The Effect of Bicarbonate on Oxidation by Peroxynitrite: Implication for Its Biological Activity

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The effect of bicarbonate on the oxidation rates and yields of the reaction of peroxynitrite with Ni^{II}(cyclam), ferrocyanide, and ABTS was studied using the stopped-flow technique. In the absence of bicarbonate, Ni^{II}(cyclam) is oxidized directly by peroxynitrous acid whereas ferrocyanide and ABTS are oxidized by intermediates that are formed during the self-decomposition of peroxynitrous acid. In the presence of sufficient concentrations of bicarbonate, the rate-determining step of these oxidation processes is the reaction of $ONOO^-$ with CO_2. The latter reaction results in the formation of an adduct $ONOOCO_2^-$, which is incapable of oxidizing directly these substrates. It is shown that about 33% of this adduct decomposes into highly oxidizing species, most probably into $^{\bullet}NO_2$ and $CO_3^{\bullet -}$ via the homolysis of the peroxo O−O bond. Under physiological conditions, where the most abundant species is $ONOO^-$ and the concentration of CO_2 exceeds 1 mM, the half-life of peroxynitrite is reduced, and consequently its diffusion distance is shortened. Therefore, indirect oxidation by peroxynitrite through intermediates that are formed during its self-decomposition will not take place *in vivo*, whereas direct oxidation by peroxynitrite may take place provided that the rate of this reaction competes efficiently with that of peroxynitrite with CO_2. We conclude that the toxicity of peroxynitrite *in vivo* is most probably governed by $ONOOCO_2^-$, which has a reactivity different from that of peroxynitrite.

Introduction

Peroxynitrous acid is an unstable and weak acid ($pK_a = 6.8$) that isomerizes to nitric acid ($1.3\ s^{-1}$ at 25 °C), whereas the anion is stable.[1] Peroxynitrite ($ONOOH/ONOO^-$) oxidizes a large variety of substrates including many biomolecules through complex mechanisms.[1,2] The oxidation may take place directly through the reaction of peroxynitrite with the substrate or through intermediates formed during the decomposition of peroxynitrous acid.[2]

Lymar and Hurst[3] have shown that $ONOO^-$ reacts rapidly with CO_2 to form the unstable nitrosoperoxycarbonate (reaction 1). They have suggested that reaction 1 must be the predominant pathway for peroxynitrite disappearance in biological systems as physiological concentrations of bicarbonate in intracellular and interstitial fluids are approximately 12 and 30 mM, respectively.[4]

$$ONOO^- + CO_2 \rightarrow ONOOCO_2^- \qquad k_1 = 3 \times 10^4\ M^{-1}\ s^{-1} \tag{1}$$

In the absence of other reactants, nitrate was found to be the major product of the reaction of CO_2 with peroxynitrite.[5] The adduct may act as a damaging species either by reacting directly with a substrate or through an intermediate formed during its decomposition. Recent studies have demonstrated that $ONOOCO_2^-$ participates in oxidation and nitration processes: (i) Bicarbonate significantly enhanced peroxynitrite-mediated nitration of aromatics,[6−8] DNA,[9] and ethyl acetoacetate.[10] (ii) It partially inhibited the oxidation by peroxynitrite of thiols,[7] dimethyl sulfoxide,[7] oxyhemoglobin,[7] and (cytochrome *c*)$^{2+}$,[7] and it had hardly any effect on the oxidation of ABTS (2,2′-azinobis(3-ethyl-1,2-dihydrobenzothiazoline-6-sulfonate))[5] and DNA.[9] (iii) It inhibited completely peroxynitrite-mediated hydroxylation of benzoate[7] and peroxynitrite-induced strand breakage in plasmid pBR322 DNA[9] and thymine−propenal formation from thymidine.[9]

In this study, the effect of bicarbonate on the kinetics and yields of the oxidation of Ni^{II}(cyclam), ferrocyanide, and ABTS was investigated in line with the above observations and the earlier studies on the oxidation of these substrates by peroxynitrite.[1,11−13]

Experimental Section

Chemicals. All chemicals used were of analytical grade and were used as received. Solutions were prepared with deionized water that was distilled and purified using a Milli-Q water purification system.

Peroxynitrite was synthesized in a quenched-flow system by the reaction of nitrite with acidified hydrogen peroxide. A syringe pump (Model SP 230IW) was used to inject 0.6 M nitrite and 0.606 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

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to the second mixing chamber where 3 M NaOH was pushed at the same flow rate through four inlets to quench the reaction. By varying the flow rates of the solutions and/or the delay volume between the two mixing chambers, we were able to control the time elapsed between starting the reaction and quenching it.[14] The yield of peroxynitrite was about 85% as determined by measuring the absorbance at 302 nm using $\epsilon = 1670\ M^{-1}\ cm^{-1}$.[15] These solutions contained about 1.5% nitrite, 2.5% H_2O_2, and 13.5% nitrate.[14] $Ni(cyclam)(ClO_4)_2$ was synthesized according to published procedures.[16]

Kinetic Measurements. Kinetic measurements were carried out using a Bio SX-17MV sequential stopped-flow apparatus from Applied Photophysics with a mixing time of less than 2 ms and a 1-cm-long mixing cell. The reactions of peroxynitrite with Ni^{II}(cyclam), ferrocyanide, and ABTS were followed at 360 nm ($\epsilon_{360} = 5100\ M^{-1}\ cm^{-1}$)[11], 420 nm ($\epsilon_{420} = 1000\ M^{-1}\ cm^{-1}$) and 660 nm ($\epsilon_{660} = 12000\ M^{-1}\ cm^{-1}$),[17] respectively, by mixing equal volumes of alkaline peroxynitrite with Ni^{II}(cyclam), ferrocyanide, or ABTS in 0.2 M phosphate or 0.2 M acetate buffers with and without bicarbonate. 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 assuming p$K = 6.2$ ($I = 0.25$ M) for the reaction $CO_2 + H_2O \rightleftharpoons HCO_3^- + H^+$.[18] Under all experimental conditions, the reaction of peroxynitrite with CO_2 was faster than CO_2 equilibration with HCO_3^-.[18,19] Therefore, the concentration of CO_2 immediately after mixing was half of that at equilibrium prior to mixing. Peroxynitrite concentration was measured before mixing by transferring a portion from the syringe into a cuvette and measuring the absorbance at 302 nm. To determine the effect of CO_2 contamination, the buffer solutions without bicarbonate were purged with argon for at least 30 min. The pH of the mixture was measured at the outlet of the flow system. Each run was repeated at least five times. The formation of the absorbance was fitted to first-order reaction kinetics, using a nonlinear regression program. The kinetic measurements were carried out at 21.7 ± 0.1 °C.

Results and Discussion

The Ni^{II}(cyclam) System. Previously,[11] we demonstrated that ONOOH rapidly oxidizes Ni^{II}(cyclam) to Ni^{III}(cyclam) in the presence of phosphate ions in a reaction that is first order in both reactants. The observed rate constant was determined to be $6.5 \times 10^4\ M^{-1}\ s^{-1}$, and it decreased with the increase in pH as $ONOO^-$ does not oxidize the metal complex.[11] The relatively rapid oxidation of Ni^{II}(cyclam) by peroxynitrite implies that the contamination of the buffer solutions with carbonate had no effect on the kinetics and yields in this system as the reaction of peroxynitrite with the metal complex is faster than reaction 1 under all of our previous experimental conditions ([Ni^{II}(cyclam)] = 0.5−3 mM, pH 5.5−8).[11] The stoichiometry of the reaction was found to be 1.69 ± 0.19 at pH 6.0−6.9, decreasing at higher pH's due to the decrease in [$H_2PO_4^-$], which is essential for stabilizing the trivalent complex.[11]

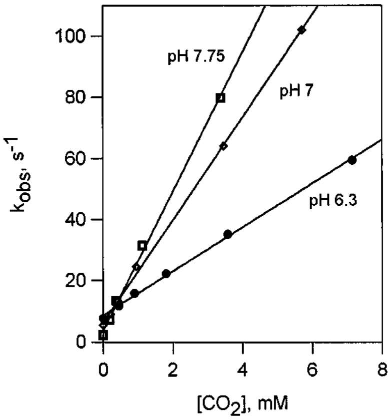

Figure 1. The observed rate constant of the formation of Ni^{III}(cyclam) as a function of [CO_2] at various pH's. The final mixtures contained 0.125−0.25 mM Ni^{II}(cyclam), 20−45 μM peroxynitrite and 0.1 M phosphate buffer.

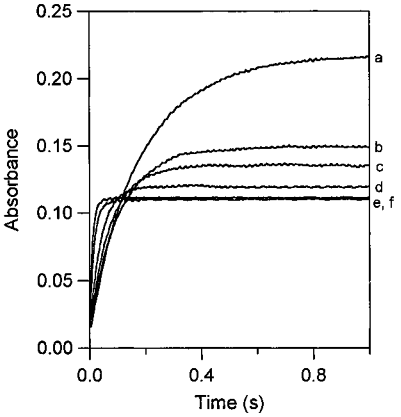

Figure 2. Kinetic traces recorded at 360 nm when 44 μM peroxynitrite in 0.01 M NaOH was mixed with 0.25 mM Ni^{II}(cyclam) in 0.2 M phosphate buffer at pH 6.8 in the absence and presence of various concentrations of bicarbonate. The pH measured at the outlet of the flow system was 7.0 ± 0.05. The final concentrations of CO_2 were (a) none, (b) 0.208 mM, (c) 0.416 mM, (d) 0.946 mM, (e) 3.45 mM, (f) 5.71 mM (f is faster than e).

The effect of bicarbonate was studied at pH 6.3, 7, and 7.75 (0.1 M phosphate buffer) in the presence of 0.125−0.25 mM Ni^{II}(cyclam), 20−45 μM peroxynitrite, and an excess of CO_2 over peroxynitrite. The rate of the formation of Ni^{III}(cyclam) increased linearly with [CO_2] (Figure 1). The observed second-order rate constants obtained from the slopes of the lines given in Figure 1 are $k = 7.19 \times 10^3$, 1.68×10^4, and $2.28 \times 10^4\ M^{-1}\ s^{-1}$ at pH 6.3, 7, and 7.75, respectively. The increase in k with the pH confirms the earlier conclusion that CO_2 reacts mainly with $ONOO^-$,[3] and therefore, $k_1 = k(K_a + [H^+])/K_a$. The best value obtained for k_1 from these three slopes was $(2.50 \pm 0.02) \times 10^4\ M^{-1}\ s^{-1}$ for p$K_a = 6.7$ (for p$K_a = 6.8$, $k_1 = (2.8 \pm 0.2 \times 10^4\ M^{-1}\ s^{-1}$).

The observed rate constant of the formation of Ni^{III}(cyclam) increased with bicarbonate concentration, while the yield of Ni^{III}(cyclam) decreased until it reached a plateau value (Figure 2). The yield of Ni^{III}(cyclam) at the plateau region was found to be [Ni^{III}(cyclam)]/[peroxynitrite]$_0$ = 0.64 ± 0.03 at pH 6.3 and 0.72 ± 0.04 at pH 7.0 as compared to 1.7 ± 0.2 in the absence of bicarbonate at both pH's.

The Ferrocyanide System. Previously,[11] we showed that the oxidation of ferrocyanide by peroxynitrite takes place via intermediates formed during the self-decomposition of peroxynitrous acid. We determined the stoichiometry of the reaction by rapidly vortexed equal volumes of peroxynitrite and ferrocyanide solutions. The stoichiometry of the reaction was about 0.8, independent of pH 5.5−9.4 and ferrocyanide concentrations

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(17) (a) Goldstein, S.; Czapski, G. *J. Am. Chem. Soc.* **1995**, *117*, 12078. (b) Upon irradiation of aerated solutions containing 0.5 M nitrate, 0.05 M nitrite, and 0.1−3 mM ABTS at pH 6.4 (2 mM phosphate buffer), the solvated electrons and hydroxyl radicals are converted into $^{\bullet}NO_2$, which subsequently reacts with ABTS to form $ABTS^+$. The spectrum of $ABTS^+$ was recorded in the presence of the various concentrations of ABTS. The absorption at 660 nm is almost unaffected by [ABTS] ($\epsilon_{660} = 12\,000 \pm 1000\ M^{-1}\ cm^{-1}$), whereas at [ABTS] > 0.5 mM, the maximum absorption at 414 nm is reduced and shifted toward 420 nm. In the presence of 0.1 mM ABTS, $\epsilon_{414}/\epsilon_{660} = 2.8$, and in the presence of 1−3 mM ABTS, $\epsilon_{420}/\epsilon_{660} = 2.3$ and $\epsilon_{404}/\epsilon_{660} = 2.1$.

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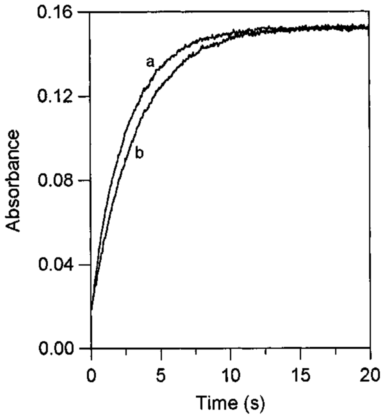

Figure 3. Kinetic traces recorded at 420 nm when 0.46 mM peroxynitrite in 0.01 M NaOH was mixed with 20 mM ferrocyanide in 0.2 M phosphate buffer: (a) air-saturated buffer solution, $k = 0.38$ s^{-1}; (b) the buffer solution was purged with argon for 1 h, $k = 0.31$ s^{-1}.

(10−50 mM).[11] Therefore, we concluded that about 60% of ONOOH isomerizes to nitric acid and about 40% is converted into a highly reactive intermediate via the indirect oxidation pathway.[2,11] This intermediate directly oxidizes ferrocyanide, most probably through the formation of $^{\bullet}NO_2$, which subsequently oxidizes ferrocyanide.[2,11]

However, if the buffer solutions were contaminated with atmospheric CO_2, which catalyzes peroxynitrite decomposition,[20] the measured rates and yields might be higher than in the absence of CO_2. Therefore, these experiments were repeated and the results obtained in air-saturated buffer solutions were compared to those obtained when the buffers were purged with argon.

The oxidation of 0.3−50 mM ferrocyanide by 0.24−0.36 mM peroxynitrite was studied at pH 4.8−8.6 in the presence of 0.1 M acetate or phosphate buffers. We found that the rate of the formation of ferricyanide decreased only around neutral pH when the buffer solutions were purged with argon, while the oxidation yields were unaffected (Figure 3). Around neutral pH, reaction 1 competes with the self-decomposition of peroxynitrite due to contamination of the solutions with atmospheric CO_2. In alkaline solutions the concentration of CO_2 is very low ($pK = 6.2$), and can be neglected. In acidic solutions reaction 1 will not compete with the rate of self-decomposition of peroxynitrite because the concentration of $ONOO^-$ is markedly reduced ($pK_a = 6.8$).

The oxidation rates and yields were reported previously to be independent of ferrocyanide concentrations.[11] In this study we found that only below 10 mM ferrocyanide were the yields independent of ferrocyanide concentration. Above 10 mM, there was a slight increase in the yields and rates, suggesting that, in the presence of relatively high concentrations of ferrocyanide, there is mixed direct and indirect oxidation of ferrocyanide by peroxynitrite.[2] The second-order rate constant of the reaction of peroxynitrite with ferrocyanide was determined to be 5.3, 2.5, and 1.5 M^{-1} s^{-1} at pH 5.2, 6.3, and 7, respectively, suggesting that peroxynitrous acid directly oxidizes ferrocyanide. The direct oxidation of ferrocyanide by peroxynitrite was not observed previously.[11] In our earlier study we used the Hi-Tech Scientific Instrument model PQ/SF-53 stopped-flow apparatus, which yielded less accurate results,[11] and the increase in the rates and yields was at that time within the limits of the experimental error.

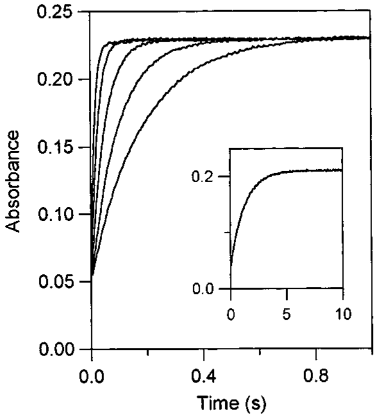

Figure 4. Kinetic traces recorded at 420 nm when 0.57 mM peroxynitrite in 0.01 M NaOH was mixed with 20 mM ferrocyanide in 0.2 M phosphate buffer at pH 6 in the absence and presence of various concentrations of bicarbonate. The pH measured at the outlet of the flow system was 6.3 ± 0.1. The final concentrations of CO_2 were 0.81, 1.62, 3.24, 6.48, and 12.96 mM.

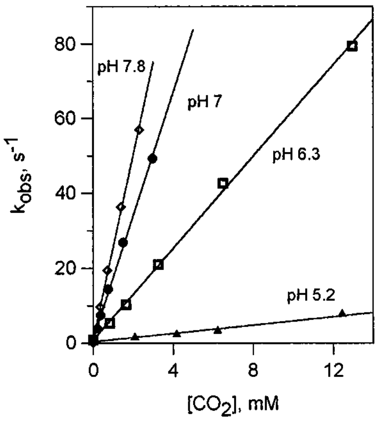

Figure 5. The observed rate constant of the formation of ferricyanide as a function of $[CO_2]$ at various pH's. The final mixtures contained 10 mM ferrocyanide, 0.27−0.32 mM peroxynitrite, and 0.1 M phosphate or acetate buffers.

The effect of bicarbonate on the oxidation rates and yields was studied in the presence of 1−50 mM ferrocyanide at pH 5.2−8.6. In the presence of an excess of CO_2 over peroxynitrite, the rate of the formation of ferricyanide increased with the increase in $[CO_2]$, while the oxidation yields were unaffected (Figure 4). The observed rate constants for the formation of ferricyanide were linearly dependent on $[CO_2]$ and highly dependent on pH (Figure 5). As in the case of Ni^{II}(cyclam), the increase in the slope of the lines in Figure 5 with the pH is due to the reaction of CO_2 with $ONOO^-$.[3] From the slopes of these lines the best value of k_1 was determined to be (2.9 ± 0.1 × 10^4 M^{-1} s^{-1} for $pK_a = 6.9$ (for $pK_a = 6.8$, $k_1 = (2.6 \pm 0.3 \times 10^4$ M^{-1} s^{-1}).

The yield of ferricyanide was determined to be $[Fe(CN)_6{}^{3-}]/[\text{peroxynitrite}]_0 = 0.66 \pm 0.06$, independent of pH, ferrocyanide, and bicarbonate concentrations (Table 1).

The ABTS System. The oxidation of ABTS by peroxynitrite takes place through a complex mechanism, where ONOOH indirectly oxidizes ABTS to $ABTS^+$ through intermediates which are formed during its decomposition.[1,12,13] However, the yield of $ABTS^+$ decreased with the increase in pH,[1,12,13] whereas in the case of ferrocyanide it is independent of pH (Table 1). The yield of $ABTS^+$ was determined to be $[ABTS^+]/[\text{peroxynitrite}]_0 = 0.63$,[12] 0.45,[11] and 0.24[5] in the presence of [ABTS] ≤ 4 mM and at pH ≤ 7.

We found that, under limiting concentrations of peroxynitrite, the yield of $ABTS^+$ was 0.42 ± 0.03 and 0.51 ± 0.03 at pH

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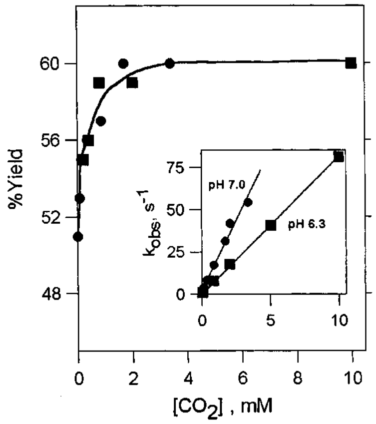


Figure 6. The yield of $ABTS^+$ ($[ABTS^+]/[peroxynitrite]_0$) as a function of $[CO_2]$ at pH 6.3 (■) and 7.0 (●). The final mixtures contained 2 mM ABTS, 72−75 μM peroxynitrite and 0.1 M phosphate buffer. Inset: The observed rate constant of the formation of $ABTS^+$ as a function of $[CO_2]$ under the same conditions as stated above.

Table 1. The Yield of Ferricyanide Obtained through the Reaction of Peroxynitrite with Ferrocyanide under Various Conditions

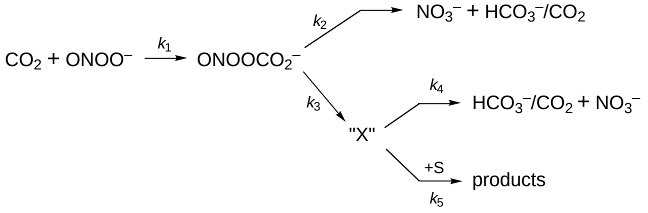
$[Fe(CN)_6^{4-}]$, mM	[peroxynitrite], mM	pH	$[CO_2]$, mM	$[Fe(CN)_6^{3-}]/[peroxynitrite]_0$
5	0.30	5.2	air	0.68
10	0.30	5.2	12.4	0.68
10	0.30	5.2	2.1	0.69
25	0.24	5.5	10.3	0.70
10	0.24	5.5	10.3	0.68
1	0.24	5.5	10.3	0.69
50	0.24	6.3	2.9	0.69
10	0.29	6.3	12.9	0.64
10	0.24	6.3	2.9	0.66
1	0.24	6.3	2.9	0.66
5	0.23	7	air	0.63
50	0.29	7	1.58	0.60
10	0.29	7	1.48	0.63
10	0.30	7	0.37	0.66
1	0.29	7	1.58	0.61
5	0.29	7.8	purged	0.66
10	0.30	7.8	2.3	0.59
10	0.30	7.8	0.69	0.66
20	0.36	8.6	1.09	0.62

6.2 and 7.0, respectively, independent of 0.1−2 mM ABTS. The oxidation yields and rates increased with the increase in [ABTS], in agreement with previous observations,[12] suggesting that under these conditions a mixed direct and indirect oxidation takes place.

The effect of bicarbonate on the oxidation yields and rates was studied at pH 6.3 and 7.0 in the presence of 0.05−10 mM ABTS. When $[CO_2] > [peroxynitrite]$, the rate of the formation of $ABTS^+$ was linearly dependent on $[CO_2]$ and pH dependent (Figure 6). From the slope of the lines in Figure 6 we calculated $k_1 = (2.7 \pm 0.2 \times 10^4$ M^{-1} s^{-1} using $pK_a = 6.8$ for peroxynitrite. The yield of $ABTS^+$ increased with $[CO_2]$ reaching a plateau value where $[ABTS^+]/[peroxynitrite]_0 = 0.60 \pm 0.03$ (Figure 6). The maximum yield was independent of pH and 0.05−10 mM ABTS.

Uppo et al.[5] reported that the yield of $ABTS^+$ increased from 0.24 to 0.27 upon the addition of 20 mM bicarbonate to 0.005−0.1 mM peroxynitrite and 2 mM ABTS at pH 7.0. However, they measured $ABTS^+$ spectrophotometrically at 404 nm using $\epsilon_{404} = 36\,800$ M^{-1} cm^{-1}.[5] We have already noted that the spectrum of $ABTS^+$ depends on [ABTS].[17] The absorption at 660 nm is almost unaffected by [ABTS] ($\epsilon_{660} = 12\,000$ M^{-1} cm^{-1}), whereas the maximum absorption at 414 nm is reduced and shifted toward 420 nm in the presence of $[ABTS] > 0.5$ mM.[17] Furthermore, Uppo et al.[5] allowed peroxynitrite to react with ABTS for 2 min, ignoring the slow decay of $ABTS^+$.[1,12] Nevertheless, the yield of $ABTS^+$ in their system increased by about 13% in the presence of bicarbonate, which correlates well with our observations at pH 7.0.

Mechanism. Under the conditions where reaction 1 competes efficiently with the oxidation of Ni^{II}(cyclam), ferrocyanide, and ABTS by peroxynitrite, the yields of the various products were determined to be 66 ± 6%, independent of pH, $[CO_2]$, and substrate concentrations. Since two molecules of these substrates are consumed by one molecule of peroxynitrite, only 33 ± 3% of added peroxynitrite is available for these oxidation processes, and we define the oxidation yield as 33%. Our results are in agreement with those reported in the case of glutathione (GSH),[21] nitrate,[22] and $Mo(CN)_8^{4-}$.[22] Zhang et al.[21] showed that the amount of GSH consumed in the presence of bicarbonate by 0.4 mM peroxynitrite was 0.23 mM. Since two GSH molecules are consumed upon reaction with peroxynitrite, we calculated that the amount of peroxynitrite available for the reaction is 29%. Hurst and Lymar[22] reported that 33−34% of added peroxynitrite in the presence of bicarbonate is available for the reaction with nitrate and $Mo(CN)_8^{4-}$. These results suggest that the oxidation by peroxynitrite in the presence of bicarbonate takes place through the following mechanism, which has already been suggested by Lymar et al.[5] for tyrosine:



The oxidation yield in the presence of sufficient concentrations of the substrates ($k_5[S] >> k_4$) is 33 ± 3%, and therefore $k_2/k_3 = 2.0 \pm 0.3$.

Lymar et al.[6] have also suggested that reaction 1 may form in parallel $ONOOCO_2^-$ and “X”. These two mechanisms are kinetically indistinguishable, and therefore neither is excluded by the data. However, it is difficult to envisage that “X” is formed directly from the reaction of $ONOO^-$ and CO_2, especially if “X” is the couple $CO_3^{\bullet-}$ and $^{\bullet}NO_2$ (see below).

At present, our data are not sufficient for identification of “X”. It might be an isomer of $ONOOCO_2^-$, which hydrolyzes slowly and hence is capable of oxidizing the substrate. Uppo et al.[5] have suggested the species $O_2NOCO_2^-$, but theoretical calculations in the gas phase imply that it is unstable as it undergoes spontaneous homolysis to give $^{\bullet}NO_2$ and $CO_3^{\bullet-}$.[23] “X” may represent the couple $^{\bullet}NO_2$ and $CO_3^{\bullet-}$, which are formed via the homolytic cleavage of the peroxo O−O bond, as both radicals are relatively long-lived and are capable of oxidizing many compounds.[24] In the absence of any substrate they will yield nitrate and CO_2 via reaction 6.[25]

$$^{\bullet}NO_2 + CO_3^{\bullet-} \rightarrow NO_3^- + CO_2 \qquad k_6 = 1 \times 10^9\ M^{-1}\ s^{-1} \qquad (6)$$

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The identity of "X" may be the couple NO_2^+ and CO_3^{2-}, which are formed via the heterolytic cleavage of the peroxo O−O bond. However, the half-life of NO_2^+ in water has been determined to be 1.4 ns,[26] which is too short to be scavenged by the relatively low concentrations of the substrates, thus eliminating this species as the oxidizing entity.

It has been shown that nitrate is the major product of the reaction of peroxynitrite with CO_2 in the absence of any other reactants.[5] Therefore, we have to assume that the decomposition of $ONOOCO_2^-$ via reaction 2 yields nitrate. However, the hydrolysis of $ONOOCO_2^-$ to nitrate and bicarbonate seems unlikely since it will yield a peroxidic species ($ONOO^-$ or $^-OOCO_2$) as one of the immediate products.[27] In addition, if water can attack the adduct, any other good nucleophile should also be able to capture all $ONOOCO_2^-$, which so far has not been reported.[27] Therefore, we suggest that the decomposition of $ONOOCO_2^-$ to form nitrate as a final product (reaction 2) takes place via the formation of an intermediate. This intermediate might be an isomer of $ONOOCO_2^-$, e.g., $O_2NOCO_2^-$, which hydrolyzes to form nitrate and bicarbonate or decomposes to NO_3^- and CO_2.

Conclusions

The reaction of peroxynitrite with CO_2 yields $ONOOCO_2^-$, which under our experimental conditions is incapable of directly oxidizing Ni^{II}(cyclam), ferrocyanide, and ABTS. We have shown that only about 33% of this adduct can oxidize the various substrates. Note that this ratio is similar to that determined earlier for the direct isomerization of peroxynitrite to nitrate and its conversion into a highly oxidizing intermediate.[2,11] However, though these ratios are similar, they are related to different reactions. The reactivity of peroxynitrite in the presence of bicarbonate differs from that of peroxynitrite as it is governed by the formation of $ONOOCO_2^-$, of which 33 ± 3%, at most, can participate in the oxidation processes.

The toxicity of peroxynitrite is usually attributed to its relatively long half-life which enables it to diffuse away from the site of its formation. However, under physiological conditions (pH 7.4, $pK_a(ONOOH) = 6.8$, $[HCO_3^-] = 12-30$ mM, 37 °C), the most abundant species is $ONOO^-$ which reacts with CO_2 with $k_1 = 5.8 \times 10^4\ M^{-1}\ s^{-1}$.[7] As the concentrations of CO_2 under these conditions are 0.7−1.8 mM, the half-life of peroxynitrite in the intracellular and interstitial fluids is reduced from about 1 s to less than 20 ms, and consequently its average diffusion distance is shortened to less than 6.3 μm ($L = (2Dt)^{0.5}$, $D \sim 10^{-5}\ cm^2\ s^{-1}$). We conclude that indirect oxidation by peroxynitrite will not take place *in vivo* in the presence of bicarbonate. The direct oxidation by peroxynitrite will take place provided that this reaction competes efficiently with reaction 1.

In conclusion, the toxicity of peroxynitrite *in vivo* is most probably governed by $ONOOCO_2^-$, which has a reactivity different from that of peroxynitrite.

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