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Direct and Indirect Oxidations by Peroxynitrite

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The mechanism of oxidation of iodide, Ni^{II}cyclam, and ferrocyanide by peroxynitrite has been studied in detail. The results demonstrate that the oxidative chemistry of peroxynitrite is highly pH dependent and can take place either directly by the peroxynitrous acid, as in the case of iodide and Ni^{II}cyclam, or indirectly by secondary reactive intermediates formed via the self-decomposition of peroxynitrous acid, as in the case of ferrocyanide. The rate constants of the oxidation of I^- and Ni^{II}cyclam by ONOOH have been determined to be $(2.3 \pm 0.1) \times 10^4$ M^{-1} s^{-1} and $(3.25 \pm 0.15) \times 10^4$ M^{-1} s^{-1}, respectively. In the indirect pathway, about 60% of ONOOH isomerizes to nitric acid and about 40% is converted into a highly reactive oxidant which directly oxidized ferrocyanide via the formation of $NO_2^{\bullet}$ as an intermediate without the involvement of hydroxyl radicals in this process.

Introduction

Nitric oxide has become in the last few years one of the most studied and fascinating molecules in biological chemistry and was chosen as "molecule of the year" by *Science*.[1] Nitric oxide, which is formed enzymatically,[2−4] is involved in a large number of biological processes, including relaxation of vascular smooth muscle,[5] platelet aggregation,[6] inhibition of protein synthesis,[7] neurotransmission,[8] mediation of glutamate neurotoxicity,[9] and lysis of tumor cells.[10] However, excess production of nitric oxide can be toxic.[11−13] Part of its toxicity is believed to be due to its reaction with superoxide. Superoxide reacts with nitric oxide at a rate of $(4.3-6.7) \times 10^9$ M^{-1} s^{-1} to form peroxynitrite ($ONOO^-$).[14,15] Peroxynitrous acid (ONOOH, $pK_a \approx 6.8$) is a relatively long-lived toxic compound with a half-life lower than 1 s, whereas the anion is stable.[16]

Anbar and Taube[17] have demonstrated, using isotopic labeling, that in weak acid (pH 2−5) nitrate is the only product of the decay of ONOOH.

$$\mathrm{ONOOH} \xrightarrow{k_1} \mathrm{H^+} + \mathrm{NO_3^-} \qquad (1)$$

The rate of the self-decomposition of peroxynitrite, as measured at 302 nm, follows first-order kinetics and is highly pH dependent. The observed rate constant is given by eq 2, where k_1 is the rate constant for the decay of ONOOH and K_a is its acid dissociation constant.[16]

$$k_{\mathrm{obs}} = k_1[\mathrm{H^+}]/([\mathrm{H^+}] + K_a) \qquad (2)$$

The oxidative chemistry of peroxynitrite is highly pH dependent. Peroxynitrous acid is capable of oxidizing Br^-,[18] I^-,[19] CN^-,[19] SCN^-,[19] sulfhydryls,[20] deoxyribose,[21] dimethyl sulfoxide,[21] DNA,[22] 2,2′-azinobis(3-ethylbenzothiazoline-6-sulfonate) (ABTS),[23,24] dihydrorhodamine-123,[25] and methionine.[26] Peroxynitrous acid also hydroxylates and nitrates aromatic compounds.[27−29] Peroxynitrite oxidizes hydrogen peroxide and hydroxylamine, provided copper ions are present,[19,30] whereas in strong basic solutions it reduces MnO_4^- to MnO_4^{2-}.[31]

The oxidation of the various substrates by either ONOOH or $ONOO^-$ can take place via multiple pathways, which are presently poorly understood: (i) ONOOH may directly oxidize the substrates, as in the case of Br^-,[18] I^-,[19] and sulfhydryls.[20] (ii) ONOOH may decompose first into highly reactive species

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which subsequently oxidize the substrates (e.g., ABTS[23,24]) or hydroxylate and nitrate aromatic compounds (e.g., tyrosine,[28] phenylalanine[29]). It has been suggested that these oxidizing species may involve hydroxyl radical and nitrogen dioxide from the homolytic dissociation of ONOOH (reaction 3)[21,23] or nitronium cation from the heterolytic dissociation of ONOOH (reaction 4).[28,29]

$$\mathrm{ONOOH} \rightarrow {}^{\bullet}\mathrm{OH} + \mathrm{NO_2}^{\bullet} \qquad (3)$$

$$\mathrm{ONOOH} \rightarrow \mathrm{OH^-} + \mathrm{NO_2}^+ \qquad (4)$$

However, thermodynamic and kinetics considerations demonstrated that neither process 3 nor process 4 is feasible,[16] though metal ions may catalyze the heterolytic cleavage of ONOOH to form a nitronium-like species.[28] It has been suggested that an intermediate closely related to the transition state for the isomerization of ONOOH to nitric acid may be the oxidizing species responsible for the hydroxyl radical-like activity.[16]

The yield of the indirect nitration of 4-hydroxyphenylacetic acid (4-HPA) by peroxynitrite is pH dependent, having a maximum of 6.9% at pH 7.5, and decreases at more acidic and basic solutions.[28] A similar pH curve was obtained in the presence of superoxide dismutase (SOD), but the maximum yield obtained was 9%.[28] From each of these two pH curves, apparent pK_a values of 6.8 and 7.9 were calculated, which were attributed to *cis*-ONOOH and *trans*-ONOOH, respectively.[28] In a recent study,[24] it was found that peroxynitrite indirectly oxidizes dimethyl sulfoxide (DMSO) and ABTS to formaldehyde and ABTS$^+$, respectively. The product yield in both cases decreased with increasing pH, resulting in an apparent pK_a of 7.9 in the case of DMSO and 8.2 in the case of ABTS. Thus, it was concluded[24,28] that the more stable *cis*-ONOOH (pK_a = 6.8) isomerizes to the unstable *trans*-ONOOH (p$K_a \approx 8$), where the latter forms a vibrationally excited intermediate that can directly rearrange to nitric acid or oxidize the substrate with formation of $\mathrm{NO_2}^{\bullet}$ during this reaction. The proposed mechanism is given by reactions 5–10:[16,24,28]

$$cis\text{-ONOOH} \rightleftharpoons cis\text{-ONOO}^- + \mathrm{H}^+ \qquad \mathrm{p}K_a = 6.8 \qquad (5)$$

$$cis\text{-ONOOH} \rightleftharpoons trans\text{-ONOOH} \qquad (6)$$

$$trans\text{-ONOOH} \rightleftharpoons trans\text{-ONOO}^- + \mathrm{H}^+ \qquad \mathrm{p}K_a \approx 8 \qquad (7)$$

$$trans\text{-ONOO}^- \rightarrow \mathrm{NO_3}^- \qquad (8)$$

$$trans\text{-ONOOH} \rightarrow \mathrm{NO_3}^- + \mathrm{H}^+ \qquad (9)$$

$$trans\text{-ONOOH} + \mathrm{S} \rightarrow \mathrm{SOH}^{\bullet} + \mathrm{NO_2}^{\bullet} \qquad (10)$$

This mechanism predicts that for the case where S = ABTS, the yield of ABTS$^+$ at pH < 7 must depend on [ABTS]$_0$ due to the competition between reactions 9 and 10. At relatively high concentrations of ABTS (where k_{10}[ABTS]$_0$ > k_9 and [ABTS]$_0$ > [peroxynitrite]$_0$), the yield of ABTS$^+$ should approach 2[peroxynitrite]$_0$ as $\mathrm{NO_2}^{\bullet}$ also rapidly oxidizes ABTS.[23] This is in contrast to the experimental results, where it was found that [ABTS$^+$]/[peroxynitrite]$_0$ = 0.63[23] and 0.45–0.55[24] at pH < 7, independent of [ABTS]$_0$. Therefore, these results do not support the above-suggested mechanism. Furthermore, one cannot obtain the pK_a of the *trans*-ONOOH from the pH-dependent curves of the product yield. These curves should depend on the pK_a of the *trans*-ONOOH and on the relative rates of the isomerization of the *trans* species to nitrate and the reaction of the *trans*-ONOOH with the substrate, i.e., on the rate constant of this reaction and on the substrate concentration.

Peroxynitrite has great physiological importance since it is a highly oxidizing agent that can also act as a mediator of free radical toxicity. However, there is no consensus on the mechanism by which peroxynitrite or peroxynitrous acid acts in its deleterious reaction, and therefore, we decided to study the reaction mechanism of this compound. We have chosen I$^-$, nickel(II) 1,4,8,11-tetraazacyclotetradecane (Ni$^{\mathrm{II}}$cyclam), and $\mathrm{Fe(CN)_6}^{4-}$ as model reductants.

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 prepared according to the standard method, where acidified hydrogen peroxide solution is added to a nitrite solution and the reaction is quenched by adding strong alkali to stabilize the peroxynitrite.[16,18] All three solutions of 20 mL of 1 M $\mathrm{NaNO_2}$, 20 mL of 1.2 M $\mathrm{H_2O_2}$ in 1 M HCl, and 10 mL of 7 M NaOH in beakers were cooled in ice water, and the nitrite solution was well stirred. The hydrogen peroxide solution was added rapidly to the nitrite solution, followed immediately by the alkali. The yellow solution was then treated with an excess of $\mathrm{MnO_2}$ and filtered. Yields of 35–40% were obtained, which were determined spectrophotometrically using $\epsilon(302)$ = 1670 M^{-1} cm^{-1}.[14,31] These solutions, which are unavoidably contaminated with nitrate and nitrite, were frozen, and the iced phase was removed. The concentration of peroxynitrite was in the range 200–250 mM, and solutions kept at −18 °C showed little decomposition over several weeks. Dilution of the peroxynitrite solution was done in slightly alkaline solutions (final pH 11–12). The concentration of peroxynitrite was determined before each experiment by measuring the absorbance at 302 nm.

[Ni(cyclam)][ClO$_4$]$_2$ was synthesized according to published procedures.[32] Ni$^{\mathrm{III}}$cyclam was formed via the oxidation of Ni$^{\mathrm{II}}$cyclam by $\mathrm{Na_2S_2O_8}$ in an aqueous solution containing 0.1 M phosphate buffer at pH 6–7.8. In the presence of $\mathrm{H_2PO_4}^-$ as a stabilizing anion,[33,34] Ni$^{\mathrm{III}}$cyclam has an absorption maximum at 284 nm (ϵ = 10 000 ± 200 M^{-1} cm^{-1}) and a shoulder at 360 nm (ϵ = 5600 ± 150 M^{-1} cm^{-1} at pH 6 and 5100 ± 100 M^{-1} cm^{-1} at pH 6.8–7.8). The trivalent complex is not stable at pH > 8.[35]

Stoichiometry. Solutions of NaI, [Ni(cyclam)][ClO$_4$]$_2$, and $\mathrm{K_4Fe(CN)_6}$ in 0.2 M phosphate buffer (unless otherwise stated) were rapidly vortexed with equal volumes of peroxynitrite solutions. The final pH was measured after completion of the reaction to account for the slight alkaline shift caused by the addition of alkali peroxynitrite. The concentrations of $\mathrm{I_3}^-$, Ni$^{\mathrm{III}}$cyclam, and $\mathrm{Fe(CN)_6}^{3-}$ were determined spectrophotometrically using the extinction coefficients of 26 400 M^{-1} cm^{-1} at 352 nm,[36] 5150–5600 M^{-1} cm^{-1} at 360 nm (see above), and 1000 M^{-1} cm^{-1} at 420 nm, respectively.

Kinetic Measurements. Stopped-flow kinetics were performed on a Hi-Tech Scientific Instrument, Model PQ/SF-53. The decomposition of peroxynitrite was followed at 302 nm by mixing alkaline peroxynitrite solutions with 0.2 M phosphate buffer at various pHs. The reactions of peroxynitrite with I$^-$, Ni$^{\mathrm{II}}$cyclam, and $\mathrm{Fe(CN)_6}^{4-}$ were followed at 352, 360, and 420 nm, respectively, by mixing alkaline peroxynitrite solutions with various concentrations of the reductants in 0.2 M phosphate buffer. The optical path length was either 1 or 0.2 cm. The pH of each reaction mixture was measured after the addition of peroxynitrite. The kinetics of peroxynitrite decomposition and the formation of $\mathrm{I_3}^-$, Ni$^{\mathrm{III}}$cyclam, and $\mathrm{Fe(CN)_6}^{3-}$ were fitted to first-order

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Table 1. Stoichiometry and the Observed Rate Constant of the Oxidation of I^- by Peroxynitrite under Various Conditions[a]

$[I^-]_0$ (M)	$[ONOO^-]_0$ M	pH	$[I_3^-]_0$ (M)	$[I^-]_0/[ONOO^-]_0$	k_{obs} (s^{-1})
6.0×10^{-3}	3.1×10^{-4}	3.4			140 ± 4
		3.4			1.0 ± 0.2[45]
0.1	6.2×10^{-5}	5.8	4.93×10^{-5}	1.59	*b*
0.1	3.5×10^{-4}	5.9	2.68×10^{-4}	1.53	*b*
0.1	1.7×10^{-4}	5.9	1.43×10^{-4}	1.68	*b*
	1.0×10^{-3}	6.4			1.15
1.0×10^{-3}	1.7×10^{-4}	6.8			19.5 ± 0.7
2.5×10^{-2}	6.2×10^{-5}	6.8	4.43×10^{-5}	1.43	nd[c]
0.1	6.7×10^{-5}	6.8	5.39×10^{-5}	1.61	*b*
0.1	3.5×10^{-4}	6.8	2.81×10^{-5}	1.61	*b*
0.5	6.7×10^{-5}	6.8	4.96×10^{-5}	1.48	*b*
0.01	6.2×10^{-5}	6.8	3.50×10^{-5}	1.13	nd
0.01 + 1 M 2-propanol	6.2×10^{-5}	6.8	3.53×10^{-5}	1.14	nd
2.0×10^{-3}	6.7×10^{-5}	6.8	1.74×10^{-5}	0.52	nd
2.0×10^{-3} + 1 M *tert*-butyl alcohol	6.7×10^{-5}	6.8	1.88×10^{-5}	0.56	nd
0.1	1.7×10^{-4}	7.9	1.28×10^{-4}	1.51	nd
6×10^{-3}	3.1×10^{-4}	7.8			15.2 ± 0.3
	1.0×10^{-3}	7.7			0.28 ± 0.03
0.1	3.5×10^{-4}	8.0	2.52×10^{-4}	1.44	nd
0.1	1.7×10^{-4}	10.2	1.38×10^{-4}	1.62	1.65 ± 0.1
		10.0			1.8×10^{-3}[45]
0.1	1.7×10^{-4}	10.8	9.6×10^{-5}	1.13	nd
0.1	1.7×10^{-4}	11.1	3.0×10^{-5}	0.35	nd

[a] All solutions contained 0.1 M phosphate buffer in the range 3.4–8.0. [b] The rate was too fast to be measured. [c] nd, not determined.

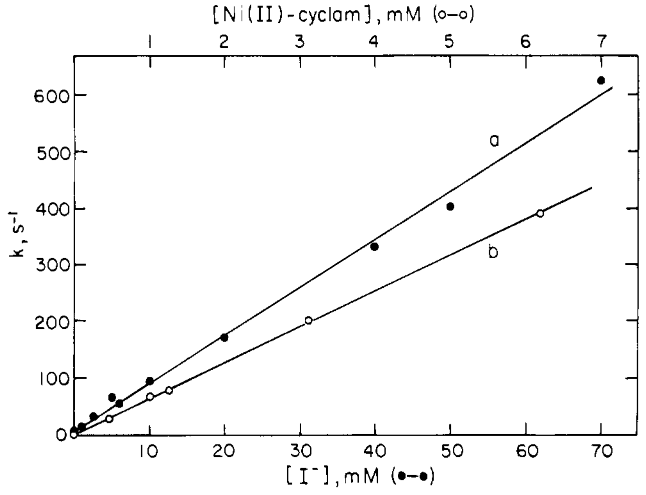

Figure 1. (a) Observed rate constant of the formation of I_3^- in the oxidation of I^- by peroxynitrite as a function of iodide concentration. Solutions contained excess concentrations of iodide over peroxynitrite and 0.1 M phosphate buffer at pH 6.8. (b) Observed rate constant of the formation of Ni^{III}cyclam in the oxidation of Ni^{II}cyclam by peroxynitrite as a function of [Ni^{II}cyclam]. Solutions contained excess concentrations of Ni^{II}cyclam over peroxynitrite and 0.1 M phosphate buffer at pH 6.0.

reaction kinetics using a nonlinear regression program. The kinetic measurements were carried out at 27 °C.

γ-Irradiation. γ-Radiolysis experiments were carried out with a ^{137}Cs source. The dose rate (10 Gy/min) was determined with the Fricke dosimeter.

Results

The Oxidation of I^-. The reaction of I^- with peroxynitrite in the presence of excess concentrations of I^- over peroxynitrite yields I_3^-. The rate of the formation of I_3^- was faster than the rate of the self-decomposition of peroxynitrite at pH 3.4–10.2 (Table 1). Below pH 3.4, the contamination of peroxynitrite with nitrite interferes due to the oxidation of I^- by nitrite.[37] At

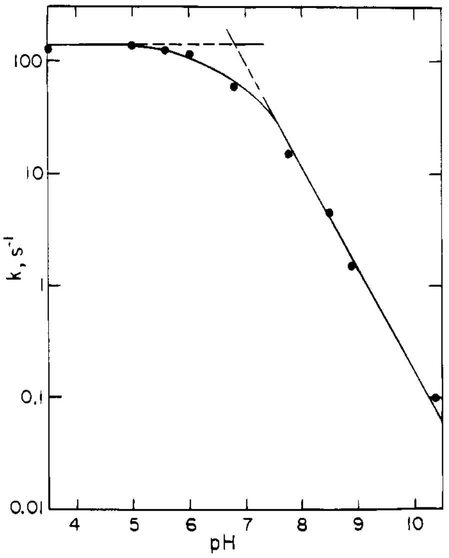

Figure 2. Observed rate constant of the formation of I_3^- in the oxidation of I^- by peroxynitrite as a function of pH. Solutions contained 6 mM iodide, 0.33 mM peroxynitrite, and 0.1 M phosphate.

pH > 10, the yield of I_3^- decreased (Table 1) due to the hydrolysis of I_3^- to IO^- (where the latter disproportionates rapidly to IO_3^- and I^-).[37] The rate of I_3^- formation obeys pseudo-first-order kinetics. The observed rate constant depends linearly on [I^-] (Figure 1a) and is highly pH dependent (Figure 2). The stoichiometry of the reaction was determined at relatively high concentrations of I^- (0.025–0.5 M) to avoid the dissociation of I_3^- to I_2 and I^- ($K = 710\ M^{-1}$).[37] It was found that 1 mol of peroxynitrite oxidizes 1.56 ± 0.12 mol of iodide, independent of $[ONOO^-]_0$, $[I^-]_0$ ($[I^-]_0 > 0.025$ M), and pH in the range 5.8–10.2 (Table 1). In solutions containing 0.002–0.01 M iodide at pH 6.6–6.8, the oxidation yield of I_3^- by peroxynitrite was unaffected by the presence of 1 M *tert*-butyl alcohol and 1 M 2-propanol, respectively (Table 1).

Oxidation of Ni^{II}Cyclam by Peroxynitrite. The reaction of peroxynitrite with Ni^{II}cyclam in the presence of phosphate ions (pH 5.5–8.0) forms Ni^{III}cyclam. At pH > 8, the trivalent complex is unstable even in the presence of phosphate.[35] The rate of the formation of Ni^{III}cyclam was faster than the rate of

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Table 2. Stoichiometry and the Observed Rate Constants of the Oxidation of Ni^{II}Cyclam by Peroxynitrite under Various Conditions

$[Ni(II)]_0$ (M)	$[ONOO^-]_0$ M	[PB][a]	pH	$[Ni(III)]_{exp}$ (M)	$[Ni(III)]_{exp}/[ONOO^-]_0$	$[Ni(III)]_{cal}$[a] (M)	$[Ni(III)]_{cal}/[ONOO^-]_0$	k_{obs} (s^{-1})
1.0×10^{-3}	3.5×10^{-5}	0.04	5.5	nd[c]				63 ± 2
1.0×10^{-3}	3.8×10^{-5}	0.18	5.5	nd				62 ± 2
1.0×10^{-3}	8.2×10^{-5}	0.10	6.0	1.3×10^{-4}	1.59	1.5×10^{-4}	1.83	68 ± 1
1.0×10^{-3}	2.5×10^{-4}	0.10	6.0	4.4×10^{-4}	1.76	4.3×10^{-4}	1.72	nd
5.0×10^{-4}	6.3×10^{-5}	0.10	6.0	1.1×10^{-4}	1.75	1.1×10^{-4}	1.75	30 ± 1
5.0×10^{-4}	2.0×10^{-4}	0.10	6.1	3.3×10^{-4}	1.65	3.2×10^{-4}	1.58	nd
5.0×10^{-4} + 1 M *tert*-butyl alcohol	2.0×10^{-4}	0.10	6.1	3.0×10^{-4}	1.50			nd
1.2×10^{-3}	9.5×10^{-5}	0.10	6.0	1.7×10^{-4}	1.79	1.8×10^{-4}	1.89	77 ± 2
3.1×10^{-3}	9.5×10^{-5}	0.10	6.0	nd				201 ± 2
6.2×10^{-3}	9.5×10^{-5}	0.10	6.0	nd				385 ± 5
5.0×10^{-4}	2.0×10^{-4}	0.10	6.8	3.2×10^{-4}	1.60	3.2×10^{-4}	1.58	nd
5.0×10^{-4}	2.0×10^{-4}	0.18	6.8	3.0×10^{-4}	1.50	3.2×10^{-4}	1.58	nd
1.0×10^{-3}	8.0×10^{-5}	0.10	6.9	1.3×10^{-4}	1.63	1.5×10^{-4}	1.88	40 ± 1
1.0×10^{-3}	2.5×10^{-4}	0.10	6.9	4.4×10^{-4}	1.76	4.3×10^{-4}	1.72	nd
5.0×10^{-4}	2.0×10^{-4}	0.10	7.5	1.4×10^{-4}	0.70			nd
5.0×10^{-4}	2.0×10^{-4}	0.18	7.5	2.4×10^{-4}	1.20			nd
1.0×10^{-3}	7.1×10^{-5}	0.10	8.0	8.2×10^{-5}	1.15			7.7 ± 0.2

[a] PB, phosphate buffer. [b] Calculated values are based on simulations of reactions 15, 16, 17, and 24, assuming $k_{17} = 9 \times 10^5\ M^{-1}\ s^{-1}$. [c] nd, not determined.

the self-decomposition of peroxynitrite at the same pH and obeyed pseudo-first-order kinetics. The observed rate constant depends linearly on [Ni^{II}cyclam] (Figure 1b) and decreases with increasing pH. The observed first-order rate constant and the stoichiometry of the reaction under various conditions are given in Table 2. It was found that, at pH 6.0–6.9, 1 mol of peroxynitrite oxidizes 1.69 ± 0.19 mol of Ni^{II}cyclam. The yield of Ni^{III}cyclam at pH 6.0 was unaffected by the presence of 1 M *tert*-butyl alcohol (Table 2).

Oxidation of Ni^{II}cyclam by $NO_2^{\bullet}$. $NO_2^{\bullet}$ was generated by γ-irradiation of deaerated aqueous solutions containing 0.01 M $NaNO_2$ and 0.05 M $NaNO_3$ at pH 6.0 (10 mM phosphate buffer). Under these conditions, all the primary free radicals formed by the radiation are converted into $NO_2^{\bullet}$ according to the following sequence of reactions 11–14:

$$H_2O \xrightarrow{\gamma} {}^{\bullet}OH\ (2.75),\ e_{aq}\ (2.75),\ H^{\bullet}\ (0.60),\ H_2O_2\ (0.75) \quad (11)$$

(The numbers in parentheses are G values, which represent the number of molecules formed per 100 eV absorbed by the solution.)[38]

$$^{\bullet}OH + NO_2^- \rightarrow OH^- + NO_2^{\bullet} \qquad k_{12} = 1.0 \times 10^{10}\ M^{-1}\ s^{-1}\ {}^{38} \quad (12)$$

$$e_{aq}^- + NO_3^- \rightarrow NO_3^{2-} \qquad k_{13} = 9.7 \times 10^{9}\ M^{-1}\ s^{-1}\ {}^{38} \quad (13)$$

$$NO_3^{2-} + H_2O \rightarrow NO_2^{\bullet} + 2OH^- \qquad k_{14} = 5.5 \times 10^{4}\ s^{-1}\ {}^{39} \quad (14)$$

The yield of Ni^{III}cyclam increased linearly with the dose and, at a constant dose, increased linearly with an increase in [Ni^{II}cyclam]. These results indicate that, under our experimental conditions, the decay of $NO_2^{\bullet}$ via reactions 15 and 16 competes with its reaction with Ni^{II}cyclam (reaction 17) or that NO_2^-, present in very high concentrations, reduces Ni^{III}cyclam back to the starting material (reaction −17). The latter suggestion was verified by adding 0.1 M $NO_2^{\bullet -}$ to 5×10^{-5} M Ni^{III}cyclam at pH 6.0 (10 mM phosphate buffer). The yellow color of the Ni^{III}cyclam disappeared.

$$2NO_2^{\bullet} \rightleftharpoons N_2O_4 \qquad 2k_{15} = 9 \times 10^{8}\ M^{-1}\ s^{-1};\quad k_{-15} = 6.9 \times 10^{3}\ s^{-1}\ {}^{39} \quad (15)$$

$$N_2O_4 + H_2O \rightarrow NO_2^- + NO_3^- + 2H^+ \qquad k_{16} = 1 \times 10^{3}\ s^{-1}\ {}^{39} \quad (16)$$

$$Ni(II)cyclam + NO_2^{\bullet} \rightleftharpoons Ni(III)cyclam + NO_2^- \quad (17)$$

Therefore, $NaNO_2$ was replaced by 1 M *tert*-butyl alcohol. Under these conditions, *tert*-butyl alcohol scavenges the hydroxyl radicals, and the solvated electrons are converted into $NO_2^{\bullet}$ according to reactions 13 and 14. It was found that G(Ni^{III}cyclam) = 2.51 ± 0.23, independent of [Ni^{II}cyclam] over the range 0.1–1 mM. This G value equals, within experimental error, G_{e^-}, indicating that all $NO_2^{\bullet}$ formed by the radiation oxidizes Ni^{II}cyclam via reaction 17.

Oxidation of $Fe(CN)_6^{4-}$. Peroxynitrite oxidizes $Fe(CN)_6^{4-}$ to $Fe(CN)_6^{3-}$. The rate of formation of $Fe(CN)_6^{3-}$ obeys first-order kinetics. The observed rate constant was found to be independent of $[Fe(CN)_6^{4-}]_0$ (Table 3) and, within experimental error, identical with the self-decomposition rate of peroxynitrite at the pH range studied (Figure 3). The yield of the oxidation of ferrocyanide by peroxynitrite was determined in the presence of excess concentrations of $Fe(CN)_6^{4-}$ over peroxynitrite. It was found that 1 mol of peroxynitrite oxidizes 0.83 ± 0.17 mol of ferrocyanide, independent of $[Fe(CN)_6^{4-}]_0$, $[ONOO^-]_0$, and pH (Table 3). At very high pHs, the yield of ferricyanide was measured at 420 nm in parallel to the decomposition of peroxynitrite at 302 nm at the same pH. In order to determine whether $^{\bullet}OH$ is the reacting entity in this system, the relative ability of typical hydroxyl radical scavengers to inhibit the oxidation of ferrocyanide by peroxynitrite was assessed (Table 4). Under the conditions where all scavengers used compete efficiently with ferrocyanide ($[Fe(CN)_6^{4-}]_0 \leq 0.1$ mM) for $^{\bullet}OH$ radicals, no inhibition at all was observed (Table 4). In the case of 2-propanol and ethanol, the inhibition should even have been twice as high as compared to *tert*-butyl alcohol and DMSO since the radicals derived from the reaction of $^{\bullet}OH$ with ethanol and 2-propanol reduce ferricyanide to ferrocyanide very

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Table 3. Stoichiometry and the Observed Rate Constants of the Oxidation of Ferrocyanide by Peroxynitrite under Various Conditions[a]

$[Fe(CN)_6{}^{4-}]_0$ (M)	$[ONOO^-]_0$ (M)	pH	$[Fe(CN)_6{}^{3-}]/[ONOO^-]_0$	k_{obs} (s^{-1})
0.01	6.5×10^{-4}	5.5	0.86	1.5 ± 0.05
0.01	4.0×10^{-4}	5.8	0.83	1.31 ± 0.03
0.01	3.8×10^{-4}	6.0	0.77	1.20 ± 0.02
0.025	4.0×10^{-4}	6.3	0.79	nd[c]
0.01	3.6×10^{-4}	6.7	0.65	0.58 ± 0.05
0.0005	7.5×10^{-4}	6.9	nd	0.70 ± 0.04
0.001	7.5×10^{-4}	6.9	nd	0.68 ± 0.03
0.01	7.5×10^{-4}	6.9	nd	0.70 ± 0.02
0.05	7.5×10^{-4}	6.9	0.79	0.67 ± 0.03
0.025	4.0×10^{-4}	7.0	0.76	nd
0.05	1.0×10^{-3}	7.0	0.80	nd
0.01	4.2×10^{-4}	8.2	0.90	0.05 ± 0.003
0.005	4.0×10^{-4}	8.5	0.88	nd
0.01	3.9×10^{-4}	8.5	0.99	0.03 ± 0.002
0.025	4.0×10^{-4}	8.5	0.80	nd
0.05	7.0×10^{-4}	8.6	0.92	0.024 ± 0.0005
0.0005	8.0×10^{-4}	8.7	nd	0.020 ± 0.001
0.001	8.0×10^{-4}	8.7	nd	0.022 ± 0.002
0.01	8.0×10^{-4}	8.7	nd	0.020 ± 0.001
0.05	6.5×10^{-4}	8.7	0.92	0.018 ± 0.001
0.05	3.5×10^{-4}	8.7	nd	0.024 ± 0.001
0.05	7.7×10^{-4}	8.8	0.92	0.014 ± 0.0007
0.01	1.2×10^{-3}	8.9	0.85	0.013 ± 0.002
0.05	8.5×10^{-4}	9.4	0.92	0.0038 ± 0.0002
0.01	5.4×10^{-4}	10.2	1.21	0.0048 ± 0.0003
0.005	3.2×10^{-4}	11.9[b]	0.86	nd
0.005	6.2×10^{-4}	12.4[b]	0.97	nd

[a] All solutions contained 0.1 M phosphate buffer. [b] No buffer added. [c] nd, not determined.

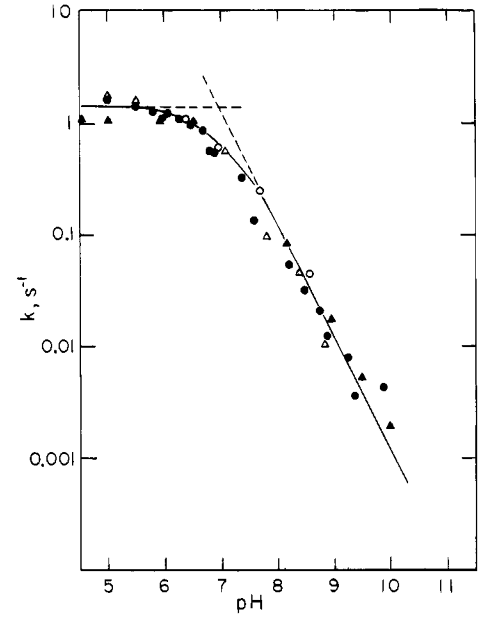

Figure 3. Observed rate constant of the oxidation of 5–50 mM $Fe(CN)_6{}^{4-}$ by 0.3–1.2 mM peroxynitrite as measured at 420 nm as a function of pH (●). The observed rate constant of the decay of 1 mM peroxynitrite alone as measured at 302 nm as a function of pH (○). (△) Data from ref 16. (▲) Data from ref 45. The line was calculated according to eq 2 using $k_1 = 1.4\ s^{-1}$ and $pK_a = 7$.

quickly.[40] There was no correlation at all between the inhibition of the oxidation of ferrocyanide by peroxynitrite in the presence of *tert*-butyl alcohol, ethanol, 2-propanol, DMSO, and mannitol and their ability as efficient •OH scavengers (Table 4).

Discussion

The oxidation of various substrates by ONOOH and/or $ONOO^-$ can take place either directly or indirectly by the highly reactive species which are formed via the self-decomposition of peroxynitrous acid. If oxidation occurs via the direct reaction of ONOOH with the substrates, the reaction rate must be faster than the self-decomposition rate of peroxynitrite. Thus, when the concentrations of either peroxynitrite or the substrates are in excess, the rate of decomposition of peroxynitrite or the rate of formation of the oxidized substrates will obey pseudo first-order kinetics. However, if the rate-determining step is the self-decomposition of peroxynitrite, the rate of formation of the oxidized products should be identical with the rate of the self-decomposition of peroxynitrite. In both the direct and indirect oxidation mechanisms by ONOOH, the *reaction rate* will depend on pH and will decrease with an increase in pH, yielding the apparent pK_a of peroxynitrous acid.

The results demonstrate that peroxynitrite can oxidize a compound either directly or after its conversion into a highly reactive oxidant. The particular reaction pathway depends on the kind of compound used as a reductant. The oxidation rates of I^- and Ni^{II}cyclam by peroxynitrite are pH dependent and are faster than the self-decomposition rates of peroxynitrite. From plots of the observed rate constant of oxidation as a function of pH, values of pK_a of 6.9 ± 0.1 in the case of I^- (Figure 2) and 7.1 ± 0.2 in the case of Ni^{II}cyclam (Table 2) are calculated in agreement with the literature value of 6.8,[16] indicating that the active oxidizing species is the peroxynitrous acid. The second-order rate constants of the formation of $I_3{}^-$ and Ni^{III}cyclam via the reaction of ONOOH with I^- and Ni^{II}cyclam are determined to be $(2.3 \pm 0.1) \times 10^4$ and $(6.5 \pm 0.3) \times 10^4\ M^{-1}\ s^{-1}$, respectively.

The stoichiometry of the oxidation of I^- and Ni^{II}cyclam by ONOOH was found to be 1.56 ± 0.12 and 1.69 ± 0.19, respectively, rather than 2.0 as expected for ONOOH as a two-equivalent oxidant. The calculation of the stoichiometry of these reactions was based on $\epsilon_{302} = 1670 \pm 50\ M^{-1}\ cm^{-1}$ for peroxynitrite, which was determined by potentiometric titration of alkaline $MnO_4{}^-$ with $ONOO^-$.[31] This value was recently verified by Huie and Padmaja[14] by comparing the known absorbance of $O_2{}^-$ to that of $ONOO^-$, assuming that $O_2{}^-$ is

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Table 4. Effect of Several •OH Scavengers on the Yield of the Oxidation of Ferrocyanide by Peroxynitrite under Various Conditions[a]

[scavenger]	$[Fe(CN)_6^{4-}]_0$ (mM)	$[ONOO^-]_0$ (mM)	pH	$[Fe(CN)_6^{3-}]_{obs}$ (mM)	$[Fe(CN)_6^{3-}]_{cal}$[b] mM
	5.9	0.29	6.0	0.213	
0.1 M *tert*-butyl alcohol				0.196	0.159
1.0 M *tert*-butyl alcohol				0.186	0.116
	0.1	0.88	6.4	0.109	
0.5 M *tert*-butyl alcohol				0.116	0.058
0.5 M 2-propanol				0.092	0.000
0.5 M ethanol				0.092	0.000
0.05 M mannitol				0.105	0.058
1.0 M DMSO				0.109	0.058
	0.2	0.85	6.9	0.205	
0.5 M *tert*-butyl alcohol				0.213	0.103
0.5 M 2-propanol				0.153	0.000
0.5 M ethanol				0.145	0.000
0.5 M DMSO				0.184	0.103
0.1 M mannitol				0.158	0.103
	5	0.42	7.0	0.303	
1 M *tert*-butyl alcohol				0.277	0.163
	10	0.45	7.1	0.312	
0.03 M 2-propanol				0.245	0.189
0.1 M 2-propanol				0.224	0.108
0.5 M 2-propanol				0.213	0.030
0.05 M ethanol				0.219	0.160
0.5 M ethanol				0.185	0.030
	5	0.50	7.9	0.417	
1 M *tert*-butyl alcohol				0.393	0.209
	5	0.34	9.8	0.409	
1.0 M *tert*-butyl alcohol				0.395	0.220

[a] All solutions contained 0.1 M phosphate buffer. [b] Oxidation yields were calculated with the assumption that •OH radicals are formed as intermediates. The following rate constants for the reaction of •OH with the various substates were taken from ref 38: 1.1×10^{10} M^{-1} s^{-1} for ferrocyanide, 6×10^8 M^{-1} s^{-1} for *tert*-butyl alcohol, 6.5×10^9 M^{-1} s^{-1} for DMSO, 1.9×10^9 M^{-1} s^{-1} for 2-propanol, ethanol, and mannitol. In the case of 2-propanol and ethanol, the reduction of ferricyanide by CH_3CH_2OH and $(CH_3)_2COH$ with rate constants of 5.3×10^9 M^{-1} s^{-1} and 4.7×10^9 M^{-1} s^{-1},[41] respectively, were taken into account. In the case of mannitol, the reduction of ferricyanide by the radical was not taken into account, though the radicals derived from the reaction of •OH radicals with glycerol and glucose reduce ferricyanide very quickly ferricyanide.[41]

fully converted into $ONOO^-$ in the presence of NO. Thus, a stoichiometry lower than 2, obtained in the case of I^- and Ni^{II}cyclam, suggests that the oxidation mechanism by peroxynitrite involves an additional pathway which does not yield I_3^- or Ni^{III}cyclam, respectively.

The direct oxidation of I^- by peroxynitrite may take place via the following sequence of reactions

$$ONOOH \rightleftharpoons ONOO^- + H^+ \qquad pK_a \qquad (5)$$

$$ONOOH + I^- \rightarrow I^\bullet + NO_2^\bullet + OH^- \qquad k_{18} = 2.3 \times 10^4\ M^{-1}\ s^{-1} \qquad (18)$$

$$NO_2^\bullet + I^- \rightleftharpoons I^\bullet + NO_2^- \qquad k_{19} = 1.1 \times 10^5\ M^{-1}\ s^{-1}\ {}^{41} \qquad k_{-19} = 8.8 \times 10^9\ M^{-1}\ s^{-1}\ {}^{41} \qquad (19)$$

$$I^\bullet + I^- \rightarrow I_2^- \qquad k_{20} = 1.1 \times 10^{10}\ M^{-1}\ s^{-1}\ {}^{41} \qquad (20)$$

$$I_2^- + I_2^- \rightarrow I_3^- + I^- \qquad k_{21} = 2.3 \times 10^9\ M^{-1}\ s^{-1}\ {}^{41} \qquad (21)$$

$$I_2 + I^- \rightleftharpoons I_3^- \qquad K_{22} = 710\ M^{-1}\ {}^{37} \qquad (22)$$

This mechanism predicts a stoichiometry of 2, which exceeds the observed value of 1.56 ± 0.12. Therefore, we have to assume that in parallel to reaction 18, another reaction occurs which does not yield I_3^-. This could possibly be reaction 23, which has already been suggested by Hughes and Nicklin[19]

$$ONOOH + I^- \rightarrow HOI + NO_2^- \qquad (23)$$

In order to account for a stoichiometry of 1.56 ± 0.12, we have to assume that $k_{18}/k_{23} \approx 4$ and that HOI is rapidly oxidized by ONOOH and does not react with I^- to yield I_3^-. Hughes and Nicklin[19] have claimed that the stoichiometry of the reaction of peroxynitrite with iodide is unity at $[OH^-] = 0.07-0.19$ M, and they determined $k_{23} \approx 1 \times 10^5$ M^{-1} s^{-1}. From their paper, it was not clear how the stoichiometry was determined.[19] The readers were referred to ref 31, where analysis of nitrite and nitrate was described. However, the same yield of nitrite is expected whether the oxidation is initiated by reaction 18 followed by 19 or by reaction 23.

The mechanism of the oxidation of Ni^{II}cyclam by peroxynitrite is given by reactions 24 and 17

$$ONOOH + Ni(II)cyclam \rightarrow Ni(III)cyclam + NO_2^\bullet + OH^- \qquad 2k_{24} = 6.5 \times 10^4\ M^{-1}\ s^{-1} \qquad (24)$$

$$Ni(II)cyclam + NO_2^\bullet \rightarrow Ni(III)cyclam + NO_2^- \qquad (17)$$

The rate constant of reaction 17 is unknown. However, when deaerated aqueous solutions containing 0.1 M $NaNO_3$, 1 M *tert*-butyl alcohol, and various concentrations of Ni^{II}cyclam at pH 6.0 (10 mM phosphate buffer) were γ-irradiated, the formation of Ni^{III}cyclam was observed. We determined $G(Ni^{III}cyclam) = 2.51 \pm 0.23$, independent of $[Ni^{II}cyclam] = 0.1-1$ mM, indicating that all $NO_2^\bullet$ formed by the radiation oxidized Ni^{II}cyclam. A value of $k_{17} > 3 \times 10^5$ M^{-1} s^{-1} is estimated from the fact that, at 0.1 mM Ni^{II}cyclam, the maximum yield of Ni^{III}cyclam is obtained. The stoichiometry of the reaction is 1.69 ± 0.19 and not 2 as predicted by reactions 24 and 17. Therefore, we have to assume that to some extent the decay of $NO_2^\bullet$ via reactions 15 and 16 also proceeds under these experimental conditions where the steady-state concentrations of $NO_2^\bullet$ are orders of magnitude higher than those obtained in the γ-radiolysis experiments. Because nitrite contamination of the peroxynitrite solutions is relatively low, reaction −17 does not take place under our experimental conditions. Simulations of a mechanism consisting of reactions 15, 16, 17, and 24, assuming that $k_{17} = 9 \times 10^5\ M^{-1}\ s^{-1}$, yield a stoichiometry similar to that obtained experimentally (Table 2). At pH > 7, the yield of Ni^{III}cyclam decreased, most probably due to decrease in $[H_2PO_4^-]$, which is essential for stabilizing the trivalent complex.[33,34] Indeed, when the concentration of the total phosphate buffer was increased, the yield of Ni^{III}cyclam increased (Table 2).

The rate of oxidation of $Fe(CN)_6^{4-}$ by peroxynitrite was found to be similar to the rate of the self-decomposition of peroxynitrite, and from Figure 3, a pK_a value of 7.0 is obtained. Therefore, the oxidation of $Fe(CN)_6^{4-}$ does not take place directly by peroxynitrite but via reactive oxidizing intermediates formed from its decomposition. The stoichiometry of the reaction demonstrates that 1 mol of peroxynitrite oxidizes 0.83 ± 0.17 mol of $Fe(CN)_6^{4-}$, independent of $[ONOOH]_0$, $[Fe(CN)_6^{4-}]_0$ and pH (Table 3). Thus, as ONOOH oxidizes only 0.8 mol of ferrocyanide rather than 2.0 mol, we have to assume that the decomposition of ONOOH proceeds in two parallel pathways. The first pathway proceeds via the direct isomerization of ONOOH to nitric acid (~60%), whereas via the second pathway (~40%) ONOOH is converted into a highly reactive oxidant, which subsequently oxidizes ferrocyanide ions.

Mahoney[42] found from the analysis of the kinetics of oxygen evolution and nitrite disappearance in mixtures of nitrite and hydrogen peroxide at pH ~ 5 that the contribution of the direct isomerization to nitrate is 68%. We found in the ferrocyanide system that the contribution of the direct isomerization of peroxynitrous acid to nitric acid is about 60%, in agreement with Mahoney's results.[42]

Competitive inhibition studies in our system with various $^\bullet OH$ scavengers showed in some cases no inhibition and in other cases only partial inhibition of the oxidation of ferrocyanide by peroxynitrite, far less than that predicted by the relevant rate constants of these $^\bullet OH$ scavengers (Table 4). Therefore, we conclude that the homolytic dissociation of ONOOH to $^\bullet OH$ and $NO_2^\bullet$ does not take place in the presence of ferrocyanide.

We suggest that about 40% of the ONOOH is converted into a highly reactive intermediate ("X") which in the presence of ferrocyanide oxidizes it directly, whereas Mahoney proposed the homolytic dissociation of ONOOH into $^\bullet OH$ and $NO_2^\bullet$ in the presence of H_2O_2.[42] Mahoney's proposal was based on the observation that the evolution of oxygen in the presence of $[H_2O_2] > 1$ M was strongly retarded by 0.05 M benzoate or 2-propanol or acetate. As $k_{OH+H_2O_2} = 2.7 \times 10^7\ M^{-1}\ s^{-1}$, $k_{OH+benzoate} = 5.9 \times 10^9\ M^{-1}\ s^{-1}$, $k_{OH+2\text{-}propanol} = 1.9 \times 10^9\ M^{-1}\ s^{-1}$, and $k_{OH+acetate} = 8.5 \times 10^7\ M^{-1}\ s^{-1}$,[38] the relative rates of all these solutes with $^\bullet OH$ under these experimental conditions are 1:11:3.5:0.15, respectively. Therefore, if $^\bullet OH$ is really formed as an intermediate and is scavenged by H_2O_2, one expects hardly any inhibition by acetate and 80−90% inhibition by 2-propanol and benzoate, respectively, in contrast to the results.[42] However, two alternative pathways may account for Mahoney's observations which agree with our mechanism: (i) the reactive intermediate ("X") reacts faster with benzoate, 2-propanol, and acetate as compared to H_2O_2, yielding products which are not O_2; (ii) the reactive intermediate ("X") is in equilibrium with $^\bullet OH$ and $NO_2^\bullet$, which strongly favors "X". Hydrogen peroxide does not react fast enough with "X", and therefore, at very high concentrations, it will scavenge $^\bullet OH$ and yield O_2 as a final product. Thus, $^\bullet OH$ scavengers will compete with H_2O_2 for $^\bullet OH$ radicals and will inhibit the evolution of O_2.

The yield of the indirect oxidation of DMSO to formaldehyde by peroxynitrite was found to be about half the yield obtained when DMSO was replaced by ABTS.[24] As $NO_2^\bullet$ is capable of oxidizing ABTS but not DMSO,[40] it was suggested that the reactive intermediate oxidizes the substrate via the formation of $NO_2^\bullet$ as a second intermediate.[24]

We suggest the following mechanism for the oxidation of $Fe(CN)_6^{4-}$ by peroxynitrite:

$$cis\text{-ONOOH} \rightleftharpoons cis\text{-ONOO}^- + H^+ \qquad pK_a = 7 \qquad (5)$$

$$cis\text{-ONOOH} \rightarrow \text{"X"} \qquad k_6 = 0.4k_1 \qquad (6)$$

$$cis\text{-ONOOH} \rightarrow NO_3^- + H^+ \qquad k_{25} = 0.6k_1 \qquad (25)$$

$$\text{"X"} + Fe(CN)_6^{4-} \rightarrow Fe(CN)_6^{3-} + NO_2^\bullet \qquad (26)$$

$$Fe(CN)_4^{4-} + NO_2^\bullet \rightarrow Fe(CN)_6^{3-} + NO_2^- \qquad k_{27} = 4.3 \times 10^6\ M^{-1}\ s^{-1}\ {}^{41} \qquad (27)$$

The independence of the yield of ferricyanide on the pH and on $[Fe(CN)_6^{4-}]_0$ indicates that the rate of the oxidation of $Fe(CN)_6^{4-}$ by "X" competes efficiently with the self-decomposition of this reactive species. This suggested mechanism predicts that the rate of the formation of $Fe(CN)_6^{3-}$ should be identical to the rate of the self-decomposition of peroxynitrite, resulting in an apparent $pK_a = 7 \pm 0.2$ of the peroxynitrous acid. Both of these predictions are in agreement with our results. Furthermore, the measured yield of the oxidation of ferrocyanide by peroxynitrite in the presence of various $^\bullet OH$ scavengers indicates that "X" reacts much faster with ferrocyanide than with these scavengers. The lack of inhibition of 0.5 M $^\bullet OH$ scavengers at 0.1 mM ferrocyanide (Table 4) requires that k_{26} is at least 10^4-fold faster than the rate constants of the reaction of "X" with the various $^\bullet OH$ scavengers used.

Supporting theoretical evidence for the formation of *trans*-ONOOH as the reactive intermediate ("X") is given and discussed elsewhere.[16,24,43,44] Thus, we assume that the reactive species, "X", is the less stable *trans*-ONOOH, which may be in equilibrium with $^\bullet OH$ and $NO_2^\bullet$, an equilibrium which strongly favors the *trans*-ONOOH. In the presence of appropriate substrates, e.g., ferrocyanide, *trans*-ONOOH directly oxidizes the substrate. In other cases, e.g., H_2O_2,[42] the rate of the reaction of the *trans*-ONOOH with the substrate is too slow, and the oxidation of the substrate may take place via its reaction with $^\bullet OH$, thus shifting the equilibrium toward the homolytic dissociation of the *trans*-ONOOH (Scheme 1). As we have no direct evidence for the identification of "X" with the *trans*-ONOOH, we cannot exclude other alternative intermediates.

According to an earlier suggested mechanism (reactions 5−10),[16,24,28] the *trans*-ONOOH is the oxidizing species as well

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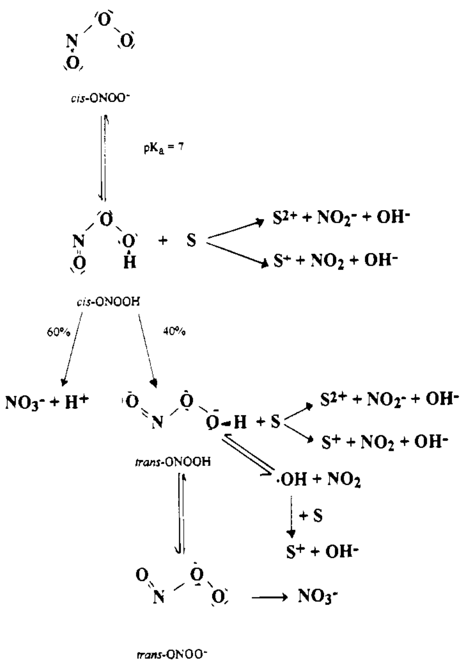
Scheme 1



as the one which isomerizes to nitric acid. As discussed earlier, the fact that ABTS$^+$ yields are independent of [ABTS]$_0$ at pH < 7,[23,24] rules out this mechanism. These results are in agreement with our mechanism, where it is suggested that peroxynitrous acid isomerizes into nitric acid and in parallel is converted into a highly reactive oxidant, which stoichiometrically is a two-electron equivalent oxidant.

Conclusions

The oxidation mechanism with peroxynitrite can take place either directly or through secondary products being formed via the self-decomposition of this oxidant. Our results indicate that the active species in the direct pathway is the peroxynitrous acid, most likely the stable *cis* isomer. In the indirect oxidation mechanism, peroxynitrous acid is partially converted into a highly reactive oxidant, most likely into the less stable *trans*-ONOOH, and in parallel, it isomerizes into nitric acid. All the relevant reactions involved in the direct and indirect pathways are shown in Scheme 1.

In the direct pathway, at sufficiently high concentrations of a substrate, isomerization should be suppressed completely. The stoichiometry of the direct oxidation may vary between 1 and 2. If the direct oxidation is a two-electron equivalent reaction, forming S^{2+}, then a stoichiometry of 1 will be obtained. However, if S^+ and $NO_2^\bullet$ are formed via a one-electron oxidation process, then the stoichiometry may vary between 1 and 2, depending on whether $NO_2^\bullet$ is capable of oxidizing the substrate. In the indirect pathway, the contribution of the isomerization of the *cis*-ONOOH to nitric acid is about 60% under all conditions, and only about 40% of the more stable *cis*-ONOOH is converted into *trans*-ONOOH, which according to some of the literature results may be in equilibrium with ${}^\bullet OH$ and $NO_2^\bullet$. Thus, the *trans*-ONOOH may oxidize the substrate either directly, as in the case of ferrocyanide, or via ${}^\bullet OH$ radicals (in cases where the substrate reacts too slow with the *trans*-ONOOH). In both pathways, $NO_2^\bullet$ will be formed as an intermediate unless the *trans*-ONOOH oxidizes the substrate via a two-electron oxidation process (Scheme 1). Thus, in the indirect pathway, one expects that the stoichiometry of the reaction will vary between 0.4 and 0.8. In cases where $NO_2^\bullet$ is formed as a second intermediate, the stoichiometry of the reaction may vary between 0.4 and 0.8, depending on the relative rates of the oxidation of the substrate by $NO_2^\bullet$ and the natural decay of $NO_2^\bullet$.

Our value for the pK_a of peroxynitrous acid is 7.0 ± 0.2, in agreement with earlier determinations.

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