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Formation of Peroxynitrate from the Reaction of Peroxynitrite with CO_2: Evidence for Carbonate Radical Production

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Abstract: The decomposition of peroxynitrite in the presence of bicarbonate and various substrates was studied using the stopped-flow technique. We have shown that peroxynitrite ($ONOOH/ONOO^-$) reacts with excess of CO_2 to form peroxynitrate (O_2NOOH/O_2NOO^-) in aerated solutions containing HCO_2^-, H_2O_2, CH_3OH, or $(CH_3)_2CHOH$. The yield of peroxynitrate increased with the increase in these substrate concentrations approaching 30−33% of added peroxynitrite. Competition kinetics study yields rate constants which are similar to those determined directly for the reactions of these substrates with the carbonate radical anion. We therefore suggest that the formation of peroxynitrate takes place via the following steps: (i) the rapid reaction of peroxynitrite with CO_2 to form $ONOOCO_2^-$; (ii) the homolytic cleavage of 30−33% of $ONOOCO_2^-$ into $^{\bullet}NO_2$ and $CO_3^{\bullet -}$; (iii) the reaction of $CO_3^{\bullet -}$ with the various substrates, yielding ultimately superoxide in aerated solutions; and (iv) the fast reaction of superoxide with $^{\bullet}NO_2$ to form O_2NOO^-.

Introduction

Peroxynitrite ($ONOOH/ONOO^-$) is formed from the very fast reaction of superoxide with $^{\bullet}NO$ ($k = (4.3-6.7) \times 10^9\ M^{-1}\ s^{-1}$).[1,2] It is a powerful oxidant,[3−5] that can be formed by activated macrophages and neutrophils, and may be the major damaging species produced after cerebral and myocardial ischemia, inflammation, sepsis, and many other pathological conditions.[3]

Peroxynitrite is unstable in the presence of carbonate,[6,7] and low concentrations of bicarbonate were shown to protect *Escherichia coli* from the toxic effect of peroxynitrite.[8] Lymar and Hurst[9] were the first to show that $ONOO^-$ reacts rapidly with CO_2 ($k = 3 \times 10^4\ M^{-1}\ s^{-1}$), apparently forming an adduct whose composition is $ONOOCO_2^-$.[9−15]

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \qquad (1)$$

The concentrations of CO_2 in vivo are relatively high due to high levels of bicarbonate in intracellular (12 mM)[16] and interstitial fluids (30 mM).[16] This suggests that the reaction of peroxynitrite with CO_2 must be the predominant pathway for peroxynitrite disappearance in biological systems.[9−15,17] Therefore, the chemical properties of this adduct are of great importance.

Recent studies have demonstrated that bicarbonate enhances peroxynitrite-mediated nitration of aromatics,[10−12,18,19] DNA,[20] and ethylacetoacetate.[13] It partially or completely inhibited the oxidation of various compounds by peroxynitrite, e.g., dimethyl sulfoxide,[18] benzoate,[18] glutathione,[15,18] Ni(II)cyclam,[21] but had hardly any effect on the oxidation yield of ferrocyanide and ABTS (2,2′-azinobis(3-ethyl-1,2-dihydrobenzothiazoline 6-sulfonate).[21]

It has been suggested that $ONOOCO_2^-$ can be a source for several reactive intermediates including the carbonate radical/nitrogen dioxide radical pair, which may be formed via the homolytic cleavage of the peroxo O−O bond of $ONOOCO_2^-$,[9−15,17−19] the nitronium ion/carbonate ion pair, which may be formed via the heterolytic cleavage of the peroxo O−O bond of $ONOOCO_2^-$,[10,12−15,18,19] or the nitrocarbonate anion ($O_2NOCO_2^-$), which may be formed via the isomerization of $ONOOCO_2^-$.[10,13−15,19] We have recently shown that the amount of peroxynitrite available for the oxidation of various substrates in the presence of excess CO_2 over peroxynitrite does not exceed

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33%.[21] Therefore, we have suggested that 30−33% of $ONOOCO_2^-$ decomposes into an intermediate, X, which either forms nitrate and bicarbonate or oxidizes/nitrates the substrate. Our data was not sufficient for the identification of X. We suggested that X may be the couple $^{\bullet}NO_2$ and $CO_3^{\bullet-}$, as both radicals are capable of oxidizing ferrocyanide, ABTS and Ni(II)cyclam.[21,22] The identity of X as the couple NO_2^+ and CO_3^{2-} was ruled out because the half-life of NO_2^+ in water is 1.4 ns,[23] which is far too short to be scavenged by the various substrates.[11−15,18−21]

In this study, the reaction of peroxynitrite with excess of CO_2 was investigated in the presence of HCO_2^-, H_2O_2, methanol, 2-propanol, and DTPA (diethylenetriaminepentaacetic acid). We will demonstrate that in the presence of these substrates up to 30−33% of added peroxynitrite is converted into peroxynitrate, and will show that X is the couple $^{\bullet}NO_2$ and $CO_3^{\bullet-}$.

Experimental Section

Chemicals. Peroxynitrite was synthesized by the reaction of nitrite with acidified H_2O_2 at room temperature. Syringe pump (Model SP 230IW) was used to inject 0.606 M nitrite and 0.60 M H_2O_2 in 0.7 M $HClO_4$ through four tangential inlets of the first mixing chamber, and the combined solutions flowed through a short connector to the second mixing chamber where 3.6 M NaOH was pushed at the same flow rate (45 mL/min) through four inlets to quench the reaction.[24] The yield of peroxynitrite was 85% (0.2 M) as determined by measuring the absorption at 302 nm using $\epsilon = 1670\ M^{-1}\ cm^{-1}$,[25] and the stock solution contained about 2.5% nitrite and 1.5% H_2O_2.[24]

Kinetic Measurements. Kinetic measurements were carried out using the Bio SX-17MV Sequential Stopped-Flow from Applied Photophysics with a 1-cm long mixing cell. The reaction of peroxynitrite with CO_2 was followed at 290 or 302 nm by mixing equal volumes of alkaline peroxynitrite with 0.2 M phosphate buffer containing bicarbonate and other reactants. Bicarbonate was added as a sodium salt to the buffer solutions 2−5 min before mixing to allow the equilibration of the various carbonated species. The concentration of CO_2 in the buffer solutions was determined by measuring the pH of these solutions and using $pK = 6.2$ ($I = 0.25$ M) for the equilibrium $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$.[26] Under all our experimental conditions, the reaction of peroxynitrite with CO_2 was faster than the hydration of CO_2.[26,27] Therefore, the concentration of CO_2, immediately after mixing, was half of that at equilibrium before mixing. Peroxynitrite concentration was measured before mixing by transferring a portion from the syringe into a cuvette and measuring the absorption at 302 nm. The pH of the mixture was measured at the outlet of the flow system. Each run was repeated for at least five times. The kinetic measurements were carried out at 21.7 ± 0.1 °C.

Results and Discussion

The reaction of peroxynitrite with CO_2 was studied under limiting concentrations of peroxynitrite at pH 4.7−9.3 in the presence of 0.1 M acetate or phosphate buffers. The decay of peroxynitrite in the presence of excess of CO_2 was first order, and k_{obs} was linearly dependent upon $[CO_2]$ and highly dependent upon pH. We determined $k_1 = (2.3 \pm 0.2) \times 10^4\ M^{-1}\ s^{-1}$ using $pK_a = 6.8$ for ONOOH[3,4] and $pK = 6.2$ for the hydration of CO_2[26] (data not shown). Our value is somewhat lower than that determined by Lymar and Hurst ($k_1 = 3 \times 10^4\ M^{-1}\ s^{-1}$)[9] due to different methods of calculations.[28]

Table 1. k_{obs} of the Decay of Peroxynitrite in the Absence and Presence of Formate Ions[a]

pH	[PN], M	$[HCO_2^-]$, M	λ, nm	ΔOD	k_{obs}, s^{-1}
4.1	2.2×10^{-4}		280	0.059	0.79
4.1	2.2×10^{-4}	0.48	280	0.061	0.83
4.5	2.1×10^{-4}		280	0.056	0.82
4.5	2.1×10^{-4}	0.42	280	0.058	0.86
5.0	2.0×10^{-4}		280	0.057	0.81
5.0	2.0×10^{-4}	0.25	280	0.054	0.81
6.3	3.6×10^{-4}		302	0.21	0.76
6.3	3.4×10^{-4}	0.25	302	0.20	0.73
7.0	3.4×10^{-4}		302	0.39	0.46
7.0	3.2×10^{-4}	0.25	302	0.39	0.53
7.0	1.3×10^{-4}	0.40	302	0.14	0.63
8.3	2.8×10^{-4}		302	0.41	0.046
8.3	1.2×10^{-4}	0.50	302	0.19	0.86
8.3	1.2×10^{-4}	1.0	302	0.20	0.94

[a] All solutions were air saturated and contained 0.1 M acetate or phosphate buffer.

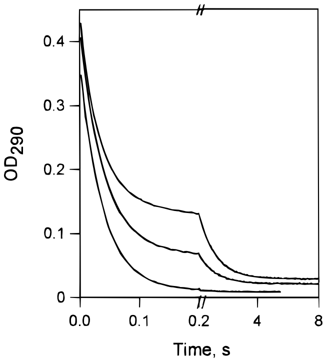

Figure 1. The decay of peroxynitrite in the presence of 1.2 mM CO_2 in solutions containing 0.1 M phosphate at pH 7.7. Lower curve: 0.26 mM peroxynitrite and no formate; middle curve: 0.28 mM peroxynitrite and 5 mM formate; upper curve: 0.29 mM peroxynitrite and 0.2 M formate.

The Formate System. It has been previously demonstrated,[3] and confirmed in this study (Table 1), that formate enhances the rate of the self-decomposition of peroxynitrite at pH > 7.[3] The mechanism of the reaction is complex and is strongly affected by the pH and by the presence of oxygen (data not shown). Peroxynitrite reacts relatively slowly with formate (Table 1), and, therefore, the contribution of this reaction to the decomposition of peroxynitrite in the presence of bicarbonate can be ignored as reaction 1 predominates.

The effect of up to 0.5 M formate on the decomposition of peroxynitrite in the presence of excess of CO_2 over peroxynitrite was studied at pH 4.8−9.5, where peroxynitrite decayed via two sequential first-order reactions. Typical kinetic traces at two concentrations of formate are given in Figure 1.

The observed rate constant of the first fast decay was linearly dependent upon $[CO_2]$, independent of $[HCO_2^-]$ and [phosphate], and decreased with the decrease in pH, suggesting that $ONOO^-$ is the reactive form. As ONOOH does not react with CO_2,[9,10] we determined the rate constant of the reaction of $ONOO^-$ with CO_2 in the presence of formate by plotting $k_{obs}(K_a + [H^+])/K_a$ as a function of $[CO_2]$ at several pH's using the value of 6.8 for the pK_a of ONOOH[4] (Figure 2). From the slope of the line we determined $k_1 = (2.2 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$, which is in excellent agreement with that determined in

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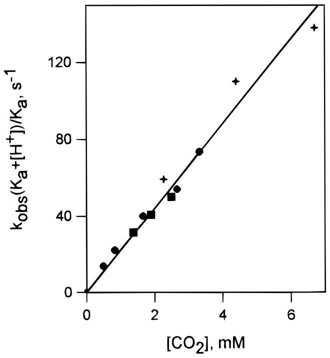

Figure 2. $k_{obs}(K_a + [H^+])/K_a$ of the first decay as a function of $[CO_2]$. (+) pH 6.27, 0.33 mM peroxynitrite, 0.25 M formate; (■) pH 7.13, 0.37 mM peroxynitrite, 0.01−0.25 M formate; (●) pH 8.35, 0.28 mM peroxynitrite, 0.2 M formate. All solutions contained 0.1 M phosphate buffer.

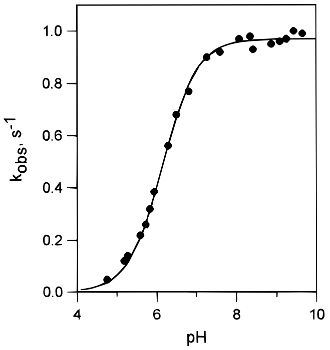

Figure 3. k_{obs} of the second decay as a function of pH. Solutions contained 0.3 mM peroxynitrite, 1 mM CO_2, 0.2−0.25 M formate and 0.1 M phosphate. The solid line is the best fit calculated using eq 2 with $k_a = 0$, $k_b = 0.97$ s^{-1}, and p$K_a = 6.15$.

the absence of formate, suggesting that the pK_a of ONOOH is unaffected by the presence of formate.

The observed first-order rate constant of the second slow decay was independent of [peroxynitrite], $[CO_2]$, $[HCO_2^-]$ and [phosphate] but decreased with the decrease in pH (Figure 3). The observed rate constant of the second decay is given by eq 2, where k_a and k_b are the rate constants of the decay of the acid and anion forms, respectively:

$$k_{obs} = (k_a[H^+] + k_bK_a)([H^+] + K_a) \qquad (2)$$

The best fit of eq 2 to the experimental results given in Figure 3 was obtained with $k_a = 0$, $k_b = 0.97 \pm 0.05$ s^{-1}, and p$K_a = 6.15 \pm 0.05$. The contribution of the second decay to the whole process increased with the increase in $[HCO_2^-]$ and leveled off at all pH's at $[HCO_2^-] > 0.15$ M. In the presence of 0.2−0.25 M formate, the contribution of the second decay was highly dependent upon pH (Figure 4). The best fit of eq 2 to the experimental results given in Figure 4, where $\Delta\epsilon_{290}$ replaces k ($\Delta\epsilon_{290} = \Delta OD_{290}/[\text{peroxynitrite}]_o$), was obtained with $\Delta\epsilon_a = 0$, $\Delta\epsilon_b = 440 \pm 30$ M^{-1}cm^{-1}, and p$K_a = 6.15 \pm 0.05$. The spectrum of $ONOO^-$, as measured immediately after the mixing, differs than that measured at the end of the first decay (Figure 5).

We conclude that a new species with a p$K_a = 6.15 \pm 0.05$ is formed at the end of the reaction of $ONOO^-$ with CO_2 in the presence of formate. The acid form of this species is relatively stable and does not absorb at 290 nm, whereas the anion form has a maximum absorption at 290 nm, and it decays with $k_b = 0.97 \pm 0.05$ s^{-1}. We have recently shown that only 30−33% of added peroxynitrite is available for the oxidation of ferrocyanide, Ni(II)cyclam, and ABTS in the presence of excess of CO_2 over peroxynitrite.[21] Therefore, we assume that 30−33% of $ONOOCO_2^-$ is also available for the oxidation of formate, and the extinction coefficient of the species formed at the end of the first decay should be 3-fold the value determined in alkali pH's (440 ± 30 M^{-1} cm^{-1}, Figure 4), i.e., $\epsilon_{290} = 1400 \pm 150$ M^{-1} cm^{-1}. All these features strongly suggest that in the presence of sufficient concentrations of formate, 30−33% of peroxynitrite is converted to peroxynitrate as the properties of the latter species are the following: *(i)* The pK_a of O_2NOOH has been determined to be 5.9 ± 0.1.[29,30] *(ii)* The rate constants of the decay of O_2NOOH and O_2NOO^- have been determined to be 7×10^{-4} s^{-1} [28] and 1.0 s^{-1},[29,30] respectively. *(iii)* O_2NOOH does not have any appreciable absorption above 280 nm,[29,31] whereas the maximum absorption of O_2NOO^- is at 285−290 nm ($\epsilon = 1500$−1650 M^{-1} cm^{-1}).[29,30]

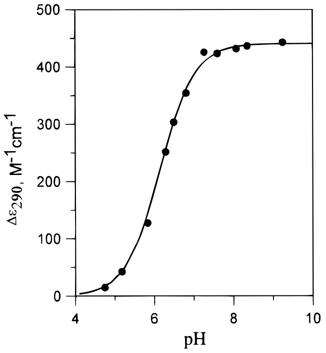

Figure 4. $\Delta\epsilon_{290}$ of the second decay as a function of pH. Solutions contained 0.3 mM peroxynitrite, 1 mM CO_2, 0.2−0.25 M formate, and 0.1 M phosphate. The solid line is the best fit calculated using eq 2 ($\Delta\epsilon$ replaces k) with $\Delta\epsilon_a = 0$, $\Delta\epsilon_b = 440$ M^{-1} cm^{-1}, and p$K_a = 6.15$.

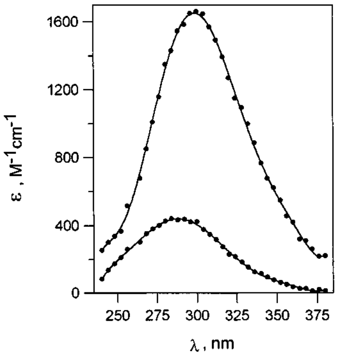

Figure 5. The spectrum obtained immediately and 0.1 s after mixing. The final solution contained 0.36 mM peroxynitrite, 1.6 mM CO_2, 0.5 M formate, and 0.1 M phosphate at pH 8.44.

The H_2O_2 System. The decomposition of 0.26 mM peroxynitrite in the presence of 1.2 mM CO_2 and 0.047−66 mM H_2O_2 was studied at pH 7.7 (0.1 M phosphate). Under these conditions, peroxynitrite decayed via two sequential first-order reactions as in the presence of formate. The rate of the first decay was independent of $[H_2O_2]$ with $k_{obs} = 27 \pm 0.2$ s^{-1}, which correlates well with $k_1 = (2.3 \pm 0.2) \times 10^4$ M^{-1} s^{-1}. The rate constant of the second decay was determined to be 0.9 ± 0.03, independent of $[H_2O_2]$, in agreement with the value determined in the presence of formate at pH 7.7 (Figure 3). The contribution of the second decay increased with increasing

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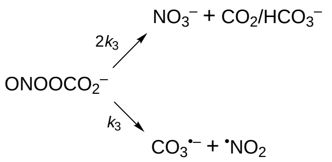
$[H_2O_2]$ and leveled off at $[H_2O_2] > 70$ mM, where $\Delta\epsilon_{290} = 460 \pm 10\ M^{-1}cm^{-1}$, which corresponds to 30−33% yield of peroxynitrate, similarly to the case of formate.

The Alcohol System. The decay of peroxynitrite was followed under identical conditions to those described for the H_2O_2 system but in the presence of up to 5 M methanol or 2-propanol. As in the former cases, the decay of peroxynitrite followed two sequential first-order reactions with the same rate constants as in the presence of H_2O_2. We found that the yield of peroxynitrate in the presence of high concentrations of these alcohols approached 30−33% of added peroxynitrite. Under anaerobic conditions, only the first decay was observed.

The DTPA System. DTPA is usually added in most peroxynitrite studies to eliminate the possible catalysis of the decomposition of peroxynitrite by traces of metal impurities.[4] We avoided the use of DTPA because it can be oxidized by the intermediates formed during the decomposition of $ONOOCO_2^-$. Indeed, the decay of 0.26 mM peroxynitrite in the presence of 1.2 mM CO_2 and various concentrations of DTPA at pH 7.7 (0.1 M phosphate) followed two sequential first-order reactions as in the presence of HCO_2^-, H_2O_2, methanol, and 2-propanol. The contribution of the second process in the presence of 0.1 and 2 mM DTPA was 3% and 15% of added peroxynitrite, respectively.

Mechanism and Competition Kinetics. We have shown that the reaction of peroxynitrite with excess of CO_2 in aerated solutions containing formate, H_2O_2, methanol, and 2-propanol yields peroxynitrate. We have previously demonstrated that only 30−33% of $ONOOCO_2^{\bullet-}$ is capable of oxidizing the substrates, and we will now show that 30−33% of $ONOOCO_2^-$ is converted into $^{\bullet}NO_2$ and $CO_3^{\bullet-}$. We suggest the following mechanism:



(3)

$$CO_3^{\bullet-} + {}^{\bullet}NO_2 \rightarrow CO_2 + NO_3^- \qquad (4)$$
$$k_4 = 1.0 \times 10^9\ M^{-1}\ s^{-1\ 22}$$

In the presence of formate reactions 5 and 6 take place:

$$CO_3^{\bullet-} + HCO_2^- \rightarrow CO_2^{\bullet-} + HCO_3^- \qquad (5)$$
$$k_5 = (1.1-1.6) \times 10^5\ M^{-1}\ s^{-1}\ (I \rightarrow 0)\ ^{22}$$

$$CO_2^{\bullet-} + O_2 \rightarrow CO_2 + O_2^{\bullet-} \qquad (6)$$
$$k_6 = (2.0-4.2) \times 10^9\ M^{-1}\ s^{-1\ 22}$$

In the presence of H_2O_2 reactions 7 and 8 take place:

$$H_2O_2 + CO_3^{\bullet-} \rightarrow HCO_3^- + HO_2^{\bullet} \qquad (7)$$
$$k_7 = 8.0 \times 10^5\ M^{-1}\ s^{-1\ 22}$$

$$HO_2^{\bullet} \rightleftharpoons H^+ + O_2^{\bullet-} \qquad (8)$$
$$pK = 4.8\ ^{32}$$

In the presence of methanol and 2-propanol (ROH) reactions 9−11 take place:

$$CO_3^{\bullet-} + ROH \rightarrow R^{\bullet} + HCO_3^- \qquad (9)$$
$$k_9(MeOH) = 5.0 \times 10^3\ M^{-1}\ s^{-1}$$
$$k_9(2\text{-}PrOH) = (3.9-5.0) \times 10^4\ M^{-1}\ s^{-1\ 22}$$

$$R^{\bullet} + O_2 \rightarrow RO_2^{\bullet} \qquad (10)$$
$$k_{10} = (3.5-4.9) \times 10^9\ M^{-1}\ s^{1\ 22}$$

$$RO_2^{\bullet} \rightarrow R^+ + O_2^{\bullet-} \qquad (11)$$
$$k_{11}(MeOH) < 3\ s^{-1}$$
$$k_{11}(2\text{-}PrOH) = 700-960\ s^{-1\ 22}$$

In the presence of DTPA, reactions 12 and 13 most probably take place, though the yield of superoxide may be lower than 100%:[33]

$$CO_3^{\bullet-} + DTPA \rightarrow CO_3^{2-} + DTPA^{\bullet} \qquad (12)$$
$$k_{12} = 1.7 \times 10^7\ M^{-1}\ s^{-1}\ \text{at pH } 11^{22}$$

$$DTPA^{\bullet} + O_2 \rightarrow O_2^{\bullet-} + \text{products} \qquad (13)$$

The reaction sequences 5−6, 7−8, 9−11, or 12−13 are followed by reactions 14−17:

$$O_2^{\bullet-} + {}^{\bullet}NO_2 \rightarrow O_2NOO^- \qquad (14)$$
$$k_{14} = 4.5 \times 10^9\ M^{-1}\ s^{-1\ 29}$$

$${}^{\bullet}NO_2 + {}^{\bullet}NO_2 \rightleftharpoons N_2O_4 \qquad (15)$$
$$k_{15} = 4.5 \times 10^8\ M^{-1}\ s^{-1} \qquad k_{-15} = 6.9 \times 10^3\ s^{-1\ 34}$$

$$N_2O_4 + H_2O \rightarrow NO_3^- + NO_2^- + 2H^+ \qquad (16)$$
$$k_{16} = 1.0 \times 10^3\ s^{-1\ 34}$$

$$O_2NOO^- \rightarrow NO_2^- + O_2 \qquad (17)$$
$$k_{17} = 1.0\ s^{-1\ 29,30}$$

The rate constants of the reactions of $^{\bullet}NO_2$ with formate and H_2O_2 have not been determined. However, since the rate constants of the reactions of $ClO_2^{\bullet}$ with HCO_2^- and H_2O_2 are lower than 0.01 and 4 $M^{-1}\ s^{-1}$, respectively,[22] it is expected that the rate constants of the reactions of $^{\bullet}NO_2$ with HCO_2^- and H_2O_2 will not exceed these values,[35] and, therefore, these reactions will not compete with superoxide for $^{\bullet}NO_2$. It is also important to note that reaction 11 is catalyzed by HPO_4^{2-} and OH^-,[22] and, therefore, superoxide is formed rapidly in the presence of 0.1 M phosphate at pH 7.7. The results in the presence of DTPA indicates that reaction 13 is also catalyzed by HPO_4^{2-}.

According to our suggested mechanism, if HCO_2^-, H_2O_2, or alcohol (RH) compete with $^{\bullet}NO_2$ for $CO_3^{\bullet-}$, peroxynitrate will subsequently be formed. We found that the yield of peroxynitrate as measured at 290 nm increased with increasing [RH]. If

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Table 2. Comparison of the Rate Constants Determined Directly with Those Determined from the Slope of the Lines in Figure 6 for the Reaction of $CO_3^{\bullet -}$ with the Various Substrates

RH	$k(CO_3^{\bullet -} + RH)$, $M^{-1}\ s^{-1}$ [a]	slope = $k_4[^\bullet NO_2]/k$, M^{-1} [b] (Figure 6)	$k \times$ slope, s^{-1}
H_2O_2	$k_{12} = 8.0 \times 10^5$	1.9×10^{-3}	1.5×10^3
HCO_2^-	$k_4 = (1.1-1.6) \times 10^5\ (I \rightarrow 0)$ $(2.2-3.2) \times 10^5\ (I \sim 0.28\ M)$ [c]	5.7×10^{-3}	$(1.3-1.8) \times 10^3$
2-PrOH	$k_{15}(\text{2-PrOH}) = (3.9-5.0) \times 10^4$	2.2×10^{-2}	$(0.9-1.1) \times 10^3$
CH_3OH	$k_{15}(\text{MeOH}) = 5.0 \times 10^3$	0.47	2.3×10^3

[a] These rate constants were determined directly and were taken from ref 21. [b] All solutions contained 0.29−0.31 mM peroxynitrite, 1.2 mM CO_2 and 0.1 M phosphate at pH 7.7. [c] Under our experimental conditions the ionic strength exceeds 0.28 M.

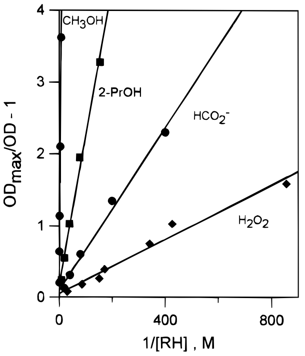

Figure 6. $OD_{max}/OD - 1$ as measured at 290 nm versus 1/[RH] for RH = H_2O_2, HCO_2^-, 2-PrOH, and CH_3OH. All solutions contained 0.29−0.31 mM peroxynitrite, 1.2 mM CO_2, and 0.1 M phosphate at pH 7.7.

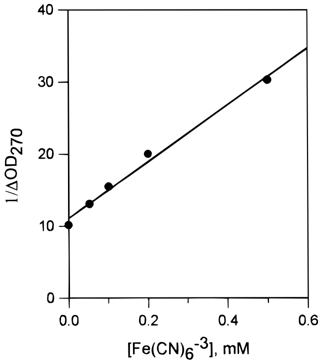

Figure 7. The dependence of $1/\Delta OD_{270}$ of the second decay on ferricyanide concentration. Solutions contained 0.28 mM peroxynitrite, 1.2 mM CO_2, 0.2 M HCO_2^-, and 0.1 M phosphate at pH 8.1.

RH competes with $^\bullet NO_2$ for $CO_3^{\bullet -}$, eq 18 will be obtained, where $k(RH + CO_3^{\bullet -})[RH] = k_5[HCO_2^-]$ or $k_7[H_2O_2]$ or $k_9[ROH]$:

$$\frac{OD_{max}}{OD} = 1 + \frac{k_4[^\bullet NO_2]}{k(RH + CO_3^{\bullet -})[RH]} \qquad (18)$$

Plots of $OD_{max}/OD - 1$ as a function of 1/[RH] under identical experimental conditions yielded straight lines with intercepts close to zero (Figure 6). The various slopes obtained for formate, H_2O_2, methanol, and 2-propanol from Figure 6, and the literature values for the rate constants of $CO_3^{\bullet -}$ with these substrates are summarized in Table 2. As the slope of these lines equals $k_4[^\bullet NO_2]/k(RH + CO_3^{\bullet -})$, the last entry in Table 2 (slope × $k(RH + CO_3^{\bullet -})$) is similar for the various RH, suggesting that the formation of peroxynitrate in all four systems is initiated by the reaction of RH with $CO_3^{\bullet -}$.

If the conversion of peroxynitrite to peroxynitrate takes place through the homolysis of $ONOOCO_2^-$ into $^\bullet NO_2$ and $CO_3^{\bullet -}$, the addition of ferricyanide should lower the yield of O_2NOO^- in the case of the formate, as it would compete with oxygen for $CO_2^{\bullet -}$ (reaction 19).

$$Fe(CN)_6^{3-} + CO_2^{\bullet -} \rightarrow Fe(CN)_6^{4-} + CO_2 \qquad (19)$$
$$k_{19} = 1.1 \times 10^9\ M^{-1}\ s^{-1}\ (I = 0.3\ M)^{22}$$

When a deaerated solution of 0.56 mM peroxynitrite at pH 12 was mixed with an aerated solution containing 2.4 mM CO_2, 0.4 M HCO_2^-, 0.2 M phosphate, and 0.105−1 mM $Fe(CN)_6^{3-}$ (the final pH was 8.1), the yield of O_2NOO^- decreased with the increase in ferricyanide concentration. The yield of peroxynitrate was measured at 270 nm, where the absorption of ferricyanide is relatively low, and is given by eq 20, where ΔOD_o is the absorption measured in the absence of ferricyanide:

$$\Delta OD = \Delta OD_o k_6[O_2](k_6[O_2] + k_{19}[Fe(CN)_6^{3-}]) \qquad (20)$$

A plot of $1/\Delta OD_{270}$ as a function of $[Fe(CN)_6^{3-}]$ yields a straight line with *intercept/slope* = $k_6[O_2]/k_{19} = (2.8 \pm 0.4) \times 10^{-4}$ M (Figure 7). As $[O_2]$ = 0.12 mM, it is calculated that $k_6/k_{19} = 2.3 \pm 0.3$, which is in agreement with the literature values.[22] The same effect is expected in the case of methanol and 2-propanol as the radicals derived from these substrates react rapidly with ferricyanide.[22]

We conclude that the competition kinetics study shows that 30−33% of $ONOOCO_2^-$ decomposes via the homolytic cleavage of the peroxo O−O bond into $CO_3^{\bullet -}$ and $^\bullet NO_2$.

Lymar and Hurst[9] synthesized peroxynitrite through the reaction of nitrite with acidified hydrogen peroxide. They destroyed the excess of H_2O_2 with MnO_2 and did not add DTPA. Therefore, it is expected that in their system peroxynitrite in the presence of excess of CO_2 would decay via a single exponent. Denicola et al.[18] synthesized peroxynitrite by the same method and used 0.1 mM DTPA in their kinetic measurements. However, they reported only on a single first-order decay of peroxynitrite. It is possible that they did not look at a longer time scale of seconds or that their preparation contained relatively high concentrations of nitrite, which might compete with DTPA for $CO_3^{\bullet -}$.[22] Uppu et al.[10] synthesized peroxynitrite by the ozonation of 0.1 M azide at pH 12 and reported only on a single first-order decay of peroxynitrite in the presence of 0.1 mM DTPA and excess of CO_2. Therefore, we have to assume that either they did not look at a longer time scale of seconds or that their solutions are contaminated with azide. The latter may compete with DTPA for the carbonate radical, and superoxide will not be formed. To test this hypothesis, we added azide to the mixture containing 0.26 mM peroxynitrite, 1.2 mM CO_2, and 0.1 mM DTPA at pH 7.7 (0.1 M phosphate buffer). Under these conditions, the contribution of the second decay in the absence of azide was ca. 3% of added peroxynitrite, and it diminished completely in the presence of $[N_3^-] > 0.1$

mM. We conclude that the use of relatively high [DTPA] as chelator for traces of metal impurities should be avoided in these kind of experiments.

Conclusions

We have demonstrated that the decomposition of peroxynitrite in the presence of excess of CO_2 and various organic and inorganic solutes yields up to 30−33% peroxynitrate. The competition kinetics study shows that the formation of peroxynitrate is initiated by the reaction of $CO_3^{\bullet-}$ with these solutes. Therefore we conclude that 30−33% of $ONOOCO_2^-$ decomposes into $CO_3^{\bullet-}$ and $^{\bullet}NO_2$. The chemical properties and reactivity of peroxynitrate is not well understood as it is difficult to prepare it in aqueous solutions.[29−31] This paper describes a new method for the preparation of the relatively stable O_2NOOH ($pK_a = 5.9$, $\tau_{1/2} \sim 30$ min)[29−31] via the reaction of peroxynitrite with CO_2 in the presence of H_2O_2, formate, or 2-PrOH, which may enable a further study on this interesting compound. The optimal conditions are around pH 6, where the concentration of CO_2 is relatively high ($pK = 6.2$), and the half-life of peroxynitrate is longer than 1 s. In the presence of 0.1 M H_2O_2, 0.2 M formate, or 2 M 2-PrOH, about 30% of peroxynitrite will be converted into peroxynitrate, and the latter can be efficiently quenched with strong acid.

It has been previously suggested that the reaction of peroxynitrite with CO_2 must be the predominant pathway for peroxynitrite disappearance in many biological systems.[9,17] Therefore, we suggest that the reaction of peroxynitrite with CO_2 is a plausible pathway for the formation of peroxynitrate in biological systems, which contain high concentrations of various solutes, which scavenge $CO_3^{\bullet-}$ and yield ultimately superoxide (e.g., DNA, glucose). This reaction can take place if peroxynitrite is formed in the absence of relatively high concentrations of $^{\bullet}NO$ and SOD as theses compounds will compete with $^{\bullet}NO_2$ for superoxide. Thus, bicarbonate will be protective or enhance the toxicity of peroxynitrite depending on the chemical properties and reactivity of peroxynitrate.

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