Table II. EPR Parameters

protein	site	$10^4A_{\parallel}$, cm^{-1}	$10^4A_{\perp}$, cm^{-1}	$g_{\parallel}$	$g_{\perp}$	$w_{\parallel}$,[a] mT	$w_{\perp}$,[a] mT	ref
^{65}Cu enriched	type 1	43	15	2.298	2.048	40	30	this work
Chinese laccase	type 2	201	18	2.250	2.050	40	35	this work
Japanese laccase	type 1	43	17	2.298	2.047	35	35	*b*
	type 2	200	18	2.237	2.053	42	42	*b*

[a] Line-width parameters. [b] Malmström, B. G.; Reinhammar, B.; Vänngard, T. *Biochim. Biophys. Acta* **1970**, *205*, 48-57.

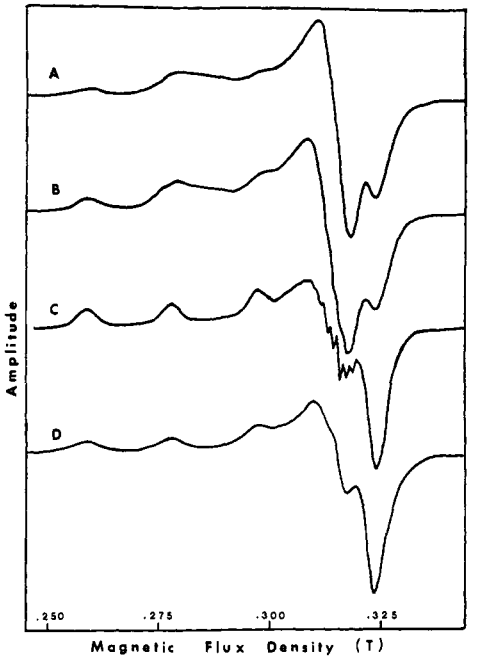

Figure 1. EPR Spectra of native laccase and mercury derivative. (A) The simulated spectrum of native laccase using the parameters presented in Table II. (B) The corresponding experimental spectrum obtained with a 2.17 mM sample of protein at -150 °C with a modulation amplitude of 1.0 mT, a power of 40 mW, and a frequency of 9.080 GHz. (C) The experimental spectrum of a 1.22 mM sample of the mercury derivative from sample V in Table I under the conditions of B except the gain was increased by a factor of 3.1. (D) The simulated spectrum of the mercury derivative using the parameters for the type 2 copper from Table II and ignoring the ligand hyperfine interaction. The ratio of the double integral of spectrum B to that of C is 2.16. All samples have been remetalated with isotopically pure ^{65}Cu. The buffer is 0.1 M pH 6 phosphate.

The results presented above argue strongly that the mercury derivative is fully metalated and that Hg(II) is bound specifically at the type 1 site. Consistent with Reinhammar's kinetic results, which indicate that the type 1 copper plays a key role in accepting electrons and funneling them to the type 3 site,[18] we estimate that the activity of the mercury derivative is less than 5% that of native laccase.[19] The precise activity is difficult to measure because of the presence of trace amounts of underivatized protein. Detailed kinetic studies of the oxidation and the reduction of the mercury derivative would be less sensitive to trace impurities and could shed new light on the role of the type 2 site in the functioning enzyme. Moreover, by analogy with native laccase[20] it may be possible to selectively remove type 2 copper from the mercury derivative. If so, it should be possible to probe the type 3 site of laccase in the absence of other copper centers using EXAFS and other techniques. In conclusion, the availability of a mixed-metal form offers new avenues to the study of laccase; the scope and limitations of the approach will only become clear as new experiments unfold.

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Nitric Oxide Dissociation from Trioxodinitrate(II) in Aqueous Solution

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The decomposition of sodium trioxodinitrate(II), $Na_2N_2O_3$ (Angeli's salt), in aqueous solution has been the subject of considerable interest since Angeli first proposed the existence of the elusive nitroxyl (HNO or NOH) as a reaction intermediate.[1] Although stable as a crystalline solid, $Na_2N_2O_3$ decomposes in neutral or alkaline media to nitrous oxide and nitrite ion stoichiometrically according to eq 1, but at pH <3 nitric oxide is the

$$2HN_2O_3^- \rightarrow N_2O + 2NO_2^- + H_2O \quad (1)$$

sole nitrogen-containing product.[2,3] First-order kinetic dependence on $[HN_2O_3^-]$ is observed for decomposition in alkaline and acidic media,[3-6] and labeling experiments have established that nitrous oxide is formed exclusively from the nitrogen bound to one oxygen[5] whereas nitrite is generated only from the nitrogen that is bound to the two oxygens[7] of the planar[8] $(ONNO_2)^{2-}$ ion. The composite data have been interpreted uniformly from the time of Angeli's first proposal by a mechanism (eq 2 and 3) in which the formation of nitroxyl and nitrite is rate limiting.

$$HN_2O_3^- \rightarrow HNO + NO_2^- \quad (2)$$

$$2HNO \rightarrow N_2O + H_2O \quad (3)$$

We have previously established that hemoglobin is a particularly sensitive reagent for the detection of free nitric oxide generated from nitric oxide donors.[9] Association of deoxyhemoglobin (Hb) with nitric oxide occurs with a rate constant of 2×10^7 M^{-1} s^{-1} and is essentially irreversible.[10] If the pathway discribed by eq 2 and 3 is an accurate accounting of $HN_2O_3^-$ decomposition, the presence of Hb should not alter the reaction process. However, treatment of Hb with $HN_2O_3^-$ in 0.05 M phosphate buffer at pH 7.0 results in an initial production of nearly equivalent amounts of nitrosylhemoglobin (HbNO) and methemoglobin (Hb^+), and

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nitrite formation is suppressed. Nitrosylhemoglobin is the sole hemoprotein product when $[HN_2O_3^-]/[Hb] > 4$. By use of limiting ratios of $HN_2O_3^-$ to Hb with quantitative spectrophotometric determination[11] of HbNO and Hb^+ and HPLC analysis[12] of NO_2^-, the stoichiometry of the initial reaction could be shown to be that described by eq 4. Secondary reactions with $HN_2O_3^-$ convert

$$2HN_2O_3^- + 2Hb \rightarrow HbNO + Hb^+ + NO_2^- + N_2O + 2OH^- \quad (4)$$

Hb^+ to HbNO but on a time scale that is a factor of 5 slower than that for initial Hb^+/HbNO formation.

The rate of reaction was first order in $[HN_2O_3^-]$ but zero order in [Hb], and zero-order kinetics for the hemoglobin transformation were followed spectrophotometrically at 430 nm through four half-lives. The rate constant for the conversion of Hb to HbNO and Hb^+ was $(1.38 \pm 0.08) \times 10^{-3}$ s^{-1} at pH 7.0 and 25.0 °C, which is a factor of 2.0 greater than the rate constant for $HN_2O_3^-$ decomposition under the same conditions[6] but in the absence of Hb. Under these conditions with $[HN_2O_3^-]/[Hb] > 4$ the reaction stoichiometry approaches $2[Hb]/[HN_2O_3^-] = 2[HbNO]$ as evidenced by nitrite analysis. Oxidation of Hb by nitrite is not competitive.[11a]

Trapping of nitric oxide by Hb is consistent with rate-limiting formation of nitric oxide and the nitrous acid radical anion from $HN_2O_3^-$ decomposition (eq 5) rather than nitrite and nitroxyl (eq 2). Sodium hydronitrite (Na_2NO_2) is a known, but unstable,[13-15] species whose reaction characteristics have not been examined. In the absence of Hb, $(HONO)^-\cdot$ is presumed to undergo electron transfer to $HN_2O_3^-$, which, in turn, combines with nitric oxide at the nitrogen bound to one oxygen to form the observation products (eq 6 and 7), although initial nitric oxide association with $HN_2O_3^-$ followed by electron transfer from $(HONO)^-\cdot$ would produce the same outcome. In the presence of Hb, which effectively sequesters nitric oxide, electron transfer from Hb to $(HN_2O_3)^{2-}$ provides the alternative pathway for decomposition that accounts for methemoglobin production (eq 8). The significance of the initial trapping of nitric oxide is clearly seen when decomposition of $HN_2O_3^-$ occurs in the presence of ferrocytochrome *c*, a hemoprotein that does not associate with nitric oxide at pH 7.[16] Ferrocytochrome *c* remains unaffected by $HN_2O_3^{2-}$ decomposition. On the basis of the stoichiometry for $HN_2O_3^-$ decomposition in the presence of Hb, under conditions employed for rate determination, the rate constant for hemoglobin conversion should be twice the rate constant for $HN_2O_3^-$ decomposition, and this is exactly what is observed.

$$HN_2O_3^- \rightarrow NO + (HONO)^-\cdot \quad (5)$$

$$(HONO)^-\cdot + HN_2O_3^- \rightarrow HONO + (HN_2O_3)^{2-} \quad (6)$$

$$(HN_2O_3)^{2-} + NO \rightarrow [ONN(OH)NO_2]^{2-} \rightarrow NO_2^- + N_2O + OH^- \quad (7)$$

$$(HN_2O_3)^{2-} + Hb \rightarrow Hb^+ + (N_2O_2)^{2-} + OH^- \quad (8)$$

Further confirmation of nitric oxide dissociation from $HN_2O_3^-$ has been obtained from investigations of reactions performed with oxymyoglobin (MbO_2). Like Hb, MbO_2 is an effective trap for nitric oxide that undergoes stoichiometric oxidation to metmyoglobin (Mb^+) and nitrate with an associated rate constant of 3.7×10^7 M^{-1} s^{-1} at 25 °C.[17] Decomposition of $HN_2O_3^-$ at pH 7.0 in the presence of an exact 2-fold excess of MbO_2[18] provided the spectral time course of Figure 1 and resulted in stoichiometric production of Mb^+, NO_2^-, NO_3^-, and, presumably, HO_2^-. Reactions using various ratios of $[HN_2O_3^-]$ to $[MbO_2]$ were employed to establish the exact reaction stoichiometry (eq 9).[12]

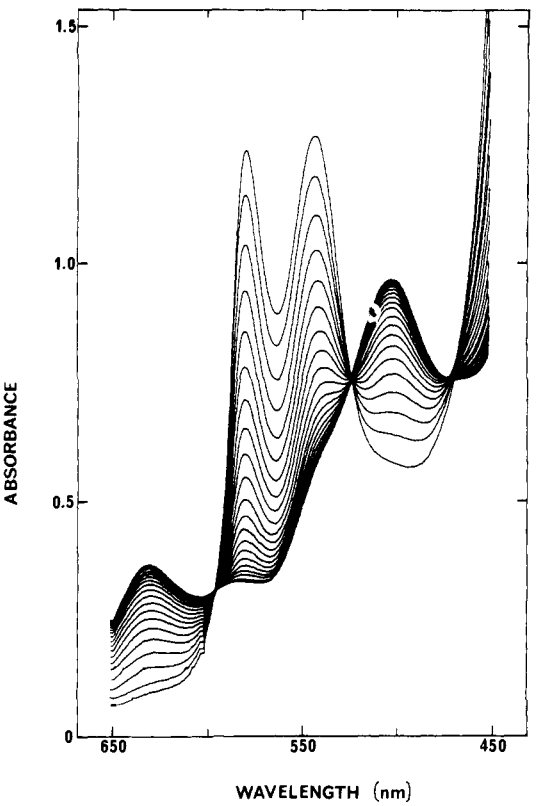

Figure 1. Spectral time course for the reaction of MbO_2 with $HN_2O_3^-$, performed in 0.05 M phosphate buffer, pH 7.0, 25.0 °C, with 8.0×10^{-5} M MbO_2 and 4.0×10^{-5} M $HN_2O_3^-$. Spectra were recorded at 2.0-min intervals (100-s scan time) after the first spectral scan taken 1.0 min following initiation of reaction.

$$HN_2O_3^- + 2MbO_2 \rightarrow 2Mb^+ + NO_2^- + NO_3^- + HO_2^- \quad (9)$$

The rate for Mb^+ formation, which was first order in $[HN_2O_3^-]$ and zero order in $[MbO_2]$, was followed spectrophotometrically at 581 nm through four half-lives. The rate constant for Mb^+ formation, $(1.51 \pm 0.10) \times 10^{-3}$ s^{-1} at pH 7.0 and 25.0 °C, was again, within experimental limits, equal to twice that for $HN_2O_3^-$ decomposition in the absence of the hemoprotein. Identical results were obtained with oxyhemoglobin: $k = (1.50 \pm 0.06) \times 10^{-3}$ s^{-1}.[19] Equations 10 and 11 adequately account for these observations.

$$MbO_2 + NO \rightarrow Mb^+ + NO_3^- \quad (10)$$

$$MbO_2 + (HONO)^-\cdot \rightarrow Mb^+ + NO_2^- + HO_2^- \quad (11)$$

The results reported here are inconsistent with dissociation of nitroxyl in the decomposition of $HN_2O_3^-$. Quantitative trapping of nitric oxide, either directly by association with Hb or indirectly through oxidation of oxyhemoproteins, and kinetic identities show that $HN_2O_3^-$ decomposition occurs by nitric oxide dissociation.

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