Peroxynitrite Induces Long-Lived Tyrosyl Radical(s) in Oxyhemoglobin of Red Blood Cells through a Reaction Involving CO_2 and a Ferryl Species[†]

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ABSTRACT: Peroxynitrite-mediated oxidative chemistry is currently the subject of intense investigation owing to the toxic side effects associated with nitric oxide overproduction. Using direct electron spin resonance spectroscopy (ESR) at 37 °C, we observed that in human erythrocytes peroxynitrite induced a long-lived singlet signal at $g = 2.004$ arising from hemoglobin. This signal was detectable in oxygenated red blood cells and in purified oxyhemoglobin but significantly decreased after deoxygenation. The formation of the $g = 2.004$ radical required the presence of CO_2 and pH values higher than the pK_a of peroxynitrous acid (p$K_a = 6.8$), indicating the involvement of a secondary oxidant formed in the interaction of $ONOO^-$ with CO_2. The $g = 2.004$ radical yield leveled off at a 1:1 ratio between peroxynitrite and oxyhemoglobin, while CO-hemoglobin formed less radical and methemoglobin did not form the radical at all. These results suggest that the actual oxidant is or is derived from the $ONOOCO_2^-$ adduct interacting with oxygenated Fe^{II}-heme. Spin trapping with 2-methyl-2-nitrosopropane (MNP) of the $g = 2.004$ radical and subsequent proteolytic digestion of the MNP/hemoglobin adduct revealed the trapping of a tyrosyl-centered radical(s). A similar long-lived unresolved $g = 2.004$ singlet signal is a common feature of methemoglobin/H_2O_2 and metmyoglobin/H_2O_2 systems. We show by spin trapping that these $g = 2.004$ signals generated by H_2O_2 also indicated trapping of radicals centered on tyrosine residues. Analysis of visible spectra of hemoglobin treated with peroxynitrite revealed that, in the presence of CO_2, oxyhemoglobin was oxidized to a ferryl species, which rapidly decayed to lower iron oxidation states. The $g = 2.004$ radical may be an intermediate formed during ferrylhemoglobin decay. Our results describe a new pathway of peroxynitrite-dependent hemoglobin oxidation of dominating importance in CO_2-containing biological systems and identify the $g = 2.004$ radical(s) formed in the process as tyrosyl radical(s).

The discovery of a role of nitric oxide ($^\bullet NO$)[1] as a signal-transducing agent in important processes such as immune response, vasorelaxation, and neurotransmission has caused considerable interest in the physiology of this radical (*1*). Moreover, it was evident that in pathological conditions $^\bullet NO$ was responsible for several beneficial effects by decreasing the adhesion of neutrophils and platelets to the endothelium, improving blood flow after reperfusion and scavenging lipid peroxyl radicals (*1*, *2*).

However, the intense synthesis of $^\bullet NO$ may also lead to a variety of undesirable toxic side effects (*2*). Some of these toxic effects are severe enough to stimulate intense research on the species involved. It was realized that $^\bullet NO$ toxicity is probably due to other nitrogen oxides formed in the reaction of $^\bullet NO$ with oxygen or reactive oxygen species. Recent studies have shown that cells activated to produce $^\bullet NO$ simultaneously produced $O_2^{\bullet -}$ (*3*) and that the inducible form of $^\bullet NO$ synthase (iNOS, type II) generates simultaneously $O_2^{\bullet -}$ and $^\bullet NO$ (*4*). The $^\bullet NO$ and $O_2^{\bullet -}$ radicals can react at nearly diffusion-controlled rates (*5*) to form a toxic species, the peroxynitrite anion $ONOO^-$, which is probably responsible for most of the cellular injuries associated with $^\bullet NO$ overproduction (*6*−*8*).

$ONOO^-$ is a relatively stable species and is capable of oxidizing biological targets through a two-electron-transfer process (*6*, *9*). Furthermore, at neutral to acidic pH $ONOO^-$ is in equilibrium with its conjugated acid, peroxynitrous acid (ONOOH, p$K_a = 6.8$, ref *10*). ONOOH is a strong oxidant but is unstable and rapidly decays to nitrate ($t_{1/2} \leq 1$ s).

During the decay process, ONOOH is converted to a potent cytotoxic intermediate (*ONOOH or the geminate pairs $^\bullet NO_2/^\bullet OH$), which is responsible for hydroxylation and one-electron oxidations (*10*−*12*). In vivo, the formation of this cytotoxic intermediate is outcompeted by the direct reactions of $ONOO^-$ or ONOOH with biological targets. Of these, CO_2 and hemoproteins are of particular importance, since

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[1] Abbreviations: $^\bullet NO$, nitric oxide, IUPAC-recommended name nitrogen monoxide; Hb, hemoglobin; Mb, myoglobin; $O_2^{\bullet -}$, superoxide anion radical; $ONOO^-$, peroxynitrite anion, IUPAC-recommended name oxoperoxonitrate (1−); ONOOH, peroxynitrous acid, IUPAC-recommended name hydrogen oxoperoxonitrate; $[Me_4N][ONOO^-]$, tetramethylammonium salt of peroxynitrite; DTPA, diethylenetriaminepentaacetic acid; diethylamine-NONOate, $C_4H_{10}N_3O_2 \cdot C_4H_{12}N$; Sod, superoxide dismutase; Cat, catalase; MNP, 2-methyl-2-nitrosopropane; DMPO, 5,5-dimethyl-1-pyrroline-*N*-oxide; DBNBS, 3,5-dibromo-4-nitrosobenzenesulfonate; ESR, electron spin resonance spectroscopy.

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these compounds are highly concentrated in biological samples (micromolar to millimolar) and their reactions with peroxynitrite are the fastest reactions known for this oxidant (*13*−*16*). Consequently, the interaction of $ONOO^-$ with CO_2 and hemoproteins not only inhibits the formation of *ONOOH but also precedes that with low molecular weight antioxidants (*13*, *17*).

Unexpectedly, the reaction of $ONOO^-$ with CO_2 does not detoxify peroxynitrite (this term refers to both the anion and the different forms of its conjugate acid), but leads to the formation of a postulated highly reactive short-lived secondary oxidant, the nitrosoperoxycarbonate adduct, $ONOOCO_2^-$ (*18*), which forms other reactive intermediates during the decay process (*18*, *19*). These reactive species have been shown either to increase or to protect some biological targets (*13*, *17*, *19*−*21*). In blood plasma, we showed by direct ESR and spin trapping experiments that CO_2 increased the formation of protein cysteinyl, tryptophanyl, and tyrosyl radicals, thus suggesting that CO_2 enhances the peroxynitrite-mediated one-electron oxidation pathway (*22*−*24*).

The aim of this study was to investigate long-lived radicals produced by peroxynitrite in human erythrocytes by direct ESR at 37 °C. We found that in the presence of CO_2 this oxidant induced a $g = 2.004$ radical of Hb attributed by spin trapping with MNP to tyrosyl radical(s). Notably, our results suggest that the radical induced in Hb was mediated by a direct bimolecular reaction between oxygenated Fe^{II}-heme and the $ONOOCO_2^-$ adduct or a species derived therefrom.

EXPERIMENTAL PROCEDURES

Materials. $[Me_4N][ONOO^-]$ was obtained from A. G. Scientific (San Diego, CA). GGYA and GGWR were from Serva (Heidelberg, Germany). Diethylamine-NONOate was obtained from Cayman (Ann Arbor, MI) and DMPO was from Aldrich (Milwaukee, WI). All other reagents were from Sigma (St. Louis, MO).

Preparation of Plasma, Red Blood Cells, Hemolysate, Ghosts, and Hemoprotein Derivatives. Heparinized fresh human blood was obtained from normal donors following informed consent. Plasma was separated by blood centrifugation for 5 min at 1000*g*. To minimize the loss to the gas phase of CO_2 dissolved in plasma, the samples were maintained at 0 °C in closed vials. After removal of plasma and buffy coat, red blood cells were washed three times with isotonic phosphate buffer (0.14 M NaCl, 3 mM KCl, and 10 mM sodium phosphate, pH 7.4) and suspended to 50% in phosphate buffer/DTPA/sodium bicarbonate (150 mM sodium phosphate, 0.1 mM DTPA, and 25 mM sodium bicarbonate, pH 7.4). To prepare a concentrated hemolysate, cells were first washed at 0 °C with 75 mM NaCl and 5 mM sodium phosphate buffer, pH 7.4, thus decreasing the salt concentration without producing hemolysis (*25*), and then lysed at 0 °C with an equal volume of 5 mM sodium phosphate containing 0.1 mM phenylmethanesulfonyl fluoride, pH 8. The hemolysate was obtained by centrifugation for 15 min at 40000*g* followed by three subsequent filtrations of the supernatant through 1.2, 0.45, and 0.2 μm disposable filter holders (Schleicher & Schuell, Dassel, Germany). Membranes were washed three times at 4 °C by centrifugation for 10 min at 40000*g* with lysis buffer. Ghosts were then obtained by resealing leaky membranes in 5 mM DTPA, 0.1 mM phenylmethanesulfonyl fluoride, 10 μg/mL aprotinin, 10 μg/mL leupeptin, 0.13 M KCl, and 5 mM phosphate buffer, pH 7.4, at 37 °C for 10 min. OxyHb free of Cat was purified from the hemolysate as described by Winterbourn (*26*) except that the ionic exchange column was a TSK-GEL DEAE-3SW (TosoHaas, GmbH, Germany). MetHb was prepared from oxyHb by the addition of potassium ferricyanide in a 1:1 molar ratio followed by extensive dialysis against isotonic phosphate buffer. CO−Hb was obtained from oxyHb by bubbling with CO gas for 2 min. OxyMb was obtained by treatment with dithionite (1:1.5 molar ratio, 30 min, 25 °C) of metMb previously degassed with argon (30 min). Dithionite in excess was removed by passage through a Sephadex G-25 column. Proteins were concentrated by centrifugation on Microcon concentrators (cutoff 3000, Amicon, Beverly, MA). To avoid metal-catalyzed oxidation of peroxynitrite (*27*), buffers were treated extensively with Chelex 100 (Bio-Rad, Richmond, CA) and all samples contained 0.1 mM DTPA.

Treatment with Peroxynitrite. All the experiments described here were performed with $[Me_4N][ONOO^-]$. In contrast to conventional preparations of peroxynitrite, which are usually obtained from acidified NO_2^- and H_2O_2, this salt of peroxynitrite is not contaminated by NO_2^- and H_2O_2 (*28*). Some experiments were repeated with a conventional preparation of peroxynitrite containing 64% NO_2^- (measured after $ONOO^-$ decomposition) and a residual 2.5% H_2O_2 (after the MnO_2 treatment). Essentially comparable results were obtained with the two peroxynitrite preparations up to a concentration of 2 mM. The concentration of peroxynitrite was determined spectrophotometrically in 1 M NaOH, with an extinction coefficient at 302 nm of 1720 M^{-1} cm^{-1} for the $[Me_4N][ONOO^-]$ and 1670 M^{-1} cm^{-1} for the conventional preparation of peroxynitrite. Peroxynitrite was added as a bolus to samples buffered with phosphate/DTPA/sodium bicarbonate buffer, pH 7.4 (150 mM phosphate final concentration and bicarbonate ranging from 0 to 100 mM) and immediately mixed by vigorous vortexing. Decomposed peroxynitrite was obtained by adding peroxynitrite to phosphate/DTPA/sodium bicarbonate buffer, pH 7.4, or by neutralization with HCl before the addition of the biological target. The two methods gave comparable results.

Measurement of Dissolved O_2 and CO_2. pCO_2, pO_2, and Hb saturation were measured with ABL 330 (Radiometer, Copenhagen, Denmark). The pO_2 in the reaction mixture was changed by bubbling into the solution 100% argon and/or 20% O_2 for variable periods of time before the addition of a small volume of 1 M sodium bicarbonate and peroxynitrite. The syringe used for hemogas analysis was extensively purged before sampling. In plasma the oxygen concentration was reduced by incubation with 20 mM glucose, 142 μg/mL glucose oxidase, and 50 μg/mL Cat (*29*). This last method cannot be used to produce anaerobic conditions in oxyHb-containing samples because it induces the formation of metHb.

ESR Spectroscopy. Spectra were measured on a Bruker ECS 106 spectrometer (Bruker, Rheinstetten, Germany) equipped with a variable-temperature unit (ER4111VT). Samples were drawn up into a gas-permeable Teflon tube with 0.81 mm internal diameter and 0.05 mm wall thickness (Zeuss Industrial Products, Raritan, NJ). The Teflon tube was folded four times, inserted into a quartz tube, and fixed to

the ESR cavity (4108 TMH). Samples were exposed to air at 37 °C. The dead time of sample preparation and ESR analysis was exactly 2 min after the last addition. All ESR spectra were corrected for baseline drift by a linear function of the software supplied by Bruker (ESP 1600 data system). To obtain an integrated relative area, we followed the two methods previously described (*24*). The concentration of $g = 2.004$ radical was estimated by using 4-amino-TEMPO nitroxide radical as standard and identical instrument settings. The spin trap MNP was suspended in 50 mM phosphate buffer, pH 7.1, and stirred overnight in the dark at 25 °C. Spectrometer conditions common to all spectra were modulation frequency, 100 kHz; microwave frequency, 9.4 GHz; and microwave power, 20 mW.

Spectrophotometric Determination of Hb Oxidation. The reaction of oxyHb and metHb with peroxynitrite was followed spectrophotometrically in the visible region (Lambda 14 P UV/vis, Perkin-Elmer, Norwalk, CT) and evaluated quantitatively by the equations described by Winterbourn (*26*). This method, however, cannot discriminate between hemichrome and ferrylHb. This last chromophore was measured after reduction with 2 mM Na_2S to form Fe^{II}-sulfHb (extinction coefficient at 617 nm = 24.0 mM^{-1} cm^{-1}) (*30*). Hb and Mb concentrations were expressed per heme group.

Fitting of Data. To draw a curve from the experimental data, nonlinear dose−response sigmoid or polynomial curves were used (Prism 2.0 software, GraphPad, San Diego, CA).

RESULTS AND DISCUSSION

Peroxynitrite in Red Blood Cells Induces a $g = 2.004$ Radical Due to OxyHb. We previously reported (*24*) that peroxynitrite induced the formation of a long-lived $g = 2.004$ radical in plasma proteins detectable by direct ESR at 37 °C (Figure 1A) (throughout this work we use the term $g = 2.004$ radical to refer to the broad unresolved signal at $g = 2.004$ arising from one or more radical centers). A radical with similar spectral characteristics was also formed in oxygenated red blood cells suspended to 50% in phosphate/DTPA/sodium bicarbonate buffer, pH 7.4, and treated with peroxynitrite (Figure 1D). The radical was detectable at peroxynitrite concentrations ≥ 1 mM and its intensity was decreased (−45%) by increasing the bicarbonate concentration to 100 mM (the CO_2 in equilibrium was 5 mM). The high concentration of peroxynitrite needed to observe the $g = 2.004$ signal in red blood cells may be due in part to the presence of intracellular antioxidants (e.g., ascorbate, glutathione) that, as previously observed in plasma (*24*), can reduce the radical after its formation. Accordingly, the observed inhibitory effect of 100 mM bicarbonate may be due to the ability of CO_2 to spare low molecular weight antioxidants in peroxynitrite-mediated oxidations (*19*−*21*). Second, CO_2 may affect the oxidation mediated by peroxynitrite by reducing its diffusion across the membrane (short lifetime of $ONOOCO_2^-$; see discussion in refs *13* and *15*). Moreover, while the intensity of the radical produced by peroxynitrite in red blood cells was significantly decreased by hypoxia (Figure 1E), the radical produced in plasma was not dependent on dissolved oxygen (Figure 1B). These $g = 2.004$ signals were not detectable if peroxynitrite decomposed before the addition of targets (Figure 1C,F), ruling out oxidative processes mediated by peroxynitrite decomposition products (nitrite and nitrate).

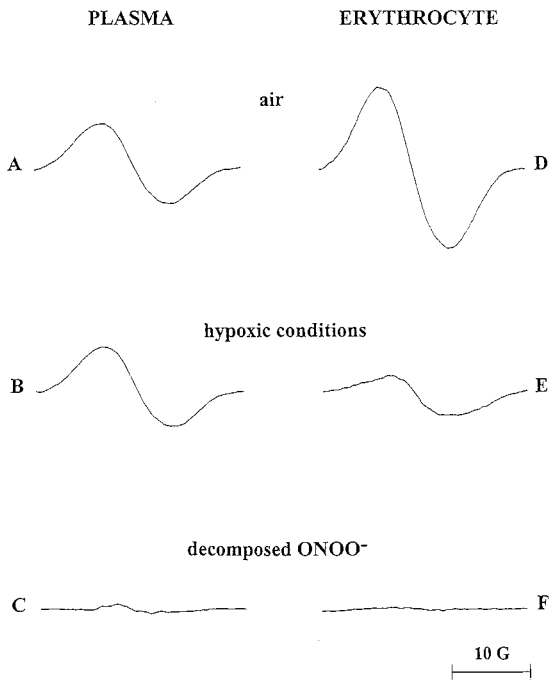

FIGURE 1: ESR spectra at 37 °C of plasma and red blood cells treated with peroxynitrite. (A) Plasma diluted to 50% (v/v) with phosphate buffer/DTPA/25 mM sodium bicarbonate, pH 7.4, and treated with 5 mM $[Me_4N][ONOO^-]$ in aerobic conditions (pO_2 = 183 mmHg). (B) Plasma as in panel A but in hypoxic conditions obtained with glucose/glucose oxidase/Cat (pO_2 = 2 mmHg). (C) Decomposed $[Me_4N][ONOO^-]$ (5 mM) added to plasma. (D) Red blood cells suspended to 50% in phosphate buffer/DTPA/25 mM sodium bicarbonate, pH 7.4, and treated with 5 mM $[Me_4N][ONOO^-]$ in aerobic conditions (pO_2 = 145 mmHg). (E) Red blood cells as in panel D but after bubbling for 15 min with argon (pO_2 = 24 mmHg). (F) Decomposed $[Me_4N][ONOO^-]$ (5 mM) added to red blood cells. Spectrometer conditions were as follows: modulation amplitude, 2.6 G; time constant, 164 ms; sweep time, 10.5 s; gain, 2×10^5; number of scans, 40.

A radical with similar spectral features has been described in nitrite-mediated oxidation of oxyHb (*31*, *32*) and appears to be similar to at least one of the radicals formed in the reaction of metHb and metMb with H_2O_2 (*33*, *34*). However, the $g = 2.004$ radical generated by peroxynitrite in red blood cells was unaffected by treatment with Cat (50 μg/mL) and was not observed when cells were treated with 0.1−5 mM $NaNO_2$, 0.1−5 mM H_2O_2, or 5 mM diethylamine-NONOate (a compound releasing $^{\bullet}NO$ with a $t_{1/2}$ of about 2 min). We concluded that the $g = 2.004$ radical produced by peroxynitrite is not directly derived from NO_2^-, H_2O_2, or $^{\bullet}NO$, although the radical may be similar to that produced by other oxidants.

Figure 2 shows the dependence on pO_2 of the $g = 2.004$ radical yield induced by peroxynitrite in red blood cells. The radical yield increased up to a pO_2 of 100 mmHg and, interestingly, followed closely the Hb saturation curve, suggesting a role for oxyHb. This hypothesis was confirmed by the observation that ghosts were unable to form the radical (Figure 3A), whereas the radical was observed in the hemolysate (Figure 3B), as well as in purified oxyHb (Figure 3C), but was undetectable in the low molecular weight fraction of the cell lysate (Figure 3D). In addition, pretreatment of oxyHb with the unspecific protease Pronase to

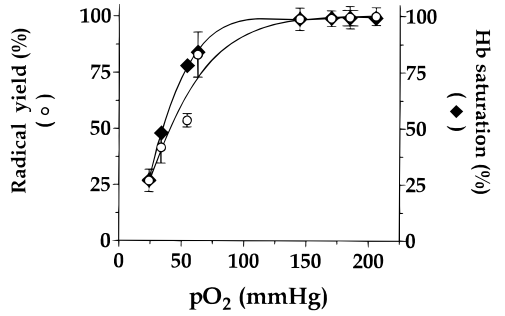

FIGURE 2: Dependence on the pO_2 of the $g = 2.004$ radical yield in red blood cells treated with peroxynitrite. Cells were suspended to 50% in phosphate buffer/DTPA/25 mM sodium bicarbonate, pH 7.4, and treated with 5 mM $[Me_4N][ONOO^-]$. Points represent mean value ± SD ($n = 3$).

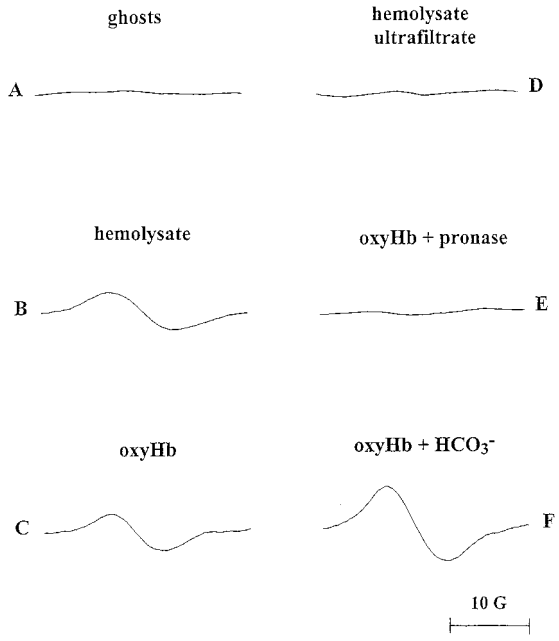

FIGURE 3: ESR spectra at 37 °C of different derivatives of red blood cell treated with peroxynitrite. $[Me_4N][ONOO^-]$ (5 mM) was added to (A) ghosts suspended to 50% (v/v); (B) hemolysate (oxyHb final concentration 3.5 mM); (C) purified oxyHb (1.5 mM) without sodium bicarbonate; (D) low molecular weight fraction ($M_r \leq 3000$) of the cell lysate; (E) purified oxyHb (1.5 mM) pretreated with 5 mg/mL Pronase (30 min, 25 °C); and (F) purified oxyHb (1.5 mM) with 50 mM sodium bicarbonate. All samples were in phosphate/DTPA buffer, pH 7.4. Spectrometer conditions were as described in the legend to Figure 1.

degrade the protein to small peptides and amino acids before addition of peroxynitrite completely prevented radical formation (Figure 3E). Collectively these results suggest localization of the radical on oxyHb. It must be remembered that the inability to form a $g = 2.004$ radical does not preclude the formation by peroxynitrite of other radical species or even of the same radical species but with a shorter lifetime. Thus, throughout this work the lack of the $g = 2.004$ signal means only that a long-lived radical stable at 37 °C is not formed.

Generation of g = 2.004 Radical Depends on the Presence of CO_2. CO_2 is an important reactant of peroxynitrite in biological samples and, in particular, inside the erythrocyte, where the CO_2 increases on oxygenation and is converted to HCO_3^- in deoxygenated cells. Without targets, the mechanism of CO_2 action is the catalytic acceleration of peroxynitrite decomposition to nitrate (lifetime of peroxynitrite in 20 mM sodium bicarbonate is 48 ms) (*13*). In the presence of a target, the reactive intermediates formed by $ONOO^-/CO_2$ reaction promotes one- or two-electron oxidation and aromatic nitration (*13*, *18*, *35*, *36*).

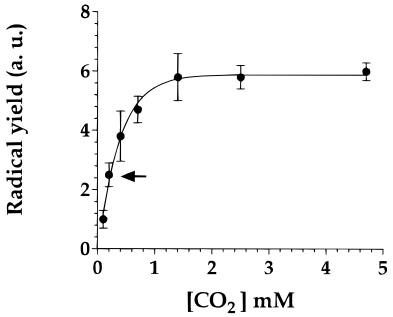

FIGURE 4: Effect of CO_2 concentration on $g = 2.004$ radical yield in peroxynitrite-treated Hb. Sodium bicarbonate was added to 1.5 mM oxyHb in phosphate buffer/DTPA, pH 7.4, and CO_2 concentration was measured before addition of 2 mM $[Me_4N][ONOO^-]$. The arrow indicates the CO_2 concentration of samples without intentionally added sodium bicarbonate, and the lower concentration was obtained by treatment with 90 milliunits/mL phospho(enol)pyruvate-carboxylase and 5 mM phospho(enol)pyruvate (1 h, 25 °C). Points represent mean value ± SD ($n = 3$); a. u. = arbitrary units.

The addition of 50 mM sodium bicarbonate (the CO_2 in equilibrium was 2.5 mM) to purified oxyHb doubled the $g = 2.004$ radical intensity (Figure 3F). The enhancing effect of CO_2 on the $g = 2.004$ radical generated by 2 mM peroxynitrite was evident at low CO_2 concentrations and leveled off at concentrations between 1 and 2 mM (Figure 4). Air-equilibrated physiologic buffers are inevitably contaminated by CO_2/HCO_3^- (*35*, *37*), although HCO_3^- is not added intentionally (without the addition of sodium bicarbonate our samples contained 0.2 mM CO_2). To decrease the CO_2 concentration we used the enzyme phospho(enol)-pyruvate-carboxylase, which reduced the CO_2/HCO_3^- concentration by catalyzing the reaction of CO_2 with phospho-(enol)pyruvate. As shown in Figure 4, the reduction of the CO_2 to 0.1 mM further decreased the radical intensity (a complete removal of CO_2 was not obtained, probably because of air contamination). These results indicate that the radical was generated mainly, if not only, in samples containing CO_2 and thus the formation of a $g = 2.004$ radical depends on an oxidant derived from $ONOO^-/CO_2$ reaction.

To further investigate the role of CO_2, we studied the $g = 2.004$ radical yield as a function of pH. Lymar and Hurst (*13*, *17*, *38*), using a pH jump method, showed that $ONOO^-$ reacts with CO_2 faster than the decomposition of peroxynitrous acid to nitrate. Furthermore, it was shown that in the presence of CO_2 the pH dependence of the yields in 3,3′-dityrosine (a marker of tyrosyl radical formation) increased at alkaline pH concomitantly with a decrease in 3-nitrotyrosine, possibly due to competitive reactions between tyrosyl radicals and tyrosine phenolate anion for $^{\bullet}NO_2$ (*17*). The CO_2 concentration present in phosphate/HCO_3^- buffers is high at acidic pH and low at alkaline pH due to the hydration reaction ($CO_2 + H_2O \rightarrow H_2CO_3 \rightarrow H^+ + HCO_3^-$, $pK_a = 6.1-6.3$). However, the equilibration of CO_2 with HCO_3^- is much slower than the formation of the $ONOOCO_2^-$ adduct and in pH jump experiments the concentration of CO_2 remains fixed during the peroxynitrite reaction time (*13*, *17*, *37*−*39*). We added 25 mM sodium bicarbonate to oxyHb in phosphate buffer at pH 6.4 (the CO_2 was 2.8 ± 0.2 mM)

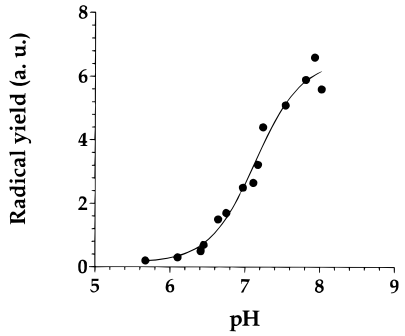

FIGURE 5: Dependence on pH of $g = 2.004$ radical yield. OxyHb (1.5 mM) was treated with 2 mM [Me_4N][$ONOO^-$] at different pH by a pH jump method (see Results). The pH values were measured after the spectra recording; a. u. = arbitrary units.

and a pH jump was performed by adding variable amounts of NaOH containing a fixed concentration of peroxynitrite (2 mM). The amount of NaOH was calibrated to obtain the desired final pH in the mixed solution. The addition of 25 mM sodium bicarbonate to oxyHb in phosphate buffers at pH < 6.4 produced a CO_2 concentration in excess with respect to peroxynitrite (>3 mM). As shown in Figure 5, the $g = 2.004$ radical yield was found to increase significantly at alkaline pH. A comparable increase of radical yield was not observed when sodium bicarbonate was added to buffer at pH = 8 without performing the pH jump (results not shown), confirming the role of CO_2. Even though the $ONOOCO_2^-$ oxidation mechanisms of free tyrosine and oxyHb appear to be different (see below), the pH profile of the yield of $g = 2.004$ radical was similar to the pH profile previously reported for 3,3′-dityrosine yield (*17*). This may be indicative of the involvement of common radical intermediates.

The g = 2.004 Radical Is Formed through a Reaction Involving Oxygenated Fe^{II}-Heme. One method of discriminating between reactions involving amino acid residues easily oxidizable by peroxynitrite and a reaction first involving the heme is to use Hb derivatives with different heme ligands or different heme oxidation states. Notably, when deoxyHb and CO−Hb were treated with peroxynitrite, they produced a $g = 2.004$ radical with significantly reduced yields, while metHb did not form the radical at all (Table 1). These results suggest a relationship between the $g = 2.004$ radical formation and the heme structure, supporting the hypothesis of a reaction first involving the heme.

The role of heme in the formation of the $g = 2.004$ radical may indicate the participation of reactive oxygen species. However, the addition of Cat, Sod, mannitol, or dimethyl sulfoxide to oxyHb before peroxynitrite treatment did not significantly modify the radical yield (Table 1), ruling out the involvement of H_2O_2, $O_2^{\bullet -}$, $^{\bullet}OH$, and also NO_2^-. This last species forms a globin-centered radical in oxyHb through the intermediary formation of H_2O_2 (*31*, *32*) as demonstrated by Cat inhibition (*40*). We considered the possibility that the inability of Cat to inhibit the $g = 2.004$ radical formation may be due to enzyme inactivation by peroxynitrite. However, the activity of 50 μg/mL Cat was reduced only from 83 to 53 ± 4 $\mu M \cdot min^{-1}$ after treatment with 2 mM peroxynitrite/CO_2. Considering that in our samples peroxynitrite would react preferentially with oxyHb (1 mM) and not with 0.2 μM Cat (*14*, *15*), these results do not support the hypothesis of the involvement of H_2O_2 in the formation of $g = 2.004$ radical.

Table 1: Yield of the $g = 2.004$ Radical from Hb and Mb Submitted to Different Treatments[a]

sample	% of control value ± SD ($n = 3$)
oxyHb (control in air)	100 ± 6
Hb ($pO_2 = 27$ mmHg)	33 ± 4
metHb	0 ± 8
CO−Hb	38 ± 7
oxyHb + Sod (15 μg/mL)	94 ± 6
oxyHb + Cat (50 μg/mL)	104 ± 5
oxyHb + dimethyl sulfoxide (100 mM)	96 ± 4
oxyHb + mannitol (50 mM)	98 ± 8
oxymb	23 ± 3
metMb	0 ± 6
oxyHb + CPA (40 units/mL) 2 h, 37 °C	76 ± 4
oxyHb + CPB (40 units/mL) 2 h, 37 °C	76 ± 6
oxyHb + CPA (40 units/mL) + CPB (40 units/mL) 2 h, 37 °C	74 ± 2

[a] Hemoproteins (1.5 mM) were treated with 2 mM [Me_4N][$ONOO^-$] in phosphate buffer/DTPA/50 mM sodium bicarbonate, pH 7.4. Spectrometer conditions were as described in the legend to Figure 1, except for the number of scans, 5.

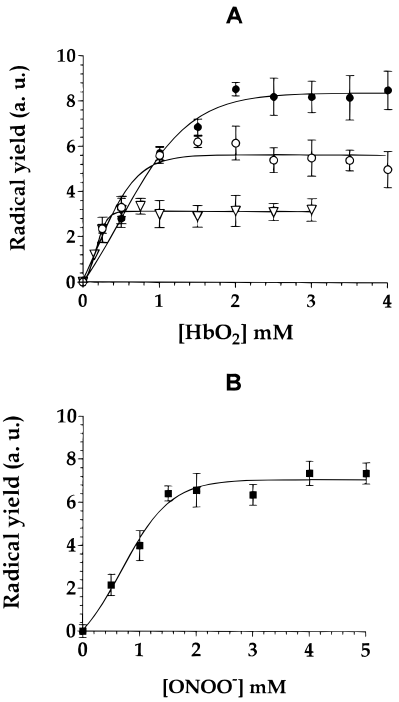

FIGURE 6: Yield of the $g = 2.004$ radical as a function of Hb (A) and peroxynitrite (B) concentration. (A) OxyHb (0−4 mM) was in phosphate buffer/DTPA/50 mM sodium bicarbonate, pH 7.4, and treated with (▽) 0.5, (○) 1.0, or (●) 2 mM [Me_4N][$ONOO^-$]. (B) OxyHb (1.5 mM) was treated with 0.5−5 mM [Me_4N][$ONOO^-$] in phosphate buffer/DTPA/100 mM sodium bicarbonate, pH 7.4. Points represent mean value ± SD ($n = 3$); a. u. = arbitrary units. Spectrometer conditions were as described in the legend to Figure 1, except for the number of scans, 5.

With the aim of clarifying whether the oxidant responsible for the $g = 2.004$ radical formation was the $ONOOCO_2^-$ adduct or a reactive intermediate derived therefrom, we studied the $g = 2.004$ radical yield as a function of oxyHb and peroxynitrite concentration (Figure 6). A direct bimolecular reaction with the $ONOOCO_2^-$ adduct should oxidize the target with a yield approaching 100%, while the reactive intermediates derived from this adduct are produced in a 20−

30% yield and, thus, can oxidize the target with up to a 20−30% yield (*17*, *20*, *35*). Figure 6A shows that at three different peroxynitrite concentrations (0.5, 1.0, and 2.0 mM) the $g = 2.004$ radical yield leveled off at an apparent 1:1 ratio between peroxynitrite and heme. A similar conclusion was deduced from data of Figure 6B, where it is shown that the yield of $g = 2.004$ radical obtained from 1.5 mM oxyHb leveled off between 1.5 and 2.0 mM peroxynitrite. These results indicate that the oxidant may be the $ONOOCO_2^-$ adduct reacting in a direct bimolecular reaction with oxygenated Fe^{II}-heme. As expected, the threshold at which the $g = 2.004$ radical was detectable with purified oxyHb was significantly lower than that with red blood cells (≥0.15 and 1.0 mM, respectively).

However, it should be pointed out that the amount of $g = 2.004$ radical formed at 1:1 peroxynitrite/Hb ratio was only 0.10% ± 0.05% the amount of Hb, and such a low relative yield cannot be ascribed simply to radical instability ($t_{1/2} = 13$ min). Also, the intensity of radical species produced by H_2O_2 oxidation of metHb reached a maximum level at a 1:1 heme:H_2O_2 ratio, but again the radical yield measured by low-temperature direct ESR was significantly lower (*33*). Probably the low yield of globin-centered radicals is the result of a complex decay process involving several protein residues (*41*, *42*).

The g = 2.004 Radical Is Due to a Tertiary Carbon-Centered Radical of Tyrosine Residue(*s*). The radical(s) involved in the formation of the $g = 2.004$ signal cannot be easily interpreted due to the absence of hyperfine structure. We used the spin trapping technique to characterize the Hb residue(s) involved in the $g = 2.004$ signal. Taking advantage of the high stability of the $g = 2.004$ radical (lifetime > 30 min) and to avoid trapping of radicals with shorter lifetimes, the spin trap was added 2 min after peroxynitrite. Under these conditions MNP trapped the radical adduct shown in Figure 7A. This spectrum is characteristic of a highly immobilized nitroxide ($2A_{zz} = 56.5$ G), consistent with trapping of a protein-bound radical(s). If MNP was added to Hb treated with peroxynitrite at low pO_2, the intensity of the resulting MNP adduct was considerably reduced (Figure 7B), confirming that the radical trapped by MNP was sensitive to Hb oxygenation.

When the MNP/Hb adduct was submitted to proteolysis with Pronase, the resulting spectrum was composed of a three-line spectrum ($a_N = 15.5 \pm 0.2$ G), superimposed on a residual immobilized adduct (Figure 7C). The apparent lack of additional hyperfine structure indicates that the radical was located on a tertiary carbon-centered atom. Unfortunately, analysis of the superhyperfine structure of each line of the Pronase-treated MNP/Hb adduct, which could have given additional information, was made impossible by the decay of the adduct.

Analysis of data in the literature suggests that the MNP adduct with $a_N = 15.5$ G is due to the trapping of a tyrosine-centered radical identical to the previously described Pronase-treated MNP/cytochrome *c* and MNP/metMb adducts generated by H_2O_2 (*29*, *41*). This tyrosine radical adduct has been shown to be localized on the C-1 tertiary carbon atom of the aromatic ring (next to the amino acid side chain) (*41*). Figure 7D shows, for comparison, the radical adduct formed by treatment of a tetrapeptide containing tyrosine (GGYR) with horseradish peroxidase/H_2O_2 in the presence of MNP.

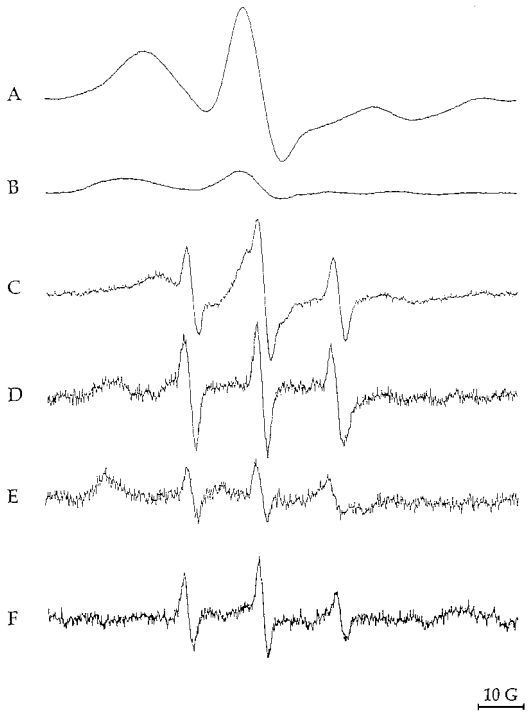

FIGURE 7: ESR spectra of MNP/Hb adduct obtained from the reaction with peroxynitrite and comparison with MNP/peptide adducts. (A) MNP (10 mM) added 2 min after treatment of oxyHb (1.5 mM) with 2 mM [Me_4N][$ONOO^-$]. (B) Sample as in panel A but in hypoxic conditions ($pO_2 = 7.5$ mmHg, Hb saturation = 4.2%). (C) Sample as in panel A but with the addition of 0.5 mg/mL Pronase. (D) Peptide GGYR (10 mM) treated with 0.1 mg/mL HRP and 0.15 mM H_2O_2 in the presence of 10 mM MNP. (E) Peptide GGYR (10 mM) treated with 5 mM [Me_4N][$ONOO^-$] in the presence of 10 mM MNP. (F) Peptide GGWA (10 mM) treated with 5 mM [Me_4N][$ONOO^-$] in the presence of 10 mM MNP. All treatments with peroxynitrite were performed in phosphate buffer/DTPA/100 mM sodium bicarbonate, pH 7.4. Spectrometer conditions were as follows: time constant, 82 ms; sweep time, 168 ms; modulation amplitude, 5 G for spectra A and B and 1 G for spectra C−F; gain, 1×10^5 for spectra A and B and 5×10^5 for spectra C−F; number of scans, 5 for spectra A, B, and D−F and 1 for spectrum C.

As expected, the tyrosyl radical trapped by MNP showed a three-line spectrum with a nitrogen splitting of 15.5 ± 0.2 G. Treatment of this tetrapeptide with peroxynitrite in the presence of MNP also resulted in the trapping of a three-line spectrum with a comparable hyperfine splitting constant (Figure 7E). The low intensity of the MNP/GGYR adduct generated by peroxynitrite may be due to competing reactions such as tyrosine dimerization and/or tyrosyl radical nitration (*13*, *17*).

Tryptophan is another easily oxidizable protein site that produces tertiary carbon-centered radicals. We have shown previously by spin trapping with DBNBS that peroxynitrite induces in proteins a tertiary carbon-centered radical on tryptophan residues (*23*). To compare the tyrosyl and tryptophanyl radical adducts of MNP we tried to trap the tryptophan radical produced by peroxynitrite. As shown in Figure 7F, peroxynitrite treatment of a tryptophan-containing tetrapeptide (GGWA) in the presence of MNP resulted in an MNP/tryptophan radical adduct indicative of a tertiary carbon-centered atom, but the nitrogen hyperfine coupling constant was larger than that of MNP/tyrosine adduct (16.0 ± 0.1 and 15.5 ± 0.2 G, respectively). Furthermore, a role

of tryptophan in the $g = 2.004$ radical is ruled out by its shorter lifetime (4 s; ref *24*) and its ability form a peroxyl radical with a characteristic line at $g = 2.037$ (*42*).

The $g = 2.004$ Generated by Peroxynitrite Is Similar to the Long-Lived $g = 2.004$ Radical Produced by MetHb/H_2O_2 and MetMb/H_2O_2. A known procedure to form globin-centered radicals is to treat metHb with H_2O_2. This results in the formation of radicals detectable by direct ESR at room temperature with a continuous-flow apparatus (*34*). At least three different radical species have been described: (i) a tryptophan peroxyl radical (*42*), (ii) a six-line tyrosyl radical, and (iii) a long-lived singlet radical at $g = 2.004$ not fully characterized and assigned to an aromatic amino acid (*34*). This last radical shows spectral features similar to those of the radical produced by peroxynitrite on oxyHb.

To limit our study to long-lived radicals, metHb was allowed to react with an equimolar amount of H_2O_2 and after 10 s Cat was added at a concentration 25-fold lower than metHb to stop the reaction and start the radical decay process (*43*). As for peroxynitrite, spectra were recorded 2 min after the addition of Cat. Figure 8A shows that under these conditions a singlet radical at $g = 2.004$ was detectable in the metHb/H_2O_2 system with spectral characteristics similar to those of the radical induced by peroxynitrite. Also the yield of $g = 2.004$ radical generated by the metHb/H_2O_2 system was low and similar to that generated by peroxynitrite (0.2% and 0.1% the amount of Hb, respectively). Notably, the $g = 2.004$ radical was not formed if H_2O_2 was added to oxyHb instead of metHb (Figure 8B). The $g = 2.004$ radical was trapped by adding MNP 2 min after H_2O_2 (Figure 8C). Proteolytic treatment of this MNP/Hb adduct with Pronase resulted in a three-line signal with $a_N = 15.5 \pm 0.1$ G (Figure 8D), which assigns the trapped radical to the C-1 tertiary carbon atom of tyrosine aromatic ring. Thus, our results provide evidences that also the long-lived singlet signal at $g = 2.004$ of metHb/H_2O_2 may be due to tyrosyl radical(s) (see discussion in ref *34*). Moreover, Gunther et al. (*41*), by using site-directed mutants of sperm whale metMb treated with H_2O_2, showed that another spin trap, DMPO, trapped only the tyrosyl radical localized on Y103 and was unable to trap the Y151 residue, while the Y146 residue of metHb appears to be untrappable. We treated with peroxynitrite horse skeletal muscle oxyMb in which the Y151 is replaced by F151 and observed the formation of a long-lived $g = 2.004$ radical, although of lower intensity if compared with oxyHb (Table 1). We trapped the $g = 2.004$ radical formed by peroxynitrite on oxyMb by adding DMPO 2 min after peroxynitrite. The DMPO radical adduct showed hyperfine splitting constants (Figure 8E) superimposable on those generated by metMb/H_2O_2 (Figure 8F). These results indicate that the long-lived $g = 2.004$ radical induced by peroxynitrite in horse oxyMb is at Y103, the same tyrosine forming a radical in metMb/H_2O_2 and nearest to the porphyrin (*44*). Similarly to metHb, peroxynitrite did not form a $g = 2.004$ radical with metMb (Table 1).

Hb possesses six tyrosine residues per $\alpha\beta$ dimer (Y24, Y42, and Y140 on the α chain and Y36, Y130, and Y145 on the β chain). The C-terminal penultimate tyrosine of both subunits could be selectively removed by treatment with carboxypeptidases (*45*). Carboxypeptidase A removes H146 and Y145 of the β chain and carboxypeptidase B removes only the C-terminal R141 of the α chain, but simultaneous

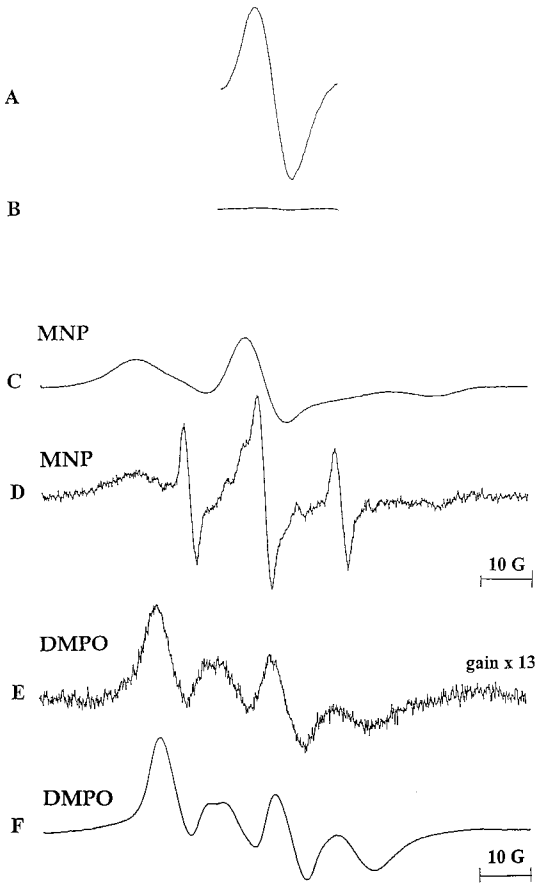

FIGURE 8: ESR spectra at 37 °C of Hb and Mb treated with H_2O_2 or peroxynitrite. (A) MetHb (1 mM) was treated with H_2O_2 (1 mM) and the reaction was stopped after 10 s by the addition of Cat (10 mg/mL). (B) Sample as in panel A but in the presence of 1 mM oxyHb. (C) MetHb treated as in panel A but receiving 10 mM MNP 2 min after H_2O_2. (D) Sample as in panel C but with the addition of Pronase (0.25 mg/mL). (E) DMPO (0.1 M) was added 2 min after the treatment of oxyMb (1.5 mM) with 2 mM [Me_4N][$ONOO^-$]. (F) OxyMb (1 mM) treated with 1 mM H_2O_2 in the presence of 0.1 M DMPO. All treatments with peroxynitrite were performed in phosphate buffer/DTPA/50 mM sodium bicarbonate, pH 7.4. Spectrometer conditions: spectra A and B were obtained as described in the legend to Figure 1 except for the number of scans, 5; spectra C, E, and F were obtained as described in the legend to Figure 7A; spectrum D was obtained as described in the legend to Figure 7C.

treatment with the two enzymes removes both Y145 and Y140 (*46*). As reported in Table 1, pretreatment of oxyHb with these proteases only marginally decreased the intensity of the $g = 2.004$ radical produced by peroxynitrite. The comparable decrease observed after treatment with carboxypeptidase A or B added individually or together suggests that the small effect may be due to a minor contamination by other proteases and not to the removal of tyrosines participating in the $g = 2.004$ radical. Excluding the penultimate tyrosines, plausible candidates for most of the $g = 2.004$ signal may be one or more of the following four tyrosine residues: Y24 and Y42 of the α chain and Y35 and Y130 of the β chain, with Y42 and Y35 nearest to the porphyrin (*47*).

Peroxynitrite in the Presence of CO_2 Produces the Intermediate Formation of FerrylHb. The finding that the $g = 2.004$ signal was observed with Hb/H_2O_2, a well-known generator of Hb ferryl species, suggests that the radical may be generated by a common mechanism. However, peroxy-

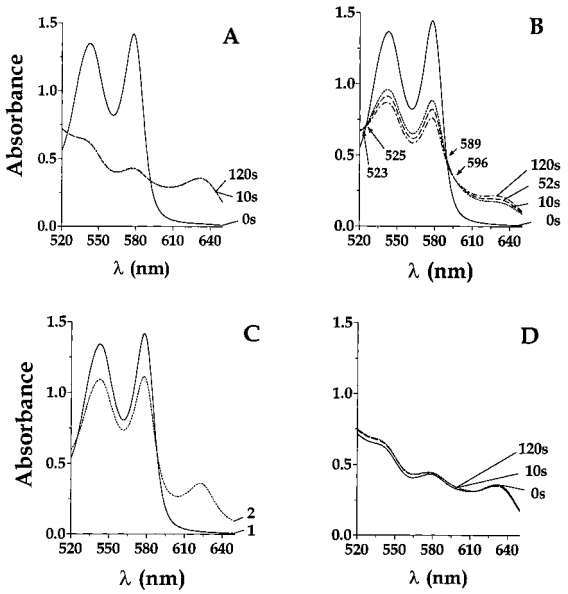

FIGURE 9: Hb spectral changes induced by peroxynitrite. (A) OxyHb after treatment with $[Me_4N][ONOO^-]$ in phosphate buffer, pH 5.8. (B) OxyHb after treatment with $[Me_4N][ONOO^-]$ in phosphate buffer, pH 7.4, containing 25 mM sodium bicarbonate. (C) OxyHb containing 2 mM Na_2S (spectrum 1) and treated with $[Me_4N][ONOO^-]$ in phosphate buffer/sodium bicarbonate, pH 7.4 (spectrum 2). (D) MetHb treated with $[Me_4N][ONOO^-]$ in phosphate buffer/DTPA/sodium bicarbonate, pH 7.4. Solid line spectra were from Hb not treated with peroxynitrite. The protein (1 mM) was treated at 1:1 ratio with peroxynitrite and samples diluted 10 times in phosphate buffer, pH 7.4. Spectra were recorded before (0 s) or 10−120 s after peroxynitrite addition.

nitrite treatment of oxyHb has been reported to form only metHb (*15*) or metHb with only trace amounts of ferrylHb (*48*) and, what is more important, the pH profile of metHb oxidation yield was higher at acidic pH and lower at alkaline pH (i.e., opposite to that shown for the $g = 2.004$ radical in Figure 5).

To clarify this apparent discrepancy, we reinvestigated the spectral changes induced by peroxynitrite in oxyHb. Figure 9A shows the spectral changes induced by peroxynitrite in oxyHb at pH 5.8 without intentionally added sodium bicarbonate. All the spectral changes occurred within the time required to mix and dilute the samples (10 s) and the spectrum was not modified in the following 2 min (Figure 9A). Analysis of this spectrum by the Winterbourn formulas (*26*) indicated the formation of 100% metHb.

A second experiment was performed at pH 7.4 in the presence of 25 mM sodium bicarbonate. As shown in Figure 9B, about half of oxyHb was oxidized within 10 s with a small increase in the following 2 min. Comparison with Figure 9A clearly shows that metHb formation was significantly inhibited by CO_2, thus strongly supporting the hypothesis that the reaction of peroxynitrite with CO_2 precedes or efficiently competes with the fast reaction between Hb and peroxynitrite (*15, 48*). A previous investigation of the interaction of peroxynitrite with oxyHb in the presence of CO_2 reported a similar extent of oxyHb oxidation and interpreted the incomplete inhibition afforded by CO_2 in terms of a secondary oxidant (i.e., the $ONOOCO_2^-$ adduct) with a reactivity different than peroxynitrite itself (*36*). Moreover, Figure 9B shows that the isosbestic points at 525 and 589 nm of the first spectrum at 10 s were not maintained in the following spectra, as would be expected if the chromophores were formed in a constant proportion during the oxidative reaction. This may indicate, but does not prove, that the reaction involves a vanishing chromophore. Analysis by the Winterbourn formulas of the spectrum obtained after 10 s indicated the presence of 48.7% oxyHb, 43.5% metHb, and 7.8% of ferrylHb or other oxidation products indistinguishable from ferryl species (*26*). To establish whether a ferryl species was formed as a transient, 2 mM Na_2S was added to oxyHb. At this concentration Na_2S did not modify the oxyHb spectrum, while the spectrum after treatment with peroxynitrite showed the formation of a maximum at 617 nm characteristic of Fe^{II}-sulfHb (Figure 9C). Since this last chromophore is a product of the reaction of Na_2S with ferrylHb (*30*), the formation of the 617 nm peak is considered prognostic of ferrylHb formation (*49*). As calculated by the extinction coefficient at 617 nm of Fe^{II}-sulfHb, the amount of ferrylHb formed by 1 mM peroxynitrite and 1 mM oxyHb was 140 μM. No change in the visible spectra of oxyHb was observed if the protein (1 mM) was added to 1 mM peroxynitrite previously decomposed (spectrum not shown).

The visible spectrum of metHb was minimally modified by peroxynitrite treatment both in the absence (*48*) and in the presence of 25 mM sodium bicarbonate (Figure 9D). At reduced pO_2 (21.7 mmHg, Hb saturation 25%), the oxidation of Hb by peroxynitrite was inhibited, since after 10 s the amounts of metHb and ferrylHb were 27.0% and 3.5%, respectively.

In summary, at least two oxidative pathways exist between oxyHb and peroxynitrite: one in the absence of CO_2 with ONOOH being the actual oxidant (*15*) and the other occurring in the presence of CO_2 with the oxidant species being $ONOOCO_2^-$.

Mechanism of g = 2.004 Radical Formation by Peroxynitrite. In the presence of CO_2, peroxynitrite induced a transient formation of ferrylHb, a species that has been identified and extensively investigated in peroxide-mediated metHb oxidation. For this reason we believe that the oxidizing species derived from $ONOO^-$ and CO_2 is a peroxide, probably the postulated $ONOOCO_2^-$ adduct (*18*). We found that both H_2O_2 and peroxynitrite induced similar long-lived $g = 2.004$ tyrosyl radical(s) on Hb and Mb, implying a similar mechanism. Nevertheless, our study reveals a major difference in the mechanism of tyrosyl radical(s) formation between H_2O_2 and peroxynitrite. H_2O_2 oxidizes metHb to a relatively stable species ($^{\bullet}XFe^{IV}{=}O$, where $^{\bullet}X$ denotes a globin-centered radical, also called compound I), which subsequently decays to yield a $XFe^{IV}{=}O$ species (compound II). In contrast, $ONOOCO_2^-$ did not form ferrylHb or stable tyrosyl radical(s) from metHb but only from oxyHb. A plausible mechanism may be, thus, a two-electron abstraction from oxygenated Fe^{II}-heme with formation of a ferrylHb species, nitrite, and bicarbonate according to

$$Hb(Fe^{II}O_2) + ONOOCO_2^- + H_2O \rightarrow Hb(Fe^{IV}{=}O) + NO_2^- + HCO_3^- + H^+ + O_2 \quad (1)$$

The ferryl species (compound II or a similar species with one oxidizing equivalent less than $^{\bullet}XFe^{IV}{=}O$) subsequently decays to lower iron oxidation states (Fe^{III} and Fe^{II}) (*50*).

The $g = 2.004$ tyrosyl radical may result from globin-dependent reduction of the initially oxidized heme and likely contributes to the rapid ferryl decay. Moreover, the NO_2^- formed, far from being an inert molecule, is able to contribute to the reduction of ferryl species (*31*, *40*) with formation of $^{\bullet}NO_2$ radical, which, in turn, disproportionate to nitrite and nitrate:

$$Hb(Fe^{IV}{=}O) + NO_2^- + 2H^+ \rightarrow Hb(Fe^{III}) + {}^{\bullet}NO_2 + H_2O \quad (2)$$

$$^{\bullet}NO_2 + {}^{\bullet}NO_2 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \quad (3)$$

Such a mechanism requires further studies based on careful analysis of reaction products, but if $^{\bullet}NO_2$ formation is confirmed, oxidation by $^{\bullet}NO_2$ of tyrosine phenolate anion with formation of tyrosyl radical (*51*) may be a reason for the observed increase in radical yield at alkaline pH (Figure 5).

From our ESR and spectrophotometric results, it seems that the $ONOOCO_2^-$ adduct in deoxyHb and CO−Hb forms significantly less ferrylHb or globin-centered radical(s). These findings may be explained by bearing in mind the rapid decay of $ONOOCO_2^-$ adduct (*13*, *17*) and the closer T-conformation of deoxyHb (*47*) or the higher affinity of heme for CO.

Biological Significance. Previous (*15*, *48*, *52*) and present results suggest that Hb in oxygenated erythrocytes is one of the most important targets of peroxynitrite generated in the vasculature. It should be considered that Hb physiologically handles both $^{\bullet}NO$ and $O_2^{\bullet -}$ (*53*, *54*). Thus, it is conceivable to assume that Hb may be exposed to peroxynitrite attack and may possess a defense mechanism. In oxygenated conditions $^{\bullet}NO$ appears to be associated with C93 of Hb β chains and the radical is released on deoxygenation (*53*). Thus, the binding of $^{\bullet}NO$ to C93 appears to be a mechanism to avoid peroxynitrite formation inside the cell. However, at high pO_2/pCO_2, oxyHb is predicted to be an important sink of peroxynitrite generated, for example, by the inducible form of $^{\bullet}NO$ synthase, an enzyme apparently with peroxynitrite synthase activity (*4*). This scavenging role of Hb is favored by (i) the rapid membrane crossing of peroxynitrite (*15*), (ii) the high intracellular concentration of Hb, (iii) the high rate constant of Hb for peroxynitrite (*15*, *48*), and (iv) the possibility of oxyHb regeneration. Although ferrylHb is a potentially damaging species (*49*), its formation may facilitate peroxynitrite detoxification since metHb is metabolically reducible and the long-lived $g = 2.004$ tyrosyl radical(s), at variance to peroxynitrite, can be easily scavenged by intracellular antioxidants (*24*, *49*).

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