significantly reduced both the intensity of the dimers and the extent of their nitration. Dimers were clearly observed at a 0.25:1 oxyHb/peroxynitrite molar ratio. A comparison of Hb dimer intensity and dimer nitration in the presence and in the absence of bicarbonate is shown in Figure 3. These results agree with the known ability of CO_2 to increase nitration and one-electron oxidations mediated by peroxynitrite.

OxyHb Protects Ala−Tyr against Peroxynitrite-Mediated Tyrosine Nitration. We first investigated the effects of purified oxyHb in peroxynitrite/CO_2-induced nitration of a target dipeptide Ala−Tyr. The formation of 3-nitrotyrosine was measured in the low molecular mass fraction (≤3 kDa) obtained by sample ultrafiltration. The results, summarized in Figure 4, showed that oxyHb was able to strongly decrease 3-nitrotyrosine formation so that 0.2 mM oxyHb provided 50% inhibition, and a complete inhibition was observed at a 1:1 oxyHb/peroxynitrite ratio or in excess of oxyHb. Decomposed peroxynitrite was unable to induce nitration of Ala−Tyr either in the absence or in the presence of oxyHb. Moreover, the effect of oxyHb cannot be ascribed to the heme-dependent nonenzymatic reduction of nitrotyrosine (*33*), since the yield of 3-nitrotyrosine did not change when oxyHb was added 60 s after peroxynitrite and Ala−Tyr separated from oxyHb immediately or after 3 h incubation at 37 °C (138 ± 3 and 140 ± 2 μM, respectively, $n = 3$). CO-Hb and metHb were significantly less efficient in the protection of Ala−Tyr against peroxynitrite/CO_2-mediated tyrosine nitration, so that the concentration of Hb able to induce 50% protection shifted to 2 and 1 mM, respectively (Figure 4). These results point to a protective role of oxyHb

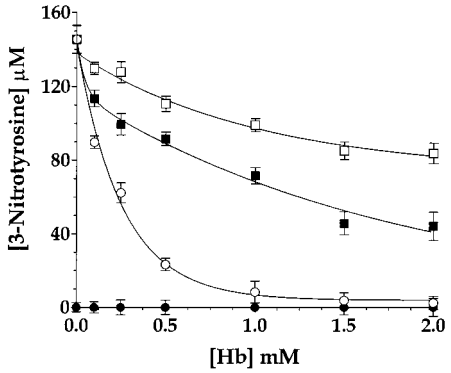


FIGURE 4: Effects of oxyHb on peroxynitrite-mediated nitration of Ala−Tyr. Ala−Tyr (1 mM) was treated with peroxynitrite (1 mM) in phosphate/DTPA/bicarbonate in the presence of different concentrations of oxyHb (○), metHb (■), and CO-Hb (□). Ala−Tyr and oxyHb treated with 1 mM decomposed peroxynitrite (●). Points represent mean value ± SD, $n = 4$.

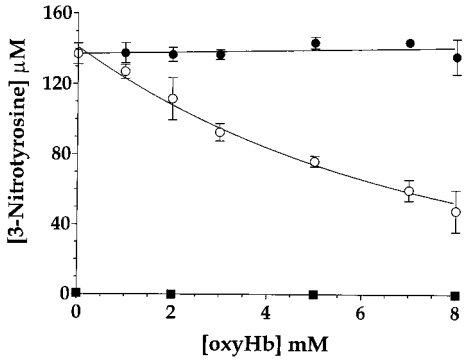


FIGURE 5: Effects of erythrocytes on the nitration of Ala−Tyr by peroxynitrite. Ala−Tyr (1 mM) in phosphate/DTPA/bicarbonate was treated with peroxynitrite (1 mM) in the presence of erythrocytes (○) or DIDS-treated erythrocytes (●) expressed as oxyHb concentration. Erythrocytes and Ala−Tyr treated with 1 mM decomposed peroxynitrite (■). Points represent mean value ± SD, $n = 4$.

mediated by a reaction between oxygenated Fe(II)heme and peroxynitrite and not to the trapping by globin amino acid residues of peroxynitrite-dependent reactive species.

We then investigated the effect of the addition of oxygenated erythrocytes on peroxynitrite/CO_2-mediated nitration of Ala−Tyr. As shown in Figure 5, the yield of 3-nitrotyrosine decreased by increasing cell density with a 50% protection at a cell density corresponding to ~38% hematocrit (6.8 mM as heme). At 8 mM oxyhemoglobin (about the physiologic 42−45% hematocrit), the nitration of Ala−Tyr was reduced by 65%. Control experiments showed that nitrotyrosines were not released from erythrocytes because of peroxynitrite treatment, since the omission of Ala−Tyr did not result in the detection of 3-nitrotyrosine (0.2 ± 0.18 μM, $n = 6$).

At pH 7.4, about 80% of peroxynitrite is present as $ONOO^-$ (*40*) and thus crosses the erythrocyte membrane mainly through the band 3 anion exchange protein (*12*, *40*). The band 3 exchanger is easily modified by stilbene derivatives such as DIDS, which, by binding to a lysine residue, inhibits the anion exchange process (*41*). To demonstrate whether the inhibitory effect of erythrocytes was due to the scavenging of peroxynitrite diffusing inside the cell, we pretreated the erythrocytes with DIDS. DIDS-modified erythrocytes were unable to protect Ala−Tyr against peroxynitrite-mediated nitration (Figure 5), thus confirming the lack of

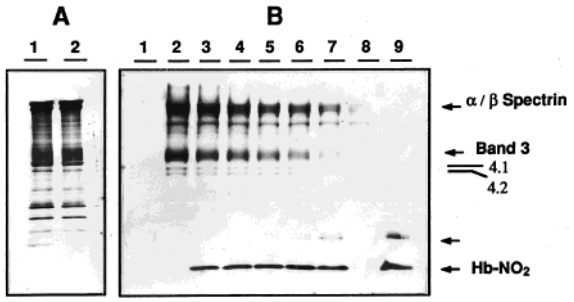


FIGURE 6: Effects of oxyHb trapped within ghosts on peroxynitrite-mediated nitration of membrane proteins. (A) Electrophoretic pattern of membrane proteins from peroxynitrite-treated ghosts visualized by Coomassie staining. Lane 1, untreated ghosts. Lane 2, ghosts (protein, 0.8 mg/mL) treated with 1 mM peroxynitrite in phosphate/DTPA/bicarbonate. (B) Western blot analysis with anti-3-nitrotyrosine antibodies of erythrocyte ghosts resealed with 0−0.6 mM oxyHb. Lane 1, untreated ghosts. Lanes 2−7, ghosts treated with 1 mM peroxynitrite in phosphate/DTPA/bicarbonate and containing 0, 0.1, 0.15, 0.2, 0.3, and 0.6 mM oxyHb, respectively. Lane 8, ghosts treated with decomposed 1 mM peroxynitrite. Lane 9, purified oxyHb (0.5 mM) treated with 1 mM peroxynitrite. Proteins were separated by 5−15% SDS−PAGE.

protection in the absence of a significant diffusion of peroxynitrite inside the cell. The remarkable difference observed between free oxyHb and oxyHb in erythrocytes (compare Figures 4 and 5) suggests that in the presence of CO_2 the membrane may represent a diffusional barrier for peroxynitrite, a result previously anticipated (*10*, *12*).

OxyHb Protects Membrane Proteins against Peroxynitrite-Mediated Nitration. To investigate whether oxyHb can protect membrane proteins from peroxynitrite-dependent nitration and to separate the contribution of oxyHb from that of other intracellular compounds, we entrapped purified oxyHb inside the ghosts and, after washing out the unincorporated Hb, treated the ghosts with 1 mM peroxynitrite in phosphate/DTPA/bicarbonate. Treatment of erythrocytes with peroxynitrite/CO_2 did not induce significant changes in the SDS−PAGE pattern of membrane proteins under reducing conditions (Figure 6A, compare lanes 1 and 2). However, Western blot analysis with anti-3-nitrotyrosine antibodies showed significant nitration in bands with apparent molecular weights corresponding to all the major membrane proteins such as α/β spectrin (220−210 kDa), band 3 (100 kDa), and proteins 4.1−4.2 (80−75 kDa), with α/β spectrin apparently more heavily nitrated. No nitration in bands corresponding to actin (43 kDa) or glyceraldehyde-3-phosphate dehydrogenase (37 kDa) was observed. As expected, no nitration was observed in untreated ghosts (Figure 6B, lane 1) or in ghosts treated with 1 mM decomposed peroxynitrite (Figure 6B, lane 8). Interestingly, 0.1−0.6 mM oxyHb trapped inside the ghosts provided dose-dependent protection against peroxynitrite-mediated protein nitration, and at the same time, a nitrated band corresponding to Hb was observed (Figure 6B, lanes 3−7). A faint nitrated band with an apparent molecular mass of 30−35 kDa was also observed in the sample containing 0.6 mM trapped oxyHb (Figure 6B, lane 7). Comparison of this nitrated band with purified oxyHb treated with peroxynitrite (Figure 6B, lane 9) suggests the possible formation of cross-linked Hb dimers. The inhibitory effect of oxyHb cannot be ascribed to heme- and thiol-dependent nonenzymatic reduction of 3-nitrotyrosine to 3-aminotyrosine (*33*), since the intensity of nitrated

bands did not change when 1 mM oxyHb was added to peroxynitrite-treated ghosts a few minutes after peroxynitrite (results not shown).

Treatment of Intact Red Blood Cells with Peroxynitrite. Western blot analysis showed no evidence of nitrotyrosine formation either in membrane proteins or in the hemolysate obtained from erythrocytes (0.75−15% hematocrit, 0.15−3.0 mM as heme) treated with 1−4 mM peroxynitrite (results not shown). In these samples, the CO_2 was always in excess to peroxynitrite (from 1.3 to 5 mM).

DISCUSSION

One of the tyrosines nitrated by peroxynitrite is αTyr42, the tyrosine nearest to the α-heme and, for this reason, supposed to be involved in tyrosyl radical generated by hydrogen peroxide (*37*). Examination of the crystal structure revealed that the α-heme-Tyr42 shortest distance was compatible with van der Waals contact (∼4 Å). Assuming the same structure in the crystal and in solution (*42*), it is conceivable to suggest that the αTyr42 radical may be due to an ET process from this tyrosyl group to ferryHb, since donor-to-acceptor distance is of primary concern in ET (*43*). However, the π-orbitals of αTyr42 are almost perpendicular to the unsaturated heme system, and this relative orientation has been shown to slow the ET kinetically, which, on the contrary, is favored by a coplanar donor-to-acceptor orientation (*44*). The unfavorable orientation of αTyr42 suggests a role for other residue(s) as intermediaries or suggests a geometry of ferrylHb different from that of oxyHb. Oxidized and reduced hemoproteins often have different conformations, which also play a key role in ET (*45*).

There is some preliminary EPR evidence that more than one tyrosyl radical species is formed in ferrylHb (*28*, *37*). MS results also suggest that βTyr130 and, to a lesser extent, αTyr24 can form tyrosyl radicals as deduced by peroxynitrite-mediated nitration. The βTyr130 is relatively distant from the β-heme (10.5 Å), but examination of residues neighboring the β-heme revealed the presence of βPhe71 (shortest distance to heme ∼4 Å), which forms a "hydrophobic area" Phe71−Trp15−Tyr130 with each residue spaced by 4−5 Å and possibly involved in coupling with β-heme. Interestingly, the orientation of π-orbitals of these residues is roughly coplanar to the β-heme.

The αTyr24 ring, which is about 10 times less nitrated than the other tyrosines, is also largely buried and relatively distant from the α-heme (10.8 Å), but its hydroxyl group is external (*46*), potentially exposing it to a direct attack by peroxynitrite. Nevertheless, ET can take place over distances greater than 10 Å (*43*), so that a coupling with α-heme cannot be discounted by the current data. The most exposed tyrosine residues of oxyHb are the penultimate αTyr140 and βTyr145, which are neither involved in tyrosyl radical formation (*28*) nor detectably nitrated. Moreover, the nonnitrated αTyr140, βTyr35, and βTyr145 are not far from the respective heme groups (8.8, 7.0, and 8.6 Å). These data confirm previous studies showing that tyrosine residues targeted for nitration cannot be simply predicted on the basis of solvent accessibility (*47*) or on the distance from metal centers (*24*). Ascorbate inhibition experiments showed that dityrosine and disulfide cross-linkings were largely due to post-addition oxidations during denaturing conditions, although we cannot completely rule out the possibility that a small amount of cross-linkings may occur following peroxynitrite treatment.

Collectively, our MS results show a major targeting of peroxynitrite to a few buried tyrosine residues, whereas no nitration of the most available tyrosines was observed. As indicated by the higher nitration yield (Table 1), we suggest that peroxynitrite oxidizes the heme to ferrylHb that, through ET processes, oxidizes tyrosyl residues centered on α42 and β130. However, the hypothesis that these tyrosines are involved in ferrylHb needs be confirmed by further studies with peroxides that are not reactive nitrogen species.

Our immunoblotting results revealed that nitration of tyrosine residues sharply increased when peroxynitrite was in molar excess, disproving a catalytic role of oxyHb. One can postulate that a major difference with $P450_{nor}$ is that oxyHb does not form a ferryl−$^{\bullet}NO_2$ complex (*21*) because the reacting species is oxygenated Fe(II)heme instead of Fe(III)heme. The formation of ferrylHb from Fe(II) suggests a peroxynitrite-mediated two-electron oxidation mechanism that would be expected to form the less reactive NO_2^- anion (eqs 1−2) rather than the $^{\bullet}NO_2$ radical.

$$\text{Hb}(\text{Fe}^{\text{II}}\text{O}_2) + \text{ONOO}^- + \text{H}_2\text{O} \rightarrow \text{Hb}(\text{Fe}^{\text{IV}}{=}\text{O}) + \text{NO}_2^- + \text{O}_2 + \text{OH}^- + \text{H}^+ \quad (1)$$

$$\text{Hb}(\text{Fe}^{\text{II}}\text{O}_2) + \text{ONOOCO}_2^- + \text{H}_2\text{O} \rightarrow \text{Hb}(\text{Fe}^{\text{IV}}{=}\text{O}) + \text{NO}_2^- + \text{O}_2 + \text{HCO}_3^- + \text{H}^+ \quad (2)$$

The formation of 3-nitrotyrosines is ascribed to the fraction of peroxynitrite escaping reactions 1 or 2 and forming $^{\bullet}NO_2$. This radical may originate from one-electron oxidation of $Hb(Fe^{IV}{=}O)$ by peroxynitrite (eq 3 or 4) or during $ONOOCO_2^-$ decay to nitrate (eqs 5 and 6).

$$\text{Hb}(\text{Fe}^{\text{IV}}{=}\text{O}) + \text{ONOOCO}_2^- \longrightarrow \text{Hb}^{\bullet}(\text{Fe}^{\text{IV}}{=}\text{O}) + {}^{\bullet}\text{NO}_2 + \text{HCO}_3^- \quad (3)$$

$$\text{Hb}(\text{Fe}^{\text{IV}}{=}\text{O}) + \text{ONOO}^- \longrightarrow \text{Hb}^{\bullet}(\text{Fe}^{\text{IV}}{=}\text{O}) + {}^{\bullet}\text{NO}_2 + \text{OH}^- \quad (4)$$

$$\text{ONOOCO}_2^- \longleftrightarrow [{}^{\bullet}\text{NO}_2 \cdots \text{CO}_3^{\bullet-}] \rightarrow \text{NO}_3^- + \text{CO}_2 \quad (5)$$

Caged radicals; $^{\bullet}NO_2 + CO_3^{\bullet-}$ (Free Radicals) + Scavengers → Products (6)

Finally, the $^{\bullet}NO_2$ recombines with tyrosyl radicals to form 3-nitrotyrosines or, together with $CO_3^{\bullet-}$, can be involved in one-electron oxidations of Hb to increase radical formation including the tyrosyl radical. Reactions 3−6 are favored by peroxynitrite in molar excess to oxyHb and are thus possible in vitro but unlikely in vivo (17−22 mM oxyHb inside the erythrocyte).

The proposed difference between oxyHb and $P450_{nor}$ would imply that the Fe(III)heme of metHb can instead catalyze tyrosine nitration, but this effect was not observed (Figure 4). Probably, one reason may be the inability of metHb to form ferrylHb (*28*). Characterization of the metHb reaction is underway, but our unpublished results show that metHb after treatment with equimolar tetramethylammonium peroxynitrite/CO_2 forms 60% less tyrosyl radical(s) as compared to oxyHb and shows a different radical dose-dependency.

We observed that erythrocytes were less effective at inhibiting Ala−Tyr nitration than free oxyHb. However, it is wrong to conclude that erythrocytes do not represent a significant target for peroxynitrite in blood. In fact, the protection of Ala−Tyr from peroxynitrite-mediated nitration at the physiologic 45% hematocrit was as high as 65%. Therefore, taking into consideration the obvious in vitro

limitations, it could be estimated that about three-fifths of extracellular peroxynitrite may be scavenged by erythrocytes, in good agreement with previously published theoretical model and experimental results (*48*). This "sink" function of red cells is remarkably facilitated by the huge amount in the membrane of band 3 (1.2×10^6 copies/cell), which is the major intrinsic membrane protein and the major route of peroxynitrite anion transport (*12*, *40*).

Moreover, oxyHb provides powerful protection against peroxynitrite-mediated tyrosine nitration of membrane proteins. Although oxyHb trapped inside ghosts did not exceed 0.6 mM as heme, extrapolation to its physiologic concentration strongly suggests that it may give a complete protection of membrane proteins from peroxynitrite-mediated tyrosine nitration, a conclusion supported by the lack of tyrosine nitration in intact erythrocytes treated with peroxynitrite/CO_2.

In tissues, a major effect of CO_2 is to limit, at least in part, the effects of peroxynitrite to the cellular compartment in which it is generated. The fraction of peroxynitrite that decays extracellularly is expected to modify extracellular targets and also to provide transmembrane redox signals, which may occur without crossing of the plasma membrane by the oxidant (*49*−*52*). For instance, in peroxynitrite-treated erythrocytes, we observed the up-regulation of phosphotyrosine signaling (*53*) under conditions in which >80% of peroxynitrite reacts with CO_2 and decays extracellularly (*48*). The up-regulation of tyrosine kinases increased the phosphorylation of band 3 cytoplasmic domain (*34*), a domain that binds and inhibits three major enzymes involved in glycolysis (*54*). The phosphorylation of band 3 reverses this binding and allows enzyme activation (*55*). The up-regulation of glycolysis supplies the cofactor NADH crucial to maintain reduced Hb. Since the peroxynitrite/oxyHb reaction forms mainly metHb (*12*, *23*, *28*, *48*), the phosphotyrosine signaling completes the cellular process involved in the inactivation of this oxidant.

We conclude that oxyHb is a peroxynitrite scavenger of physiologic relevance, and this pathway protects blood and vascular targets from oxidation/nitration. An important inhibitory role of erythrocytes in inflammatory reactions mediated by peroxynitrite is predictable.

ACKNOWLEDGMENT

We are grateful to Prof. G. Girelli, Centro Trasfusionale, Università La Sapienza, Roma, for providing blood samples.

REFERENCES

1. Kahn, J., Brennan, D. M., Bradley, N., Gao, B., Bruckdorfer, R., and Jacobs, M. (1998) *Biochem. J. 330*, 795−801.
2. Beckman, J. S. (1996) *Chem. Res. Toxicol. 9*, 836−844.
3. Ischiropoulos, H. (1998) *Arch. Biochem. Biophys. 356*, 1−11.
4. Kooy, N. W., Lewis, S. J., Rojall, J. A., Ye, Y. Z., Kelly, D. R., and Beckman, J. S. (1997) *Crit. Care Med. 25*, 812−819.
5. Eiserich, J. P., Hristova, M., Cross, C. E., Jones, A. D., Freeman, B. A., Halliwell, B., and van der Vliet, A. (1998) *Nature 391*, 393−397.
6. Huie, R. E., and Padmaja, S. (1993) *Free Radical Res. Commun. 18*, 195−199.
7. Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997) *Chem. Res. Toxicol. 10*, 1285−1292.
8. Beckman, J. S., and Koppenol, W. (1996) *Am. J. Physiol. 271*, C1424−C1437.
9. Lymar, S. V., and Hurst, J. K. (1995) *J. Am. Chem. Soc. 117*, 8867−8868.
10. Lymar, S. V., and Hurst, J. K. (1996) *Chem. Res. Toxicol. 9*, 845−850.
11. Thomson, L., Trujillo, M., Telleri, R., and Radi, R. (1995) *Arch. Biochem. Biophys. 319*, 491−497.
12. Denicola, A., Souza, J. M., and Radi, R. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 3566−3571.
13. Denicola, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996) *Arch. Biochem. Biophys. 333*, 49−58.
14. Goldstein, S., and Czapski, G. (1998) *J. Am. Chem. Soc. 120*, 3458−3463.
15. Bonini, M. G., Radi, R., Ferrer-Sueta, G., Ferreira, A. M., and Augusto, O. (1999) *J. Biol. Chem. 274*, 10802−10806.
16. Lehnig, M. (1999) *Arch. Biochem. Biophys. 368*, 303−318.
17. Uppu, R. M., Squadrito, G. L., and Pryor, W. A. (1996) *Arch. Biochem. Biophys. 327*, 335−343.
18. Pietraforte, D., and Minetti, M. (1997) *Biochem. J. 321*, 734−750.
19. Pietraforte, D., and Minetti, M. (1997) *Biochem. J. 325*, 675−684.
20. Scorza, G., and Minetti, M. (1998) *Biochem. J. 329*, 405−413.
21. Mehl, M., Daiber, A., Herold, S., Shoun, H., and Ullrich, V. (1999) *Nitric Oxide 3*, 142−152.
22. Floris, R., Piersma, S. R., Yang, G., Jones, P., and Wever, R. (1993) *Eur. J. Biochem. 215*, 767−775.
23. Alayash, A. I., Brockner Ryan, B. A., and Cashon, R. E. (1998) *Arch. Biochem. Biophys. 349*, 65−73.
24. MacMillan-Crow, L. A., Crow, J. P., and Thompson, J. A. (1998) *Biochemistry 37*, 1613−1622.
25. McCormick, M. L., Gaut, J. P., Lin, T.-S., Britigan, B. E., Buettner, G. R., and Heinecke, J. W. (1998) *J. Biol. Chem. 273*, 32030−32037.
26. Ostdal, H., Andersen, H. J., and Davies, M. J. (1999) *Arch. Biochem. Biophys. 362*, 105−112.
27. Herold, S. (1999) *FEBS Lett. 443*, 81−84.
28. Minetti, M., Scorza, G., and Pietraforte, D. (1999) *Biochemistry 38*, 2078−2087.
29. Winterbourn, C. C. (1990) *Methods Enzymol. 186*, 265−272.
30. Beckman, J. S., Ischiropoulos, H., Zhu, L., van der Woerd, M., Smith, C., Chen, J., Harrison, J., Martin, J. C., and Tsai, M. (1992) *Arch. Biochem. Biophys. 298*, 438−445.
31. Radi, R., Beckman, J. S., Bush, K., and Freeman, B. A. (1991) *J. Biol. Chem. 266*, 4244−4250.
32. Spagnuolo, C., Rinelli, P., Coletta, M., Chiancone, E., and Ascoli, F. (1987) *Biochim. Biophys. Acta 911*, 59−65.
33. Balabanli, B., Kamasaki, Y., Martin, E., and Murad, F. (1999) *Proc. Natl. Acad. Sci. U.S.A. 96*, 13136−13141.
34. Mallozzi, C., Di Stasi, A. M. M., and Minetti, M. (1997) *FASEB J. 11*, 1281−1290.
35. Crow, J. P., and Ischiropoulos, H. (1996) *Methods Enzymol. 269*, 185−194.
36. Wardman, P., and von Sonntag, C. (1995) *Methods Enzymol. 251*, 31−45.
37. Giulivi, C., and Cadenas, E. (1998) *Free Radical Biol. Med. 24*, 269−279.
38. Sturgeon, B. E., Sipe, H. J., Jr., Barr, D. P., Corbett, J. T., Martinez, J. G., and Mason, R. P. (1998) *J. Biol. Chem. 273*, 30116−30121.
39. Salvemini, D., Wang, Z.-Q., Stern, M. K., Currie, M. G., and Misko, T. P. (1998) *Proc. Natl. Acad. Sci. U.S.A. 95*, 2659−2663.
40. Macfadyen, A. J., Reiter, C., Zhuang, Y., and Beckman, J. S. (1999) *Chem. Res. Toxicol. 12*, 223−229.
41. Schopfer, L. M., and Salhany, J. M. (1995) *Biochemistry 34*, 8320−8329.
42. Tame, J. R. (1999) *Trends Biochem. Sci. 24*, 372−377.
43. Majo, S. L., Ellis, W. R., Jr., Crutchley, R. J., and Gray, H. B. (1986) *Science 233*, 948−952.
44. Makinen, M. W., Schichman, S. A., Hill, S. C., and Gray, H. B. (1983) *Science 222*, 929−931.
45. Gray, H. B., and Winkler, J. R. (1996) *Annu. Rev. Biochem. 65*, 537−561.
46. Perutz, M. F., Muirhead, H., Cox, J. M., and Goaman, L. C. G. (1968) *Nature 219*, 131−139.

47. Souza, J. M., Daikhin, E., Yudkoff, M., Raman, C. S., and Ischiropoulos, H. (1999) *Arch. Biochem. Biophys. 371*, 169−178.
48. Romero, N., Denicola, A., Souza, J. M., and Radi, R. (1999) *Arch. Biochem. Biophys. 368*, 23−30.
49. Mishra, R. K., and Passow, H. (1969) *J. Membr. Biol. 1*, 214−224.
50. Reglinski, J., Hoey, S., Smith, W. E., and Sturrock, R. D. (1988) *J. Biol. Chem. 263*, 12360−12366.
51. Ciriolo, M. R., Paci, M., Sette, M., De Martino, A., Bozzi, A., and Rotilio, G. (1993) *Eur. J. Biochem. 215*, 711−718.
52. May, J. M., Qu, Z.-C., and Whitesell, R. R. (1995) *Biochemistry 34*, 12721−12728.
53. Mallozzi, C., Di Stasi, A. M. M., and Minetti, M. (1999) *FEBS Lett. 456*, 201−206.
54. Tsai, I.-H., Murthy, S. N. P., and Steck, T. L. (1982) *J. Biol. Chem. 257*, 1438−1442.
55. Low, P. S., Allen, D. P., Zioncheck, T. F., Chari, P., Willardson, B. M., Geahlen, R. L., and Harrison, M. L. (1987) *J. Biol. Chem. 262*, 4592−4596.

BI9927991