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Formation of Peroxynitrite from the Nitrosation of Hydrogen Peroxide by an Oxygenated Nitric Oxide Solution

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Peroxynitrite is formed when $^{\bullet}$NO is added to oxygenated solutions of hydrogen-peroxide. The formation rates and the yields of peroxynitrite were determined using the stopped-flow technique at pH 7.5−11.7. The stoichiometry of this process has been determined, and is given by $4^{\bullet}NO + O_2 + 2H_2O_2 \rightarrow 2ONOO^- + 2NO_2^- + 4H^+$. Kinetic studies show that the rate law of the nitrosation process is given by $-d[O_2]/dt = k_1[^{\bullet}NO]^2[O_2]$ with $k_1 = (2.4 \pm 0.3) \times 10^6$ M^{-2} s^{-1}. The kinetic results are identical to those obtained for the autoxidation of $^{\bullet}$NO, indicating that the rate determining step of the nitrosation process is the formation of ONOONO (or $ONONO_2$ or O_2NNO_2), which is the precursor of $^{\bullet}NO_2$ and of N_2O_3. The stoichiometry of the nitrosation process suggests that $^{\bullet}NO_2$ and/or N_2O_3 are the reactive species. Competitive kinetic studies demonstrate that the yield of peroxynitrite is independent of $^{\bullet}$NO concentrations, indicating that the reactive intermediate is N_2O_3. The rate constant of the reaction of N_2O_3 with HO_2^- was determined to be $(1.0 \pm 0.6) \times 10^9$ M^{-1} s^{-1}, whereas that with H_2O_2 is at least 4 orders of magnitude lower. The nitrosation of H_2O_2 by $^{\bullet}$NO takes place only in the presence of oxygen, and under the conditions of this study, we found no evidence for the direct nitrosation of H_2O_2 by $^{\bullet}$NO.

Introduction

Peroxynitrite is a toxic compound that oxidizes a large variety of biomolecules such as sulfhydryls,[1] lipids,[2] enzymes,[3] and DNA.[4] It is a weak acid ($pK_a = 6.8$) that decays rapidly to NO_3^- and H^+ at a rate of 1.3 s^{-1} at 25 °C.[5,6] Peroxynitrite ion ($ONOO^-$) is relatively stable species, which exhibits a broad absorption spectrum with a maximum at 302 nm ($\epsilon = 1670 \pm 50$ M^{-1} cm^{-1}).[7]

Peroxynitrite can be formed through the diffusion-controlled reaction of $^{\bullet}$NO with $O_2^{\bullet -}$ ($k = (4.3-6.7) \times 10^9$ M^{-1} s^{-1}),[8,9] through the reaction of nitroxyl anion (NO^-) with molecular oxygen,[10−12] and through the nitrosation of H_2O_2.[13−15] Only the occurrence of the reaction of $^{\bullet}$NO with O_2^- has been demonstrated in biological systems.[16,17]

Halfpenny and Robinson[13] reported that the reactions of $^{\bullet}$NO and $^{\bullet}NO_2$ with alkaline H_2O_2 give peroxynitrite. They have also reported that the reaction of $^{\bullet}$NO with H_2O_2 takes place in alkaline solutions in the absence of oxygen.[13] Petriconi and Papee[18] have shown that $^{\bullet}NO_2$ does not react with alkaline H_2O_2 to form peroxynitrite, whereas the reaction of $^{\bullet}$NO with aerated alkaline H_2O_2 gives low yields of peroxynitrite (∼2%). Blough and Zafiriou[19] found no evidence for such a reaction. The direct addition of $^{\bullet}$NO to deaerated alkaline solutions of H_2O_2 did not produce the characteristic absorption of peroxynitrite at 302 nm.[19] Furthermore, Seddon and Sutton[20] have demonstrated that a thermal reaction between $^{\bullet}$NO and H_2O_2 occurs at room temperature ($H_2O_2 + 2^{\bullet}NO \rightarrow 2HNO_2$) and that the rate is so slow in neutral solutions that, in mixtures of 100 μM H_2O_2 and 2 mM $^{\bullet}$NO, the reaction consumes less than 2% of the H_2O_2 in 3 h. At pH 12, the thermal reaction consumes 100 μM H_2O_2 in saturated $^{\bullet}$NO solution in less than 30 min.[21]

Since $^{\bullet}$NO is not a nitrosating agent, the formation of peroxynitrite by $^{\bullet}NO/H_2O_2$ most probably requires oxygen, and proceeds through nitrosating intermediates that are formed during the autoxidation of $^{\bullet}$NO.[22−24] In this study the kinetics and yields of the nitrosation of H_2O_2 by oxygenated $^{\bullet}$NO solution were investigated. Our results demonstrate that peroxynitrite is formed in this system via the reaction of N_2O_3 with the hydroperoxide anion (HO_2^-).

Experimental Section

Materials. All chemicals were of analytical grade and were used as received. Nitric oxide, CP, was purchased from Matheson Gas Products. Solutions were prepared with deionized water that was distilled and purified using a Milli-Q water purification system. Oxygen-saturated solutions (1.2 mM at 22 °C and 690 mmHg, which is the barometric pressure in Jerusalem)[25] were prepared by bubbling gastight syringes with oxygen for 30 min. The $^{\bullet}$NO gas was purified

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Table 1. Typical Results Obtained under Various Experimental Conditions

$[O_2]_o$, M	$[^{\bullet}NO]_o$, M	$[H_2O_2]_o$, M	pH	ΔOD_{302}	$k_{obs} = k/\epsilon l$, s^{-1}(2nd order)	k_{obs}, s^{-1} (1st order)
1×10^{-3}	1.5×10^{-4}	7.2×10^{-2}	9.4	0.097	16.4	
1×10^{-3}	3.0×10^{-4}	6.0×10^{-2}	10.0	0.225	14.6	
1×10^{-3}	1.5×10^{-4}	7.2×10^{-2}	10.4	0.101	11.4	
1×10^{-3}	3.0×10^{-4}	7.2×10^{-2}	10.4	0.192	12.3	
1×10^{-3}	9.2×10^{-5}	7.2×10^{-2}	10.4	0.060	10.4	
1×10^{-3}	1.5×10^{-4}	1.8×10^{-2}	10.4	0.099	12.9	
5×10^{-4}	1.5×10^{-4}	1.8×10^{-2}	10.4	0.095	6.8	
2×10^{-4}	6.0×10^{-5}	1.8×10^{-2}	10.4	0.036	2.5	
1×10^{-3}	1.5×10^{-4}	3.6×10^{-2}	11.7	0.102	8.8	
1×10^{-3}	1.5×10^{-4}	1.2×10^{-2}	11.7	0.098	9.5	
4×10^{-5}	1.5×10^{-3}	7.2×10^{-2}	10.3	0.110		5.6
4×10^{-5}	1.5×10^{-3}	9.0×10^{-3}	10.0	0.101		4.8
4×10^{-5}	7.5×10^{-4}	9.0×10^{-3}	10.0	0.106		1.5
4×10^{-5}	5.0×10^{-4}	9.0×10^{-3}	10.0	0.097		0.53
4×10^{-5}	1.5×10^{-3}	0.11	9.0	0.107		5.9
4×10^{-5}	1.5×10^{-3}	0.144	8.1	0.108		5.9

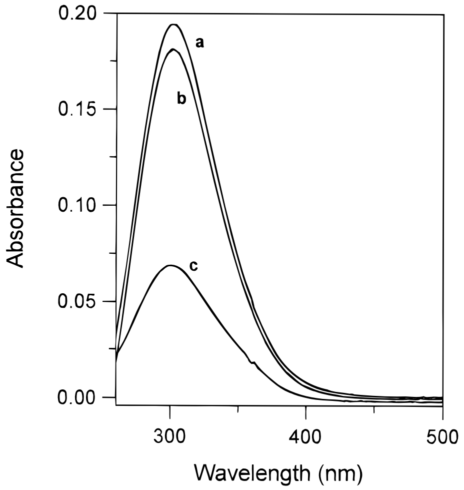

Figure 1. Spectrum of the species measured 30 s after the mixing of $^{\bullet}NO$-saturated solutions with oxygenated H_2O_2 solutions. The pH as measured at the outlet was 10.0, and the final concentrations were 0.3 mM $^{\bullet}NO$, 1 mM O_2, 5 mM borate buffer, 100 μM EDTA and (a) 60 mM H_2O_2, (b) 30 mM H_2O_2, or (c) 0.85 mM H_2O_2. The blank solution was the same mixture without H_2O_2.

by passing it through a series of scrubbing bottles containing 50% NaOH and distilled water in this order. The solutions in the traps were first deaerated by purging them with Helium for an hour. Nitric oxide solutions were prepared in gastight syringes by purging first the solutions (containing 2 mM phosphate or 5 mM borate buffers) with helium to remove O_2, followed by bubbling for 30 min with $^{\bullet}NO$. The $^{\bullet}NO$-saturated solutions (1.8 mM at 22 °C and 690 mmHg)[25] were stored in syringes and subsequently diluted with helium-saturated solutions to the desired concentrations by the syringe technique.

The concentration of H_2O_2 was determined with the Fricke dosimeter (10 mM Fe^{II} in 0.8 N H_2SO_4) using $\epsilon_{302}(Fe^{III}) = 2200\ M^{-1}\ cm^{-1}$.[26]

Stopped-flow kinetic measurements were carried out using a Bio SX-17MV sequential stopped-flow instrument from Applied Photophysics. A xenon lamp (Osram XBO 150 W) produced the analyzing light, and a Hammamatsu R928 photomultiplier was used for the measurements. The formation of peroxynitrite was followed at 302 nm. The optical path length was 1 cm. Each value given is an average of at least five measurements. All measurements were carried out at 21.5 °C. Phosphate and borate buffers were used, and unless otherwise stated, the final mixtures contained 100 μM EDTA. The latter had no effect on the measured yields and rates in our system. The pH was measured at the outlet.

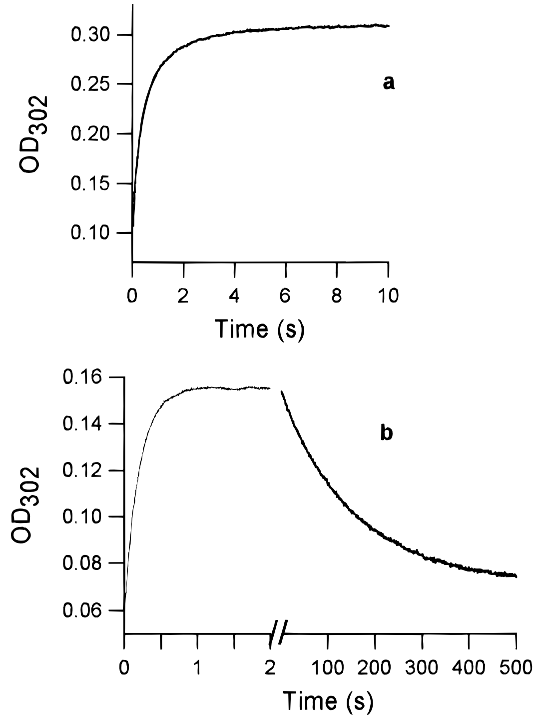

Figure 2. (a) Kinetic trace of the formation of the absorbance at 302 nm under limiting concentration of $^{\bullet}NO$ at pH 10. The final concentrations after the mixing were 0.3 mM $^{\bullet}NO$, 1 mM O_2 , 60 mM H_2O_2, 5 mM borate buffer, and 100 μM EDTA. (b) Kinetic traces of the formation and decay of the absorbance at 302 nm under limiting concentration of O_2 at pH 10. The final concentrations after the mixing were 1.5 mM $^{\bullet}NO$, 40 μM O_2, 9 mM H_2O_2, 5 mM borate buffer, and 100 μM EDTA.

Results

(i) Limiting Concentrations of $^{\bullet}$NO. When oxygen-saturated solutions containing H_2O_2 and EDTA were mixed with $^{\bullet}NO$ solutions to yield final concentrations of 0.2−1 mM O_2, 0.06−0.3 mM $^{\bullet}NO$, 0.0825−72 mM H_2O_2, and 100 μM EDTA at pH 9.1−11.7 (5 mM borate buffer at pH 9.1−10.4), a rapid formation of an absorption with a maximum at 302 nm was observed (Figure 1). The rate of the formation was second order. A typical kinetic trace is given in Figure 2a. The observed rate constant was linearly dependent on $[O_2]_o$ and decreased slightly with the increase in pH (Table 1). The yields of the absorbance increased with $[H_2O_2]$ (Figure 3). The

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Table 2. Observed First Order Decay of the Absorbance at 302 nm under Various Conditions

$[O_2]_o$, M	$[^{\bullet}NO]_o$, M	$[H_2O_2]_o$, M	pH	k_{decay}, s^{-1}	
				measd	calcd
4×10^{-5}	1.5×10^{-3}	7.2×10^{-2}	7.5 (10 mM phosphate)	0.19	0.21[a]
4×10^{-5}	1.5×10^{-3}	1.44×10^{-1}	7.7 (10 mM phosphate)	0.13	0.14[a]
4×10^{-5}	1.5×10^{-3}	2.2×10^{-3}	8.0 (10 mM phosphate)	0.080	0.077[a]
4×10^{-5}	1.5×10^{-3}	7.2×10^{-2}	8.1 (10 mM phosphate)	0.066	0.062[a]
1×10^{-3}	1.5×10^{-4}	9.0×10^{-3}	9.1 (5 mM borate)	0.028	0.025[b]
1×10^{-3}	1.5×10^{-4}	7.2×10^{-2}	9.4 (5 mM borate)	0.012	0.013[b]
4×10^{-5}	1.5×10^{-3}	3.3×10^{-3}	10.0 (5 mM borate)	0.0061	0.0032[b]

[a] The literature values for the rate constant of decay of peroxynitrite in phosphate buffer were calculated assuming that the rate constant of the decay of peroxynitrite is 1.3 s^{-1} and pK = 6.8.[5,6] [b] In the presence of high concentrations of various buffers, including borate, the pK is shifted toward 7.9−8.0 whereas the rate constant remains 1.3 s^{-1}.[27] The specific concentrations of the buffers were not indicated.[27] The best fit of the measured rate constants in the presence of 5 mM borate is to a rate of 1.3 s^{-1} and pK = 7.4.

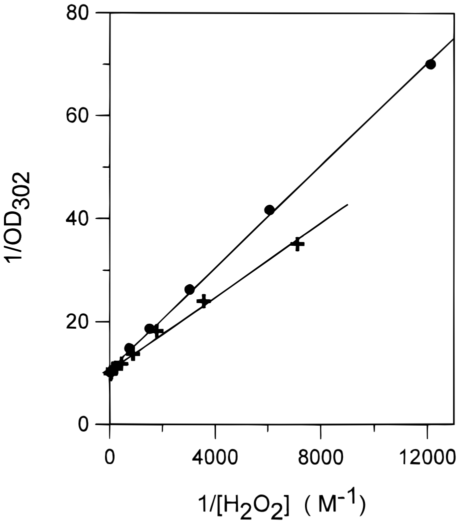

Figure 3. Double reciprocal plots of the change in the absorbance at 302 nm with varying the concentration of H_2O_2: (+) pH 10.4, 5 mM borate buffer; (●) pH 11.7. All solutions contained 0.15 mM •NO, 1 mM O_2 and 100 μM EDTA.

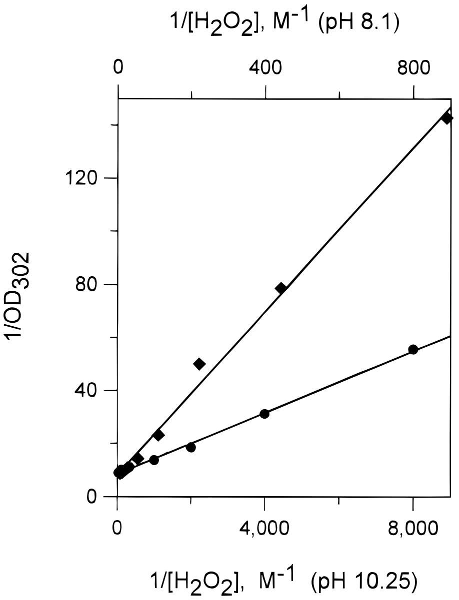

Figure 4. Double reciprocal plots of the change in the absorbance at 302 nm with varying the concentration of H_2O_2: (◆) pH 8.1, 10 mM phosphate buffer; (●) pH 10.25, 5 mM borate buffer. All solutions contained 1.5 mM •NO, 40 μM O_2, and 100 μM EDTA.

asymptotic value at constant [•NO] was the same at all pH's, but was achieved at different concentrations of H_2O_2 (Figure 3). The maximum yields were linearly dependent on $[^{\bullet}NO]_o$, resulting $\Delta OD_{302}/[^{\bullet}NO]_o = 700 \pm 40$ M^{-1} cm^{-1} (Table 1). The absorption decayed back to its initial value via a first-order kinetics with a highly pH-dependent rate constant (see below). The kinetics of the formation of the absorbance could not be determined below pH 9 because the decay of the absorbance is already within the time scale of the formation process.

(ii) Limiting Concentrations of O_2. When air-saturated solutions containing H_2O_2 and EDTA were mixed with •NO solutions to yield final concentrations of 0.5−1.5 mM •NO, 40 μM O_2, and 1.44×10^{-4} to 0.144 M H_2O_2 at pH 8.1−10.0 (10 mM phosphate or 5 mM borate buffers), the rate of the formation of the absorbance was first-order. Typical kinetic traces of the formation and decay of the absorbance at 302 nm are given in Figure 2b. The observed first order rate constant was linearly dependent on $[^{\bullet}NO]_o^2$ (Table 1), yielding a third order rate constant of $(2.4 \pm 0.3) \times 10^6$ M^{-2} s^{-1}. The yields of the absorbance depended on pH and increased with $[H_2O_2]$ (Figure 4). The asymptotic value was the same at all pH's, yielding $\Delta OD_{302}/[O_2]_o = 2750 \pm 250$ M^{-1} cm^{-1} (Figure 4). The absorption decayed back to its initial value by a first order kinetics, with a highly pH-dependent rate constant (Figure 2b, Table 2). The kinetics of the formation was not studied below pH 8.0 as the decay of the absorbance is already within the time scale of the formation process.

The yield of the absorbance in the absence of oxygen was very small. In mixtures containing 44−88 mM H_2O_2 and 0.15−0.3 mM •NO at pH 8.3−9.3 (10 mM phosphate or 5 mM borate buffers, 100 μM EDTA), ΔOD_{302} = 0.01−0.05. The rate of the formation of the absorbance was second order in [•NO] and zero order in $[H_2O_2]$. The absorbance decayed via a first-order process, and the observed rate constants were identical to those measured in the presence of oxygen at the same pH. These results indicate that the formation of the absorbance is due to contamination of the solutions with O_2, which is most probably formed from slow decomposition of H_2O_2. We calculated from the comparison of the yields in deaerated and oxygenated mixtures that under our conditions, the contamination of the final mixtures with oxygen is 4−18 μM.

Discussion

When •NO is added to oxygenated H_2O_2 solutions at pH > 7.5, an unstable species is formed. The spectrum (Figure 1) and the stability of this species as a function of pH (Table 2) indicate that peroxynitrite is formed. Peroxynitrite oxidizes H_2O_2 via an indirect oxidation pathway, where the rate of the oxidation is zero order in $[H_2O_2]$ at acid pH's.[28] It has been demonstrated that the rate of the decay of peroxynitrite decreased by 50% in the presence of high $[H_2O_2]$ at alkaline pH's,[28] but we find no evidence for such a decrease in our system (Table 2).[28]

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The rate of the formation of peroxynitrite in the $^{\bullet}\mathrm{NO}/\mathrm{O_2}/\mathrm{H_2O_2}$ system is second order in [$^{\bullet}$NO] and first order in [O_2]. The third order rate constant measured under limiting concentrations of O_2 is $(2.4 \pm 0.3) \times 10^6\ \mathrm{M^{-2}\ s^{-1}}$, which is identical within experimental error to that of the autoxidation of $^{\bullet}$NO.[22−24] This result shows that the rate determining step of the nitrosation of H_2O_2 in oxygenated $^{\bullet}$NO solution is the same as that of the autoxidation of $^{\bullet}$NO. The ratio between the nitrosation yields under limiting concentrations of O_2 ($\Delta\mathrm{OD}_{302}/[\mathrm{O_2}]_\mathrm{o} = 2750 \pm 250\ \mathrm{M^{-1}\ cm^{-1}}$) and under limiting concentrations of $^{\bullet}$NO ($\Delta\mathrm{OD}_{302}/[^{\bullet}\mathrm{NO}]_\mathrm{o} = 700 \pm 40\ \mathrm{M^{-1}\ cm^{-1}}$) is 3.9 ± 0.5. Therefore, the stoichiometry of the whole nitrosation process is given by eq 1, whereas that of the autoxidation of $^{\bullet}$NO is

$$4^{\bullet}\mathrm{NO} + \mathrm{O_2} + 2\mathrm{H_2O_2} \rightarrow 2\mathrm{ONOO^-} + 2\mathrm{NO_2^-} + 4\mathrm{H^+} \qquad (1)$$

given by eq 2.[22−24]

$$4^{\bullet}\mathrm{NO} + \mathrm{O_2} + 2\mathrm{H_2O} \rightarrow 4\mathrm{NO_2^-} + 4\mathrm{H^+} \qquad (2)$$

We have recently shown that the rate determining step of the autoxidation of $^{\bullet}$NO is the formation of ONOONO (or $\mathrm{ONONO_2}$ or $\mathrm{O_2NNO_2}$), and that the overall process can be described by reactions 3−7.[22]

$$^{\bullet}\mathrm{NO} + \mathrm{O_2} \rightleftharpoons \mathrm{ONOO}^{\bullet}\ (\text{or } \mathrm{NO}^{\bullet\bullet}\mathrm{O_2})^{29} \qquad (3)$$

$$\mathrm{ONOO}^{\bullet}\ (\text{or } \mathrm{NO}\cdots\mathrm{O_2}) + {}^{\bullet}\mathrm{NO} \rightarrow \mathrm{ONOONO}\ (\text{or } \mathrm{ONONO_2} \text{ or } \mathrm{O_2NNO_2}) \qquad (4)$$

$$\mathrm{ONOONO} \rightarrow 2^{\bullet}\mathrm{NO_2} \qquad (5)$$

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

$$\mathrm{N_2O_3} + \mathrm{H_2O} \rightarrow 2\mathrm{NO_2^-} + 2\mathrm{H^+} \qquad (7)$$

According to this mechanism, and under the condition where $k_{-3} > k_4[^{\bullet}\mathrm{NO}]$, rate eq 8 is obtained for which $k_1 = k_3k_4/k_{-3} = (2.0-2.9) \times 10^6\ \mathrm{M^{-2}\ s^{-1}}$.[22−24]

$$-\frac{1}{4}\frac{\mathrm{d}[^{\bullet}\mathrm{NO}]}{\mathrm{d}t} = -\frac{\mathrm{d}[\mathrm{O_2}]}{\mathrm{d}t} = \frac{1}{4}\frac{\mathrm{d}[\mathrm{NO_2^-}]}{\mathrm{d}t} = \frac{k_3k_4[^{\bullet}\mathrm{NO}]^2[\mathrm{O_2}]}{k_{-3} + k_4[^{\bullet}\mathrm{NO}]} = k_1[^{\bullet}\mathrm{NO}]^2[\mathrm{O_2}] \qquad (8)$$

As the stoichiometry of the nitrosation process is given by eq 1, rate eq 9 is obtained irrespective of the detailed mechanism.

$$-\frac{1}{4}\frac{\mathrm{d}[^{\bullet}\mathrm{NO}]}{\mathrm{d}t} = -\frac{\mathrm{d}[\mathrm{O_2}]}{\mathrm{d}t} = \frac{1}{2}\frac{\mathrm{d}[\mathrm{ONOO^-}]}{\mathrm{d}t} = k_1[^{\bullet}\mathrm{NO}]^2[\mathrm{O_2}] \qquad (9)$$

The rate of the nitrosation process was determined by following the formation of $\mathrm{ONOO^-}$ at 302 nm. Under limiting concentrations of O_2, each O_2 yields 2 $\mathrm{ONOO^-}$, and therefore rate eq 10 is obtained for the formation of peroxynitrite. Under

$$\mathrm{d}[\mathrm{ONOO^-}]/\mathrm{d}t = 2k_1[^{\bullet}\mathrm{NO}]^2[\mathrm{O_2}] = 2k_1[^{\bullet}\mathrm{NO}]^2 0.5([\mathrm{ONOO^-}]_\infty - [\mathrm{ONOO^-}]) = k_1[^{\bullet}\mathrm{NO}]^2([\mathrm{ONOO^-}]_\infty - [\mathrm{ONOO^-}]) \qquad (10)$$

limiting concentrations of $^{\bullet}$NO, each $^{\bullet}$NO yields 0.5 $\mathrm{ONOO^-}$, and therefore rate eq 11 is obtained for the formation of

$$\mathrm{d}[\mathrm{ONOO^-}]/\mathrm{d}t = 2k_1[^{\bullet}\mathrm{NO}]^2[\mathrm{O_2}] = 2k_1[\mathrm{O_2}]\{2([\mathrm{ONOO^-}]_\infty - [\mathrm{ONOO^-}])\}^2 = 8k_1[\mathrm{O_2}]\,([\mathrm{ONOO^-}]_\infty - [\mathrm{ONOO^-}])^2 \qquad (11)$$

peroxynitrite. Thus, irrespective of the detailed mechanism of the nitrosation process, the rate constant determined by following the formation of peroxynitrite under limiting concentrations of O_2 or $^{\bullet}$NO is k_1 or $8k_1$, respectively. We have determined $k_1 = (2.4 \pm 0.3) \times 10^6\ \mathrm{M^{-2}\ s^{-1}}$ under limiting concentrations of O_2 at pH 8.1−10.3, which is in excellent agreement with the values determined for the autoxidation of $^{\bullet}$NO.[22−24] Under limiting concentrations of $^{\bullet}$NO, and under the condition where the maximum nitrosation yields were obtained, $k_\mathrm{obs}/[\mathrm{O_2}] = 8k_1/\epsilon_{302}l = 1.32 \times 10^4\ \mathrm{M^{-1}\ s^{-1}}$ (Table 1). The value of $8k_1$ was determined to be $(2.2 \pm 0.5) \times 10^7\ \mathrm{M^{-2}\ s^{-1}}$, using the literature value of $\epsilon_{302} = 1670\ \mathrm{M^{-1}\ cm^{-1}}$.[7] The value of $8k_1$ obtained under limiting concentrations of $^{\bullet}$NO is in agreement with the value of k_1 determined under limiting concentrations of O_2.

The stoichiometry of the nitrosation process (eq 1) indicates that both $^{\bullet}\mathrm{NO_2}$ and $\mathrm{N_2O_3}$ may be the reactive intermediates. The formation of peroxynitrite via $^{\bullet}\mathrm{NO_2}$ can take place through reactions 12 and 13. However, the feasibility of reaction 12 is unknown. According to this mechanism, the nitrosation yield

$$^{\bullet}\mathrm{NO_2} + \mathrm{H_2O_2} \rightarrow \mathrm{NO_2^-} + \mathrm{O_2^{\bullet-}} + 2\mathrm{H^+} \qquad (12)$$

$$^{\bullet}\mathrm{NO} + \mathrm{O_2^{\bullet-}} \rightarrow \mathrm{ONOO^-} \qquad (13)$$

will depend on [$^{\bullet}$NO] due to the competition between reactions 12 and 6, 7 (eq 14).

$$\frac{[\mathrm{ONOO^-}]}{[\mathrm{ONOO^-}]_\mathrm{max}} = \frac{k_{12}[\mathrm{H_2O_2}]_\mathrm{o}}{k_{12}[\mathrm{H_2O_2}]_\mathrm{o} + k_6k_7[^{\bullet}\mathrm{NO}]_\mathrm{o}/(k_{-6} + k_7)} \qquad (14)$$

In such a case, a plot of $1/\Delta\mathrm{OD}_{302}$ vs $1/[\mathrm{H_2O_2}]_\mathrm{o}$ yields a straight line with intercept/slope $= I/S = k_{12}(k_{-6} + k_7)/k_6k_7[^{\bullet}\mathrm{NO}]_\mathrm{o}$.

If the nitrosation takes place via $\mathrm{N_2O_3}$ (eq 15), the yield of peroxynitrite will be independent of [$^{\bullet}$NO] as the competition is between reactions 7 and 15 (eq 16).

$$\mathrm{N_2O_3} + \mathrm{H_2O_2} \rightarrow \mathrm{ONOO^-} + \mathrm{NO_2^-} + 2\mathrm{H^+} \qquad (15)$$

$$\frac{[\mathrm{ONOO^-}]}{[\mathrm{ONOO^-}]_\mathrm{max}} = \frac{k_{15}[\mathrm{H_2O_2}]_\mathrm{o}}{k_{15}[\mathrm{H_2O_2}]_\mathrm{o} + k_7} \qquad (16)$$

In this case, a plot of $1/\Delta\mathrm{OD}_{302}$ vs $1/[\mathrm{H_2O_2}]_\mathrm{o}$ yields a straight line with $I/S = k_{15}/\mathrm{k}_7$.

The results demonstrate that under all experimental conditions, plots of $1/\Delta\mathrm{OD}_{302}$ vs $1/[\mathrm{H_2O_2}]_\mathrm{o}$ yield straight lines. Typical plots are given in Figures 3 and 4. The values of I/S are independent of $[^{\bullet}\mathrm{NO}]_\mathrm{o}$ (Table 3), indicating that the nitrosating species is $\mathrm{N_2O_3}$, and hence $I/S = k_{15}/k_7$. From the dependence of the values of I/S on pH (Table 3), it is concluded that both k_7 and k_{15} are pH-dependent (eqs 17 and 18), where

$$k_7 = k_{7\mathrm{a}} + k_{7\mathrm{b}}[\mathrm{OH^-}] \qquad (17)$$

$$k_{15} = k_{15\mathrm{a}}\frac{K_\mathrm{a}}{K_\mathrm{a} + [\mathrm{H^+}]} + k_{15\mathrm{b}}\frac{[\mathrm{H^+}]}{K_\mathrm{a} + [\mathrm{H^+}]} \qquad (18)$$

$k_{15\mathrm{a}}$ and $k_{15\mathrm{b}}$ are the rate constants of the reactions of $\mathrm{N_2O_3}$ with

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Table 3

$[O_2]_o$, M	$[^{\bullet}NO]_o$, M	pH	$I/S = k_{15}/k_7$ M^{-1} [a]	k_{15}, M^{-1} s^{-1} ($k_7 = 2 \times 10^3 + 10^8[OH^-]$, s^{-1})[31]	$K_a/(K_a + [H^+])$ [b]
4×10^{-5}	1.5×10^{-3}	8.1	51.9	1.04×10^5	0.00025
4×10^{-5}	1.5×10^{-3}	9.0	410	1.22×10^6	0.002
4×10^{-5}	1.5×10^{-3}	10.0	1086	1.30×10^7	0.0196
1×10^{-3}	3.0×10^{-4}	10.0	881	1.06×10^7	0.0196
4×10^{-5}	1.5×10^{-3}	10.25	1435	2.84×10^7	0.0343
1×10^{-3}	1.5×10^{-4}	10.4	2864	7.77×10^7	0.048
1×10^{-3}	1.5×10^{-4}	11.7	2127	1.07×10^9	0.5

[a] I and S are the intercepts and the slopes of the straight lines obtained for plots of $1/\Delta OD_{302}$ vs $1/[H_2O_2]_o$ as in Figures 3 and 4. [b] The fraction of HO_2^- at a given pH ($K_a = 2 \times 10^{-12}$ M).

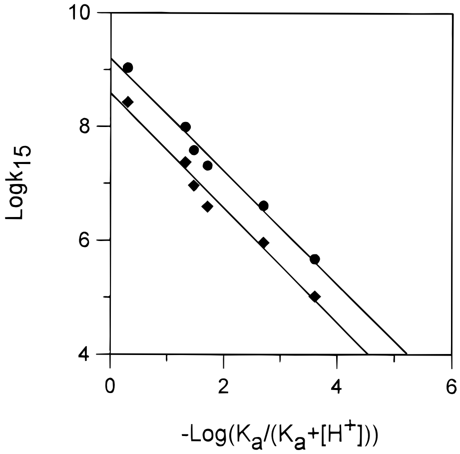

Figure 5. log k_{15} as a function of $-\log(K_a/(K_a + [H^+]))$, where $K_a = 2 \times 10^{-12}$ M is the dissociation constant of H_2O_2. k_{15} was calculated from the values of I/S (Table 3) using (◆) $k_7 = (2 \times 10^3 + 2.5 \times 10^7[OH^-])$ s^{-1} and (●) $k_7 = (9 \times 10^3 + 10^8[OH^-])$ s^{-1} in order to get the best fit of the data to a straight line with slope −1.

HO_2^- and H_2O_2, respectively, and $K_a = 2 \times 10^{-12}$ M is the dissociation constant of H_2O_2.

The rate constant of the hydrolysis of N_2O_3 (k_7) has been determined earlier using the pulse radiolysis and flash photolysis techniques.[30,31] Gratzel et al.[30] determined $k_7 = 530$ s^{-1} at pH < 8 and 20 °C, whereas Treinin and Hayon[31] reported that $k_7 = (2 \times 10^3 + 10^8[OH^-])$ s^{-1} at 25 °C. As noted above, both k_{15} and k_7 are pH-dependent. Therefore, we used Treinin and Hayon's value for calculating k_{15} from the I/S values (Table 3). The variation in k_{15} with pH (Table 3) demonstrates that the rate constant of the reaction of N_2O_3 with H_2O_2 (k_{15b}) is considerably lower than that with HO_2^- (k_{15a}).

The rate constant of the reaction of N_2O_3 with HO_2^- was determined from the dependence of k_{15} on $K_a/(K_a + [H^+])$, assuming that the reaction of N_2O_3 with H_2O_2 can be neglected (eq 18). A plot of log k_{15} vs $-\log(K_a/(K_a + [H^+]))$ should yield a straight line with slope −1 and intercept log k_{15a}. However, using Treinin and Hayon's value for k_7 (Table 3), the plot of log k_{15} vs $-\log(K_a/(K_a+[H^+]))$ yielded a straight line, but with a slope −1.21 rather than −1. Thus, we made two best fits for plots with slope −1 by varying Treinin and Hayon's value of k_7. The first plot was obtained for $k_7 = (2 \times 10^3 + 2.5 \times 10^7[OH^-])$ s^{-1}, whereas the second plot was obtained for $k_7 = (9 \times 10^3 + 10^8[OH^-])$ s^{-1} (Figure 5). The rate constant of the reaction of N_2O_3 with HO_2^- was determined from the intercepts of these lines to be 3.9×10^8 and 1.6×10^9 M^{-1} s^{-1}, respectively (Figure 5).

N_2O_3 is also formed in the presence of high concentrations of HNO_2 (ca. 0.1 M) and at moderate acidities.[32]

$$2HNO_2 = N_2O_3 + H_2O$$

$$K = k_{-7}/k_7 = 3.0 \times 10^{-3} \text{ to } 0.2\ M^{-1}\ {}^{32-34}$$

The recent value $(3.03 \pm 0.23) \times 10^{-3}$ M^{-1},[33] is in agreement with the value calculated thermodynamically, $(2.3 \pm 1.0) \times 10^{-3}$ M^{-1}.[34] The rate constant of reaction −7 has been determined in several systems to be 9−13.9 M^{-1} s^{-1} at 25 °C.[32,35,36] As these measurements were carried out in acid solutions, $k_{7a} = 2970{-}4590$ s^{-1}, which are higher than those determined directly.[30,31] We have no explanation for this discrepancy. We can only argue that our data is more consistent with $k_{7a} > 2 \times 10^3$ s^{-1} and that Gratzel's value is too low.

Due to the uncertainty in the values of k_7, we cannot decide which of the values of k_{15a} (3.9×10^8 or 1.6×10^9 M^{-1} s^{-1}) is better. Therefore, we have chosen for k_{15a} the average value of $(1.0 \pm 0.6) \times 10^9$ M^{-1} s^{-1}, and hence $k_{15b} < 1.0 \times 10^5$ M^{-1} s^{-1}. These results are in agreement with the literature data, which demonstrate that N_2O_3 (an electrophilic agent) nitrosates the free base form of the substrate (a nucleophilic agent) with rate constants that vary between 1.4×10^7 and 5.9×10^9 M^{-1} s^{-1}.[32,36−39]

Under the conditions of this study, we found no evidence for the formation of peroxynitrite via the direct reaction of $^{\bullet}NO$ with H_2O_2. The very low yields of peroxynitrite that were obtained in deaerated solution are attributed to the contamination of the solutions with oxygen since the rate of the formation of peroxynitrite under these conditions was second order in $[^{\bullet}NO]$ and zero order in $[H_2O_2]$.

Conclusions

We have demonstrated that the nitrosation of H_2O_2 by $^{\bullet}NO$ takes place only in the presence of oxygen. The rate-determining step of this process is the formation of ONOONO (or $ONONO_2$ or O_2NNO_2), and therefore the rate of the autoxidation of $^{\bullet}NO$ is unaffected by the presence of H_2O_2. Competitive kinetic studies have shown that the nitrosating species is N_2O_3, which is one of the intermediates formed during the autoxidation of $^{\bullet}NO$. The rate constant of the reaction of N_2O_3 with HO_2^- has been determined to be $(1.0 \pm 0.6) \times 10^9$ M^{-1} s^{-1}, whereas that with H_2O_2 is lower than 1×10^5 M^{-1} s^{-1}.

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The reaction of $^{\bullet}NO$ with H_2O_2 has been used in the detection of $^{\bullet}NO$ in the picomolar range from perfused organ, based on the chemiluminescence reaction of peroxynitrite with luminol.[40] However, we found no evidence for the direct reaction of $^{\bullet}NO$ with H_2O_2 to yield peroxynitrite. As the nitrosating agent is formed from the autoxidation of $^{\bullet}NO$, it might also react with other substrates that are present in the medium (e.g., thiols, amines), and the measured chemiluminescence will be only a low estimate of the $^{\bullet}NO$ concentration.

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