J. Am. Chem. Soc. **2000,** *122,* 1229−1230

A Totally Synthetic Peroxynitritase Model That Is a Postfunctional Suicide Catalyst

Xiaoping Zhang and Daryle H. Busch*

Department of Chemistry, University of Kansas
Lawrence, Kansas 66045

Received July 12, 1999

Peroxynitrite ($OONO^-$) and its conjugate acid (HOONO, pK_a = 6.83 ± 0.05[1]) are highly noxious oxidants produced by the combination of nitric oxide (NO) and superoxide anion ($O_2^{\bullet -}$),[2−4] a reaction that destroys the blood vessel dilator nitric oxide and may generate significant levels of $OONO^-$ in vivo.[5] The anion ($OONO^-$) is thermally stable, but HOONO isomerizes to HNO_3 with a half-life of slightly less than 1 s at 37 °C.[6−8] The destructive capability of $OONO^-$ has been much discussed,[4,9−16] with $OONO^-$ implicated in cell death and tissue injury in many human diseases. Understanding the mechanisms of reaction of $OONO^-$ will increase the possibility of using such catalysts in therapeutic strategies.[16−18]

Floris et al.[19] showed that HOONO oxidizes peroxidases, and Jensen, Stern, and Riley identified the peroxynitratase activity of peroxidases.[20] Stern et al.[17] reported certain water-soluble iron porphyrins as the first biomimics for those putative peroxynitritases. Groves et al. studied the mechanisms of catalytic decomposition of $OONO^-$ by iron[16] and manganese[18] porphyrins. We report here the first totally synthetic (non-porphyrin) model for the peroxynitritase enzymes, the macrocyclic iron(III) complex *cis*-$[Fe(L)Cl_2]BF_4$, where L is *meso*-2,12-dimethyl-3,7,11−17-tetraazabicyclo[11.3.1]heptadeca-1(17),13,15-triene (**1**). In harmony with the peroxynitritase activity of peroxidases, this complex has long been recognized as an outstanding synthetic catalase and peroxidase model.[21,22] The peroxynitritase activity of *cis*-$[Fe(L)Cl_2]BF_4$ approaches the rates of the porphyrin models, and 97% of the $OONO^-$ is isomerized to NO_3^- (ion chromatography, pH 6.85), firmly establishing the peroxynitrite isomerase action of this new catalyst.[23] Unlike porphyrins, the ligand L is folded,[22] and the folded structure is critical to the catalysis; catalyst suicide occurs upon conversion of the cis complex into its trans isomer.

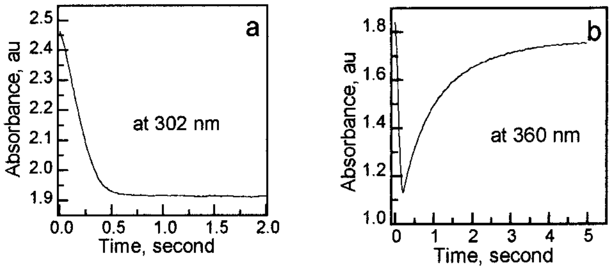

Figure 1. Reaction traces for the decomposition of peroxynitrite in the presence of 500 μM *cis*-$[Fe(L)Cl_2]^+$. $T = 25$ °C; $[2,6\text{-lutidine}]_T = 0.2$ M; pH = 6.2; $[OONO^-]_0 = 7.36 \times 10^{-4}$ M. (a) Typical trace recorded at 302 nm (λ_{max} for peroxynitrite, $\epsilon = 1.67 \times 10^3$ M^{-1} cm^{-1}) showing an initial induction followed by zero-order loss of $OONO^-$; (b) typical trace recorded at 360 nm (λ_{max} for trans-$[Fe(L)Cl_2]^+$ in water, $\epsilon = 2.6 \times 10^3$ M^{-1} cm^{-1}) showing the same rapid destruction of peroxynitrite, followed by isomerization of the catalyst.

The time-resolved absorption spectra of solutions containing $OONO^-$ and *cis*-$[Fe(L)Cl_2]BF_4$ undergo a rapid decrease in absorbance at 302 nm, the wavelength of maximum absorbance for $OONO^-$, followed by a smaller absorbance increase at 360 nm, assignable to formation of the trans isomer of the catalyst. Kinetic traces for the reaction of the new catalyst with $OONO^-$ (Figure 1) show three distinct steps: sequentially, the catalyst activation step (an induction period we have studied with initial rates and identified with the rate constant k_1 and eqs 1 and 2 below), the steady-state catalytic process during which the concentration of substrate diminishes to zero (k_2, eqs 3−4), and finally, the suicidal isomerization of the catalyst, which occurs only when the concentration of substrate is nearly exhausted (eq 5).

Potentiometric titrations (this work) showed that fresh aqueous solutions of [*cis*-$Fe(L)Cl_2]BF_4$ contain the ions *cis*-$[Fe(L)(H_2O)_2]^{3+}$, *cis*-$[Fe(L)(H_2O)(OH)]^{2+}$, and *cis*-$[Fe(L)(OH)_2]^+$, and pK_1 and pK_2 are 2.29 ± 0.12 and 7.34 ± 0.03, respectively. Upon aging solutions of the complex for 24 h, an inactive trans isomer is formed with pK_1' and pK_2' = 3.74 ± 0.08 and 7.64 ± 0.06, respectively.

The Induction Period: Catalyst Activation.[24] With an excess of $OONO^-$, the initial reaction rate is linearly dependent on the concentrations of both *cis*-$[Fe(L)Cl_2]^+$ and $OONO^-$ in various biological buffers, obeying the rate law $V^1_{initial} = (a + b[cis\text{-}[Fe(L)Cl_2]^+]_o)[OONO^-]_o$, where $[cis\text{-}[Fe(L)Cl_2]^+]_o = [cis\text{-}[Fe(L)(H_2O)(OH)]^{2+}] + [cis\text{-}[Fe(L)(OH)_2]^+]$. The second-order rate constants, b (M^{-1} s^{-1}), exhibit a bell-shaped pH profile (Figure

---

(1) Pryor, W. A.; Squadrito, G. L. *Am. J. Physiol.* **1995**, *268* (5), L699−L720.

(2) Beckman, J. S.; Beckman, T. W.; Chen, J.; Marshall, P. A.; Freeman, B. A. *Proc. Natl. Acad. Sci. U.S.A.* **1990**, *87*, 1620−1624.

(3) Huie, R. E.; Padmaja, S. *Free Radicals Res. Commun.* **1993**, *18*, 195−199.

(4) Koppenol, W. H.; Moreno, J. J.; Pryor, W. A.; Ischiropoulous, H.; Beckman, J. S. *Chem. Res. Toxicol.* **1992**, *5*, 834−842.

(5) Carreras, M. C.; Pargament, G. A.; Catz, S. D.; Poderoso, J. J.; Boveris, A. *FEBS Lett.* **1994**, *341*, 65−68.

(6) Richeson, C. E.; Mulder, P.; Bowry, V. W.; Ingold, K. U. *J. Am. Chem. Soc.* **1998**, *120*, 7211−7219.

(7) Alvarez, B.; Ferrer-Sueta, G.; Freeman, B. A.; Radi, R. *J. Biol. Chem.* **1999**, *274* (2), 842−848 and references therein.

(8) Lymar, S. V.; Hurst, J. K. *Chem. Res. Toxicol.* **1998**, *11*, 714−715 and references therein.

(9) Ischiropoulos, H.; Zhu, L.; Chen, J.; Tsai, M.; Martin, J. C.; Smith, C. D.; Beckman, J. S. *Arch. Biochem. Biophys.* **1992**, *298*, 431−437.

(10) King, P. A.; Jamison, E.; Strahs, D.; Anderson, V. E.; Brenowitz, M. *Nucleic Acids Res.* **1993**, *21*, 2473−2478.

(11) Rubbo, H.; Radi, R.; Trujillo, M.; Teller, R.; Kalyanaraman, B.; Barnes, S.; Kirk, M.; Freeman, B. A. *J. Biol. Chem.* **1994**, *269*, 26066−26075.

(12) Radi, R.; Beckman, J. S.; Bush, K. M.; Freeman, B. A. *J. Biol. Chem.* **1991**, *266* (7), 4244−4250.

(13) Halfpenny, E.; Robinson, P. L. *J. Chem. Soc. A* **1952**, 939.

(14) Ischiropoulos, H.; Al-Mehdi, A. B. *FEBS Lett.* **1995**, *364* (3), 279−282.

(15) Szabo, C.; Salzman, A. L.; Ischiropoulos, H. *FEBS Lett.* **1995**, *363*, 235−238 and references therein.

(16) Lee, J.; Hunt, J. A.; Groves, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 7493−7501 and references therein.

(17) Stern, M. K.; Jensen, M. P.; Kramer, K. *J. Am. Chem. Soc.* **1996**, *118*, 8735−8736.

(18) Lee, J.; Hunt, A.; Groves, J. T. *J. Am. Chem. Soc.* **1998**, *120*, 6053−6061.

(19) Floris, R.; Piersma, R. R.; Yang, G.; Jones, P.; Wever, R. *Eur. J. Biochem.* **1993**, *215*, 767−775.

(20) Jensen, M. P.; Stern, M. K.; Riley, D. P. Private communication, June 1997.

(21) Melnyk, A. C.; Kjeldahl, N. K.; Rendina, A. R.; Busch, D. H. *J. Am. Chem. Soc.* **1979**, *101*, 3232−3240.

(22) Cairns, C. J.; Heckman, R. A.; Melnyk, A. C.; Busch, D. H.; Davis, W. M. *J. Chem. Soc., Dalton Trans.* **1987**, *24*, 2505−2510.

(23) Riley, D. P.; Busch, D. H.; Zhang, X. Patent Publication (PCT), WO 98-US5567, March 26, 1998.

(24) Definition of parameters: $V_{initial} = -d[OONO^-]/dt = (-dA/dt)(1/\epsilon b)$; $K_a = [OONO^-][H^+]/[HOONO]$; $K_1 = [cis\text{-}Fe(L)(H_2O)(OH)^{2+}][H^+]/[cis\text{-}Fe(L)(H_2O)_2^{3+}]$; $K_2 = [cis\text{-}Fe(L)(OH)_2^+][H^+]/[cis\text{-}Fe(L)(H_2O)(OH)^{2+}]$; $K_1' = [trans\text{-}Fe(L)(H_2O)(OH)^{2+}][H^+]/[trans\text{-}Fe(L)(H_2O)_2^{3+}]$; $K_2' = [trans\text{-}Fe(L)(OH)_2^+][H^+]/[trans\text{-}Fe(L)(H_2O)(OH)^{2+}]$.

(25) See: Gopinath, E.; Bruice, T. C. *J. Am. Chem. Soc.* **1991**, *113*, 4657−4665.

(26) For similar cases, see: (a) Panicucci, R.; Bruice, T. C. *J. Am. Chem. Soc.* **1990**, *112*, 6063−6071. (b) Zhang, X. van Eldik, R. *Inorg. Chem.* **1995**, *34*, 5606−5614.

(27) (a) Rush, J. D.; Koppenol, W. H. *J. Am. Chem. Soc.* **1988**, *110*, 4957−4963. (b) Zipplies, M. F.; Lee, W. A.; Bruice, T. C. *J. Am. Chem. Soc.* **1986**, *108*, 4433−4445.

10.1021/ja9924344 CCC: $19.00

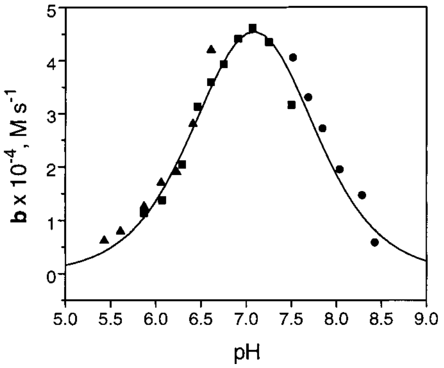

Figure 2. pH Dependence of the second-order rate constant *b* for the initial reaction: $T = 25.0$ °C; $\lambda = 302$ nm; [*cis*-Fe(L)Cl_2^+] = 50 μM; [buffer] = 0.20 M; ionic strength = 0.187 M (KCl); $[OONO^-]_o = 8.1 \times 10^{-4}$ M (2,6-lutidine buffer), 1.05×10^{-3} M (hepes buffer), and 8.88×10^{-4} M (mes buffer). Key: (■) 2,6-lutidine buffer, (●) hepes buffer, (▲) mes buffer. Solid line calculated from eq 2 (pK_a = 6.83, pK_2 = 7.34, $k_1 = 1.1 \times 10^5$ M^{-1} s^{-1}).

2)[25] with a maximum at pH ~ 7.1. Thus, both protonation and deprotonation of kinetically involved species slow the reaction. Further, both species must have acid p*K* values near neutral pH. This is completely consistent with assuming that the two reactants are *cis*-[Fe(L)(H_2O)(OH)]$^{2+}$ and $OONO^-$ and that the induction process is formation of the peroxynitrito complex, *cis*-[Fe(L)-($OONO^-$)(OH)]$^+$. The fact that the reaction is inhibited by competing ligands (CN^-, SCN^-, N_3^-, or HPO_4^{2-}) supports the view that the reaction in question is properly represented by eq 1.

$$cis\text{-}[Fe(L)(H_2O)(OH)]^{2+} + OONO^- \xrightarrow{k_1} cis\text{-}[Fe(L)(OONO^-)(OH)]^+ + H_2O \quad (1)$$

The corresponding rate law is given by eq 2.

$$c = k_1 \frac{K_a}{[H^+] + K_a} \frac{[H^+]}{[H^+] + K_2} \quad (2)$$

Fitting the rate data to eq 2 gives $k_1 = (1.1 \pm 0.1) \times 10^5$ M^{-1} s^{-1}; $K_a = 1.96 \times 10^{-7}$ and pK_a = 6.71 ± 0.07 (literature value[1] for HOONO, 6.83); and pK_2 = 7.49 ± 0.08 (experimental value for *cis*-[Fe(L)(H_2O)(OH)]$^{2+}$ from titrations, 7.34 ± 0.03). Clearly, the peroxynitrite anion replaces a water molecule in the coordination sphere of the iron(III) during this initiation step.

Catalytic Destruction of OONO$^-$. The second process involves isomerization of bound $OONO^-$ to bound NO_3^-, and it is zero-order in [$OONO^-$], indicating saturation of the preequilibrium formation of *cis*-[Fe(L)($OONO^-$)(OH)]$^+$. Other investigators[17,18] suggested that the mechanism involves the breaking of the O−O bond in *cis*-[Fe(L)($OONO^-$)(OH)]$^+$, generating an iron(IV) intermediate, and momentarily retaining the accompanying species, $^\bullet NO_2$, within the solvent cage (eq 3).

$$cis\text{-}[Fe^{III}(L)(OONO^-)(OH)]^+ \xrightleftharpoons{k_2} \{cis\text{-}[(L)(OH)Fe^{IV}{=}O] + {}^\bullet NO_2\}^+ \quad (3)$$

Then, rapid rearrangement within the solvent cage produces bound nitrate (eq 4).

$$\{cis\text{-}[(L)(OH)Fe^{IV}{=}O] + {}^\bullet NO_2\}^+ \rightarrow cis\text{-}[Fe^{III}(L)(NO_3^-)(OH)]^+ \quad (4)$$

The pseudo-zero-order reaction rate constant, k°_{obs}, is linearly dependent on $[cis\text{-}[Fe(L)Cl_2]]_o$ and increases with increasing pH, becoming constant between pH 7 and 7.5. The following equation and parameters describe this pH dependence: $k^\circ_{obs} = k^\circ K_a/([H^+] + K_a)$, where $k^\circ = k_2[cis\text{-}Fe(L)Cl_2^+]$, p$K_a$ = 6.64 ± 0.02, and $k_2 = 38 \pm 2$ s^{-1}.[26] This observed pK_a value is comparable to those of HOONO (6.83)[1] and *cis*-[Fe(L)(H_2O)(OH)]$^{2+}$ (7.34) and almost certainly reflects the ionization of *cis*-[(L)(H_2O)Fe^{III}($OONO^-$)]$^{2+}$, since its zero-order dependence indicates that free peroxynitrite is not involved in this second reaction. On this basis, the rate-determining step is attributed to the rearrangement of peroxynitrite into nitrate within the hydroxy complex, *cis*-[Fe^{III}(L)($OONO^-$)-(OH)]$^+$ (eqs 3 and 4). The rate constant of 38 s^{-1} is an enhancement by a factor of ~6×10^4 over the spontaneous isomerization of $OONO^-$.[1,6,7] The catalytic cycle is completed by direct replacement of the coordinated product NO_3^- with reactant $ONOO^-$.

Postfunction Catalyst Suicide. When most of the $ONOO^-$ has been decomposed by the catalyst, an absorbance peak attributable to *trans*-[Fe(L)$(H_2O)_2$]$^{3+}$, and its pH dependent partners, grows in at 360 nm. The corresponding pseudo-first-order rate constant decreases as the pH increases: $k^i_{obs} = k^i\{[H^+]/([H^+] + K_2')$, with p$K_2'$ = 7.55 ± 0.04 and $k^i = 2.1 \pm 0.3$ s^{-1}. The increase in the rate of isomerization of the catalyst with decreasing pH implicates the protonated form of *cis*-[Fe^{III}(L)-(OH_2)(NO_3^-)]$^{2+}$ in the rate-determining step (eq 5), since it should have a pK_a value similar to those of the water molecules in *cis*- and *trans*-[Fe(L)(H_2O)(OH)]$^{2+}$.

$$cis\text{-}[Fe^{III}(L)(OH_2)(NO_3^-)]^{2+} + H_2O \rightleftharpoons trans\text{-}[Fe(L)(H_2O)_2]^{3+} + NO_3^- \quad (5)$$

Further, *cis*-[Fe^{III}(L)(NO_3^-)(OH)]$^+$ should lose nitrate more rapidly than *cis*-[Fe^{III}(L)(OH_2)(NO_3^-)]$^{2+}$. Also, many rapid substitution events (of substrate $OONO^-$ for product NO_3^-) have occurred in the course of the catalysis without conversion of the cis complex into its trans isomer.

It follows that the events leading to suicidal catalyst isomerization are mechanistically different from those associated with propagation of the catalytic cycle. The catalytic propagation (replacement of NO_3^- by $OONO^-$) probably occurs by a dissociative pathway involving the deprotonated species, *cis*-[Fe^{III}(L)(NO_3^-)(OH)]$^+$, with the incoming $OONO^-$ approaching from the near side, thereby conserving the cis structure. Similarly, *cis*-[Fe^{III}(L)(NO_3^-)(OH)]$^+$ may exchange water and nitrate at a substantial rate without creating a kinetically measurable event. In contrast, catalyst isomerization occurs with the protonated form, *cis*-[Fe^{III}(L)(OH_2)(NO_3^-)]$^{2+}$, and in order to produce the trans isomer a water molecule must enter the coordination sphere on the far side of the iron; that is, opposite to the side from which the nitrate departs.

Intermediates Are Oxidants. The oxidizing power of the intermediates was examined by experiments with the often used reagent ABTS, which undergoes one electron oxidation to the green ABTS$^{\bullet+}$ radical cation.[27] At 25 °C, with [ABTS] > $30[OONO^-]_o$ > 100[*cis*-[Fe(L)Cl_2]$^+$], absorbance−time traces recorded at 660 nm showed an initial induction period, followed by an increase in absorbance. As observed in the absence of ABTS, the induction period is associated with the initial formation of *cis*-[Fe(L)($OONO^-$)(OH)]$^+$ (eq 1). The rapid production of color in the second process is assignable to the scavenging by ABTS of the intermediates, proposed[16−18] to be {*cis*-[(L)(OH)-Fe^{IV}=O] + $^\bullet NO_2$}$^+$, yielding 2ABTS$^{\bullet+}$, the iron(III) complex, and nitrite.

Acknowledgment. The financial support of the Monsanto Co. is gratefully acknowledged.

Supporting Information Available: Table 1 of catalytic rate constants, Table 2 of spontaneous rate constants, Supporting Figures 1 and 2, and the derivation of eq 2 (PDF). This material is available free of charge via the Internet at http://pubs.acs.org.

JA9924344