Carbon Dioxide Stimulates the Production of Thiyl, Sulfinyl, and Disulfide Radical Anion from Thiol Oxidation by Peroxynitrite*

Received for publication, September 15, 2000, and in revised form, December 20, 2000
Published, JBC Papers in Press, December 27, 2000, DOI 10.1074/jbc.M008456200

Marcelo G. Bonini and Ohara Augusto‡
From the Departamento de Bioquímica, Instituto de Química, Universidade de São Paulo, CP 26077, CEP 05513-970, São Paulo, SP, Brazil

Reaction of peroxynitrite with the biological ubiquitous CO₂ produces about 35% yields of two relatively strong one-electron oxidants, CO₂ and NO₂, but the remaining of peroxynitrite is isomerized to the innocuous nitate. Partial oxidant deactivation may confound interpretation of the effects of HCO₃⁻/CO₂ on the oxidation of targets that react with peroxynitrite by both one- and two-electron mechanisms. Thiols are example of such targets, and previous studies have reported that HCO₃⁻/CO₂ partially inhibits GSH oxidation by peroxynitrite at pH 7.4. To differentiate the effects of HCO₃⁻/CO₂ on two- and one-electron thiol oxidation, we monitored GSH, cysteine, and albumin oxidation by peroxynitrite at pH 5.4 and 7.4 by thiol disappearance, oxygen consumption, fast flow EPR, and EPR spin trapping. Our results demonstrate that HCO₃⁻/CO₂ diverts thiol oxidation by peroxynitrite from two- to one-electron mechanisms particularly at neutral pH. At acid pH values, thiol oxidation to free radicals predominates even in the absence of HCO₃⁻/CO₂. In addition to the previously characterized thiyl radicals (RS⁻), we also characterized radicals derived from them such as the corresponding sulfinyl (RSO⁻) and disulfide anion radical (RSSR⁻) of both GSH and cysteine. Thiyl, RSO⁻ and RSSR⁻ are reactive radicals that may contribute to the biodamaging and bio-regulatory actions of peroxynitrite.

Peroxynitrite¹ (ONOO⁻ + ONOOH), which is formed by the fast reaction between NO and O₂, has been receiving increasing attention as a mediator of human diseases and as a toxin against invading microorganisms (1–5). The compound is a potent oxidant that is able to oxidize and nitrate a variety of biological targets by mechanisms that are presently being elucidated (6–16). In general terms, peroxynitrite-mediated oxidations are either bimolecular, first order on peroxynitrite and biomolecule concentration, or unimolecular, first order on peroxynitrite and independent of biomolecule concentration. Bimolecular processes can result in product yield either around stoichiometry and over, as is the case for thiol oxidation (17), or around 35%, as is the case for carbon dioxide (CO₂) oxidation (10, 14–16). Presently, most investigators accept that product yields around 30% are characteristic of peroxynitrite-mediated free radical processes. Indeed, it has been established that ONOO⁻ protonation (pKₐ = 6.6) leads to its fast decomposition (k = 0.17 s⁻¹ at pH 7.4) to yield approximately 70% nitrite and 30% hydroxyl radical (OH) and nitrogen dioxide (NO₂) (Fig. 1, path 1) (7–9, 11–14). These radicals are the species responsible for the potentiation of peroxynitrite-mediated unimolecular oxidations. In the presence of HCO₃⁻, ONOO⁻ decomposes much faster because of its reaction with CO₂ (k = 2.6 × 10⁴ m⁻¹ s⁻¹ at pH 7.4, 25 °C) (18, 19) to produce approximately 65% nitrate and 35% carbonate radical anion (CO₃⁻) and NO₂ (Fig. 1, path 2) (10, 14–16). The fast rate constant of this reaction and the ubiquity of the HCO₃⁻/CO₂ pair in biological environments indicate that CO₃⁻ and NO₂ are likely to play relevant roles in peroxynitrite-mediated oxidations in vivo.

Recent detailed studies have established the role of CO₃⁻ in the potentiating effects of HCO₃⁻/CO₂ on peroxynitrite-mediated tyrosine nitration (20–23). This process has a zero order dependence on the target, being exclusively dependent on free radicals produced from peroxynitrite decay (20). The higher fluxes of free radicals produced in the presence of HCO₃⁻/CO₂ and the more specific reactivity of the CO₂ compared with the OH radical toward tyrosine result in higher yields of the tyrosyl radical and its recombination products with NO₂ (nitrotyrosine) and itself (dityrosine) (21–23). The effects of HCO₃⁻/CO₂ on the oxidation of targets that compete with CO₂ for the oxidant by reacting with peroxynitrite through second order processes have yet to be elucidated.

Among these targets, thiols are particularly relevant because they are present in high concentrations in biological environments and react relatively fast with peroxynitrite (k = 6.6 × 10⁵ m⁻¹ s⁻¹ for GSH at pH 7.4, 25 °C) (Fig. 1, path 3) (17, 24–26). Additionally, GSH is an important antioxidant, and thiol groups play a major role in maintaining the native conformation of proteins and in regulating enzyme activity. Previous studies have shown that HCO₃⁻/CO₂ partially inhibited GSH and albumin thiol oxidation at pH 7.4, as monitored by thiol disappearance (19, 26). However, this parameter does not differentiate between two- and one-electron mechanisms, and thiyl radicals (RS⁻) have been shown to be produced during the oxidation of thiols by peroxynitrite (6, 25, 27–29). In this context, it can be hypothesized that HCO₃⁻/CO₂ is likely to inhibit thiol oxidation by two-electron mechanisms while stimulating their oxidation to free radicals (Fig. 1). To test this hypothesis, we studied the effects of HCO₃⁻/CO₂ on thiol disappearance and free radical production during the oxidation of GSH, cysteine, and albumin by peroxynitrite.
Carbon Dioxide Enhances Radical Production from Thiols

EXPERIMENTAL PROCEDURES

Materials—All reagents were purchased from Sigma, Merck, or Fisher and were analytical grade or better. Peroxynitrite was synthesized from 0.6 mM sodium nitrite and 0.65 mM hydrogen peroxide in a quenched flow reactor; excess hydrogen peroxide was used to minimize nitrite contamination. To eliminate excess hydrogen peroxide, the peroxynitrite solution was treated with manganese dioxide. Synthesized peroxynitrite contained low levels of contaminating hydrogen peroxide (<1%) and nitrite (10–30%) which were determined as described previously (29) by the thiyl method and absorbance measurements at 354 nm (ε = 24.6 M⁻¹ cm⁻¹), respectively. The concentration of peroxynitrite stock solutions was determined spectrophotometrically at 302 nm using an extinction coefficient of 1.670 M⁻¹ cm⁻¹.

Oxygen uptake studies were performed using an oxygen monitor (Gilson 560 oxygraphy) at 25 °C ± 1 °C. The saturation oxygen concentration at this temperature was taken as 250 μM.

EPR Experiments—The EPR fast flow spectra were recorded at room temperature (25 ± 2 °C) on a Bruker EMX spectrometer operating at 9.65 GHz and 100 KHz field modulation equipped with a Bruker ER4117 D-MTV dielectric mixing resonator with a 9-mm distance between the mixing cell and the resonator center. Thiols solutions were pre-prepared in appropriate buffers to which sodium bicarbonate was added or not; in the former case, the solutions were left undisturbed for 5 min to permit HCO₃⁻/CO₂ equilibration. Peroxynitrite solutions were prepared with water. Thiols and peroxynitrite solutions were transferred to 60-ml plastic syringes mounted on a syringe infusion pump (Harvard apparatus pump 22). Spectra were recorded 3.5 and 12 ms after mixing at continuous flow of 20 ml/min and 6 ml/min, respectively. The dispensed mixtures were collected for EPR measurement at the end of the experiments to detect changes caused by mixing with alkaline solutions of peroxynitrite. The magnetic field was calibrated with 4-hydroxy-2,2,6,6-tetramethyl-1-piperidinyloxy (g = 2.0056) (15). Computer simulation of spectra was performed using a program written by Duling (31). In the EPR spin trapping experiments, the incubation mixtures were transferred to a flat cell and the spectra recorded at room temperature, immediately after the addition of peroxynitrite.

Calculations—The concentrations of expected products during the oxidation of GSH and cysteine by peroxynitrite were estimated by calculating the percentage of the oxidant that decomposes to NO₂⁻/OH⁻ and NO₂⁻ (Fig. 1, path 1; k), reacts with thiols (Fig. 1, path 3; kRSH), and reacts with CO₂ producing CO₃²⁻, CO₂, and NO₂⁻ (Fig. 1, path 2; kCO₂) under our experimental conditions (1 mM RSH, 1 mM CO₂, and 0.5 mM peroxynitrite at 25 °C) by Equation 1 (32).

\[
-k_\text{RSH}[\text{RSH}] + k_\text{CO}_2[\text{CO}_2][\text{ONOO}_2^-] \]

\[
\frac{d[\text{ONOO}_2^-]}{dt} = (k + k_\text{RSH}[\text{RSH}] + k_\text{CO}_2[\text{CO}_2][\text{ONOO}_2^-]).
\] (Eq. 1)

The rate constants were k = 1.1 and 0.17 s⁻¹ (11, 21), k_{RSH} = 1.4 x 10² and 6.6 x 10⁻³ s⁻¹, k_{CO₂} = 6.4 x 10⁻³ and 2.0 x 10⁻² s⁻¹ (24, 25), and k_{CO₂} = 1.8 x 10⁻³ and 2.6 x 10⁻² s⁻¹ (18) at pH 5.4 and 7.4, respectively. The calculated ONOO⁻ concentration that decays through path 3 (Fig. 1) was taken as the expected RSOH concentration. Because paths 1 and 2 (Fig. 1) produce radical and nonradical products, the calculated ONOO⁻ concentrations were multiplied by 0.3 and 0.35 to estimate the produced ‘OH and NO₂⁻ and CO₃²⁻ and NO₂⁻, respectively (Table I).

RESULTS

Oxidation of GSH and Cysteine—To evaluate the effects of HCO₃⁻/CO₂ on peroxynitrite-mediated oxidation of thiols, we monitored cysteine and GSH oxidation at pH 5.4 and 7.4 by both thiol disappearance (Table I) and oxygen consumption (Fig. 2). Oxygen uptake provides a nonspecific measurement of radicals that react fast with oxygen such as RS· (33, 34). The results show that HCO₃⁻/CO₂ inhibited GSH and cysteine thiol disappearance at pH 7.4 (Table I) but in parallel increased the amount of consumed oxygen (Fig. 2). At pH 5.4, HCO₃⁻/CO₂ had marginal effects on both thiol depletion (Table I) and oxygen consumption (Fig. 2). These results indicate that HCO₃⁻/CO₂ diverts peroxynitrite-mediated thiol oxidation from two- to one-electron mechanisms as initially hypothesized (Fig. 1). At pH 5.4 the CO₂ effects were marginal because at this pH most of the thiol oxidation occurs by free radical mechanisms even in the absence of HCO₃⁻/CO₂. These conclusions are well supported by comparison of the experimental yields of depleted thiol with the yield of products, radicals (‘OH/NO₂⁻/CO₃²⁻/NO₂⁻), and thiol oxidized by two electrons (RSOH), which can be estimated from the known rate constants of the main competing reactions occurring under the experimental conditions used (Fig. 1, paths 1–3) (see “Experimental Procedures” and Table I). The data presented in Table I show that GSH oxidation at pH 5.4 occurs through the radicals produced from peroxynitrite in the presence or absence of HCO₃⁻/CO₂. The produced radicals are different, but they are expected to be formed in similar yields (Table I), and all of them (‘OH/NO₂⁻/CO₃²⁻/NO₂⁻) react quickly with GSH to produce GS (Table II). Relevantly, the experimental values of depleted thiol (0.30 and 0.26 mM) were the same as the calculated total radical yields in the presence (0.10 + 0.20 mM) and absence (0.26 mM) of HCO₃⁻/CO₂, respectively (Table I). Compared with GSH, a larger fraction of cysteine is oxidized by two-electron mechanisms at pH 5.4 in the absence of HCO₃⁻/CO₂ because of the higher second order rate constant of its reaction with peroxynitrite (24, 25). In this case, HCO₃⁻/CO₂ inhibited total thiol disappearance to some extent, but the oxidized fraction should result from the produced radicals whose expected total yield is, again, similar to the measured depleted thiol (Table I). At pH 7.4 both thiols are oxidized mainly by two-electron mechanisms in the absence of HCO₃⁻/CO₂. In this case, the experimental value of depleted thiol is considerably higher than the calculated one, confirming that the direct reaction between peroxynitrite and RSH consumes more than one thiol (Fig. 1, path 3) (17). At pH 7.4 in the presence of HCO₃⁻/CO₂, total depleted thiol decreased, but most of it should result from free radical mechanisms because of the similar concentration values of produced radicals and depleted thiols (Table I). In agreement, consumed oxygen increased in the presence of HCO₃⁻/CO₂, particularly in the case of GSH (Fig. 2).

Although the yield of depleted thiol by free radical mechanisms was roughly similar to total radical yields (Table I), it is not possible to infer that RS· are decaying mainly by reaction with themselves (Reaction 1) because a fast consumption of...
Carbon Dioxide Enhances Radical Production from Thiols

TABLE I
Comparison of the experimental yields of depleted thiol with calculated yields of products formed during the oxidation of 1 mM GSH or 1 mM cysteine by 0.5 mM peroxynitrite in the presence and in the absence of 1 mM carbon dioxide at 25 °C

| System                      | Measured depleted thiol | Calculated product concentrations |
|-----------------------------|-------------------------|-----------------------------------|
|                             | mM                      | OH + NO2 | CO3 + NO2 | RSOH |
| GSH, pH 7.4                 | 0.72 ± 0.03             | <0.02    | 0.06     | 0.40 |
| GSH + CO₂, pH 7.4           | 0.36 ± 0.03             | 0.26     | 0.02     | 0.01 |
| GSH, pH 5.4                 | 0.26 ± 0.02             | 0.10     | 0.20     | 0.02 |
| GSH + CO₃, pH 5.4           | 0.30 ± 0.01             | 0.02     | 0.32     | 0.02 |
| Cys, pH 7.4                 | 0.60 ± 0.05             | <0.02    | 0.32     | 0.04 |
| Cys + CO₂, pH 7.4           | 0.36 ± 0.01             | 0.20     | 0.20     | 0.18 |
| Cys, pH 5.4                 | 0.37 ± 0.02             | 0.10     | 0.20     | 0.09 |
| Cys + CO₃, pH 5.4           | 0.24 ± 0.02             |          |          |      |

* The inhibitory effects of CO₂ on thiol depletion were concentration-dependent but attained the maximum at 1.0 mM CO₂ under the employed experimental conditions.

** Calculations are described under “Experimental Procedures.” The concentration of each radical in the pairs (OH/NO₂ and CO₃/NO₂) corresponds to half of the shown values.

** A**

**B**

![Graph showing the effects of HCO₃/CO₂ on oxygen consumption during the oxidation of 1 mM GSH (A) and 1 mM cysteine (B) by 0.5 mM peroxynitrite at 25 °C at pH 7.4 (full bars) and pH 5.4 (open bars). The reactions were started by the addition of peroxynitrite, which triggered an extremely fast consumption of oxygen. Concentrations of CO₂ were calculated from the added HCO₃⁻ by using pH = 6.4.**

Reactions shown in the text for a general thiol (RSH) are listed with their corresponding available rate constants for GSH and cysteine

| Reaction                      | Rate constant | Ref. |
|-------------------------------|---------------|------|
| GSH + OH                      | 2.3 × 10¹⁰    | 35   |
| GSH + CO₃                     | 5.3 × 10⁸     | 36   |
| GS' + GS'                    | 1.5 × 10⁹     | 37   |
| GS' + O₂                      | 1.6 × 10⁸     | 38   |
| GSSG' + O₂                   | 4.5 × 10⁶     | 38   |
| CysH + OH                     | 4.7 × 10¹⁰    | 39   |
| CysH + CO₃                   | 4.6 × 10⁷     | 36   |
| CysH + NO₂                    | 5.0 × 10⁶     | 40   |
| CysS' + O₂                    | 8.0 × 10⁶     | 34   |
| CysS' + CysS'                | 1.2 × 10⁸     | 38   |

Oxygen was associated with thiol oxidation by peroxynitrite under all experimental conditions tested (Fig. 2) (see also Ref. 25). Because oxygen consumption was lower than oxidized thiol (Fig. 2 and Table I), it is likely that RS⁻ is decaying by at least three competing routes, i.e. dimerization, reaction with oxygen, and reaction with excess thiolate (RS⁻) (Reactions 1–3).

\[
\text{RS}^- + \text{RS}^- \rightarrow \text{RSSR}
\]

\[
\text{RS}^- + \text{O}_2 \rightarrow \text{RSOO}^- \rightarrow \text{RSO}^- + [\text{O}]
\]

\[
\text{RS}^- + \text{RS}^- \rightarrow \text{RSSR}^-
\]

** Reactions 1–3**

Other decay routes also occur as indicated by the detection of low levels of GSNO₂ (41) and GSNO (42) in incubations of GSH with peroxynitrite. The importance of Reactions 2 and 3 arises from the production of sulfonyl (RSO₂⁻) and disulfide radical anions (RSSR⁻), respectively (Table II). In previous studies of thiol oxidation by peroxynitrite, we detected RS⁻ radicals by EPR spin trapping and obtained indirect evidence for their conversion to both RSO⁻ and RSSR⁻ (25). Here, we present direct EPR evidence for the formation of these species and for the stimulatory effects of HCO₃⁻/CO₂ on their yields.

** EPR Detection of Thiyl, Sulfinyl, and Disulfide Radical Anion**—We have demonstrated previously that fast flow EPR of concentrated solutions of peroxynitrite and CO₂ produces detectable concentrations of CO₃⁻ (15). In the presence of 5 mM cysteine (Fig. 3) or 5 mM GSH (Fig. 4), the one-line EPR signal of CO₂ produced from 5 mM peroxynitrite and 5 mM CO₂ became barely detectable (indicated by a in Figs. 3 and 4) and new EPR signals appeared, particularly at pH 7.4. Cysteine oxidation produced a three-line signal (aₚ = 9.3 G; line width = 3.5 G; g = 2.0107) (Fig. 3) which has been characterized previously as the corresponding sulfinyl radical (CysSO⁻) by fast flow EPR studies of cysteine oxidation by Ti(III)-H₂O₂ (43). GSH oxidation produced a four-line signal (aₚ = 7.1 G and aₙ = 10.7 G; line width = 2.6 G; g = 2.0109) whose EPR parameters are also consistent with the corresponding sulfinyl radical (GSO⁻) (Fig. 4).

Both CysSO⁻ and GSO⁻ have two β-methylene hydrogens, but in the latter, a hindered rotation of the methylene group that is adjacent to a chiral center is likely to result in the magnetic nonequivalence (44, 45) reflected in the different hyperfine splitting constants (aₚ and aₙ) obtained for its two hydrogens (Fig. 4B).

Direct EPR detection of RSO⁻ during peroxynitrite-mediated oxidation of thiols is consistent with the initial formation of RS⁻ that reacts with oxygen to produce RSO⁻ which, in turn, as metastable intermediates, yield RSO⁻ (Reaction 2) (46). Thiyl radicals cannot be detected by direct EPR in aqueous solutions at room temperature because of the large anisotropy in their g tensors which broadens the EPR signal beyond detection (43). They are detectable in aqueous solutions by EPR spin trapping. Indeed, addition of the spin trap DMPO to the fast flow mixtures containing the thiols, HCO₃⁻/CO₂ and peroxynitrite led to the substitution of the RSO⁻ spectra by those characteristic of DMPO/SCys (aₚ = 15.2 G; aₙ = 17.4 G) (Fig. 3E) and DMPO/’SG (aₚ = 14.9 G; aₙ = 15.6 G) (Fig. 4E) radical adducts (47), confirming that RS⁻ are the RSO⁻ precursors (Reaction 2).

The effects of HCO₃⁻/CO₂ in increasing the concentrations of RSO⁻ radicals detected from cysteine and GSH oxidation by peroxynitrite are shown in Figs. 3 and 4. In agreement with the conclusion that thiols are oxidized mainly by two-electron mechanisms at pH 7.4 in the absence of HCO₃⁻/CO₂ (Fig. 2 and Table I), EPR signals were not detectable under these condi-
Carbon Dioxide Enhances Radical Production from Thiols

Fig. 3. EPR continuous flow spectra of cysteine-derived radicals produced from mixing 5 mM peroxynitrite and 5 mM cysteine with or without 5 mM CO₂, A, in 0.3 M phosphate buffer, pH 7.4; B, in 0.3 M acetate buffer, pH 5.4; C, in 0.3 M phosphate buffer, pH 5.4, equilibrated with 5 mM CO₂; D, same as B but in the presence of 20 mM DMPO. Detectable traces of CO₃²⁻ are indicated by ● in B. The spectrum in E was displaced to the left because of the marked difference in the g values of CysSO₂⁻ and DMPO/SCys. The specified concentrations are those in the final reaction mixture. The flow rate was 20 ml/min. Instrumental conditions: microwave power, 2 mW; time constant, 81.9 ms; scan rate, 0.6 G/s; modulation amplitude, 2 G; gain, 8.93 × 10⁵ except for E, where 1.78 × 10⁵ was used.

Fig. 4. EPR continuous flow spectra of GSH-derived radicals produced from mixing 5 mM peroxynitrite and 5 mM GSH with or without 5 mM CO₂, A, in 0.3 M phosphate buffer, pH 7.4; B, in 0.3 M acetate buffer, pH 5.4; C, in 0.3 M acetate buffer, pH 5.4, equilibrated with 5 mM CO₂; D, same as B but in the presence of 20 mM DMPO. Detectable traces of CO₃²⁻ are indicated by ● in B. The spectrum in E was displaced to the left because of the marked differences in the g values of GSO₂⁻ and DMPO/SG. The specified concentrations are those in the final reaction mixture. The flow rate was 20 ml/min. Instrumental conditions: microwave power, 2 mW; time constant, 81.9 ms; scan rate, 0.6 G/s; modulation amplitude, 2 G; gain, 1.78 × 10⁵ except for E, where 3.56 × 10⁵ was used.

Detection of RSO⁻ was possible in incubations containing equimolar concentrations of peroxynitrite and RSH (Figs. 3 and 4). Increases in thiol molar ratios led to composite EPR spectra similar to those at pH 5.4 in any condition (Table I), the detectable radical concentrations varied considerably (Figs. 3, B–D, and 4, B–D). This is because EPR flow experiments detect instantaneous radical concentrations whose values depend not only on radical yields but also on the observation time and rates of radical formation and decay. Rates of radical formation become more important at the short observation time used to detect RSO⁻ (3.5 ms) (48). Consequently, the higher RSO⁻ concentrations detected in the presence of HCO₃⁻/CO₂ at pH 7.4 than at pH 5.4 (Figs. 3, B and D, and 4, B and D) are likely to be due to the usually higher rate constants of thiol oxidation at alkaline pH values (25, 36, 49).

Detection of RSO⁻ was possible in incubations containing equimolar concentrations of peroxynitrite and RSH (Figs. 3 and 4). Increases in thiol molar ratios led to composite EPR spectra whose partial characterization was possible with a 5 molar excess thiol. Such excess, low oxygen tensions, and the presence of HCO₃⁻/CO₂ led to the detection of EPR spectra dominated by the spectra of GSSG⁻ (Fig. 5B) or CysSCys⁻ (Fig. 5D). These spectra have been characterized previously in flow mixtures of horseradish peroxidase/¹⁸H₂O/acetyaminophen and the corresponding thiols (50). The spectra shown in Fig. 5 were obtained with four times lower concentrations of thiols and consequently were less resolved than those published previously (50). Computer simulation (noiseless lines in Fig. 5) of the experimental spectra obtained in mixtures of excess thiol, peroxynitrite, and HCO₃⁻/CO₂ confirmed that GSSG⁻ (α₂₁G = 6.9 G; α₂₁H = 7.0 G) and CysSCys⁻ (α₂₁H = 6.6 G; α₂₁L = 7.7 G) are the predominant species produced under these conditions.

Again, RSSR⁻ attained detectable concentrations only in the presence of HCO₃⁻/CO₂ (Fig. 5).

Oxidation of Albumin Thiol—The main target of peroxynitrite in albumin is its free thiol group (17, 51). It has been reported that HCO₃⁻/CO₂ partially inhibits albumin thiol oxidation by peroxynitrite (19). This result was confirmed under our experimental conditions where 0.5 mM peroxynitrite at pH 7.4 depleted 0.68 ± 0.02 mM and 0.56 ± 0.02 mM albumin thiols in the absence and in the presence of 1 mM CO₂, respectively. Although it inhibited total thiol depletion, 1 mM CO₂ increased about two times the yield of albumin thyl radical that was trapped by POBN (Fig. 6, A and B). All POBN/protein radical adducts have similar EPR spectra, a broad triplet characteristic of high molecular weight nitroxides (52). Thus, to prove that albumin-thyl is the main radical produced under the experimental conditions employed, parallel experiments were performed with albumin pretreated with N-ethylmaleimide. EPR signals were barely detectable from albumin whose thiol group was previously blocked with N-ethylmaleimide (Fig. 6, C and D). Under conditions of high molar excess of peroxynitrite over albumin thiol, other protein-derived radicals are produced and detected (53), as expected from the reactivity of peroxynitrite-derived radicals.

DISCUSSION

Presently, most investigators agree that CO₂ is likely to be the major sink of peroxynitrite in most physiological environments (2, 5, 10). Carbon dioxide deactivates about 65% of peroxynitrite because it catalyzes isomerization of the oxidant to the innocuous nitrate, although the remaining products...
Carbon Dioxide Enhances Radical Production from Thiols

And the effects of HCO$_3$/CO$_2$ in diverting peroxynitrite reactivity from two- to one-electron mechanisms may apply to other important biological targets that react directly with the oxidant such as hemoproteins (54, 55). The demonstration that at acid pH values peroxynitrite acts as an one-electron thiol oxidant in the presence and in the absence of HCO$_3$/CO$_2$ (Figs. 2–4) was also relevant. Acid pH values are important for phagocyte function and ischemic tissue pathology but have been overlooked in studies addressing peroxynitrite reactivity in biological environments. Because the three radicals that can be produced from peroxynitrite are strong (‘OH, $E = 2.3$ V (24); and CO$^\bullet_2$, $E = 1.8$ V (15, 56–58) or moderately strong (‘NO$_2$, $E = 0.99$ V) (24) one-electron oxidants, they are likely to mediate some of the biological damage attributed to peroxynitrite such as nitration of protein tyrosine residues (2, 5, 20–23) and protein thiol oxidation (27, 59, 60).

Formation of RS$^\bullet_2$ radicals during the oxidation of low and high molecular weight thiols by peroxynitrite has been reported in the literature by our group and others (6, 25, 27–29). However, this report provides the first direct EPR detection of RSO (Figs. 3 and 4) and RSSR$^\bullet_2$ (Fig. 5) formed from the initially produced RS$^\bullet_2$ (Reactions 1–3). All low molecular weight radicals, GSO, GSSG$^\bullet_2$, CysSO$,^\bullet_2$ and CysSSCys$^\bullet_2$ were unambiguously characterized by the parameters of their corresponding EPR spectra (see “Results”). Moreover, it was shown that the presence of HCO$_3$/CO$_2$ stimulated the production of RS$^\bullet_2$ and of all the radicals derived from them (Figs. 3–6). Among the identified radicals those that may have greater physiological consequences are GSSG$^\bullet_2$ (Fig. 5B) and protein-S$^\bullet_2$ (Fig. 6). Formation of protein-S$^\bullet_2$ and protein-SO$^\bullet_2$ may alter protein structure and function (61, 62), and relevantly, protein-thiol oxidation is a recurrent event in cell signaling cascades (63). On the other hand, because GSSG$^\bullet_2$ is produced under conditions of excess GSH over peroxynitrite, it is likely to be the prevalent radical produced from peroxynitrite attack on thiols in aqueous intracellular environments where oxidant concentration is expected to be much lower than the GSH and HCO$_3$/CO$_2$ concentration. The GSSG$^\bullet_2$ radical reacts fast with oxygen, producing superoxide anion (Table II), which can further react with thiols, although not particularly fast, to produce RS$^\bullet_2$. Consequently, intracellular GSH oxidation by peroxynitrite-derived radicals may trigger an oxygen-dependent free radical chain reaction, as was shown to occur in vitro (25). This possibility argues against GSH being particularly effective in directly counteracting the oxidant properties of peroxynitrite-derived radicals. In contrast, an antioxidant such as ascorbate, which is oxidized to a radical that reacts very slowly with oxygen is expected to be more effective. Moreover, ascorbate reacts faster with CO$_2$ than GSH (65). Consequently, in aqueous environments, ascorbate may be more important than GSH in counteracting the oxidant action of free radicals. This has been shown to occur in the case of the acetaminophen-derived radical (50) and more recently, in the case of peroxynitrite-derived radicals (65).

In conclusion, our results demonstrate that HCO$_3$/CO$_2$ diverts low and high molecular weight thiol oxidation by peroxynitrite from two- to one-electron mechanisms particularly at neutral pH. At acid pH values, thiol oxidation to free radicals predominates in the presence and absence of HCO$_3$/CO$_2$. The produced thiol-derived radicals were identified as RS$^\bullet_2$, RSO$^\bullet_2$.

The reduction potential of CO$_2^\bullet_2$ has usually been assumed to be 1.6 V. The determined value was 1.58 ± 0.02 V (56, 58) and corresponds to the pair CO$_2$/CO$_2^\bullet_2$. At pH 7.0, the relevant pair is CO$_2$/HCO$_3$ (pK$_a$ of HCO$_3^\bullet_2 = 10.32$) whose reduction potential can be calculated as 1.78 V ($E_{\text{CO}_2^\bullet_2}/\text{HCO}_3^\bullet_2 - E_{\text{CO}_2}/\text{HCO}_3^\bullet_2 = 0.059 \times (pK_a - pH)$).
and RSSR. These are reactive radicals that may contribute to the biodamaging and bioregulatory actions of peroxynitrite.

Acknowledgments—We thank Drs. Ronald P. Mason, Rafael Radi, Sergei V. Lymar, and Roger Bisby for helpful discussions.

REFERENCES

1. Beckman, J. S., Beckman, T. W., Chen, J., Marshall, P. A., and Freeman, B. A. (1990) Proc. Natl. Acad. Sci. U. S. A. 87, 1620–1625
2. Squadrito, G. L., and Pryor, W. A. (1998) Free Radic. Biol. Med. 25, 392–403
3. Ischiropoulos, H. (1998) Arch. Biochem. Biophys. 356, 1–11
4. Giorgio, S., Linares, E., Ischiropoulos, H., Zuben, F. J. V., Yamada, A., and Augusto, O. (1998) Infect. Immun. 66, 867–814
5. Hodges, G. R., and Ingold, K. U. (1999) Arch. Biochem. Biophys. 368, 303–318
6. Augusto, R., Beckman, J. S., and Freeman, B. A. (1999) Arch. Biochem. Biophys. 368, 303–318
7. Santos, C. X. C., Bonini, M. G., and Augusto, O. (2000) Arch. Biochem. Biophys. 377, 147–152
8. Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) Biochem. Biophys. Res. Commun. 333, 49–58
9. Goldenfeld, S., Cristol, G., and Lymar, S. V. (1999) Inorg. Chim. Acta 320, 303–309
10. Goldstein, S., and Czapski, G. (1999) Arch. Biochem. Biophys. 377, 87–96
11. Goldstein, S., and Czapski, G. (1999) J. Am. Chem. Soc. 121, 2444–2447
12. Coddington, J. W., Hurst, J. K., and Lymar, S. V. (1999) J. Am. Chem. Soc. 121, 2438–2443
13. Gerasimov, O. V., and Lymar, S. V. (1999) Inorg. Chem. 38, 4317–4321
14. Lehninger, A. L. (1999) Biochem. Biophys. Res. Commun. 266, 4244–4250
15. Lymar, S. V., and Hurst, J. K. (1995) J. Am. Chem. Soc. 117, 8867–8868
16. Denicolao, A., Freeman, B. A., Trujillo, M., and Radi, R. (1996) Arch. Biochem. Biophys. 333, 49–58
17. Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) Biochem. 35, 7855–7861
18. Santos, C. X. C., Bonini, M. G., and Augusto, O. (2000) Arch. Biochem. Biophys. 377, 147–152
19. Lymar, S. V., Jiang, Q., and Hurst, J. K. (1996) Biochem. 35, 7855–7861
20. Pfeiffer, S., Schmidt, K., and Mayer, B. (2000) J. Biol. Chem. 275, 6346–6352
21. Coppolari, H., Pryor, W. A., Moreno, J. J., Ischiropoulos, H., and Beckman, J. S. (1992) Chem. Res. Toxicol. 5, 834–842
22. Quijano, C., Alvarez, B., Lymar, S. V., and Augusto, O. (1997) Biochem. J. 322, 167–173
23. Zang, H., Squadrito, G. L., Uppu, R. M., Lemercier, J. N., Cueto, R., and Pryor, W. A. (1997) Arch. Biochem. Biophys. 339, 183–189
24. Gattis, R. M., Radi, R., and Augusto, O. (1994) FEBS Lett. 348, 287–290
25. Karou, H., Hogg, N., Frejaville, C., Tords, P., and Kalyanaraman, B. (1996) J. Biol. Chem. 271, 6000–6009
26. Scorz, G., and Minetti, M. (1996) Biochem. J. 329, 405–413
27. Kissner, R., Nauser, T., Bugnon, P., Lye, P. G., and Koppenol, W. H. (1997) Chem. Res. Toxicol. 10, 1285–1292
28. Duling, D. R. (1994) J. Magn. Reson. 104, 105–110
29. Radi, R. (1996) Methods Enzymol. 264, 354–366
30. Quintiliani, M., Badiello, R., Tamba, M., Estefandi, A., and Gorin, G. (1977) J. Chem. Soc. Faraday Trans. 2, 247–252
31. Barton, J. P., and Packer, J. E. (1970) Int. J. Radiat. Phys. Chem. 2, 159–166
32. Music, V., Mak, I. T., Stafford, R. E., and Weglicki, W. B. (1993) Free Rad. Biol. Med. 15, 611–619
33. Chen, S. N., and Hoffman, M. Z. (1973) Radiat. Res. 54, 40–47
34. Hoffman, M. Z., and Hayon, E. (1973) J. Chem. Phys. 57, 990–996
35. Zhang, R., Lymar, S. V., and Eriksen, T. E. (1994) J. Am. Chem. Soc. 116, 12010–12015
36. Nucifora, G., Smaller, B., Remko, R., and Avery, E. C. (1972) Radiat. Res. 49, 96–111
37. Fornt, L. G., Mora-Arellano, V. O., Parker, J. E., and Wilson, R. L. (1996) J. Chem. Soc. Perkin Trans. 2, 1–6
38. Balasy, M., Pawel, M. K., Kajyuz, M., Jianzhen, T., and Wolin, M. S. (1998) J. Biol. Chem. 273, 32009–32015
39. Van der Vliet, A., Hoen, P. A. C., Wong, P. S. Y., Bast, A., and Cross, C. E. (1998) J. Biol. Chem. 273, 30255–30262
40. Gilbert, B. C., Lake, H. A. H., Norman, R. O. C., and Sealy, R. C. (1974) J. Chem. Soc. Perkin Trans 2, 892–900
41. Gilbert, B. C., Larkin, J. P., and Norman, R. O. C. (1972) J. Chem. Soc. Perkin Trans 2, 1272–1279
42. Sealy, R. C., Harman, L., West, P. R., and Mason, R. P. (1975) J. Am. Chem. Soc. 107, 3401–3406
43. Sevilla, M. D., Becker, D., Swarts, S., and Herrington, J. (1987) Biochem. Biophys. Res. Commun. 144, 1037–1042
44. Li, A. S. W., Cummings, K. B., Roething, H. P., Buettnner, G. R., and Chignell, C. F. (1998) J. Magn. Reson. 79, 140–142
45. Borg, D. C. (1976) in Free Radical in Biology (Pryor, W. A., ed) Vol. 1, pp. 69–147, Academic Press, New York
46. Chen, S. N., and Hoffman, M. Z. (1975) Radiat. Res. 62, 18–27
47. Rao, D. N. R., Fischer, V., and Mason, R. P. (1990) J. Biol. Chem. 265, 844–847
48. Radi, R. (1996) Chem. Res. Toxicol. 9, 828–835
49. Groves, J. T. (1999)curr. Opin. Chem. Biol. 3, 226–235
50. Huie, R. E., Clifton, C. L., and Neta, P. (1991) Radiat. Phys. Chem. 38, 477–481
51. Bishy, R. H., Johnson, S. A., Parker, A. W., and Tavender, S. M. (1998) J. Am. Chem. Soc. Faraday Trans. 94, 2069–2072
52. Radi, R. (1996) Radiat. Biophys. 25, 1–6
53. Winterbourn, C. C., and Metodiewa, D. (1999) Free Rad. Biol. Med. 27, 322–328
54. Kirsch, M., and de Groot, H. (2000) J. Biol. Chem. 275, 16702–16708
55. Kirsch, M., and de Groot, H. (2000) J. Biol. Chem. 275, 16702–16708
