Reaction of Human Hemoglobin with Peroxynitrite

ISOMERIZATION TO NITRATE AND SECONDARY FORMATION OF PROTEIN RADICALS*

Received for publication, June 4, 2003, and in revised form, July 22, 2003
Published, JBC Papers in Press, August 13, 2003, DOI 10.1074/jbc.M305895200

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Peroxynitrite, a strong oxidant formed intravascularly in vivo, can diffuse onto erythrocytes and be largely consumed via a fast reaction (2 × 104 M s−1) with oxyhemoglobin. The reaction mechanism of peroxynitrite with oxyhemoglobin that results in the formation of methemoglobin remains to be elucidated. In this work, we studied the reaction under biologically relevant conditions using millimolar oxyhemoglobin concentrations and a stoichiometric excess of oxyhemoglobin over peroxynitrite. The results support a reaction mechanism that involves the net one-electron oxidation of the ferrous heme, isomerization of peroxynitrite to nitrate, and production of superoxide radical and hydrogen peroxide. Homolytic cleavage of peroxynitrite within the heme iron allows the formation of ferrylhemoglobin in ~10% yields, which can decay to methemoglobin at the expense of reducing equivalents of the globin moiety. Indeed, spin-trapping studies using 2-methyl-2-nitroso propane and 5,5 dimethyl-1-pyrroline-N-oxide (DMPO) demonstrated the formation of tyrosyl- and cysteinyl-derived radicals. DMPO also inhibited covalently linked dimerization products and led to the formation of DMPO-hemoglobin adduct. Hemoglobin nitration was not observed unless an excess of peroxynitrite over oxyhemoglobin was used, in agreement with a marginal formation of nitrogen dioxide. The results obtained support a role of oxyhemoglobin as a relevant intravascular sink of peroxynitrite.

* This work was supported by grants from Consejo Nacional de Investigación Científica y Tecnológica (to N. R.), Comisión Sectorial de Investigación Científica and Third World Academy of Science (to A. D.), The Howard Hughes Medical Institute (to R. R.), and Fundación de Amparo à Pesquisa do Estado de São Paulo (FAPESP) (to O. A.). The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked “advertisement” in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

† Partially supported by a fellowship from Programa de Desarrollo de las Ciencias Básicas (PEDECIBA, Uruguay).
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Peroxynitrite,1 a strong oxidant formed intravascularly in vivo by the diffusion-limited reaction between nitric oxide (NO) and superoxide (O2−) radicals (1–5), rapidly oxidizes human oxyhemoglobin (oxyHb)2 (κ = 2 × 104 M−1 s−1 at 37 °C and pH 7.4 (6,7)) to yield methemoglobin (metHb) as the final product. Peroxynitrite can behave as either a one- or two-electron oxidant during its direct reaction with transition metal centers; therefore, it is not apparent how metHb is formed. Recently, an initial two-electron oxidation process has been proposed leading to the formation of a high oxidation state intermediate, ferrylhemoglobin (ferrylHb) (8, 9). However, the ferrylHb intermediate detected during reaction of oxyHb with peroxynitrite has a very short half-life (i.e. milliseconds), and it can only be clearly observed when trapped with sodium sulfide (8, 9). Even under excess of sodium sulfide, the yields of sulf-hemoglobin obtained were low (~10–15% of metHb yield). This contrasts with the two-electron oxidation product obtained with oxyHb under excess of hydrogen peroxide (H2O2), which yields a relatively stable ferrylHb intermediate (i.e. minutes) that can be observed directly by spectrophotometric techniques (10).

If peroxynitrite oxidizes oxyHb by a two-electron oxidation process, nitrite and molecular oxygen should be produced in addition to ferrylHb (Equation 1):

\[
Hb(Fe^2+O_2 + ONOOH → Hb(Fe^3+)O + O_2 + NO_2^- \quad \text{(Eq. 1)}
\]

Neither nitrite nor molecular oxygen yields have been assessed yet, so the mechanism proposed in Equation 1 cannot be confirmed with the available data. Other authors have previously proposed that reaction of oxyHb with peroxynitrite leads to a rapid one-electron oxidation of the heme iron to the ferric form (11). The latter (11) reported the appearance of a ferryl-like spectrum toward the end of the reaction period that was proposed to have been caused by concomitant peroxide production as a result of the presence of nitrite in the peroxynitrite preparations.

Considering that the observed final product of the reaction is metHb (6, 8, 9, 11) and that the ferryl intermediate is transient and detected at low yields, other reaction mechanisms could be

1 IUPAC recommended names for peroxynitrite anion (ONOO−) and peroxynitrous acid (ONOOH) are oxoperoxonitrite (1−) and hydrogen oxoperoxonitrite, respectively. The term peroxynitrite is used to refer to the sum of ONOO− and ONOOH.
2 The abbreviations used are: oxyHb, oxyhemoglobin; metHb, methemoglobin; ferrylHb, ferrylhemoglobin; PHPA, p-hydroxyphenylacetic acid; MNP, 2-methyl-2-nitroso propane; NEM, N-ethylmaleimide; DMPO, 5,5-dimethyl-1-pyrroline-N-oxide; DTPA, diethylenetriaminepentaacetic acid; EPR, electron paramagnetic resonance; RBC, red blood cells; MNP-d4, perdeuterated 2-methyl-2-nitroso propane.
considered that involve the formation of intermediate hemoglobin-NO\textsubscript{2} complexes, such as those observed during the oxidation of oxyHb by NO \textsuperscript{-} (12) and probably by NO\textsubscript{2} \textsuperscript{-} and/or NO\textsubscript{2} \textsuperscript{-} (13). Indeed, in the oxyHb-mediated oxidation of NO\textsuperscript{-}, a transient metHb-peroxynitrite complex is detected that rapidly evolves to metHb and nitrate (12).

\[ \text{Hb(Fe}^{III}\text{)O}_2 + \text{NO} \rightarrow \text{Hb(Fe}^{III}\text{)ONO} \rightarrow \text{Hb(Fe}^{III}\text{)} + \text{NO}_2^{-} \]  \hspace{0.5cm} \text{(Eq. 2)}

A similar complex was also proposed for the isomerization of peroxynitrite catalyzed by metmyoglobin or iron-porphyrins (14, 15).

The alternative scenarios for the peroxynitrite/oxyHb reaction are further complicated by the fact that the reactions of nitrite (NO\textsubscript{2} \textsuperscript{-}) and nitrogen dioxide (NO\textsubscript{2}) with oxyHb could promote the formation of hydrogen peroxide (13, 16) which in turn can secondarily react with oxy- and metHb to yield ferriHb (Equation 3) or ferrylHb with a second oxidation equivalent centered in the globin moiety, respectively (10, 17, 18).

\[ \text{Hb(Fe}^{III}\text{)O} + \text{H}_2\text{O}_2 \rightarrow \text{Hb(Fe}^{IV}\text{)} + \text{O}_2 + \text{H}_2\text{O} \]  \hspace{0.5cm} \text{(Eq. 3)}

Regardless of the mechanism considered, a transient ferrylHb is formed during the reaction of peroxynitrite with oxyHb (8, 9) that could be reduced back to metHb by peroxynitrite (Equation 4), nitrite (Equation 5), or globin amino acid residues adjacent to the heme moiety (Equation 6).

\[ \text{Hb(Fe}^{IV}\text{)} + \text{ONO}_2 \rightarrow \text{Hb(Fe}^{IV}\text{)O}_2 + \text{H}^+ \]  \hspace{0.5cm} \text{(Eq. 4)}

\[ \text{Hb(Fe}^{IV}\text{)} + \text{NO} + \text{O}_2 + \text{H}_2\text{O} \]  \hspace{0.5cm} \text{(Eq. 5)}

\[ \text{X} - \text{Hb(Fe}^{IV}\text{)}\rightarrow \text{X} - \text{Hb(Fe}^{III}\text{)} + \text{OH}^{-} \]  \hspace{0.5cm} \text{(Eq. 6)}

The reduction of ferrylHb by peroxynitrite has been proposed (9), but this reaction will only occur under conditions of excess of peroxynitrite with respect to oxyHb. The oxidation of nitrite by ferrylHb can also occur (16), and a kinetic constant for this reaction \((7.5 \times 10^{7} \text{ M}^{-1} \text{ s}^{-1} \text{ at pH } 7.0 \text{ and } 20^\circ \text{C})\) was recently reported (19). Finally, it is possible that the oxo-ferryl moiety oxidizes amino acid residues, in which case globin-derived radicals should be detected. This latter possibility is in line with previous results obtained with hydrogen peroxide (10). Thus, the mechanism by which 1) oxyHb is oxidized, 2) ferrylHb is formed, and 3) ferrylHb is reduced back to metHb still remain to be defined.

The reaction of peroxynitrite with oxyHb is relevant in the context of peroxynitrite reactions and diffusion in the intravascular compartment, because red blood cells and oxyHb may constitute a "sink" for intravascularly formed peroxynitrite (7, 20, 21). Indeed, peroxynitrite can readily cross erythrocyte membranes via anion channel-dependent and simple diffusion mechanisms, and we have previously established that significant amounts of peroxynitrite can reach inside red blood cells, even in the presence of physiological concentrations of carbon dioxide and other fast reacting biotargets of peroxynitrite present in plasma and the extracellular milieu (6, 7, 20). Thus, the diffusion of peroxynitrite to red blood cells competes with other routes of peroxynitrite consumption in the intravascular space. Importantly, once in the erythrocyte, the large concentration of hemoglobin (20 mM heme), together with a relatively high rate constant (for a comparison of rate constants of peroxynitrite with biotargets, see Ref. 4), results in a rapid and almost complete intraerythrocytic consumption of peroxynitrite (7). It is important to notice that the reaction of peroxynitrite with intracellular oxyHb \((20 \text{ mM heme, } k = 2 \times 10^{6} \text{ M}^{-1} \text{ s}^{-1})\) is five to six times faster than that with CO\textsubscript{2} \((1.3 \text{ mM, } k = 4.6 \times 10^{4} \text{ M}^{-1} \text{ s}^{-1})\) at 37 °C, which implies that more than 80% of the peroxynitrite inside red blood cells will react directly with hemoglobin and supports the importance of studying this reaction.

In addition to oxidation reactions, peroxynitrite also promotes the nitration of aromatic compounds such as protein tyrosine residues, a process that requires the intermediate formation of secondary nitrating species. The overall process involves free radical mechanisms in which one-electron oxidants derived from peroxynitrite attack the phenolic ring, leading to formation of a tyrosyl radical, followed by a coupling of the amino acid-derived radical with NO\textsubscript{2} \textsuperscript{-} to yield 3-nitrotyrosine (22). Yields of nitration by peroxynitrite can be significantly enhanced by ferric iron complexes, which reduce peroxynitrite by one electron, resulting in the formation of NO\textsubscript{2} \textsuperscript{-} and an oxo-iron complex that could then act as the one-electron oxidant to the phenolic ring. Another iron-dependent nitration mechanism is the one catalyzed by peroxidases, in which ferric heme promotes an initial two-electron reduction of peroxynitrite to yield peroxidase complex I and NO\textsubscript{2} \textsuperscript{-} (23). Complex I can then react quickly with NO\textsubscript{2} \textsuperscript{-} to generate NO\textsubscript{2} \textsuperscript{-} and compound II (24), both of which can participate in sequential steps that lead to nitration. According to these considerations, the coexistence of significant amounts of ferrylHb and NO\textsubscript{2} \textsuperscript{-} could promote tyrosine nitration as well (6).

The kinetics of peroxynitrite decomposition and secondary reactions is largely influenced by target concentration (4). Under biologically relevant conditions, oxyHb will be present in large excess over peroxynitrite, a condition that may not always be accomplished when performing mechanistic experiments \textit{in vitro}. Thus, in this article, we investigate the reaction of peroxynitrite with pure human oxyHb and define the mechanisms that lead to the formation of metHb under experimental conditions that are relevant to the biological chemistry of this reaction \textit{in vivo}.

**MATERIALS AND METHODS**

**Chemicals**—The following reagents were purchased from Sigma: sulfanilamide, N-\(\text{N}(\text{naphthyl})\text{ethylenediamine dihydrochloride}, p\)-hydroxyphenylacetic acid, V-\(\text{N}(\text{ethylmaleimide})\) and Pronase. Vanadium (II) chloride was from Aldrich; hydrogen peroxide was from Fluka; 5,5-dimethyl-1-pyrroline-N-oxide (DMPO) was from Alexis; and the anion exchanger DEAE-Sepharose CL-6B was obtained from Pharmacia. Perdeuterated MNP \((\text{MNP-d^7})\) was kindly provided by Dr. B. Kalyanaraman \(\text{(Medical College of Wisconsin, Milwaukee, WI). All other reagents were of research grade quality. Peroxynitrite was synthesized from hydrogen peroxide and nitrous acid as described previously (25–27). Contaminating hydrogen peroxide was eliminated with manganese dioxide, and peroxynitrite concentration was determined at 302 nm \((\varepsilon_{302} = 1.67 \text{ mm}^{-1} \text{ cm}^{-1})\). The concentration of nitrate in the peroxynitrite prepurification steps was determined by the Griess method (28) using peroxynitrite previously decomposed under acidic conditions (200 mM KH\textsubscript{2}PO\textsubscript{4} that undergoes completely isomerization to nitrate (4). Nitrite contamination was typically less than 30% of the peroxynitrite concentration. The absence of hydrogen peroxide was confirmed by decomposing peroxynitrite in buffer before the assay with horseradish peroxide plus iodide as the second substrate \((\varepsilon_{355} = 25,000 \text{ M}^{-1} \text{ cm}^{-1})\); Ref. 29). For all experimental conditions, control runs were performed with peroxynitrite previously decomposed in buffer \(\text{(reverse order addition experiments). These controls allow discounting the contribution of contaminants of the peroxynitrite preparation and decomposition products in the observed results.}

**Preparation of Human Oxyhemoglobin—**OxyHb was prepared from human erythrocytes (RBC) as described previously (30). Briefly, cells were obtained by centrifugation \((800 \times g \text{ for } 10 \text{ min})\) of freshly drawn blood from a healthy donor with heparin as anticoagulant. After the RBC had been washed with 0.15 M NaCl, lysis was achieved by diluting with 3 volumes of distilled water. After 15 min at room temperature, the
lysozyme was centrifuged and the supernatant was diluted in 0.2 volumes of 250 mM Tris-HCl, pH 8.3, and a 0.5 mM EDTA. The lysozyme was then loaded onto a DEAE-Sephacel column (2.5 × 20 cm) previously equilibrated with 50 mM Tris-HCl, pH 8.3, and 0.1 mM EDTA. OxyHb was eluted, applying a pH gradient from 8.3 to 7.0 in the same buffer. The purity of the preparations was assessed by SDS/15% polyacrylamide gel electrophoresis with silver staining, yielding a single protein band around 15.5 kDa, corresponding to the α and β chains of human hemoglobin. The solution of hemoglobin obtained was more than 95% oxyHb and neither superoxide dismutase (31) nor catalase (32) was detected. FerrylHb was prepared from oxyHb (100 μM in 100 mM phosphate buffer and 0.1 mM DTPA, pH 7.4) by addition of a 5-fold excess of hydrogen peroxide at room temperature. Conversion of oxyHb to ferrylHb was confirmed spectrophotometrically by measuring the absorbance at 577 and 630 nm as described by Winterbourn (30): oxyHb concentration of hemc, μM = 66 A630 − 80 A577; metHb concentration of hemc, μM = 279 A577 − 3 A630. In the experiments where ferrylHb was also present in the samples, the concentration of each of the three species (concentration of hem, μM) was determined by measuring the absorbance at 560, 577, and 630 nm and using the following equations derived from the corresponding molar extinction coefficients of each species (14 μM−1 cm−1 for oxyHb, 76 μM−1 cm−1 for metHb, 154 μM−1 cm−1 for ferrylHb). Absorption spectra were collected on a Shimadzu 2100 spectrophotometer.

Concentration of Hemoglobin—The concentration of oxy- and metHb was determined spectrophotometrically by measuring the absorbance at 577 and 630 nm as described by Winterbourn (30): oxyHb concentration of hemc, μM = A630/A680 = 68 A630 − 82 A577 + 42 A577 − 319 A630; ferrylHb = 96 A577 − 55 A630 − 47 A630. Spectra were collected on a Shimadzu 2100 spectrophotometer. Modification of Cystine Residues—β-93 Cys residues were blocked by incubating hemoglobin with 10-fold excess of NEM for 1 h at room temperature and then passed through a Sephadex G-25 column. The amount of total sulphydryls was quantified before and after treatment by spectrophotometric titration with p-chloromercuribenzoic acid (33).

Briefly, aliquots of 5–10 μL of 1 mM p-chloromercuribenzoic acid were added to 1 ml of hemoglobin solutions (20–30 μM) in 50 mM Tris-HCl, pH 7.0, and 1.0% SDS, and the increase in the absorbance at 250 nm was determined. The presence of SDS assured measurement of the total number of sulfhydryls present in the molecule. Generally, six thiols/hemoglobin (tetramer) were quantified after hemoglobin purification and four thiols/hemoglobin after treatment with NEM.

Determination of Nitrite and Nitrate—Determination of nitrate was performed by the Griess method (28). To determine total nitrate plus nitrite (NO2), a solution of vanadium (III) chloride (25 mM, final concentration) was added to the Griess reagents to reduce nitrite to nitrate as described previously (34). To avoid secondary reactions during NO2 determination, hemoglobin was previously separated by gel filtration. Immediately after each treatment, 100 μL of the sample was passed through a Hi-Trap Desalting column (Amersham Biosciences) and eluted using 50 mM phosphate buffer, pH 7.3, at a flow rate of 5.0 ml/min. Aliquots of the sample, and fractions that eluted between 20–40 s (hemoglobin) and 50–70 s (total NO2) were collected for further analysis. Control experiments were performed to assure that under our experimental conditions, the reaction of oxyHb with nitrite present in peroxynitrite preparations does not occur; i.e. all nitrite was recovered and no metHb was observed after sample separation. Dilution of the samples after passage through the column was 20-fold, and it was checked in every experiment with appropriate standard solutions.

Oxymetry Studies—Oxygen evolution studies were performed polarographically using a Cole Parmer oximeter with a Clark type electrode (YSI model 5300). Reactions were performed at 37 °C.

Detection of Hydrogen Peroxide as a Reaction Product—Hydrogen peroxide was detected by chemiluminescence with luminol using an assay based on the pseudo-peroxidase activity of hemoglobin (35). This method was the most appropriate because of its sensitivity and because it overcomes the loss of H2O2 from reactions with oxy- and metHb that occurs during the sample processing that other techniques would require. First, metHb (250 and 500 μM) was added to oxyHb (50 μM) in 100 mM phosphate buffer, and 0.1 mM DTPA, pH 7.3; immediately after, 100 μL of the sample was placed in a tube containing 3.0 ml of luminol (10 μM) in 200 mM phosphate buffer, pH 7.8, and 0.3 mM DTPA. Chemiluminescence was monitored using a Thorn-Emi photo counter equipped with an EMI Fact 50 MK II Photomultiplier cooled to −10 °C. The sample was stored at 1350 V. The reactions were always carried out in glass tubes that were kept at 25 °C by a temperature-controlled water bath.

EPR Experiments—The EPR spectra were recorded at room temperature (25 °C) on a Bruker EMX EPR spectrometer. The reaction mixtures (200 μL, final volume) were transferred to 100-μL flat cells immediately after peroxynitrite addition and the spectra recorded within 1 min. The magnetic field was calibrated with 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinylxyl radical (g = 2.0056). MNP stock solution (1.0 M, monomer) was prepared in acetoniitrile (high pressure liquid chromatography quality). The MNP-Hb radical concentration was estimated by using a 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinylxyl radical standard curve (145 μM) in the α, γ glycine. The calibration experiments were performed by using a fingertip Dewar flask containing liquid nitrogen.

SDS-PAGE and Western Blot Analysis—All experiments were carried out at 25 °C unless otherwise indicated. To rule out any contribution of nitrite present in peroxynitrite preparations, samples were reacted immediately after peroxynitrite addition and then stored at 4 °C until preparation for electrophoresis. Reaction mixtures containing human hemoglobin samples were separated on 15% polyacrylamide gels using SDS-PAGE. The gels were either silver-stained for protein band visualization or electrophoretically transferred (100 V, 1 h) to nitrocellulose membranes (Hybond-C extra; Amersham Biosciences) for Western blot analysis.

Protein bands transferred to nitrocellulose membranes were stained and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water. Immunodetection of DMPO nitro oxide adducts on the hemoglobin was measured using a recently developed anti-DMPO nitro oxide antibody and fixed using non-permanent 2% Ponceau S in 30% trichloroacetic acid for 20 min, scanned, and then destained overnight with deionized water.
tion of hydrogen peroxide with oxyHb (which gives ferrylHb) in the presence of nitrite showed the predictable stoichiometric consumption of 0.5 nitrite per metHb formed (Table I). So, the reactions proposed in Equations 1 and 5 could not explain the absence of nitrite detection observed in the experiment and a higher than stoichiometric formation of nitrate.

Fig. 1 shows the decay of ferrylHb (25 μM) preformed with an excess of H₂O₂. FerrylHb alone was rather stable, and the decay was moderately enhanced in the presence of NO₂⁻ or an equivalent amount of nitrite at the reaction times between ferrylHb and oxyHb (10). Oxygen within the response time of the oxygen sensor) followed by a phase, consistent with the reaction time of peroxynitrite (i.e. detected (Fig. 2).

TABLE I

| Condition | [metHb] | Δ(NO₂⁻) | [NO₃⁻] | Oxidation yield |
|-----------|---------|---------|---------|---------------|
| ONOO⁻ (150 μM) | 130 ± 10 | -33 ± 17 | 181 ± 33 | 87 |
| ONOO⁻ (250 μM) | 263 ± 5 | -56 ± 13 | 265 ± 42 | 105 |
| ONOO⁻ (420 μM) | 366 ± 10 | -95 ± 18 | 434 ± 25 | 87 |
| ONOO⁻ (250 μM) + PHPA (200 mM) | 193 ± 5 | -58 ± 11 | 272 ± 46 | 77 |
| H₂O₂ (300 μM) + NO₂⁻ (600 μM) | 180 ± 20 | -150 ± 17 | 115 ± 20 | 60 |

Detection of Hydrogen Peroxide—Because the reaction of peroxynitrite with oxyHb mainly yielded nitrate (an isomerization product) and oxygen yields were significantly less than predicted from a two-electron oxidation, we explored alternative mechanisms to account for the redox reaction leading to metHb. In this context, we evaluated the potential reduction of oxygen bound to the ferrous heme. For this, we studied the formation of hydrogen peroxide by chemiluminescence techniques. Hydrogen peroxide could arise secondary to the dismutation of superoxide radical anion formed from a one-electron transfer from the ferrous heme to bound oxygen or directly if a two-electron transfer to oxygen occurred. The reaction of H₂O₂ with oxy- or metHb results in a ferryl species similar to peroxynitrite (35). Fig. 3 shows chemiluminescence obtained when peroxynitrite (250, 500 μM) was added to oxyHb (1.0 mM) and the reaction mixture was immediately placed in a tube containing luminol. Light emission was indicative of ferrylHb formation. The signal obtained was ~50% inhibited when catalase was added 5 s after peroxynitrite. The intensity of the catalase-inhibitable signal was similar to that obtained by addition of 250 μM H₂O₂ to the oxyHb solution. No signal was obtained by pre-decomposed peroxynitrite, disproving the formation of hydrogen peroxide from the direct reaction of oxyHb with contaminant nitrite present in peroxynitrite preparations. The data supports the idea that ferrylHb is formed as a consequence of both the reaction with peroxynitrite and secondary to hydrogen peroxide.
peroxide formation, which is produced in about 50% yield with respect to peroxynitrite.

**Spin Trapping of Globin Radicals**—Fig. 4 shows EPR spectra of MNP radical adducts obtained during incubation of oxyHb with peroxynitrite followed by addition of MNP 15 s after peroxynitrite. This order of addition was intended to minimize direct interactions of peroxynitrite and secondary reactive species with the spin trap. The immobilized signal obtained (Fig. 4A) is characteristic of an adduct between MNP and an amino acid-derived radical. The presence of CO added in the same concentration ratio as observed in RBC (oxyHb; CO = 15) did not affect the immobilized signal obtained (data not shown). Calibration experiments using 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy radical as standard indicated a ~6% yield of trapped protein radicals with respect to added peroxynitrite. These results suggest that formation of protein radicals represent a secondary pathway during oxyHb oxidation. The spin adduct was subjected to nonspecific proteolysis with Pronase (final concentration, 20 mg/ml), resulting in the conversion of the spectrum into an isotropic three-line spectrum with a hyperfine coupling constant of 15.4 G (Fig. 4B). An identical signal has previously been reported for the adduct formed between MNP-cytochrome c (42) or MNP/metMb (43) treated with H2O2 and was assigned to a radical located on a tertiary carbon atom. The experiment was then repeated using MNP-d9 to increase the resolution of the superhyperfine structure. A similar three-line spectrum was detected when MNP-d9 was used as the spin trap (data not shown). When the low-field line of this triplet was scanned using a smaller modulation amplitude, the superhyperfine structure shown in Fig. 4C was obtained. Comparison of this signal with that obtained with authentic MNP-d9/Tyr generated by oxidation of the free amino acid by horseradish peroxidase (44, 45) strongly supports the idea that tyrosyl radical is formed during oxyHb reaction with peroxynitrite. Addition of H2O2 or H2O2 plus pre-decomposed peroxynitrite yields an EPR signal after 60 s that is significantly lower (<10%) than the one obtained with peroxynitrite (data not shown). These results are in agreement with an early formation of amino acid-derived radicals, mainly because of peroxynitrite-dependent ferrylHb formation and not secondary reactions caused by H2O2 production.

To obtain further information concerning the identity of the protein radicals generated by the reaction of oxyHb with peroxynitrite, experiments were also performed with the spin trap DMPO. When oxyHb was treated with peroxynitrite in the presence of 100 mM DMPO, a four-line spectrum was obtained, with a β-hydrogen splitting constant value of 15.4 G, which is consistent with those previously assigned to other DMPO/protein-thyl radical adducts (Fig. 5) (46, 47). No immobilized signal was observed with DMPO when reactive Cys-93 were blocked with NEM; the weak signal observed is compatible with the idea that Cys-93 is oxidized by peroxynitrite. Indeed, dimers of hemoglobin chains were observed during oxyHb oxidation by ferrylHb. To further investigate secondary oxidative modifications in the globin moiety, we evaluated the formation of cross-links between hemoglobin subunits. Indeed, dimers of hemoglobin chains were obtained after peroxynitrite treatment in a dose-dependent manner (Fig. 6). Importantly, 1) the dimers were obtained with less than a stoichiometric amount of peroxynitrite and 2) no dimer formation was observed during oxyHb oxidation by ferricyanide (data not shown). Thus, the data is consistent with ferrylHb-dependent formation of globin-derived radicals followed by radical recombination/dimerization. In this regard, two types of cross-linked species were detected (i.e. mercapto-
ethanol reducible (Fig. 6, lanes 6–9) and non-reducible (Fig. 6, lanes 2–5), in agreement with the formation of intermolecular disulfide bridges and dityrosine, respectively. Covalent linkage between β-93 cysteines and α-tyrosines 42 and 24 has been reported after reactions of peroxynitrite and oxyHb in the presence of CO2 (21). No differences were observed when 100 μM ascorbate was added as a reductant to the electrophoresis loading buffer, ruling out cross-linking caused by oxidation during the denaturation process (data not shown) (21).

Immunochromatographic Detection of DMPO-Hemoglobin Adducts—To further support the role of amino acid-derived radical recombination reactions in the formation of dimers of hemoglobin chains, it was observed that peroxynitrite oxidation of oxyHb in the presence of DMPO leads to an inhibition of dimer formation (Fig. 7A). The inhibitory effect of DMPO (5–225 μM) was concentration-dependent. Because there is no significant direct reaction of peroxynitrite with DMPO (50) and because DMPO-globin radical adducts were detected by EPR (Fig. 5), the decrease in dimer formation can be attributed to the trapping of tyrosyl and thyl radicals. The formation of DMPO-hemoglobin adducts was evaluated immunochromatographically by the use of an anti-DNPO nitroso globin antibody, which has just been developed (37). This antibody recognizes the one-electron oxidation product of the initial nitroxyl spin adduct, the nitroso of DMPO, which is an EPR-silent but stable product (37). Using this antibody with the oxyHb samples treated with peroxynitrite in the presence of DMPO, we found the formation of DMPO-globin monomer adducts (Fig. 7C). Interestingly, when oxyHb was pretreated with NEM, peroxynitrite addition in the presence of DMPO led to a larger amount of adduct detected and complete inhibition of dimer formation (Fig. 7, B and C). This indicates that although under our experimental conditions only DMPO-thyl globin radicals were detected by EPR, DMPO can also add to amino acid-derived radicals different from cysteinyl and is consistent with the antibody avidly recognizing DMPO nitrotyrosyl hemoglobin adducts (51).

Immunochromatographic Detection of Nitrotyrosine—Under excess of oxyHb over peroxynitrite, no 3-nitrotyrosine in hemoglobin was detected, as assessed immunochromatographically (Fig. 8) even under longer film exposure time. However, at low hemoglobin concentrations (where peroxynitrite homolysis to OH and NO2 becomes relevant) or as the peroxynitrite/hemoglobin ratio increases, nitration can be observed. Nitration requires the formation of tyrosyl radicals and NO2, which necessarily involve the participation of ferrylHb for oxidation of tyrosine residues and/or nitrite or homolysis of peroxynitrous acid (4, 22). The fact that no 3-nitrotyrosine was detected with an excess of oxyHb over peroxynitrite supports the concept that the yields of ferrylHb and/or NO2 are modest. Together, the data indicate that under biologically relevant conditions, no significant peroxynitrite-dependent nitration of hemoglobin will be expected.

**DISCUSSION**

The studies presented herein show that oxyHb oxidation by peroxynitrite results in the stoichiometric formation of nitrate and metHb (Table I), and a fractional formation (~50%) of
oxygen (Fig. 2) and hydrogen peroxide (Fig. 3). A small percentage (−6%) of protein radicals were trapped (Figs. 4, 5, and 7) although no nitrite was formed; in fact, pre-existing nitrite in peroxynitrite preparations was partially consumed (Table I). These observations are consistent with a reaction mechanism that implies the isomerization of peroxynitrite to nitrate and oxyHb oxidation at the expense of the reduction of the molecular oxygen bound to the ferrous heme:

$$\text{HbFe}^{2+}\text{O}_2 \rightleftharpoons \text{Hb(Fe}^{3+}\text{)}\text{O}_2$$  \hspace{1cm} (Eq. 7)

$$\text{Hb(Fe}^{3+}\text{)}\text{O}_2 + \text{ONO}^\bullet \rightarrow \text{Hb(Fe}^{3+}\text{)}\text{O}_2^- + \text{H}_2\text{O}$$  \hspace{1cm} (Eq. 8)

$$\text{Hb(Fe}^{3+}\text{)}\cdot \cdot \cdot \text{ONO}^\bullet \rightarrow \text{Hb(Fe}^{3+}\text{)}\cdot \cdot \cdot \text{ONO}$$  \hspace{1cm} (Eq. 9)

$$\text{Hb(Fe}^{3+}\text{)}\cdot \cdot \cdot \text{ONO} \rightarrow \text{Hb(Fe}^{3+}\text{)} + \text{NO}_3^-$$  \hspace{1cm} (Eq. 10)

$$\text{Hb(Fe}^{3+}\text{)}\cdot \cdot \cdot \text{ONO} \rightarrow \text{Hb(Fe}^{3+}\text{)}\cdot \cdot \cdot \text{NO}_2^-$$  \hspace{1cm} (Eq. 11)

$$2\text{O}_2^- + 2\text{H}^+ \rightarrow \text{H}_2\text{O}_2 + \text{O}_2$$  \hspace{1cm} (Eq. 12)

In this mechanism, peroxynitrite reacts with oxyHb (best described as an intermediate form between iron(II)-dioxygen and an iron(II)-superoxide complex Equation 7; Refs. 52, 53) to yield superoxide radical anion and a metHb-peroxynitrite int-

termediate (Equation 8), followed by a rapid ferric heme-catalyzed peroxynitrite decomposition via O–O bond homolysis to “caged” ferrylHb and 'NO$_2$ (Equation 9). Recombination of the 'NO$_2$ radical with ferrylHb within the heme cavity would mainly yield metHb and NO$_3$ (Equation 10), whereas a small fraction of the radicals could diffuse out (Equation 11) and yield NO$_2$ and ferrylHb. Ferric iron could act as a Lewis acid and catalyze the isomerization of peroxynitrite to nitrate (4, 54) as has been already reported for iron(III) porphyrins and metmyoglobin (14, 15). A similar transient metHb-peroxynitrite complex was detected during the NO-mediated oxidation of oxyHb, which rapidly evolves to metHb and nitrate (12). Importantly, oxidation of oxymyoglobin by NO also yields an intermediate complex, which promotes the oxidation of ethylene-1,2,3-13C as detected by NMR (13); this result indicates that a hydroxyl radical-like species arises at low yields from the iron-catalyzed homolysis of complexed peroxynitrite and that it can escape the solvent cage to initiate free radical chemistry (13). In addition, superoxide anion released in the first step of the reaction (Equation 8) would spontaneously dismutate to O$_2$ and H$_2$O$_2$ (Equation 12), which would initiate secondary oxidation reactions in oxy- or metHb to ferrylHb (10, 17, 18) and promote consumption of pre-existing nitrite in peroxynitrite preparations (Table I), which is facilitated by autocatalytic mechanisms (16)

The oxidation of oxyHb by peroxynitrite yields a transient ferrylHb intermediate (8, 9). This observation contrasts with the stability of the ferrylHb intermediate formed under excess of H$_2$O$_2$ (10, 55), which can be observed even in the presence of nitrite (Fig. 1). However, although the reported H$_2$O$_2$-derived ferrylHb probably arises from the two-electron oxidation of metHb and therefore contains a second oxidation equivalent in the globin moiety, the ferrylHb formed from the reaction of limiting oxidant (e.g. peroxynitrite) with oxyHb involves only a two-electron oxidation of the heme. Peroxynitrite-derived ferrylHb can be observed only if trapped with excess sulfide at relatively low yields (−15%), and it ultimately undergoes a one-electron reductive step to metHb. Under biologically relevant conditions, the reduction of ferrylHb by peroxynitrite is unlikely because the rate constants of peroxynitrite with oxyHb and ferrylHb (9) are similar ($8.8 \times 10^7$ and $9.4 \times 10^9$ M$^{-1}$ s$^{-1}$ at pH 7 and 20°C), and the concentration of ferryl- compared with oxyHb is negligible under any circumstance. Reduction of ferrylHb by nitrite (16) is not expected to represent a relevant pathway because of kinetic limitations (Fig. 1) (19). The decay of the ferryl intermediate that can occur by reaction with nearby amino acids such as tyrosine, cysteine, or tryptophan would result in the formation of globin-derived radicals that favor some oxygen consumption and intermolecular cross-links.
In this regard, cysteinyl and tyrosyl radicals were spin-trapped as detected by EPR and/or immunochemically (Figs. 4, 5, and 7) and intermolecular cross-linked products were formed (Fig. 6). Formation of amino acid-derived radicals by direct reactions of cysteine or tyrosine with peroxynitrite are ruled out because: 1) the second-order rate constant of β-Cys-93 with peroxynitrite has been determined as 1.5 × 10⁻³ m⁻³ s⁻¹ per thiol group, a value nearly 25 times smaller than reaction of oxyhemoglobin with peroxynitrite, considering that there are two reactive cysteines per hemoglobin tetramer; 2) direct reaction of peroxynitrite with thiols result in two-electron oxidation products (e.g., cysteine sulfenic acid), not the cysteinyl radical (27, 57); and 3) there is no direct reaction between peroxynitrite and tyrosine (58). Note that another two-electron oxidant of oxyHb, such as hydrogen peroxide, also leads to substoichiometric oxygen detection, whereas a one-electron oxidant, such as ferricyanide, which does not yield ferrylHb, results in 100% oxygen release (Fig. 2B).

In RBC, another mechanism of ferrylHb consumption should be considered, such as its one-electron reduction by glutathione (5 mM inside RBC), which yields metHb and glutathionyl radical without initial formation of globin-derived radicals (59). Alternatively, glutathionyl radicals may be produced by the reaction of glutathione with globin-derived radicals that would then be repaired (59).

Two important kinetic considerations must be taken into account when interpreting the data presented herein. First, under our experimental conditions of oxyHb ≥ 1 mM and equal or lower concentrations of peroxynitrite, most peroxynitrite will react directly with oxyHb and minimal amounts will undergo homolysis to ‘OH and NO₂⁻ (k = 0.26 s⁻¹ at 25 °C and pH 7.4) (41). Thus, the radical pathway depends on the presence of ferrylHb and not on secondary radicals arising from peroxynitrite decomposition. Second, DMPO, which was added as a spin trap for both EPR and immunochimical studies, does not react directly with peroxynitrite (50); therefore, the DMPO concentrations used (up to 100 mM) do not influence the initial reaction of oxyHb with peroxynitrite. Thus, the effects of DMPO are related to the trapping of secondary globin-derived radicals (Figs. 5 and 7) and perhaps some minor reaction with the ferryl intermediate (Fig. 5) (45, 48). We cannot define which of the ferrylHb-oxidized amino acids detected using the spin traps MNP and DMPO (i.e. tyrosyl and cysteinyl radicals) are the initial radicals or whether they are formed after intramolecular electron transfer (60). Recent observations using peroxynitrite-treated RBC lysates have also suggested the occurrence of both inter- and intramolecular electron transfer processes leading to the detection of different amino acid-derived radicals depending on the spin trap or hemoglobin concentration used (59).

In contrast to what is observed during peroxynitrite-dependent, one-electron oxidation of hemeperoxidases and other transition metal-containing centers, which yield NO₂⁻ and favor nitration reactions (23, 61), the mechanism of oxidation of oxyHb by peroxynitrite revealed herein circumvents nitration reactions because NO₂⁻ and nitrite are scarcely or not formed, respectively. Despite this, oxyHb nitration can be observed if excess peroxynitrite over oxyHb is present or under conditions of low oxyHb concentrations in which peroxynitrite can yield ‘OH and ‘NO²⁻. In summary, oxyHb reacts fast and isomerizes peroxynitrite to nitrate with the concomitant net one-electron oxidation of the ferrous heme to the ferric state and production of O₂/H₂O₂. There is an accompanying substoichiometric formation of transient ferrylHb and protein-derived radicals and a negligible incidence of nitrating species. Inside RBC, the H₂O₂ produced after peroxynitrite reactions will not significantly react with oxyHb because this reaction is quite slow (k = 100 m⁻¹ s⁻¹ (10)) but would be mainly removed by the action of glutathione peroxidase and catalase. Thus, the present results are compatible with a mechanism of oxyHb oxidation by peroxynitrite under biologically relevant conditions as indicated in Fig. 9 and point to oxyHb as a relevant intravascular sink of peroxynitrite.

Acknowledgments—We thank Mary J. Mason for her language assistance in the preparation of the manuscript, Dr. Carlos Batthyany (Facultad de Medicina, Universidad de la Republica) for providing expertise on nitrite/nitrate measurements, and Dr. Jin-Jie Jiang (NIH/NIEHS) for assistance in the calibration of the protein-MNP radical adduct signal.

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