1, methine), 1.71 (br s, 1, OH), 1.61 (s, 6, C1, C2 methyls), 1.60-2.30 (br m, 6, C3, C5, C6 methylenes); $^{13}$C NMR ($CDCl_3$) $\delta$ 125.2, 123.0 ($sp^2$), 67.4 (COH), 40.6 (C3), 31.6, 30.0 (C5, C6), 19.2, 18.6 (C1, C2 methyls). An analytically pure sample was obtained by preparative VPC (25% DEGS, 8 ft × $^1/_4$ in., 120 °C, He flow 75 mL/min).

**1,2-Dimethyl-1-cyclohexen-4-yl tosylate**[17] was prepared by treatment of the alcohol (4.0 g, 0.032 mol) with tosyl chloride (6.15 g, 0.032 mol) in 57% yield:[13] $^1$H NMR ($CDCl_3$) $\delta$ 7.25-7.90 (d of d, 4, aromatic), 4.70 (m, 1, methine), 2.47 (s, 3, tolyl methyl), 1.58 (br s, 6, C1, C2 methyls), 1.70-2.30 (br m, 6, C3, C5, C6 methylenes).

**4-Halo-1,2-dimethyl-1-cyclohexene.** Treatment of the tosylate with LiCl (0.40 g, 9.4 mmol), LiBr (0.50 g, 5.8 mmol), or NaI gave the respective halide compounds.[13] Chloride: 45%; $^1$H NMR ($CDCl_3$) $\delta$ 4.20 (m, 1, methine), 1.61 (s, 6, C1, C2 methyls), 1.80-2.50 (br m, 6, C3, C5, C6 methylenes). Bromide: 60%; $^1$H NMR ($CDCl_3$) $\delta$ 4.38 (m, 1, methine), 1.60 (s, 6, C1, C2 methyls), 1.80-2.65 (br m, 6, C3, C5, C6 methylenes). Iodide: 35%; $^1$H NMR ($CDCl_3$) $\delta$ 4.48 (m, 1, methine), 2.62 (br s, 2, C3 methylene), 2.10 (m, 4, C5, C6 methylenes), 1.61 (s, 6, C1, C2 methyl groups).

**4-Methoxy-$d_3$-1,2-dimethyl-1-cyclohexene** was prepared from 1,2-dimethyl-1-cyclohexen-4-ol (0.50 g, 4 mmol) by treatment with NaH and $CD_3I$:[12] $^1$H NMR ($CDCl_3$) $\delta$ 3.42 (m, 1, methine), 1.59 (s, 6, C1, C2 methyls), 1.75-2.35 (br m, 6, C3, C5, C6 methylenes).

**1,2-Dimethyl-1-cyclohexen-4-yl acetate** was prepared by the Diels-Alder reaction of 2,3-dimethyl-1,3-butadiene (5 mL, 0.044 mol) with vinyl acetate (Aldrich, 20.5 mL, 0.22 mol).[13] The product was distilled (bp 105-109 °C (25 mm Hg)) and determined to be a 3.7/1 mixture of two products, the desired acetate and the dimer of 2,3-dimethyl-1,3-butadiene, 1,2,4-trimethyl-4-(2-propenyl)cyclohex-1-ene. The two materials were separated by preparative VPC (25% DEGS on Chromosorb W, 8 ft × $^3/_4$ in., 135 °C, He flow 70 mL/min). The acetate had $^1$H NMR ($CDCl_3$) $\delta$ 4.95 (m, 1, methine), 2.03 (s, 3, $OCOCH_3$), 1.60 (s, 6, C1, C2 methyls), 1.50-2.30 (br m, 6, C3, C5, C6 methylenes).

**4-(Trimethylsilyloxy)-1,2-dimethylcyclohex-1-ene.** A solution of 1,2-dimethylcyclohexen-4-ol (1.0 g, 8.0 mmol) in dry pyridine (3 mL) was placed in a 25-mL round-bottomed flask. The flask was secured to the receiving end of a distillation apparatus, and trimethylsilyl chloride (approximately 2 mL, 16.0 mmol) was distilled from pyridine directly into the stirred reaction mixture under $N_2$. Pentane (25 mL) and $H_2O$ (25 mL) were added, and the contents were transferred to a separatory funnel. The organic layer was separated and washed with saturated $CuSO_4$ (2 × 50 mL) and $H_2O$ (50 mL). The pentane solution was dried ($Na_2SO_4$) and filtered, and the pentane was distilled. The colorless, oily residue was purified by preparative VPC (10% Carbowax on Chromosorb W 60/80, 8 ft × $^1/_2$ in., 120 °C, He flow 80 mL/min): $^1$H NMR ($CDCl_3$) $\delta$ 3.78 (m, 1, methine), 1.56 (s, 6, C1, C2 methyls), 1.65-2.10 (br m, 6, C3, C5, C6 methylenes), 0.10 (s, 9, $OSi(CH_3)_3$); $^{13}$C NMR ($CDCl_3$) $\delta$ 125.0, 123.4 ($sp^2$), 68.5 (C4), 41.3 (allylic C3), 35.8, 32.5 (C5, C6), 19.1 (C1, C2 methyls).

**4-Cyano-1,2-dimethylcyclohex-1-ene** was prepared by the Diels-Alder reaction of 2,3-dimethyl-1,3-butadiene (5 mL, 3.63 g, 0.044 mol) with acrylonitrile (Aldrich, 3.0 mL, 0.046 mol):[13] 5.5 g (92%), bp 45 °C (0.06 mm Hg) (lit.[19] bp 217-222 °C (760 mm Hg)); $^1$H NMR ($CDCl_3$) $\delta$ 2.80 (m, 1, methine), 1.62 (s, 6, C1, C2 methyls), 1.80-2.25 (br m, 6, C3, C5, C6 methylenes); $^{13}$C NMR ($CDCl_3$) $\delta$ 125.8 (C≡N), 122.7, 122.3 ($sp^2$ carbons), 34.2 (C4), 29.3 (C3 allylic), 26.0, 25.5 (C5, C6), 18.9, 18.8 (C1, C2 methyls).

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# Metmyoglobin and Methemoglobin as Efficient Traps for Nitrosyl Hydride (Nitroxyl) in Neutral Aqueous Solution

**Dennis A. Bazylinski and Thomas C. Hollocher***

*Contribution from the Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254.* *Received March 7, 1985*

**Abstract:** The reactions of metmyoglobin ($Mb^+$, ferric myoglobin) and methemoglobin ($Hb^+$, ferric hemoglobin) with trioxodinitrate monoanion ($HN_2O_3^-$) in neutral aqueous solution have been studied at 25 °C under anaerobic conditions. The sole heme product of the reaction is nitrosylmyoglobin (MbNO) or nitrosylhemoglobin (HbNO). The reactions are approximately first-order in [$HN_2O_3^-$] and zero-order in ferric heme protein concentration, and the rate of formation of the nitrosyl (ferrous) heme protein product is always less than that of $HN_2O_3^-$ decomposition. The $HN_2O_3^-$/ferric heme protein mole ratio required for quantitative conversion to the nitrosyl heme protein is about 1.4 by titration and, in the case of $Mb^+$, 1.25 by kinetic analysis. Product analyses show that nitrosyl heme protein formation occurs at the expense of $N_2O$ production, but not of nitrite production. The results are consistent with the view that $HN_2O_3^-$ decomposes in the rate-determining step into nitrosyl hydride (nitroxyl, HNO) plus nitrite and that HNO then partitions in fast reactions between dimerization/dehydration to form $N_2O$ and reaction with ferric heme protein to form nitrosyl heme protein. $Hb^+$ shows kinetic evidence for cooperativity in the latter reaction. In an alternative possibility, $HN_2O_3^-$ may decompose into NO and $(HONO)^-$ (Doyle, M. P.; Mahapatro, S. N. *J. Am. Chem. Soc.* **1984**, *106*, 3678-3679). Subsequently $(HONO)^-$ reduces $Mb^+$ or $Hb^+$ to Mb or Hb, and the latter captures NO to form MbNO or HbNO. This pathway is held to be unlikely on chemical grounds.

Nitrosyl hydride (HNO), commonly referred to as nitroxyl, is a reactive $N^{1+}$ species that can be produced by pulse radiolysis of NO in aqueous solutions[1] and by photolysis of $H_2$/NO and other systems in the gas phase.[2] Nitroxyl is subject to matrix isolation.[3] In the gas phase, nitroxyl rapidly dimerizes and dehydrates to form $N_2O$;[2] in aqueous solutions, $NO^-$ reacts rapidly with NO to form $N_2O_2^-$, $N_3O_3^-$, and their conjugate acids.[1] $N_3O_3^-$ and $HN_3O_3$ decay rapidly into $N_2O$ and nitrite.[1] The decomposition of sodium trioxodinitrate (Angeli's salt, $Na_2N_2O_3$) in aqueous solution has been the subject of several reports.[5-10] Under neutral or mildly alkaline conditions, the monoanion, $HN_2O_3^-$, spontaneously de-

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composes in a first-order reaction to $N_2O$ and nitrite (eq 1).

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

Angeli[9] first proposed that nitroxyl may be an intermediate in the reaction, and except for one recent report to the contrary,[10] a body of kinetic and isotopic evidence has developed in support of this idea.[5-8] One of the most convincing results in support of nitroxyl as an intermediate in the decomposition of $HN_2O_3^-$ is the concomitant conversion of tetracyanonickel(II) to the violet-colored tricyanonitrosylnickel (eq 2). Tetracyanonickel was

$$Ni(CN)_4^{2-} + NO^- \rightarrow Ni(NO)(CN)_3^{2-} + CN^- \quad (2)$$

originally judged to be a nitroxyl trap on the basis of nitroxyl-generating reactions involving decomposition of substituted hydroxylamines.[11-13] In the case of $HN_2O_3^-$ decomposition, $N_2O$ and tricyanonitrosylnickel were formed as competitive products,[8] just as $N_2O$ and $N_2$ were competitive products during $HN_2O_3^-$ decomposition in the presence of $NH_2OH$.[5,8] There is isotope data to suggest that nitroxyl may be the direct precursor of $N_2O$ in denitrifying bacteria.[14]

It occurred to us that a nominally 5-coordinated ferric heme product, such as methemoglobin or metmyoglobin, might act as an efficient trap for nitroxyl in neutral aqueous solutions. We report herein experiments designed to test this idea.

## Experimental Procedures

**Materials.** Sodium trioxodinitrate ($Na_2N_2O_3$) was prepared at 90% purity from butyl nitrate and hydroxylamine in anhydrous methanol/sodium methoxide by the method of Hunt et al.:[15] $E_{248nm}$ obsd, 7358 $M^{-1}$ $cm^{-1}$ in 1 N NaOH; [lit.[6] 8147 $M^{-1}$ $cm^{-1}$]. Gas chromatographic quantitative analysis of $N_2O$ resulting from decomposition of the salt at pH 7.0 indicated a purity of $\geq$90% $Na_2N_2O_3$. $NaNO_2$ contamination was $\leq$3% by weight. The remaining contaminant is likely to be $Na_2CO_3$. The first-order rate constant for $HN_2O_3^-$ decomposition at 25 °C in 50 mM potassium phosphate buffer, pH 7.0, was $6.6 \pm 0.4 \times 10^{-4}$ $s^{-1}$ (obsd), $6.9 \times 10^{-4}$ $s^{-1}$ (lit.[7]). Storage and stability of the salt in this laboratory have been described.[14] $Na_2N_2O_3$ was dissolved in anaerobic 0.1 N NaOH under argon just prior to use. The addition of alkaline $Na_2N_2O_3$ to reaction mixtures brought the final pH to 7.00 ± 0.05. Commercial NO (Matheson) was passed through a column of NaOH pellets to remove the higher oxides of nitrogen and collected over 0.1 M NaOH. The transfer of NO to reaction vessels was by means of gas-tight syringes previously purged with argon.

Methemoglobin ($Hb^+$) was prepared by oxidizing human oxygen-free hemoglobin (Hb) (adult, type IV, Sigma) with 5% molar excess of potassium ferricyanide in 50 mM potassium phosphate buffer, pH 7.0. Ferrocyanide and excess ferricyanide were removed by passing the $Hb^+$ solution through a G-25 Sephadex column (Pharmacia) using 50 mM potassium phosphate buffer, pH 7.0, as the eluting buffer. Sperm whale myoglobin (Mb) (Mann) was oxidized to metmyoglobin ($Mb^+$) similarly. Concentrated solutions of $Hb^+$ and $Mb^+$ (1-2 mM heme) were stored in sealed flasks under argon and used within several hours. No reduction of heme iron was detected during the storage period. Heme concentrations were determined from the extinction coefficients of Banerjee et al.[16] for Hb and Willick et al.[17] for Mb. Cyanomet derivatives of Mb and Hb ($Mb^+CN^-$, $\lambda_{max}$ = 540 nm; $Hb^+CN^-$, $\lambda_{max}$ = 542 nm) were prepared by addition of a 2-3-fold molar excess of anaerobic NaCN solution to $Mb^+$ and $Hb^+$.

**Spectrophotometry.** Reactions were carried out anaerobically under argon in 1-cm (3 mL) cuvettes fitted with silicone rubber septa and were initiated by the injection of anaerobic $Na_2N_2O_3$ solution. Rates of nitrosylmyoglobin (MbNO) formation from $Mb^+$ were determined at 25 °C by monitoring the increase in absorbance at 578 nm spectrophotometrically. Rates of nitrosylhemoglobin (HbNO) production were measured similarly at 572 nm.

**Table I.** Rates of Trioxodinitrate Monoanion Decomposition and Nitrosylmyoglobin and Nitrosylhemoglobin Production at Two $HN_2O_3^-$/Heme Ratios, pH 7.0, 25 °C

| time, s | A calcd rate of HNO production,[a] $\mu M \times min^{-1}$ | B obsd rate of MbNO Production,[b] $\mu M \times min^{-1}$ | A/B | obsd rate of HbNO production,[b] $\mu M \times min^{-1}$ |
|---|---|---|---|---|
| | [Heme] = 50 $\mu$M; [$HN_2O_3^-$] = 125 $\mu$M | | | |
| 60 | 4.8 | 4.0 | 1.2 | 2.2 |
| 120 | 4.6 | 4.0 | 1.1 | 2.2 |
| 240 | 4.2 | 3.8 | 1.1 | 3.2 |
| 270 | 4.1 | 3.7 | 1.1 | 3.7 |
| 300 | 4.1 | 3.4 | 1.2 | 3.5 |
| 360 | 3.9 | 3.2 | 1.2 | 3.4 |
| | [Heme] = 50 $\mu$M; [$HN_2O_3^-$] = 500 $\mu$M | | | |
| 15 | 19.6 | 14.2 | 1.4 | 5.6 |
| 30 | 19.4 | 14.2 | 1.4 | 5.8 |
| 60 | 19.0 | 14.2 | 1.3 | 7.9 |
| 120 | 18.3 | 11.5 | 1.6 | 17.8 |
| 180 | 17.6 | 7.2 | 2.5 | 12.0 |
| 240 | 16.9 | 4.2 | 4.0 | 6.4 |

[a] Calculated from the observed first-order rate constant $6.6 \times 10^{-4}$ $s^{-1}$, at pH 7.0, 25 °C. [b] The experimental error and the error of graphical analysis of the data combined are about ±5%.

**GC/MS.** For analysis of $N_2O$ and NO, conditions were the same as described above, except that reactions were carried out under helium in sealed 9-mL vials in order to make use of the greater headspace. Argon was used as an internal standard. Gas samples of 0.15-mL volume were removed from the vial, expanded into a GC sample loop as previously described,[18] and analyzed by a Hewlett-Packard 5992A GC/MS equipped with a Porapak Q column (2.4 m × 2.2 mm).[19] Column temperature was 40 °C. Absolute amounts of NO and $N_2O$ were determined with reference to external standards. Total amounts of these products were calculated by use of Henry's law and published values of aqueous solubility coefficients.[20]

**Nitrite Analysis.** To make nitrite analyses possible, it was necessary to remove any NO from reaction vessels by sparging the system with oxygen-free argon. Protein was removed by centrifugation at 6800*g* for 30 min at 4 °C in a Centricon 10 microconcentrator (Amicon), and nitrite in the filtrate was determined colorimetrically by the method of Snell and Snell.[21]

## Results

As shown in Figure 1A and 1C, the reactions between $HN_2O_3^-$ and $Mb^+$ or $Hb^+$ at pH 7 yield the respective ferrous nitrosyl compounds, MbNO and HbNO, as the only discernible products. Reference spectra of MbNO and $Mb^+NO$ and of HbNO and $Hb^+NO$ are shown in Figure 1B and 1D, respectively. The spectra numbered 1 in Figure 1A and 1C are reference spectra of $Mb^+$ and $Hb^+$, respectively. Isosbestic points for the reaction with $Mb^+$ lie at 602, 522, and 480 nm and those with $Hb^+$ at 596, 520, and 475 nm. $Mb^+CN^-$ in the presence of a 100-fold molar excess of NaCN is not converted to MbNO by $HN_2O_3^-$, but that in the presence of a 2-fold molar excess is converted. $Hb^+CN^-$ behaves similarly. Data are not shown.

Figure 2 represents a titration of 50 $\mu$M $Mb^+$ or $Hb^+$ with $HN_2O_3^-$ and shows that the $HN_2O_3^-$/oxidized heme mole ratio for stoichiometric conversion of $Mb^+$ or $Hb^+$ to MbNO or HbNO is about 1.4. For each point in the titration curves, reaction was initiated with the indicated mole ratio of reactants and allowed to run to completion, at which time the extent of production of MbNO or HbNO was determined spectrophotometrically. At low ratios, the titration curve for $Hb^+$ (but not that for $Mb^+$) may be slightly sigmoidal. We believe that this effect may be related to the sigmoidal progress reaction curves shown by $Hb^+$ (Figure 3).

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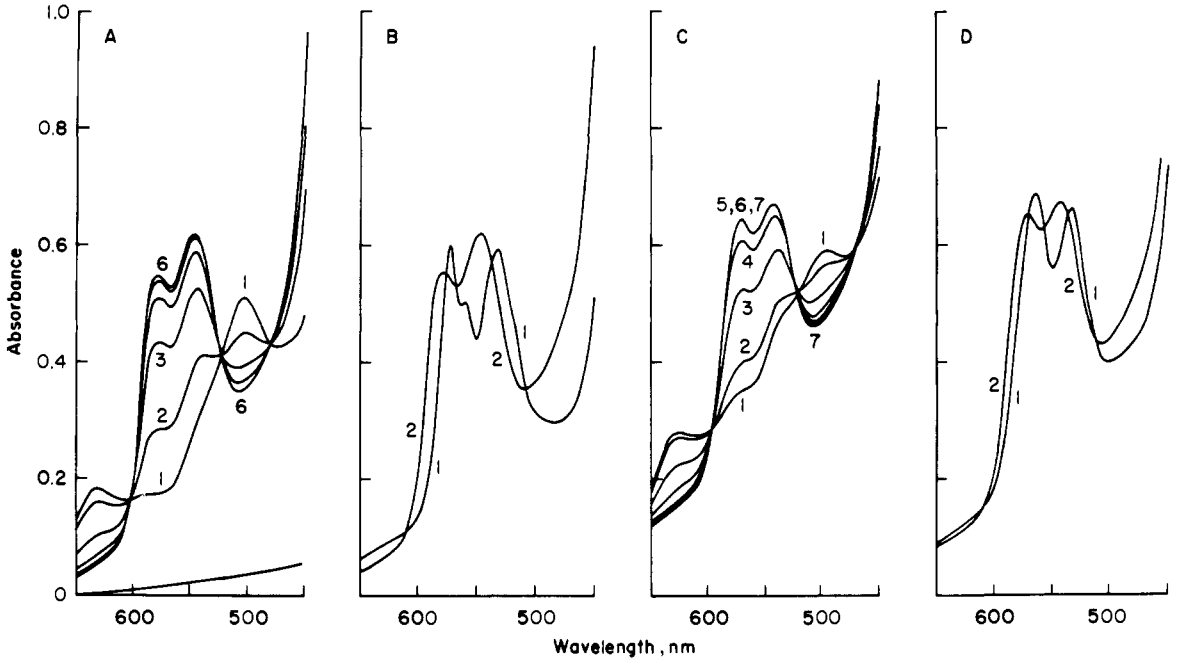


**Figure 1.** Spectral time course for the reactions of sperm whale metmyoglobin (A) and human methemoglobin (C) with trioxodinitrate monoanion at pH 7.0, 25 °C. Reaction mixtures contained 50 $\mu$M heme and 250 $\mu$M $HN_2O_3^-$ in 50 mM potassium phosphate buffer, pH 7.0, under anaerobic conditions. Reaction was initiated by injection of $Na_2N_2O_3$. With $Mb^+$, spectrum A2 was recorded at 1.5 min after initiation of the reaction and at 3.5-min intervals thereafter. For $Hb^+$, spectrum C2 was recorded at 1.0 min after initiation and at 3.0-min intervals thereafter. Scan time was 2.0 min in both cases. Spectra of $Mb^+$ and $Hb^+$ are shown in A1 and C1, respectively. $Mb^+NO$ (B1) and $Hb^+NO$ (D1) were formed by the injection of 20 $\mu$mol of gaseous NO into anaerobic cuvettes containing 3 mL of 50 $\mu$M $Mb^+$ or $Hb^+$ in 50 mM potassium phosphate buffer, pH 7.0. MbNO (B2) and HbNO (D2) were formed by reducing the oxidized NO derivatives with 300 $\mu$M ascorbic acid/10 $\mu$M *N,N,N',N'*-tetramethylphenylenediamine. This reducing system does not reduce free NO. Spectra of MbNO and HbNO prepared in this way were identical with those obtained by direct addition of NO to Mb and Hb. The buffer blank is shown at the bottom of A.

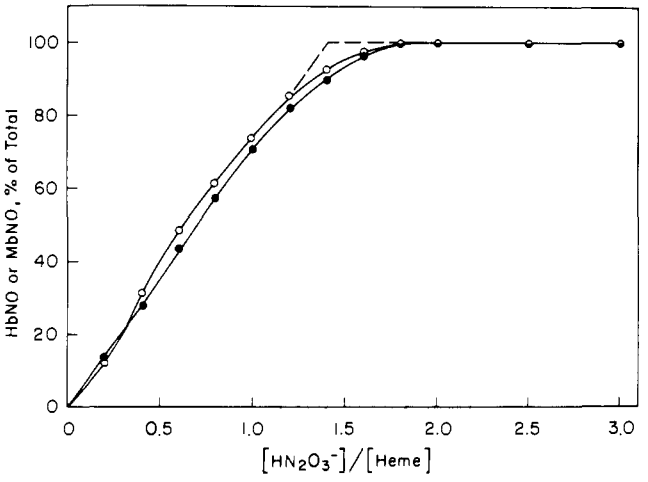


**Figure 2.** Stoichiometric titrations of 50 $\mu$M metmyoglobin (●) and 50 $\mu$M methemoglobin (○) with trioxodinitrate monoanion at pH 7.0, 25 °C. The conditions and method of Figure 1 applied. The percentages of MbNO and HbNO formed were determined from the absorbance at 578 and 572 nm, respectively.

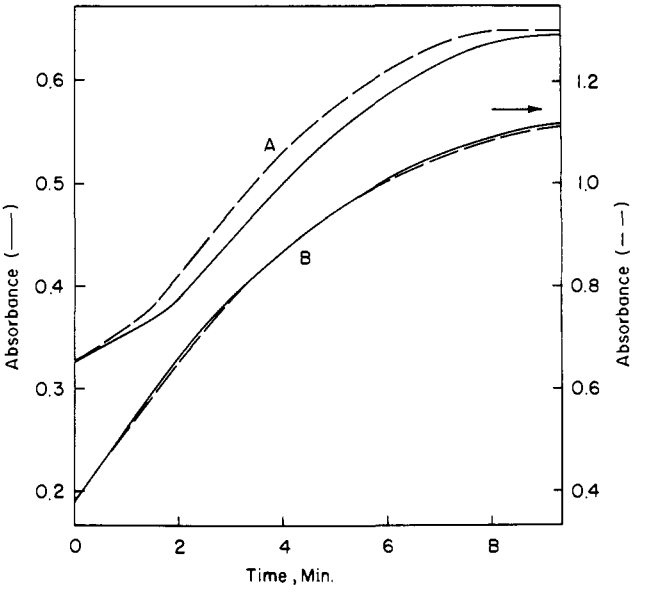


**Figure 3.** Progress curves for the reaction of methemoglobin (A) and metmyoglobin (B) with trioxodinitrate monoanion at pH 7.0, 25 °C. HbNO and MbNO production were monitored at 572 and 578 nm, respectively. Reaction mixtures contained 50 $\mu$M heme and 250 $\mu$M $HN_2O_3^-$ (—) or 100 $\mu$M heme and 500 $\mu$M $HN_2O_3^-$ (- - -) in 50 mM potassium phosphate buffer. Arrow denotes final absorbances for the reaction of $Mb^+$ on both scales.

The production of MbNO from $HN_2O_3^-$ and $Mb^+$ progresses without an inflection point (Figure 3, curves B), whereas production of HbNO shows sigmoidal progress curves (Figure 3, curves A). We assume that the sigmoidicity represents a cooperative reactivity effect attributed to the tetrameric nature of $Hb^+$. Cooperativity in the dissociation of $O_2$ or CO from Hb is well-known and that for the dissociation of NO has also been reported.[22] It is shown in Table I that the rates of $HN_2O_3^-$ breakdown, as calculated from the initial concentration and the known first-order rate constant, always exceed those for formation of MbNO and HbNO, and this has been confirmed over a range of $HN_2O_3^-$/heme protein ratios of 1–10 ([heme] = 50 or 100 $\mu$M).

Demonstration that the reactions are nearly zero-order in $Mb^+$ and $Hb^+$ was obtained by observing that a doubling of $[HN_2O_3^-]$ and $[Mb^+]$ or $[Hb^+]$ increased the reaction rate by a factor of about 2, not 4, over virtually the entire course of the reaction (Figure 3). That is to say, the two progress curves in each set in Figure 3 are nearly superimposable when the absorbance scale is changed by a factor of 2. In other experiments (not shown), a doubling of $[Mb^+]$ or $[Hb^+]$ without change in $[HN_2O_3^-]$ had

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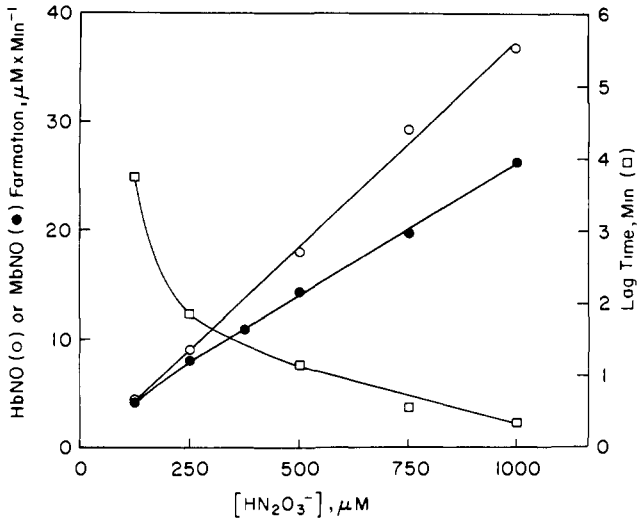


**Figure 4.** Dependence of rates of nitrosylmyoglobin and nitrosylhemoglobin production on trioxodinitrate monoanion concentration at pH 7.0, 25 °C. Reaction mixtures contained 50 μM heme, and the conditions and method of Figure 1 applied. Rates refer to the initial rates of MbNO formation or to the maximum rates of HbNO formation at the inflection point of progress curves, such as those of Figure 3. The lag time is the time required to reach the maximum rates of HbNO formation.

**Table II.** Distribution of Products of Trioxodinitrate Monoanion Decomposition at pH 7.0, 25 °C, in the Presence and Absence of Metmyoglobin and Methemoglobin[a]

| | | ng-atom of N | | | |
|---|---|---|---|---|---|
| treatment | free NO | bound NO[b] | $NO_2^-$ | $N_2O$ | total |
| control | 12 | | 1193 | 950 | 2155 |
| 1 mM $Hb^+$ | 80 | 847 | 1076 | 184 | 2187 |
| 1 mM $Mb^+$ | 94 | 727 | 986 | 357 | 2164 |

[a] Reaction was initiated at pH 7.0, 25 °C, by the injection of 1 μmol of anaerobic $HN_2O_3^-$. Final volume was 1 mL. The error in the amounts of $NO_2^-$ and $N_2O$ is about ±6%. Those for NO and bound NO are about ±15 and 5%, respectively. [b] Refers to NO as HbNO or MbNO, calculated from absorbance at 627, 572, and 544 nm for HbNO and 630, 578, and 547 nm for MbNO. The value reported is the average value.

virtually no effect on initial rates of MbNO formation or on maximum rates of HbNO formation. In comparison with other experiments, we judge the differences between the curves A of Figure 3 not to be significant.

Figure 4 shows that the reaction rates are nearly linear in $[HN_2O_3^-]$ based on initial rates for $Mb^+$ and on the maximal rates with $Hb^+$. Although the curves of Figure 4 may not quite intersect the origin, as would be expected for rigorously first-order kinetics, we believe that failure to intersect exactly is due largely to technical problems associated with running the reactions at low $[HN_2O_3^-]$. We conclude that the reactions are approximately first-order in $[HN_2O_3^-]$.

Product analysis (Table II) following reaction of 1 mM $HN_2O_3^-$ with 1 mM $Mb^+$ or $Hb^+$ shows that formation of MbNO and HbNO is competitive with $N_2O$ formation and not with nitrite formation. One mole (2 g-atom) of $HN_2O_3^-$ gives rise to about 1 g-atom of nitrite and 1 g-atom of MbNO (or HbNO) plus $N_2O$. A small amount of free NO is formed in reactions containing $Mb^+$ or $Hb^+$ but only a trace in the control without heme protein. The error of the GC/MS determinations does not allow us to decide whether the free NO arises from the singly oxygenated N of $HN_2O_3^-$ that gives rise to heme nitrosyl plus $N_2O$, from the doubly oxygenated N that gives rise to nitrite, or from both. We believe that formation of small amounts of free NO is not mechanistically significant.

## Discussion

On the basis of kinetic and product analyses, the reaction between $HN_2O_3^-$ and $Mb^+$ or $Hb^+$ can be represented most simply according to eq 3–5. Decomposition of $HN_2O_3^-$ to form NO plus

$$HN_2O_3^- \xrightarrow{\text{r.d.s.}} HNO + NO_2^- \quad (3)$$

$$Mb^+ + HNO \xrightarrow{\text{fast}} MbNO + H^+ \quad (4)$$

$$2HNO \xrightarrow{\text{fast}} N_2O + H_2O \quad (5)$$

$(HONO)^-$, as recently proposed by Doyle and Mahapatro,[10] would require eq 6–8. Although eq 6–8 cannot be entirely ruled out

$$HN_2O_3^- \xrightarrow{\text{r.d.s.}} NO + (HONO)^- \quad (6)$$

$$Mb^+ + (HONO)^- \xrightarrow{\text{fast}} Mb + NO_2^- + H^+ \quad (7)$$

$$Mb + NO \xrightarrow{\text{fast}} MbNO \quad (8)$$

by this study, we argue against the pathway as follows. If eq 6–8 were to apply, $(HONO)^-$ must serve as a kinetically efficient reductant of $Mb^+$ or $Hb^+$. Otherwise eq 7 and 8 would not overwhelm competing reactions that are postulated[10] to convert NO plus $(HONO)^-$ into $N_2O$ and $NO_2^-$ (eq 9–11). The objection

$$(HONO)^- + HN_2O_3^- \rightarrow H^+ + NO_2^- + (HN_2O_3)^{2-} \quad (9)$$

$$(HN_2O_3)^{2-} + NO \rightarrow [ONN(OH)NO_2]^{2-} \quad (10)$$

$$[ONN(OH)NO_2]^{2-} \rightarrow NO_2^- + N_2O + OH^- \quad (11)$$

to $(HONO)^-$ arises because it must serve at pH 7 simultaneously as both a reductant and indirectly as an oxidant of the heme protein. We show in the companion paper which follows,[23] in agreement with Doyle and Mahapatro,[10] that an efficient oxidizing agent for Mb or Hb is formed in the pathway of $HN_2O_3^-$ decomposition. According to Doyle and Mahapatro,[10] that oxidizing agent is $(HN_2O_3)^{2-}$ of eq 9, and it reacts with Mb or Hb according to eq 12 and 13. Thus, $(HONO)^-$ must be viewed as both a

$$(HN_2O_3)^{2-} + Mb \rightarrow Mb^+ + (N_2O_2)^{2-} + OH^- \quad (12)$$

$$(N_2O_2)^{2-} + H^+ \rightarrow N_2O + OH^- \quad (13)$$

reductant of $Mb^+$ (eq 7) and a reagent for producing an oxidant of Mb (eq 9 and 12), and it is necessarily very efficient kinetically in both of these opposing redox reactions. These attributes of the hypothetical $(HONO)^-$ species are, we believe, mutually contradictory and unprecedented. No such problems arise if $HN_2O_3^-$ decomposes into HNO plus $NO_2^-$.

Structural considerations also argue against eq 6. N-Sulfonated hydroxylamines ($HONH-SO_3^-$,[13] $HONH-SO_2Ph$[24]) under alkaline conditions and trioxodinitrate monoanion ($^-ONH-NO_2$)[8] at neutrality decompose to form an apparently identical N species that can substitute at tetracyanonickel to yield tricyanonitrosylnickel. The species is not NO, and it is derived from the hydroxylamino N in the case of trioxodinitrate. In the absence of tetracyanonickel, this species converts to $N_2O$. A common mechanism of decomposition is clearly implied. Thus, if eq 6 and 9–11 were to describe the decomposition of trioxodinitrate, then an analogous set of reactions would need to be evoked for N-sulfonated hydroxylamines, in spite of their inability to generate $(HONO)^-$. Analogous reactions for eq 9–11 are therefore problematical. No such difficulty arises if the above compounds all undergo heterolytic cleavage to yield nitroxyl as the common intermediate and some reasonably good leaving group, such a sulfite, benzenesulfinate, or nitrite.

Our results strongly suggest that ferric heme proteins can serve as kinetically competitive traps for nitroxyl in aqueous solution at neutrality. $Mb^+$ and $Hb^+$ are analogous therefore to tetracyanonickel which can function as an efficient nitroxyl trap at somewhat higher pH values.[8] Although NO complexes of heme proteins are well-known, it has been possible only recently to demonstrate nitrosyl ($NO^+$) transfer from ferrous nitrite reductase.[18] It would appear therefore that reactions of $NO^-$ (or HNO)

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and $NO^+$ have biochemical precedence.

In the latter phases of the reaction, during which the rate of MbNO formation decreases relative to that for $HN_2O_3^-$ decomposition (Table I), one can consider whether the decrease is due to a change in the rate-determining step from $HN_2O_3^-$ breakdown (zero-order in [$Mb^+$]) to nitroxyl trapping (presumably first-order in [$Mb^+$]) or to an increasingly unfavorable partition between fast trapping and fast $N_2O$ production. In the second case, the reaction should remain zero-order in [$Mb^+$] throughout. The experiments of Figure 3 suggest that the second case is the more likely, because the reactions appear to remain zero-order in [$Mb^+$] or [$Hb^+$] (2-fold rate difference throughout) and do not tend to show a transition from a 2-fold to a 4-fold rate difference toward the end of the reaction. It is reasonable to expect nitroxyl trapping to be a fast reaction, because nitroxyl dimerization/dehydration in the gas phase is known to be a fast reaction[2] and because, in our systems, the reaction must compete with addition of an anionic ligand to $Mb^+$ or $Hb^+$ for which rate constants $\geq 10^4$ $M^{-1}$ $s^{-1}$ are not unreasonable.[25] The rate constant for dimerization/dehydration of nitroxyl in neutral aqueous solution is probably $\geq 10^9$ $M^{-1}$ $s^{-1}$.[26]

The stoichiometric ratio of about 1.4:1 established in Figure 2 for 50 $\mu$M heme represents an average over the entire course of the reaction and includes the latter phases during which time nitroxyl capture by heme protein would be decreasingly competitive against $N_2O$ formation. We believe that a better measure is the ratio A/B in Table I, which is about 1.25 for $Mb^+$. Thus, in the 50 $\mu$M range, $Mb^+$ can trap something in the order of 80% of the nitroxyl generated in $HN_2O_3^-$ breakdown. The rather high efficiency for trapping can be attributed to lack of a firmly bound sixth protein ligand to iron in $Mb^+$ and $Hb^+$, the dissociation of which might be expected to be slow, as is the case generally with cytochromes. This view is supported by the fact that certain concentrations of HCN can block the conversion of $Mb^+CN^-$ to MbNO by $HN_2O_3^-$, whereas lower concentrations either cannot or are only partially effective. The observation suggests that HNO and HCN can compete with each other for the sixth ligand position of $Mb^+$ and $Hb^+$.

The sigmoidal progress curves observed with $Hb^+$ are likely due to a cooperative reactivity of the $Hb^+$ tetramer. Cooperativity in ligand binding or redox reactions of $Hb^+$ is unknown at present,[27] and reaction with nitroxyl may be the first example. Alternatively, the sigmoidicity may result from a high molecular weight inhibitor which is destroyed during the course of the reaction. This possibility seems very unlikely but has not been entirely ruled out.

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# On the Reaction of Trioxodinitrate(II) with Hemoglobin and Myoglobin

**Dennis A. Bazylinski, Joanne Goretski, and Thomas C. Hollocher***

*Contribution from the Department of Biochemistry, Brandeis University, Waltham, Massachusetts 02254.* *Received March 7, 1985*

**Abstract:** The reaction of hemoglobin (Hb) or myoglobin (Mb) with the monoanion ($HN_2O_3^-$, **1**) of sodium trioxodinitrate was studied in anaerobic 50 mM potassium phosphate buffer at pH 7.0, 25 °C. The reaction showed two kinetic phases, and the initial more rapid reaction is the reaction of interest. That reaction was first-order in **1** and zero-order in [Hb]. The rate of disappearance of Hb was nearly twice that of **1**, and in titrations, 1 mol of **1** consumed about 2 mol of Hb. The unimolecular rate-determining step in the disappearance of **1** in the presence of Hb is apparently identical with that in its absence. At 50-100 $\mu$M Hb, the products were methemoglobin ($Hb^+$) and nitrosylhemoglobin (HbNO) in approximately equimolar amounts. At 1 mM Hb, HbNO was the chief product. Similar results to above were obtained with Mb. Formation of $Hb^+$ and HbNO could suppress formation of both nitrite and $N_2O$, which are the normal decomposition products of **1** in the absence of Hb. The results are explained for the most part by rate-determining conversion of **1** to a reactive form (or to primary decomposition products), **1***, followed by reduction of **1*** by Hb or Mb. The composition of products would appear to depend on the efficiency with which newly formed $Hb^+$ traps nitrosyl hydride (nitroxyl, HNO), or related species, to form HbNO, relative to dimerization of HNO to form $N_2O$. The stoichiometry reported recently (Doyle, M. P.; Mahapatro, S. N. *J. Am. Chem. Soc.* **1984**, *106*, 3678-3679) for the reaction of Hb with **1** is apparently incorrect and the mechanism inferred therefrom in question.

There exists kinetic and isotopic evidence[1-6] to suggest that the primary products of decomposition of the monoanion (**1**)[7] of sodium trioxodinitrate (Angeli's salt,[8] $Na_2N_2O_3$) in neutral aqueous solution are nitrosyl hydride (nitroxyl, HNO) from N(1) and nitrite from N(2) (eq 1).

Nitroxyl is thought then to dimerize and dehydrate rapidly (eq 2) to form $N_2O$ as final product, just as is known to occur in the gas phase.[9] In support of nitroxyl formation in eq 1 are reactions which can compete with dimerization to trap nitroxyl nitrogen.

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