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Nitration and Nitrosation by Peroxynitrite: Role of CO_2 and Evidence for Common Intermediates

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Abstract: Peroxynitrite reacts with morpholine (MorH) to give both *N*-nitromorpholine (Mor-NO_2) and *N*-nitrosomorpholine (Mor-N═O). The pH profile of Mor-NO_2 formation shows a bell-shaped curve with a maximum yield of 1.6 mol % (relative to peroxynitrite) centered around pH 9.0. Yields of Mor-N═O, on the other hand, increase until the pH reaches 10.0 and then reach a plateau; the maximum yield of Mor-N═O is 12 mol % relative to peroxynitrite. Both the nitration and nitrosation of MorH are catalyzed by low levels of CO_2; however, excess CO_2 dramatically reduces the yields of Mor-N═O but not Mor-NO_2, and the combined yields of Mor-N═O and Mor-NO_2 are about the same under conditions of high and low concentrations of CO_2. These data indicate that both nitration and nitrosation by peroxynitrite are free radical processes. The morpholine radical (Mor$^\bullet$), formed from the reactions of carbonate and/or hydroxyl radicals with MorH, reacts with either $^\bullet NO$ or $^\bullet NO_2$ and serves as a common precursor for Mor-N═O and Mor-NO_2.

Peroxynitrite is a versatile oxidant[1,2] which is thought to mediate the toxic effects of nitric oxide ($^\bullet$NO).[3] Nitric oxide itself cannot cause either nitration or nitrosation,[4] and both of these reactions are thought to involve the intermediacy of peroxynitrite;[3,5] in fact, nitration is widely considered to be the footprint of peroxynitrite formation in vivo.[6,7] Nitration of tyrosine residues can alter phosphorylation/dephosphorylation of proteins, an important regulatory aspect of signal transduction.[8] Nitrosation of biological nucleophiles also can have pathophysiological consequences; e.g., nitrosation of purine and pyrimidine bases in DNA can produce deamination.[9] *S*-Nitrosothiols, which are present in blood plasma in concentrations up to 0.5−1.3 μM,[10a] have long been known to have $^\bullet$NO-like activity.[10b−c]

Peroxynitrite and the products derived from its reaction[11] with CO_2 react with phenol to give substantial yields of 4-nitrosophenol[5c,12] in addition to 2- and 4-nitrophenols and several other hydroxylated and quinone products.[12,13] Maximum yields of nitrosation are obtained at pH > 10,[5c] where little or no nitration of phenolic compounds generally is observed.[13,14] At first, the

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(1) The IUPAC-recommended name for peroxynitrite anion ($ONOO^-$) is oxoperoxonitrate(1−), and hydrogen oxoperoxonitrate for peroxynitrous acid (ONOOH; pK_a = 6.2−6.8). We use the term peroxynitrite to refer to the sum of $ONOO^-$ and ONOOH.

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(11) Carbonate is present in concentrations up to 25 mM in all physiological fluids, and about 7% of carbonate exists as free CO_2 at pH 7.2. Peroxynitrite anion reacts with CO_2 to form $ONOOCO_2^-$ ($k_2 = 6 \times 10^4$ M^{-1} s^{-1} at 37 °C). In the absence of peroxidases, virtually all of the peroxynitrite generated in biological environments will react with CO_2 before it can react with any other molecules. Therefore, it has become imperative to study the reactions of peroxynitrite with biomolecules in the presence of purposefully added carbonate; it also is wise to do experiments that do not have added carbonate but contain only adventitious carbonate. See: (a) Lymar, S. V.; Hurst, J. K. *J. Am. Chem. Soc.* **1995**, *117*, 8867. (b) Uppu, R. M.; Squadrito, G. L.; Pryor, W. A. *Arch. Biochem. Biophys.* **1996**, *327*, 335. (c) Denicola, A.; Freeman, B. A.; Trujillo, M.; Radi, R. *Arch. Biochem. Biophys.* **1996**, *333*, 49. (d) Floris, R.; Piersma, S. R.; Yang, G.; Jones, P.; Wever, R. *Eur. J. Biochem.* **1993**, *215*, 767. (e) Sies, H.; Sharov, V. S.; Klotz, L.-O.; Briviba, K. *J. Biol. Chem.* **1997**, *272*, 27812. (f) Lymar, S. V.; Hurst, J. K. *Chem. Res. Toxicol.* **1996**, *9*, 845.

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requirement for high pH was thought to arise from the limited availability of the phenolate ion at lower pH values (the pK_a of phenol is 9.9). However, a similar pH requirement was observed for nitrosation of aromatic primary amines[15] whose conjugate acids (protonated amines) have pK_a values of ca. 4.5.[16] The present study of the nitration and nitrosation reactions of peroxynitrite using morpholine (MorH) as a probe was undertaken since MorH gives fewer side reactions than do phenols and forms well-characterized nitration and nitrosation products. Our results suggest that both nitration and nitrosation are free radical processes requiring the simultaneous presence of a strong one-electron oxidant (such as $HO^{\bullet}$ or $CO_3^{\bullet-}$) and $^{\bullet}NO$ or $^{\bullet}NO_2$.

Experimental Section

Chemicals and Reagents. 2,2′-Azinobis(3-ethylbenzthiazoline-6-sulfonic acid, ammonium salt) (ABTS), diethylenetriaminepentaacetic acid (DTPA), MorH, and *N*-nitrosomorpholine (Mor-N═O) were purchased from Sigma (St. Louis, MO). All other chemicals were of analytical grade, and all solutions were prepared using deionized water of high resistivity (≥18 MΩ cm^{-1}). Peroxynitrite was synthesized by the ozonation of 0.1 M NaN_3 (pH ∼12) as described earlier.[17] *N*-Nitromorpholine (Mor-NO_2) was synthesized by the oxidation of Mor-N═O according to the method of Emmons[18] using 30% H_2O_2 and trifluoroacetic acid. The product Mor-NO_2 was purified by reversed-phase (RP) HPLC (described below), and the GC/MS/EI analysis gave a molecular ion at *m*/*z* 132.

Reaction of Peroxynitrite with Morpholine. Peroxynitrite[19] (2 mM) was allowed to react with 5 mM MorH in 2 mL of 0.1 M phosphate buffer (PB), pH 6.2−10.6, that also contained 0.1 mM DTPA and 0−50 mM added carbonate. The reactions were performed by adding aliquots (130−160 μL) of stock peroxynitrite solution (25−30 mM) to the MorH/PB solutions over a period of 10 s, with the contents being constantly stirred and the stirring continued for an additional 10 s. To minimize the adsorption of CO_2 from the air, the reaction mixtures were kept in sealed vials until further analysis (see below).

To study the effects of high and low concentrations of CO_2 on the peroxynitrite/MorH reaction, we used a pH-jump technique,[5c,14c] allowing rapid mixing of 0.1 M $NaHCO_3/Na_2CO_3$ (preequilibrated at pH 7.0 or 10.0 in 0.2 M PB; also contained 10 mM MorH) with solutions of 4 mM peroxynitrite that also contained 0 or 0.14 N added NaOH. Addition of NaOH was necessary to change the pH of carbonate solutions from 7.0 to 10.2. In assays where carbonate was preequilibrated at pH 10.0, no NaOH was added, and yet there was a slight jump in the pH from 10.0 to 10.2 upon mixing of individual solutions. Mixing of the reactant solutions was performed using a vortex mixer, and the final concentrations of peroxynitrite, MorH, and carbonate were 2, 5, and 50 mM, respectively. In assays where the carbonate species were preequilibrated at pH 7.0 and 10.0, the initial concentrations of CO_2 after mixing of all reagents were 5 mM and ca. 1 μM, respectively (Table 1).

Reversed-Phase HPLC and GC/MS/EI Analysis. Upon complete decay of peroxynitrite (assessed by the absence of ABTS oxidation[5c]), the reaction mixtures were filtered (pore size, 0.2 μm) and then analyzed by RP-HPLC using a Perkin-Elmer series 410 liquid chromatograph (Perkin-Elmer, Norwalk, CT) provided with a Perkin-Elmer LC-95 UV/vis spectrophotometer and a Waters Spherisorb S3 ODS2 column (2.0 × 100 mm; Waters Corp., Taunton, MA). The separation of products was achieved using an isocratic solvent system that consisted of 5% (v/v) acetonitrile in 27 mM acetate/30 mM citrate buffer (pH 3.2) at a flow of 0.5 mL/min (sample volume was 20 μL, and the eluent was monitored at 254 nm). The products were identified by matching their retention times with those of authentic compounds, and Labcalc software (Galactic Industries, Salem, NH) was used to integrate the peak areas.

In some cases, the products of the peroxynitrite/MorH reaction, separated previously by RP-HPLC, were extracted into dichloromethane and then analyzed using a Hewlett-Packard 5890 gas chromatograph equipped with a mass-selective detector 5970 and a DB-5MS column (12.5 m length × 0.20 mm internal diameter × 0.33 μm film thickness). Helium gas was used as the carrier at a flow of 0.85 mL/min. The conditions for chromatography were as follows: injection port, 250 °C; detector, 280 °C; oven, 40 °C for 3 min, 20 °C/min up to 280 °C, and 280 °C for 10 min; split, 25:1; and solvent delay, 3 min. Retention times of Mor-N═O and Mor-NO_2 were 8.4 and 9.2 min, respectively. The ion fragmentation patterns were compared with those of authentic Mor-N═O and Mor-NO_2.

Table 1. Formation of Mor-N═O and Mor-NO_2 in the Reaction of 2 mM Peroxynitrite with 5 mM MorH in the Presence of High and Low Concentrations of CO_2 at pH 10.2

assay conditions	yields of Mor-N═O (μM)	yields of Mor-NO_2 (μM)	combined yields of Mor-N═O and Mor-NO_2 (μM)
high CO_2 (5 mM)[a]	27 ± 3 (n = 7)	236 ± 12 (n = 7)	263 ± 13 (n = 7)
low CO_2 (∼1 μM)[b]	195 ± 3 (n = 4)	32 ± 6 (n = 4)	227 ± 8 (n= 4)

[a] Peroxynitrite (2 mM) was allowed to react with ca. 2.5-fold excess CO_2 (5 mM) at a final pH of 10.2. For this, a solution of 10 mM MorH plus 0.1 M carbonate was preequilibrated for 2 min at pH 7.0 in 0.2 M PB (T = 25 °C) and then mixed with an equal volume of 4 mM peroxynitrite in 0.14 N NaOH to reach the desired pH of 10.2. [b]The reaction was performed similarly to that described in footnote *a* but using solutions of 10 mM Mor plus 0.1 M carbonate (preequilibrated at pH 10.0 in 0.2 M PB, T = 25 °C) and 4 mM peroxynitrite (pH ∼12, contains no added NaOH). The concentration of CO_2 in the final reaction mixture was ca. 1 μM and the pH was 10.2. Other assay conditions were as described in the Experimental Section, and the products Mor-N═O and Mor-NO_2 were estimated by RP-HPLC. Values represent mean ± SD of 4−7 determinations.

Results

Reaction of Peroxynitrite with Morpholine in the Absence of Purposefully Added Carbonate. Figure 1 shows the RP-HPLC analysis of the products of peroxynitrite/MorH reaction at pH 7.4 in the absence of purposefully added carbonate. As can be seen, the reaction at this pH mainly produces two products. The first product elutes with a retention time (1.5 min) identical to that of Mor-N═O. The second product, which was formed in somewhat lower yield compared to that of Mor-N═O, has a retention time identical to that of Mor-NO_2 (2.3 min). The nitrated and nitrosated products from several RP-HPLC runs were extracted into dichloromethane, and the organic layer was subjected to GC/MS/EI analysis to conclusively establish their structures. We find that the product that elutes with a retention time of 1.5 min has fragmentation identical to that of Mor-N═O with ions at *m*/*z* 116, 86, and 56, corresponding to the molecular ion (M^+) ($C_4H_8N_2O_2^{\bullet+}$), and fragments with successive loss of $^{\bullet}NO$ ($C_4H_8NO^+$) and HCHO ($C_3H_6N^+$), respectively. The GC/MS/EI analysis of the product that elutes with a retention time of 2.3 min is the same as that of Mor-NO_2 with ions at *m*/*z* 132, 86, and 56, corresponding to

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(19) We have examined the yields of nitrosation of MorH at various low and high initial concentrations of peroxynitrite (0−2 mM) at pH 10.2. Nitrosation of MorH occurs at all concentrations of peroxynitrite, much in the same way as described for phenol[5c] and 1,2-phenylenediamine.[15] In these systems, the dependence of nitrosation on peroxynitrite concentration is somewhat curvilinear (for example, see Figure 3A in ref 5c), particularly in reactions performed without added carbonate but supposedly containing some adventitious carbonate.[11b,13a]

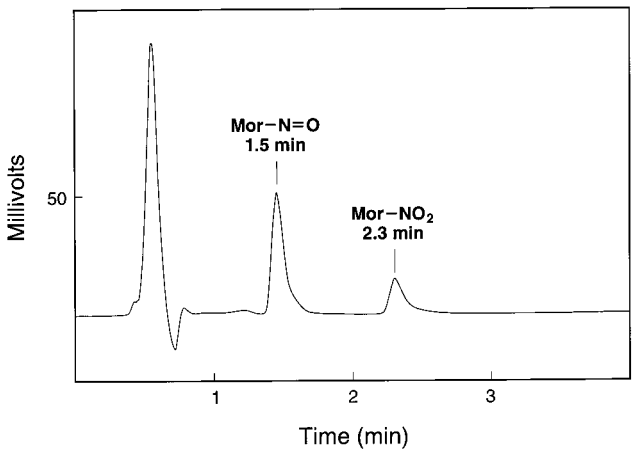

Figure 1. RP-HPLC analysis of the products of peroxynitrite reaction with MorH at pH 7.4. The reactions were carried out in a final volume of 2 mL of 0.1 M PB that also contained 0.1 mM DTPA, 2 mM peroxynitrite, and 5 mM MorH. The reaction was initiated with the addition of peroxynitrite, and all other conditions were as described in the Experimental Section.

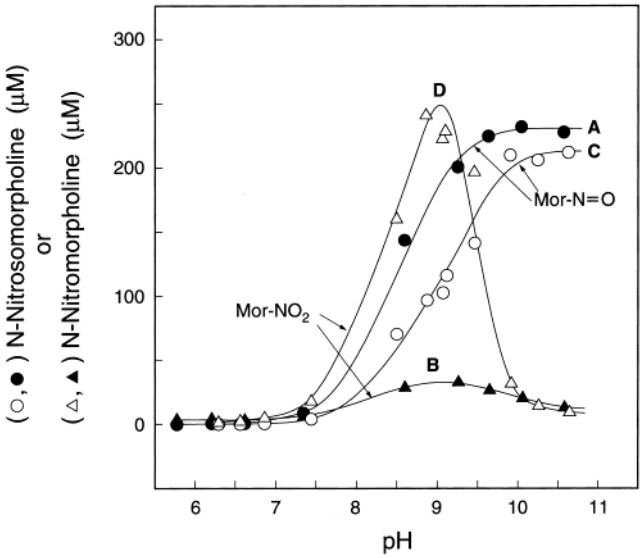

Figure 2. Yields of Mor-N═O and Mor-NO_2 in the peroxynitrite/MorH reaction in the presence and in the absence of purposefully added carbonate at pH 6.2−10.6. Peroxynitrite (2 mM) was allowed to react with 5 mM MorH in 2 mL of 0.1 M PB and 0 (●, ▲) or 20 mM added carbonate (○, △). The solutions also contained 0.1 mM DTPA. The reaction in all cases was initiated with the addition of peroxynitrite, and the products Mor-N═O (●, ○) and Mor-NO_2 (▲, △) were analyzed by RP-HPLC as described in the Experimental Section.

M^+ ($C_4H_8N_2O_3^{\bullet+}$), and fragments with successive loss of $^\bullet NO_2$ ($C_4H_8NO^+$) and HCHO ($C_3H_6N^+$).

The pH dependence of Mor-N═O formation in the peroxynitrite/MorH reaction is different from that of Mor-NO_2 (Figure 2). We detect Mor-N═O at pH values as low as 7.0, and the yields reach a maximum of about 12 mol % (relative to peroxynitrite) at pH ≥ 10 (curve A). The formation of Mor-N═O parallels the availability of unprotonated MorH[20] ($MorH_2^+ \rightleftharpoons MorH + H^+$; $pK_a = 8.5$). The formation of Mor-NO_2, on the other hand, follows a bell-shaped profile with a maximum yield of about 1.6 mol % (relative to peroxynitrite) occurring at pH 9.0 (curve B).

Although the yields of both nitration and nitrosation products are small, they are significant and typical of peroxynitrite-mediated reactions that are zero-order in substrate and involve either the activated peroxynitrous acid (ONOOH*)[21−23] or the products derived from the reaction of peroxynitrite with adventitious and purposefully added carbonate.[2,5b−d,12−15,24] The yields of products in all these reactions range from 0.1 to 30 mol % relative to peroxynitrite. These low yields suggest that the reactive intermediates from peroxynitrite primarily decompose to the innocuous products NO_3^- and NO_2^- in competition with nitration, nitrosation, and other oxidation reactions.

Catalytic Role of CO_2 in the Nitrosation of Morpholine by Peroxynitrite. Although the reactions in Figure 2 (curves A and B) were performed in the absence of added carbonate, these reaction mixtures do contain adventitious carbonate[11b] (ca. 0.1 mM).[13a] Two possible sources for carbonate contamination are the stock solutions of peroxynitrite (maintained at pH ≥ 12 for reasons of stability) and the buffers used in these reactions. In the pH range of 6.2−10.6 used in these reactions (Figure 2, curves A and B), at equilibrium, concentration of CO_2 ranges from 50 μM to 4 nM. (The molar fractions of CO_2 relative to total carbonate are 0.5 and 4×10^{-6} at pH 6.2 and 10.6, respectively.[25]) Carbon dioxide is a catalyst for the decomposition of peroxynitrite[26] and mostly is regenerated at the end of the catalytic cycle.[27] Therefore, these low concentrations of CO_2 may play a major role in nitration as well as nitrosation.

Indeed, low levels of CO_2 catalyze the nitrosation of MorH by peroxynitrite. As shown in Figure 3, the accumulation of Mor-N═O is much faster in reactions containing 50 mM added carbonate (curve A) than in those that contain small amounts of adventitious carbonate[13a] (ca. 0.1 mM) (curve B). Assuming a molar fraction of 2×10^{-5} for CO_2 at pH 10.2,[25] the concentration of CO_2 in assays without any added carbonate and with 50 mM added carbonate can be calculated to be 0.002 and 1 μM, respectively.

Modulation of Nitrosation and Nitration Potentials of Peroxynitrite by CO_2. To investigate the nature of reactive species in the peroxynitrite/CO_2 system that cause nitration and nitrosation, we designed pH-jump experiments along the lines suggested by Lymar et al.[14c] and Uppu et al.[5c] We chose two sets of experimental conditions. In the first set of conditions, to obtain high initial concentrations of CO_2 at alkaline pH, we used a rapid pH jump of 0.1 M carbonate from pH 7.0 to 10.2. Under these conditions, $[CO_2]_0 >$ [peroxynitrite]$_0$ (Table 1), and this allows a rapid and nearly total reaction of peroxynitrite with CO_2, forming $ONOOCO_2^-$. (The decay of CO_2 by competing reactions with water ($k = 0.05\ s^{-1}$) and HO^- ($k = 8500\ M^{-1}\ s^{-1}$) is a slow process at pH 10.2.[25a]) Under these conditions, we find significant formation of Mor-NO_2 with small yields of Mor-N═O (Table 1), suggesting that the products from homolysis of $ONOOCO_2^-$ ($CO_3^{\bullet-}$ and $^\bullet NO_2$) can cause nitration but not nitrosation. We find significant yields of Mor-N═O but not Mor-NO_2 in the second set of conditions in which peroxy-

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(22) For the sake of simplicity, we show $HO^\bullet$ in Scheme 1. However, the mechanism does not necessarily depend on $HO^\bullet$; it only requires an activated species from ONOOH.
(23) There is recent evidence in favor of ONOOH homolysis, giving $HO^\bullet$ and $^\bullet NO_2$ free radicals in yields up to 30 mol %: (a) Merényi, G.; Lind, J. *Chem. Res. Toxicol.* **1998**, *11*, 243. (b) Coddington, J. W.; Hurst, J. K.; Lymar, S. V. *J. Am. Chem. Soc.* **1999**, *121*, 2438. (c) Goldstein, S.; Meyerstein, D.; van Eldik, R.; Czapski, G. *J. Phys. Chem. A* **1999**, *103*, 6587.
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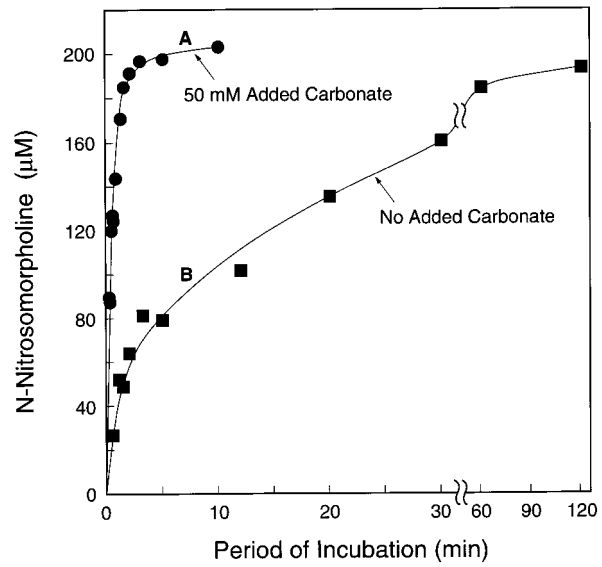
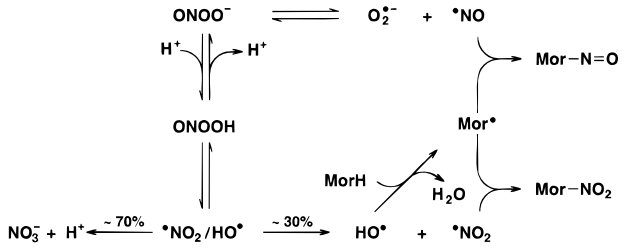


Figure 3. Time course of the formation of Mor-N═O in the peroxynitrite/MorH reaction at pH 10.2 in the (A) presence and (B) absence of 50 mM purposefully added carbonate. Peroxynitrite (2 mM) was allowed to react with 5 mM MorH in 2 mL of 0.1 M PB containing 0.1 mM DTPA and 0 or 50 mM added carbonate. All reactions were initiated with the addition of peroxynitrite, and the product Mor-N═O was estimated at various intervals of time by RP-HPLC. Other assay conditions were as described in the Experimental Section.

Scheme 1. Postulated Reactions of Peroxynitrite with MorH Leading to the Formation of Mor-N═O and Mor-NO_2 in the Absence of Carbonate[a]



[a] Another possible source of •NO involves the 1-e⁻ oxidation of $ONOO^-$ by HO• (eq 3) followed by dissociation of the product ONOO• to •NO plus O_2 (eq 4) (see text).

nitrite (2 mM) was allowed to react with MorH (5 mM) in the presence of low, catalytic concentrations of CO_2 (ca. 1 μM) at pH 10.2 (Table 1), similar to the nitrosation of phenol[5c] and 1,2-phenylenediamine[15] by peroxynitrite/CO_2. Unlike the reactions with high $[CO_2]_0$, the reactions with low $[CO_2]_0$ take several minutes to complete (see Figure 3), allowing for maximal interaction between the products of homolysis of $ONOOCO_2^-$ and $ONOO^-$.

Discussion

During the past decade, following seminal work of Beckman et al.,[28] numerous reports have been published on the oxidative and free radical reactions of peroxynitrite.[2,3,5,11−15,21−24,26−29] A vast majority of these reports center on the nitration of tyrosine and model compounds (e.g., phenol), since nitration both marks the presence of peroxynitrite and is a new type of posttranslational modification that affects phosphorylation/dephosphorylation of tyrosine residues in proteins.[8]

In contrast to nitration, only a few examples of nitrosation by peroxynitrite have been reported,[5b−d,12,15] and these reactions occur in alkaline solutions where little or no nitration is observed.[13,14] We have postulated[15] that nitrosation is a free radical process requiring the simultaneous presence of a strong one-electron oxidant (such as HO• or $CO_3^{\bullet-}$) and •NO. The higher yields of nitrosation under alkaline conditions are consistent with the formation of the free radicals HO• (eq 1)[23] and $CO_3^{\bullet-}$ (eq 2)[30] and their secondary reactions with $ONOO^-$ (eq 3),[5c,23,31,32] giving ONOO• and its dissociation products •NO and O_2 (eq 4). [33]

$$ONOOH \rightarrow {}^{\bullet}NO_2 + HO^{\bullet} \quad (1)$$

$$ONOOCO_2^- \rightarrow {}^{\bullet}NO_2 + CO_3^{\bullet-} \quad (2)$$

$$HO^{\bullet}/CO_3^{\bullet-} + ONOO^- \rightarrow HO^-/CO_3^{2-} + ONOO^{\bullet} \quad (3)$$

$$ONOO^{\bullet} \rightleftharpoons {}^{\bullet}NO + O_2 \quad (4)$$

In alkaline solutions, in addition to the reactions of HO• and $CO_3^{\bullet-}$ (eq 3), some •NO can be formed from the dissociation of $ONOO^-$ (eq 5).[23a,34]

$$ONOO^- \rightleftharpoons {}^{\bullet}NO + O_2^{\bullet-} \quad (5)$$

In model systems such as ours, HO• (eq 1) and •NO (eqs 3 and 5) come from slow reactions of peroxynitrite[11] that would not be expected to occur in vivo. In contrast, in biological systems, •NO is continuously produced by nitric oxide synthase, NOS,[35] and so the formation of •NO from peroxynitrite (eqs 3−5) is not required. Nevertheless, in vitro systems such as ours have the advantage of providing a conceptual framework for understanding the mechanisms of nitration and nitrosation in •NO-producing biological systems (see below).

Possible Mechanisms: (A) At Very Low Concentrations of Carbonate. Scheme 1 exemplifies the reactions that can

(28) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1620.

(29) (a) Vasquez-Vivar, J.; Santos, A. M.; Junqueira, V. B. C.; Augusto, O. *Biochem. J.* **1996**, *314*, 869. (b) Uppu, R. M.; Winston, W. W.; Pryor, W. A. *Chem. Res. Toxicol*. **1997**, *10*, 1331. (c) Kirsch, M.; Lomonosova, E. E.; Korth, H.; Sustmann, R.; De Groot, H. *J. Biol. Chem.* **1998**, *273*, 12716. (d) Hodges, G. R.; Ingold, K. U. *J. Am. Chem. Soc*. **1999**, *121*, 10695. (e) Yang, D.; Tang, Y.-C.; Chen, J,; Wang, X.-C.; Bartberger, M. D.; Houk, K. N.; Olson, L. *J. Am. Chem. Soc*. **1999**, *121*, 11976.

(30) (a) Goldstein, S.; Czapski, G. *J. Am. Chem. Soc*. **1998**, *120*, 3458. (b) Lymar, S. V.; Hurst, J. K. *Inorg. Chem.* **1998**, *37*, 294. (c) Bonini, M. G.; Radi, R.; Ferrer-Sueta, G.; Ferreira, A. M. D. C.; Augusto, O. *J. Biol. Chem.* **1999**, *274*, 10802

(31) (a) Pfeiffer, S.; Gorren, A. C. F.; Schmidt, K.; Werner, E. R.; Hansert, B.; Bohle, D. S.; Mayer, B. *J. Biol. Chem.* **1997**, *272*, 3465. (b) Goldstein, S.; Saha, A.; Lymar, A. V.; Czapski, G. *J. Am. Chem. Soc*. **1998**, *120*, 5549.

(32) A reviewer asked if $ONOOCO_2^-$ could react with $ONOO^-$, forming •NO and other products as shown in eq i

$$ONOOCO_2^- + ONOO^- \rightarrow CO_3^{2-} + NO_2^- + O_2 + {}^{\bullet}NO \quad (i)$$

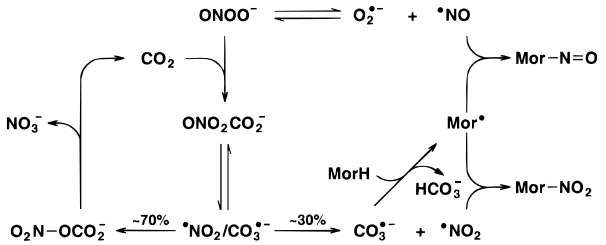
The adduct $ONOOCO_2^-$ has a very short half-life[11a−c,14c] and a similar adduct, RCH(O⁻)OONO, formed in reactions of $ONOO^-$ with aldehydes, does not have significant oxidative capability.[29b] Therefore, we believe that $ONOOCO_2^-$ does not oxidize $ONOO^-$. Even if $ONOOCO_2^-$ would react with peroxynitrite directly, the reaction is indistinguishable from those of HO• and $CO_3^{\bullet-}$ with $ONOO^-$ (eq 3).

(33) Frears, E.; Nazhat, N.; Blake, D.; Symons, M. *Free Radical Res*. **1997**, *27*, 31.

(34) The dissociation constant for $ONOO^-$ giving •NO plus $O_2^{\bullet-}$ is 0.017 s^{-1},[23a] whereas the rate constants for reactions of HO• and $CO_3^{\bullet-}$ with $ONOO^-$ are (4−5) × 10^9 [23b,c] and 8 × 10^7 M^{-1} s^{-1},[23c] respectively. Therefore, the relative yields of •NO from reactions 3 and 4 depend on pH, carbonate and buffer concentrations, and impurities present in the system.

(35) (a) Vallance, P.; Patton, S.; Bhagat, K.; MacAllister, R.; Radomski, M.; Moncada, S.; Malinski, T. *Lancet* **1995**, *346*, 153. (b) Pinsky, D. J.; Patton, S.; Mesaros, S.; Brovkovych, V.; Kubaszewski, E.; Grunfeld, S.; Malinski, T. *Circ. Res.* **1997**, *81*, 372.

Scheme 2. Schematic Representation of the Formation of Mor-N═O and Mor-NO_2 in the Peroxynitrite/MorH Reaction in the Presence of Purposefully Added Carbonate[a]



[a] Another possible source of •NO involves the 1-e⁻ oxidation of $ONOO^-$ by $CO_3^{\bullet-}$ (eq 3) followed by the dissociation of the product ONOO• to •NO plus O_2 (eq 4) (see text).

occur in the absence of purposefully added carbonate. Preparations of peroxynitrite contain ONOOH and $ONOO^-$ and low levels of their respective homolysis products $^\bullet NO_2$ plus HO• (eq 1) and •NO plus $O_2^{\bullet-}$ (eq 5).[23] Considering the highly reactive and unselective nature[36] of HO• and the abundance of MorH (5 mM), the principal target for the reaction of HO• in our system is MorH. The radical Mor• then reacts with •NO or $^\bullet NO_2$, forming Mor-N═O or Mor-NO_2 (Scheme 1).

The direct reaction of $^\bullet NO_2$ with •NO results in the formation of N_2O_3 (eq 6),[23,31a,37] and this could account for some nitrosation of MorH by peroxynitrite (eq 7).

$$^\bullet NO_2 + {}^\bullet NO \rightleftharpoons N_2O_3 \qquad (6)$$

$$N_2O_3 + \text{MorH} \rightarrow NO_2^- + \text{Mor-N═O} + H^+ \qquad (7)$$

However, in systems such as ours which use alkaline buffers and high concentrations of carbonate and phosphate, the hydrolysis of N_2O_3 (eq 8) is markedly accelerated.[38]

$$N_2O_3 + HO^- \rightarrow 2NO_2^- + H^+ \qquad (8)$$

This raises doubts regarding the contribution of N_2O_3 or other nitrosonium ion (NO^+) donors[5c] (such as N_2O_4 or a peroxy isomer of N_2O_5) to the overall yields of nitrosation of MorH by peroxynitrite under alkaline conditions.[39]

(B) In the Presence of Purposefully Added Carbonate. Scheme 2 shows the reactions that can occur in the peroxynitrite/MorH system in the presence of purposefully added carbonate. We suggest that nitration and nitrosation occur as a result of the complex interplay of the free radical species $CO_3^{\bullet-}$, $^\bullet NO_2$, •NO, and $O_2^{\bullet-}$, species which are formed in reactions 2−5.[5c,23,30−34] Nitration occurs when the radicals $CO_3^{\bullet-}$ and $^\bullet NO_2$ react with MorH. Since $CO_3^{\bullet-}$ is a more potent oxidant [$E°(CO_3^{\bullet-}/CO_3^{2-}) = 1.5$ V] than is $^\bullet NO_2$ [$E°(^\bullet NO_2/NO_2^-) = 1.04$ V],[40] nitration can involve an initial H-atom abstraction (or one-electron oxidation) from MorH by $CO_3^{\bullet-}$,[41] followed by the combination of Mor• with $^\bullet NO_2$ (Scheme 2).[42] Nitrosation occurs from the trapping of Mor• by •NO in competition with $^\bullet NO_2$ (Scheme 2).

Another possible mechanism for nitrosation by peroxynitrite/CO_2 involves the reaction of $CO_3^{\bullet-}$ with •NO (eqs 9 and 10) and the subsequent NO^+ transfer[5c] from O═N−O−CO_2^- to MorH (eq 11).

$$^\bullet NO + CO_3^{\bullet-} \rightarrow \text{O═N−O−}CO_2^- \qquad (9)$$

$$\text{O═N−O−}CO_2^- \rightarrow NO_2^- + CO_2 \qquad (10)$$

$$\text{MorH} + \text{O═N−O−}CO_2^- \rightarrow \text{Mor-N═O} + CO_3^{2-} + H^+ \qquad (11)$$

The reaction of •NO with $CO_3^{\bullet-}$ was studied in detail by Czapski et al.,[43] who find that the overall rate constant for reactions 9 and 10 is about 3.5×10^9 M^{-1} s^{-1}. This shows that the intermediate O═N−O−CO_2^-, if formed, would be extremely short-lived and incapable of nitrosating MorH (or any substrate) at the concentrations used in our experiments.[39]

Predictions Based on Free Radical Mechanisms and Their Experimental Validation. At pH 10.2, the yields of nitration are about 10-fold higher in reactions performed using high initial concentrations of CO_2 than in reactions performed with very low concentrations of CO_2 (Table 1). The yields of nitrosation, on the other hand, are much smaller in reactions performed using high CO_2 than in reactions performed with low CO_2 (Table 1). In both conditions, the H^+-catalyzed decomposition of peroxynitrite[21] (eq 1) and the autoxidation[3] of •NO (eq 12) are extremely slow; therefore, peroxynitrite reacts primarily with CO_2 and decomposes via the intermediate formation of $ONOOCO_2^-$ (eq 2).

$$2^\bullet NO + O_2 \rightarrow 2^\bullet NO_2 \qquad (12)$$

This means peroxynitrite gives comparable yields of $CO_3^{\bullet-}$ under both high and low concentrations of CO_2. If nitration and nitrosation of MorH by peroxynitrite/CO_2 proceed via a common radical intermediate Mor• (Scheme 2), the combined yields of nitration and nitrosation should be about the same under conditions of high and low concentrations of CO_2, as is observed (see Table 1).

The free radical mechanism in Scheme 2 is consistent with the high yields of Mor-N═O and low yields of Mor-NO_2 that we observe under alkaline conditions at low concentrations of

(36) Ross, A. B.; Mallard, W. G.; Helman, W. P.; Buxton, G. V.; Huie, R. E.; Neta, P. *NDRL-NIST Solution Kinetics Database*, Ver. 2.0; NIST: Gaithersburg, MD, 1994.

(37) Pfeiffer et al.[31a] postulated the formation of N_2O_3 to explain the high yields of NO_2^- and O_2 during the alkaline decomposition of peroxynitrite in the absence of added reactants, RH (in our system RH = MorH). The direct reaction of HO• with $ONOO^-$, as postulated by Pfeiffer et al., is less likely in the presence of RH and other reactive impurities (such as buffers and NO_2^-) because of competing reactions that allow for faster removal of HO• (Scheme 1). However, considering the recent evidence that $^\bullet NO_2$ and •NO can be formed (respectively) from the homolysis of ONOOH (eq 1) and $ONOO^-$ (eq 5)[23] and that the rates of R• reaction with •NO and $^\bullet NO_2$ are comparable to that of the •NO/$^\bullet NO_2$ reaction,[36] some N_2O_3 may be formed (eq 6) in reactions of peroxynitrite in the absence of purposefully added carbonate.

(38) (a) Caulfield, J. L.; Singh, S. P.; Wishnok, J. S.; Deen, W. M.; Tannenbaum, S. R. *J. Biol. Chem.* **1996**, *271*, 25859. (b) Lewis, R. S.; Tannenbaum, S. R.; Deen, W. M. *J. Am. Chem. Soc.* **1995**, *117*, 3933.

(39) One of the reviewers suggested using N_3^- to abate nitrosation by N_2O_3 and related NO^+ donors. We studied the effect of N_3^- (0−66 mM) on the yields of nitration and nitrosation in the peroxynitrite/CO_2/MorH system at pH 10.2. Our results show that N_3^- inhibits both nitration and nitrosation to a similar extent (0−70%), suggesting that N_3^- acts as a general free-radical quencher rather than as a specific scavenger of NO^+-carrier(s) in the peroxynitrite/CO_2/MorH system. This is consistent with our hypothesis that peroxynitrite-mediated oxidations are predominantly free radical processes.

(40) Stanbury, D. M. *Adv. Inorg. Chem.* **1989**, *33*, 69.

(41) The second-order rate constant for the reaction of $CO_3^{\bullet-}$ with MorH is not known. However, based on the reaction of $CO_3^{\bullet-}$ with piperidine, *N*-methylpiperidine, and 1,4-diazabicyclo[2, 2, 2]octane (Elango, T. P.; Ramakrishnan, V.; Vancheesan, S.; Kuriacose, J. C. *Tetrahedron* **1985**, *41*, 3837), we presume that this rate could be in the order of $(3-10) \times 10^6$ M^{-1} s^{-1} under the conditions employed in our assays.

(42) There also might be minor products (arising from β-scission or other processes) from the reaction of Mor• with O_2 that we could not observe in our RP-HPLC analysis of the products.

(43) Czapski, G.; Holcman, J.; Bielski, B. H. J. *J. Am. Chem. Soc.* **1994**, *116*, 11465.

CO_2 (Table 1). As a first approximation, we calculate that the formation of $^{\bullet}NO_2$ at low concentrations of CO_2 is limited by the rate at which CO_2 is replenished from HCO_3^- ($k_{HCO_3^-}[HCO_3^-] = (2 \times 10^{-4}\ s^{-1}) \times (0.016\ M) = 3.2 \times 10^{-6}\ M^{-1}\ s^{-1}$). In these reactions, $^{\bullet}NO_2$ and $CO_3^{\bullet-}$ are formed predominantly from the homolysis of $ONOOCO_2^-$ (eq 2), and, therefore, the rate of $^{\bullet}NO$ formation from the reaction of $CO_3^{\bullet-}$ with $ONOO^-$ (eqs 3 and 4) [23c,33] is about the same as or somewhat smaller than the rate of $^{\bullet}NO_2$ formation. A major source of $^{\bullet}NO$ under these alkaline conditions is the dissociation of ONOO− (eq 5); for example, at early stages of these reactions, the rate of $^{\bullet}NO$ production from the spontaneous dissociation of 2 mM peroxynitrite ($k_f[ONOO^-] = (0.017\ s^{-1}) \times (0.002\ M) = 3.4 \times 10^{-5}\ M\ s^{-1}$) is about 10 times larger than the rate of $^{\bullet}NO_2$ formation. This explains why nitrosation but not nitration is favored under alkaline conditions at low concentrations of CO_2 (Table 1).[44]

The pH profiles for nitration of MorH by peroxynitrite are bell-shaped both in the presence and in the absence of purposefully added carbonate (Figure 2, curves B and D). This pattern also is typical in several other reactions of peroxynitrite forming stable nitration products.[12−14] According to these free radical mechanisms (Schemes 1 and 2), the lower yields of nitration, in part, can be explained as due to scavenging of key intermediates (in the present case, Mor$^{\bullet}$ and $^{\bullet}NO_2$; eqs 13−15) by $^{\bullet}NO$ and $O_2^{\bullet-}$ formed from the homolysis of $ONOO^-$ (eq 5) and/or the reaction of $CO_3^{\bullet-}$ with $ONOO^-$ (eqs 3 and 4).

$$Mor^{\bullet} + {}^{\bullet}NO \rightarrow Mor\text{-}N{=}O \qquad (13)$$

$$Mor^{\bullet} + O_2^{\bullet-} \rightarrow Mor^- + O_2 \qquad (14)$$

$$^{\bullet}NO_2 + O_2^{\bullet-} \rightarrow O_2NOO^- \rightarrow NO_2^- + O_2 \qquad (15)$$

In fact, with increases in pH, both the homolysis of HOONO (eq 1) and the CO_2-catalyzed decomposition of peroxynitrite (eq 2), which are the major sources of HO$^{\bullet}$, $CO_3^{\bullet-}$, and $^{\bullet}NO_2$, become rate limiting, while the homolysis of $ONOO^-$, giving $^{\bullet}NO$ plus $O_2^{\bullet-}$ (eq 5), and the reaction of $CO_3^{\bullet-}$ with $ONOO^-$ (eq 3) become more favorable.[23,34] This, together with the fact that both $^{\bullet}NO$ and $^{\bullet}NO_2$ react with R$^{\bullet}$ at about the same rates,[36] explains why the yields of nitration but not nitrosation decrease at alkaline pH, giving a bell-shaped profile (Figure 2, curves B and D).

At high initial concentrations of CO_2, in contrast to MorH (Table 1), phenol[5c] and tyrosine (TyrH)[14c] give lower yields of nitration at pH >10. These reduced yields probably result from scavenging of $^{\bullet}NO_2$ by the phenolate ion (eq 16), resulting in poor coupling of the intermediate phenoxyl[5c] or tyrosinyl[14c] radicals (eq 17) with $^{\bullet}NO_2$ (eq 18). For example, a change in pH from 7.5 to 11.3 increases the rate of $^{\bullet}NO_2$/tyrosine reaction by about 100 times[45] without causing a major change in the reactivity of $CO_3^{\bullet-}$ toward the phenol substrate.[46] (The pK_a values of the hydroxyl in phenol and tyrosine are 9.9 and 10.1, respectively.)

$$Tyr^- + {}^{\bullet}NO_2 \rightarrow Tyr^{\bullet} + NO_2^- \qquad (16)$$

$$TyrH/Tyr^- + CO_3^{\bullet-} \rightarrow Tyr^{\bullet} + HCO_3^-/CO_3^{2-} \qquad (17)$$

$$Tyr^{\bullet} + {}^{\bullet}NO_2 \rightarrow 3\text{-}NO_2\text{-}Tyr \qquad (18)$$

Nitrogen dioxide does not react with either $MorH_2^+$ or MorH,[47] and this explains why the yields of Mor-NO_2 remain high under alkaline conditions at high initial concentrations of CO_2 (Table 1).

General Conclusions and Implications. Peroxynitrite and the products derived from its reaction with CO_2 can nitrate as well as nitrosate MorH over a wide range of pH in vitro. Both nitration and nitrosation can be explained by free radical pathways involving an initial H-atom abstraction (or one-electron oxidation) from MorH by HO$^{\bullet}$ or $CO_3^{\bullet-}$. Depending on the relative availability of $O_2^{\bullet-}$ (eq 5), $^{\bullet}NO$ (eqs 4 and 5) and $^{\bullet}NO_2$ (eqs 1 and 2), the Mor$^{\bullet}$ produced in these reactions can react further to give regenerated MorH (eq 14), Mor-N═O (eq 13), or Mor-NO_2 (Schemes 1 and 2). In addition, some MorH may be nitrosated by N_2O_3 (eq 7) formed from the reaction of $^{\bullet}NO_2$ with $^{\bullet}NO$ (eq 6).

These in vitro free radical mechanisms for peroxynitrite suggest how nitration and nitrosation may occur in vivo. In biological systems, $^{\bullet}NO$ produced from NOS is down-regulated by the reactions of $O_2^{\bullet-}$ and oxygenated heme proteins, giving peroxynitrite.[28,48] Peroxynitrite then isomerizes to NO_3^- (~70%) via the reaction[11a−c] with CO_2 with small but significant yields of free radicals $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (~30%) from the peroxynitrite/CO_2 reaction.[2c,27,30] (Since autoxidation of $^{\bullet}NO$ to $^{\bullet}NO_2$ is slow in biological systems,[3] the peroxynitrite/CO_2 reaction could be a source of $^{\bullet}NO_2$ in vivo.) Therefore, in vivo, the free radicals $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ in the presence of $^{\bullet}NO$ (from NOS) mediate nitration and nitrosation.

Nitrogen dioxide is a less potent oxidant than $CO_3^{\bullet-}$,[40] and most of the in vitro studies of peroxynitrite/CO_2 are performed using substrates (e.g., phenol and tyrosine) that $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ react with at different rates.[36] Generally, at pH 7.0−7.5, $CO_3^{\bullet-}$ reacts with RH at rates that are 10^2-10^3 times higher than those of RH with $^{\bullet}NO_2$;[36] however, the product R$^{\bullet}$ formed in either of these reactions reacts with $^{\bullet}NO_2$ at rates close to the diffusion limit.[45,49] Therefore, in vitro reactions by peroxynitrite/CO_2 at and around the neutral pH give mainly nitration products via the one-electron oxidation of RH by $CO_3^{\bullet-}$ followed by the reaction of R$^{\bullet}$ with $^{\bullet}NO_2$. However, in biological systems, where there is a continuous formation[35] of $^{\bullet}NO$ from NOS, $^{\bullet}NO$ and $^{\bullet}NO_2$ compete for reaction with R$^{\bullet}$, which can arise from autoxidation of RH as well as from the reaction of RH with $CO_3^{\bullet-}$. In vivo, in contrast with our in vitro system, sequential reactions of $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ are not necessary to form nitration products. In vivo, the yields of nitration and nitrosation products critically depend on the relative concentrations of $^{\bullet}NO_2$ and $^{\bullet}NO$.

Acknowledgment. This work was supported by Grant ES-06754 from the Environmental Health Sciences Institute of the National Institutes of Health. We thank Dr. Sara Goldstein of the Department of Physical Chemistry, The Hebrew University of Jerusalem, for helpful discussions.

JA000270H

(44) A reviewer suggested the addition of $^{\bullet}NO$ to alkaline peroxynitrite solutions to test if nitrosation is enhanced. We are not sure to what extent an experiment of this kind can lend support to our hypothesis that a mixture of $^{\bullet}NO$ and $ONOO^-$ (or, more correctly, $^{\bullet}NO$ plus $CO_3^{\bullet-}$) causes nitrosation in vivo. $^{\bullet}NO$ autoxidation generates nitrosating intermediates (like N_2O_3), and these can react directly with $ONOO^-$ (Goldstein, G.; Czapski, G.; Lind, J.; Merényi, G. *Chem. Res. Toxicol.* **1999**, *12*, 132). Thus, by adding $^{\bullet}NO$, one would introduce a peroxynitrite-independent pathway for nitrosation as well as potentially interfering cross reactions. The system the reviewer proposes is even more complex than our system, and finding increased nitrosation, as one would expect, could not be easily interpreted.

(45) Prutz, W. A.; Monig, H.; Butler, J.; Land, E. J. *Arch. Biochem. Biophys.* **1985**, *243*, 125.

(46) Chen, S.-N.; Hoffman, M. Z.; Parsons, G. H., Jr. *J. Phys. Chem.* **1975**, *79*, 1911.

(47) Goldstein, S.; Czapski, G. *J. Am. Chem. Soc.* **1996**, *118*, 3419.

(48) (a) Radi, R. *Chem. Res. Toxicol.* **1996**, *9*, 828. (b) Wade, R. S.; Castro, C. E. *Chem. Res. Toxicol.* **1996**, *9*, 1382.

(49) Elliot, A. J.; Simsons, A. S. *Can. J. Chem.* **1984**, *62*, 1831.