tions. The absorbance was monitored after each injection to assure that phenyl acetate was intact before the enzymic reaction began. The reaction was initiated with the injection of the enzyme in a 50-μL volume to bring the total to 1 mL.

The irreversible inhibition of AChE in the presence of the substrate was studied under pseudo-first-order conditions with >1000-fold excess of inhibitor over enzyme concentration to obtain a signal equal to <10% decomposition of the substrate. First-order rate constants were calculated by a least-squares fit of absorbance time coordinates. Substrate-independent, second-order inhibition constants were calculated by a linear least-squares fit of the inverse observed rate constants to substrate concentration.

Least-squares fit of the second-order rate constants to the Eyring equation was computed with the use of a BMDP statistical program.[13]

Oxidation and Reduction of Hemoproteins by Trioxodinitrate(II). The Role of Nitrosyl Hydride and Nitrite

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Received January 23, 1987

Abstract: The reactions of hemoglobin (Hb), oxyhemoglobin (HbO_2) and oxymyoglobin (MbO_2), methemoglobin (Hb^+), metmyoglobin (Mb^+), and ferricytochrome *c* (Cyt c^+) with the monoanion of trioxodinitrate(II) were investigated in phosphate-buffered solutions at pH 7.0 and 25 °C. Oxymyoglobin is oxidized to Mb^+ by the decomposition products of $HN_2O_3^-$ in a stoichiometric transformation that requires a 2:1 $[MbO_2]$:$[HN_2O_3^-]$ reactant ratio and produces nitrite, nitrate, and peroxide. Methemoglobin undergoes reductive nitrosylation with nitroxyl formed by $HN_2O_3^-$ decomposition, and its kinetic complexity, identified as due to a competing reaction of HNO with β-93 cysteine sulfhydryl groups of Hb^+, has been unraveled. Nitroxyl causes sulfhydryl oxidation to disulfide and, in an example of the entrapment of nitroxyl by reduction, is converted to hydroxylamine. Ferricytochrome *c* is reduced to ferrocytochrome *c* by apparent outer-sphere electron transfer from HNO. Hemoglobin is converted to Hb^+ and HbNO by $HN_2O_3^-$, and evidence is presented that HNO, through coordination with Hb, is a catalyst for nitrite oxidation of hemoglobin. The composite data demonstrate that dissociation of nitrosyl hydride from nitrite occurs in $HN_2O_3^-$ decomposition, and they suggest a greater diversity of transformations for nitroxyl than has been previously recognized.

We recently reported that decomposition of trioxodinitrate ($HN_2O_3^-$) in the presence of deoxyhemoglobin resulted in the production of methemoglobin and nitrosylhemoglobin.[1] This unprecedented transformation led us to suggest that decomposition of trioxodinitrate occurred by dissociation of nitric oxide from the nitrous acid radical anion (eq 1), and results obtained from

$$HN_2O_3^- \rightarrow NO + (HONO)^{\bullet -} \quad (1)$$

reactions with oxymyoglobin and oxyhemoglobin, in which the ferrihemoprotein was formed together with nitrite and nitrate, were interpreted similarly. Extensive studies by Bonner,[2-6] Hughes,[7,8] and others[9-11] had previously defined trioxodinitrate decomposition by a pathway involving the production of nitrite and nitrosyl hydride (eq 2) that adequately explained available data but appeared to be unsuitable to account for the transformations observed with hemoproteins.

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

As a result of this report, with its conflicting interpretation of the mechanistic pathway for trioxodinitrate decomposition, Hollocher and co-workers reinvestigated the reaction of the monoanion of sodium trioxodinitrate with deoxyhemoglobin and deoxymyoglobin, confirming the rate law and hemoprotein product ratios but differing from us in the stoichiometry for production of nitrite and nitrous oxide.[12] Their results, and particularly the reaction stoichiometry, were consistent with the direct intervention of an activated form of $HN_2O_3^-$ or its primary breakdown products. Complimentary studies of trioxodinitrate decomposition in the presence of nitric oxide were also performed to evaluate eq 2 as the only viable dissociative pathway, and together with Gratzel's prior report of nitroxyl association with nitric oxide,[13] Bazylinski and Hollocher concluded that eq 2, and not eq 1, was the primary pathway for trioxodinitrate decomposition.[14] More recently, the photochemical decomposition of trioxodinitrate was shown to produce triplet nitroxyl and nitrate in apparent agreement with eq 2.[15]

We have investigated trioxodinitrate decomposition in the presence of oxyferrohemoproteins and ferrihemoproteins with the expectation that results obtained from this study would further clarify their redox mechanisms. Our results with methemoglobin correspond to those reported by Bazylinski and Hollocher[16] with some relevant exceptions that are described in this publication. That methemoglobin is reduced during $HN_2O_3^-$ decomposition while oxyhemoglobin and deoxyhemoglobin are oxidized by the same reagent is one reason for our interest in the mechanism of

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these reactions. New information concerning the chemical characteristics of nitroxyl has been obtained from the oxidation of thiols and the reduction of ferricytochrome *c* that have direct bearing on the mechanism of trioxodinitrate decomposition in the presence of hemoproteins.

Experimental Section

Reagents. Sodium trioxodinitrate(II) was prepared from hydroxylamine and *n*-butyl nitrate by the method of Smith and Hein.[17] The crude product was dissolved in 0.1 M sodium hydroxide solution and reprecipitated by addition of methanol, and this procedure was repeated until the sample reached a molar absorptivity valve of 8.2×10^3 M^{-1} cm^{-1} at 248 nm in 1.0 M aqueous sodium hydroxide.[7] A stock solution of sodium trioxodinitrate(II) (0.02–0.04 M) in deoxygenated 0.10 M aqueous sodium hydroxide, prepared immediately before use, was employed for the transfer of aliquots to the reaction solutions.

Human hemoglobin A (type IV), sperm whale myoglobin, and horse heart cytochrome *c* (type VI), obtained from Sigma Chemical Co., were reduced with excess sodium dithionite and further purified by passing the resulting aqueous solutions through G-25 Sephadex columns with 0.05 M phosphate buffer at pH 7.0. The ferrihemoproteins were prepared by oxidation of the commercial reagents with excess sodium ferricyanide[18] and further purified on a G-25 Sephadex column with 0.05 M phosphate buffer at pH 7.0. Concentrated solutions were degassed under reduced pressure (less than 0.5 Torr), and the resultant deoxygenated solutions were maintained in sealed air-tight flasks at atmospheric pressure under nitrogen. Heme concentrations for oxyhemoglobin and oxymyoglobin samples were calculated from the molar absorptivities of Moore and Gibson.[19] Reaction of β-93 cysteine residues of the reactant hemoglobin was performed at pH 7.8 with oxyhemoglobin and a 10-fold molar excess of iodoacetamide, relative to heme, according to the procedure of Winterbourn and Carrell.[20] The β-93 sulfhydryl-blocked hemoglobin was separated from excess iodoacetamide on a G-25 Sephadex column. The absence of accessible sulfhydryl groups was determined with 5,5′-dithiobis(2-nitrobenzoic acid) by the method of Ellman.[21] Initial concentrations of methemoglobin were determined from molar absorptivities of its cyanide complex,[22] and those of ferrocytochrome *c* and were calculated from their known absorption spectra.[23]

Kinetic Methods. Reactions were initiated with the injection, using a gas-tight syringe, of a concentrated trioxodinitrate solution (usually 20–30 μL) into the hemoprotein sample (usually 40–70 μM) contained in 0.05 M phosphate buffer at pH 7.0. Rates for reactions between deoxyhemoglobin (Hb) and $HN_2O_3^-$ were followed at 430 nm, those for reactions between oxyhemoglobin (HbO_2) or oxymyoglobin (MbO_2) and $HN_2O_3^-$ were determined at 576 and 581 nm, respectively, and those for nitrosylhemoglobin formation from reactions between methemoglobin (Hb^+) and $HN_2O_3^-$ were determined by monitoring the increase in absorbance at 570 nm on a Hewlett-Packard Model 8451A diode array spectrophotometer or a Cary Model 118 spectrophotometer. Rates for decomposition of sodium trioxodinitrate in the absence of hemoprotein were followed at 237 nm; the conversion of thiophenol to phenyl disulfide was observed at 260 nm. Duplicate kinetic determinations were performed and average values for rate constants are reported. Rates for ferrocytochrome *c* formation were determined by monitoring the increase in absorbance at 550 nm.

Stoichiometric Determinations. Concentrations of nitrosylhemoglobin and methemoglobin or ferrocytochrome *c* and ferricytochrome *c* were calculated from the measured absorbances at two or more wavelengths by previously described methods.[24] Slow conversion of nitrosylhemoglobin to methemoglobin in the presence of nitrite required that these measurements be taken within 6 half-lives of the rate for decomposition of trioxodinitrate. Deviations in product yields from multiple determinations were generally less than ±5%.

Nitrite and nitrate concentrations were determined by HPLC analysis on a Licrosorb-NH_2 reversed-phase column monitored at 210 nm. Separation of nitrite and nitrate with retention volumes of 5.7 and 9.3 mL, respectively, from hemoprotein (retention volume <3.6 mL) occurred in 0.05 M phosphate buffer at pH 3. Phenyl disulfide yields were determined directly from the reaction solutions monitored at 254 nm on a C_{18} reversed-phase column with 80:20 methanol–water. Nitrogen oxides were analyzed by GC analysis on a Varian Model 2700 GC with thermal conductivity detectors using a molecular sieve 5A column and further characterized by mass spectral analysis on a Hewlett-Packard Model 5995C GC/MS system. Hydroxylamine concentrations were determined by the standard indooxine test using 5-amino-8-hydroxyquinoline.[25]

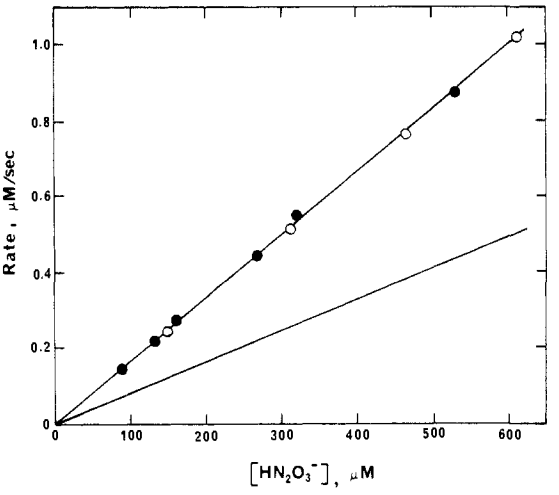

Figure 1. Rates of reaction of oxyhemoglobin (●) and oxymyoglobin (○) with the trioxodinitrate monoanion at pH 7.0 and 25 °C. Reactions were initiated by the injection of $Na_2N_2O_3$ into 50 μM heme protein ([heme]) dissolved in oxygen-saturated 0.05 M phosphate buffer at pH 7.0. The lower line is the rate for spontaneous $HN_2O_3^-$ decomposition calculated from the measured rate constant.

Table I. Products from the Reaction of the Trioxodinitrate Monoanion with Oxymyoglobin at pH 7.0, 25 °C[a]

10^5[reactants], M		anal.	10^5[products], M		
$[MbO_2]$	$[HN_2O_3^-]$	time, min	$[NO_2^-]$	$[NO_3^-]$	$\sum N^b$ (%)
0	7.8		7.15	0.30	7.45 (48)[c]
11.0	5.5	60	5.02	5.80	10.82 (98)
		120	4.30	6.50	10.80 (98)
7.8	7.8	60	7.02	5.46	12.48 (80)[d]
		120	5.45	7.02	12.47 (80)[d]

[a] Reactions performed in oxygen-saturated solutions. [b] Total concentration of nitrite and nitrate (percent yield based on initial concentration of trioxodinitrate). Estimated precision from duplicate runs, ±5%. [c] Nitrous oxide was produced in amounts corresponding to 42–49% of reactant trioxodinitrate. [d] Nitrous oxide is the residual nitrogenous product.

Results and Discussion

Oxidation of Oxyhemoglobin and Oxymyoglobin. As we communicated earlier,[1] decomposition of $HN_2O_3^-$ in the presence of oxyhemoglobin or oxymyoglobin occurs with first-order rate dependence on $[HN_2O_3^-]$ and zero-order rate dependence on the oxyhemoprotein concentration. Zero-order kinetics for the conversion of HbO_2 to Hb^+ or MbO_2 to Mb^+ were observed through more than 2 half-lives for each hemoprotein, and the resulting rate constants exhibited first-order dependence on trioxodinitrate concentration. Figure 1 describes this dependence and compares the rates for oxyhemoprotein oxidation in the presence of $HN_2O_3^-$ with the rates of spontaneous decomposition of $HN_2O_3^-$, which are based on the first-order rate constant, independently measured as 8.3×10^{-4} s^{-1},[26] for trioxodinitrate decomposition in oxygen-saturated phosphate-buffered aqueous solution at pH 7.0 and 25.0 °C. The ratio of the two slopes is exactly 2.0, which demonstrates that the reactant stoichiometry is 2:1 for HbO_2 or MbO_2 to $HN_2O_3^-$.

In addition to the methemoprotein, which was the only hemoprotein product,[1] both nitrite and nitrate were formed. Ap-

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proximately equal amounts of these two anions were detected at both 1:1 and 2:1 $[MbO_2]:[HN_2O_3^-]$ molar reactant ratios (Table I), but their relative amounts varied, depending on the time for analysis. The results presented in Table I were obtained through analysis of the reaction mixtures immediately after 5 half-lives for $HN_2O_3^-$ decomposition (1 h) and then again at 2 h after initiation of the reaction. At longer times nitrite was nearly completely converted to nitrate. These results can be explained if the stoichiometry for the reaction between MbO_2 and $HN_2O_3^-$ is that shown in eq 3. Nitrite is oxidized to nitrate under these

$$2MbO_2 + HN_2O_3^- \rightarrow 2Mb^+ + NO_2^- + NO_3^- + HO_2^- \quad (3)$$

conditions.[27] Thus, the nitrite to nitrate ratio can be expected to vary with the time for analysis so that, even with the reaction between equimolar amounts of reactants, product accountability is consistent with eq 3, decomposition of $HN_2O_3^-$ to nitrous oxide and nitrite (eq 4), and the methemoprotein-catalyzed oxidation of nitrite (eq 5) that we have previously demonstrated.[28] Similar results were obtained with oxyhemoglobin.

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

$$NO_2^- + H_2O_2 \xrightarrow{Mb^+} NO_3^- + H_2O \quad (5)$$

We previously presented the mechanism for this transformation by a sequence of events resulting from nitric oxide dissociation from the nitrous acid radical anion (Scheme I).[1] The proposal

Scheme I

$$HN_2O_3^- \rightarrow NO + (HONO)^{\bullet -} \quad (1)$$

$$MbO_2 + NO \rightarrow Mb^+ + NO_3^- \quad (6)$$

$$MbO_2 + (HONO)^{\bullet -} \rightarrow Mb^+ + NO_2^- + HO_2^- \quad (7)$$

was based, in part, on results that were obtained with deoxyhemoglobin and on the known ability of MbO_2 to undergo rapid stoichiometric oxidation with nitric oxide to metmyoglobin and nitrate (eq 6). This latter reaction has been extensively investigated, and its second-order rate constant is reported to be 31×10^6 M^{-1} s^{-1}.[29] However, particularly in view of Hollocher's mechanistic explanation of the reaction of deoxyhemoglobin with $HN_2O_3^-$, an alternate pathway result from trioxodinitrate decomposition to nitrosyl hydride and nitrite (Scheme II) must be considered. In either Scheme I or Scheme II, the rate law for metmyoglobin formation is predicted and observed to be $d[Mb^+]/dt = 2k[HN_2O_3^-]$, where k is the first-order rate constant for spontaneous decomposition of the trioxodinitrate monoanion.

Scheme II

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

$$MbO_2 + HNO \rightarrow Mb^+ + NO + HO_2^- \quad (8)$$

$$MbO_2 + NO \rightarrow Mb^+ + NO_3^- \quad (6)$$

The reaction depicted by eq 8 can be regarded as a one-electron donation by nitroxyl to the bound dioxygen of myoglobin (or hemoglobin). Similar processes involving nitrite and phenols are well-defined.[27,30] The second-order rate constant for nitrite oxidation of HbO_2 is only 8.6 M^{-1} s^{-1} but, given the expected lower potential for electron transfer from nitroxyl, the rate for eq 8 can be expected to be much faster than the rate for decomposition of $HN_2O_3^-$.

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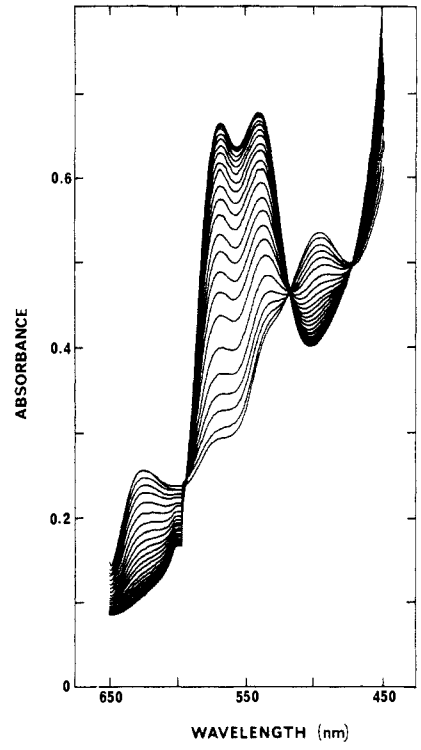

Figure 2. Spectral time course for the reaction of methemoglobin with $HN_2O_3^-$ in deoxygenated 0.05 M phosphate buffer at pH 7.0 and 25.0 °C: $[Hb^+] = 4.68 \times 10^{-5}$ M, $[HN_2O_3^-] = 1.9 \times 10^{-4}$ M. Spectra were recorded at 1.0-min intervals after the initial scan (t_0 = 30 s). Scan time was 40 s. Isosbestic points are at 598, 517, and 472 nm.

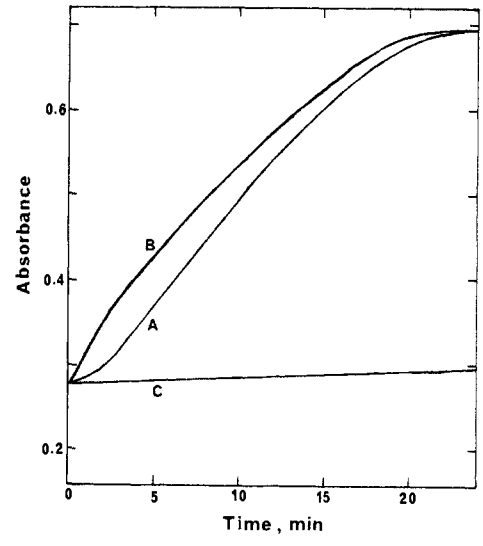

Figure 3. Progress of reaction between Hb^+ and $HN_2O_3^-$ under anaerobic conditions in 0.05 M phosphate buffer at pH 7.0 and 25 °C, monitored at 572 nm: $[Hb^+] = 4.5 \times 10^{-5}$ M, $[HN_2O_3^-] = 1.8 \times 10^{-4}$ M. Key: (A) control, unblocked methemoglobin; (B) sulfhydryl-blocked methemoglobin; (C) unblocked methemoglobin + cysteine, [CysSH] = 1.8×10^{-4} M.

Reductive Nitrosylation of Methemoglobin. Having observed that methemoglobin was converted to nitrosylhemoglobin during investigations of the reaction of deoxyhemoglobin with $HN_2O_3^-$,[1] we focused on Hb^+ as a trap for reaction intermediates formed from the decomposition of $HN_2O_3^-$. Our results agree with those reported by Bazylinski and Hollocher,[16] but we have performed additional experiments that explain certain observations that they have attributed to other causes.

Unlike reactions between $HN_2O_3^-$ and Hb or HbO_2, reaction rates for nitrosylhemoglobin formation from methemoglobin do not follow simple zero-order kinetics throughout their reaction time courses. However, initial rates are zero order in $[Hb^+]$ and

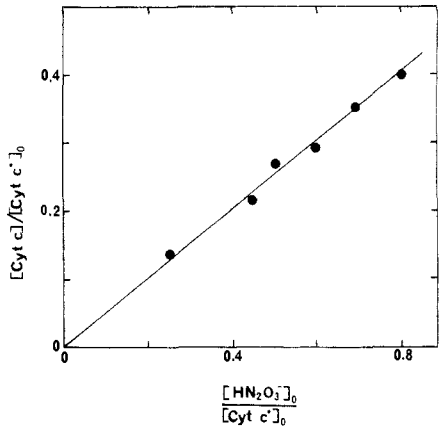

Figure 4. Plot of mole fraction of Cyt *c* versus the molar ratio of reactants for reactions performed in deoxygenated 0.05 M phosphate buffer at pH 7.0 and 25 °C: $[\text{Cyt } c^+]_0 = 4.0 \times 10^{-5}$ M.

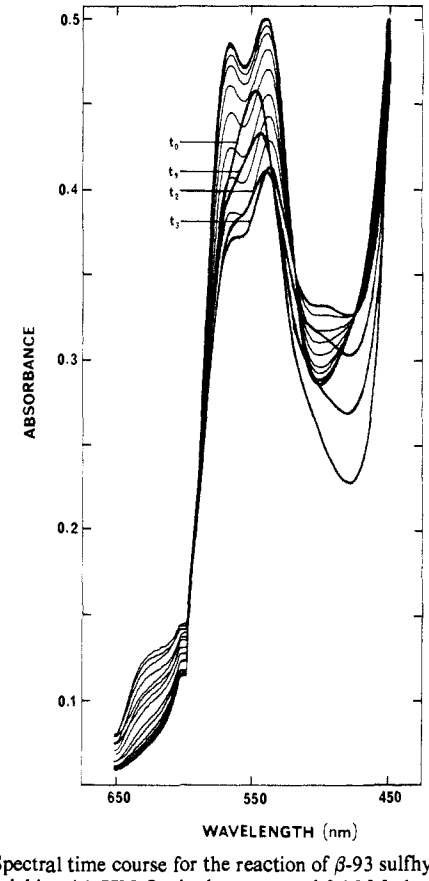

Figure 5. Spectral time course for the reaction of β-93 sulfhydryl-blocked deoxyhemoglobin with $HN_2O_3^-$ in deoxygenated 0.05 M phosphate buffer at pH 7.0 and 25.0 °C: [Hb] = 3.30×10^{-5} M, $[HN_2O_3^-] = 1.64 \times 10^{-4}$ M. Spectra were recorded at 1.0-min intervals after the initial scan (t_0 = 30 s). Scan time was 40 s. Isosbestic points after t_3 are at 598, 519, and 475 nm.

first order in $[HN_2O_3^-]$. There is an initial slow phase followed by a more rapid conversion (Figure 2) in reactions between methemoglobin and trioxodinitrate (curve A of Figure 3). The same initial slow phase is not observed for reactions performed with Mb^+, and zero-order kinetic dependence on $[Mb^+]$ characterizes this reductive nitrosylation reaction through nearly 1 half-life of the total conversion. The kinetics of methemoprotein reactions with $HN_2O_3^-$ are further complicated by nitrite association with the reactant hemoprotein[31] resulting from nitrite accumulation during the course of the reaction. The accumulated data provided by Bazylinski and Hollocher,[16] which we have

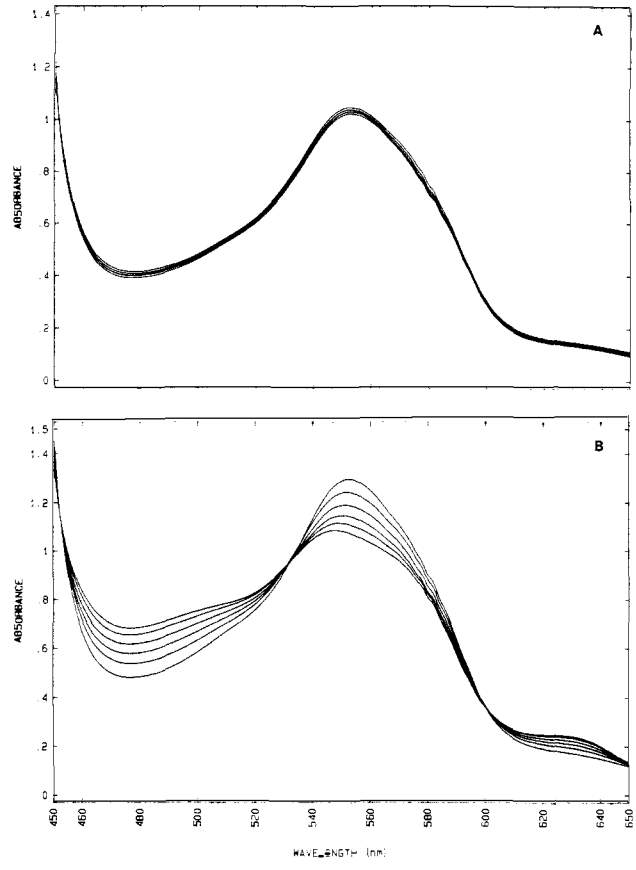

Figure 6. Spectral time courses for the reactions of deoxyhemoglobin with $HN_2O_3^-$ in deoxygenated 0.05 M phosphate buffer at pH 7.0 and 25.0 °C. (A) Absence of nitrite: [Hb] = 7.7×10^{-5} M, $[HN_2O_3^-] = 0.77 \times 10^{-5}$ M. (B) Presence of nitrite: [Hb] = 9.3×10^{-5} M, $[HN_2O_3^-] = 0.93 \times 10^{-5}$ M, $[NO_2^-] = 9.3 \times 10^{-5}$ M. Spectral scans were repeated at 10-min intervals.

confirmed, suggest that nitroxyl causes reductive nitrosylation of Mb^+ in competition with N_2O formation (Scheme III). The more complex behavior of Hb^+ has been attributed to a cooperative reactivity effect due to the tetrameric nature of Hb^+.[16]

Scheme III

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

$$HNO + Mb^+ \rightarrow MbNO + H^+ \quad (9)$$

$$2HNO \rightarrow N_2O + H_2O \quad (10)$$

Conversion of the β-93 sulfhydryl group of Hb^+ to its thioacetamide derivative completely eliminates the initial slow phase for reductive nitrosylation (Figure 3B). Furthermore, addition of cysteine increases the time span of the slow phase (Figure 3C) relative to the control (Figure 3A). Thus, the slow initial phase for the rate of conversion of Hb^+ to HbNO appears to be due to a competing reaction between the protein-bound sulfhydryl group and HNO rather than to a cooperative reactivity effect. However, the free β-93 sulfhydryl group of hemoglobin is unchanged after reaction with $HN_2O_3^-$, suggesting that either this functionality is unreactive toward HNO, and that thioacetamide formation alters allosteric interactions in the protein to eliminate the slow phase of the reaction time course (curve A → curve B in Figure 3), or this sulfhydryl group undergoes reversible addition to HNO (vide infra), which would have the same kinetic influence and also maintain complete nitrogenous product accountability.

The accessible cysteine sulfhydryl group of hemoglobin, which is not present in myoglobin, alters kinetic patterns in a variety of redox transformations.[20,27,32,33] In these studies, conversion

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of the hemoglobin β-93 sulfhydryl group to a derivative sulfide removes kinetic ambiguity to produce kinetic behavior that is identical with that observed with myoglobin, and we have demonstrated that by decreasing the reactivity of the hemoprotein relative to that of the sulfhydryl group, deviation from expected kinetic behavior is enhanced.[32] These investigations and their conclusions were based on the assumption that alteration of the β-93 sulfhydryl group by reaction with iodoacetamide did not have secondary structural effects that modified allosteric interactions, and results from experiments designed to identify conformational influences on these processes suggest the validity of this assumption.[27,33,34]

Oxidation of Sulfhydryl Compounds. Thiophenol was oxidized to phenyl disulfide by trioxodinitrate, and both nitrite and hydroxylamine were characterized products. The stoichiometry of this reaction (eq 11) was established by product determinations

$$2C_6H_5SH + HN_2O_3^- \rightarrow C_6H_5SSC_6H_5 + NO_2^- + H_2NOH \quad (11)$$

from reactions between thiophenol and $HN_2O_3^-$ in 40% aqueous acetonitrile at pH 7.8 using reactant ratios ranging from 1.0:0.25 to 1.0:1.0. Phenyl disulfide was isolated as a white crystalline solid in 98% yield from reactions between equimolar quantities of reactants. At lower reactant ratios, the yields of phenyl disulfide were 90% (1.0:0.50), 68% (1.0:0.375), and 46% (1.0:0.25).[35] Nitrous oxide was not observed in the stoichiometric reaction, and only minor amounts could be detected from reactions performed between equimolar amounts of reactants. An 88% yield of nitrite and 68% yield of hydroxylamine (mol/mol of $HN_2O_3^-$) were detected when stoichiometric quantities of reactants were employed.

The decomposition of $HN_2O_3^-$ in 40% aqueous acetonitrile at pH 7.8 and 25.0 °C has a first-order rate constant of 2.0×10^{-3} s^{-1}. In the presence of thiophenol under the same reaction conditions, the decomposition of trioxodinitrite remains first order in $[HN_2O_3^-]$ and is zero order in $[C_6H_5SH]$. Reaction rates were followed at 237 nm ($-d[HN_2O_3^-]/dt$) and at 260 nm ($-d[C_6H_5SH]/dt$) to give calculated rate constants that were identical, within experimental error, with that for trioxodinitrate decomposition in the absence of thiophenol.

The composite data suggest a reaction mechanism (Scheme IV) that involves nitroxyl formation and its subsequent reduction by thiophenol. The reductive transformation depicted in eq 12 and 13 has ample precedent in the formation of disulfides from azodicarboxylates and thiophthalimides,[36,37] but this is the first example of the reduction of nitroxyl to hydroxylamine. An alternative mechanism through which $HN_2O_3^-$ undergoes decomposition by nitric oxide dissociation (eq 1) is inconsistent with the formation of hydroxylamine.[38]

Scheme IV

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

$$HNO + C_6H_5SH \rightarrow [C_6H_5SNHOH] \quad (12)$$

$$C_6H_5SH + [C_6H_5SNHOH] \rightarrow C_6H_5SSC_6H_5 + H_2NOH \quad (13)$$

The detection of hydroxylamine in less than quantitative yield, based on the stoichiometry of eq 11, is probably due to the competitive reaction of nitroxyl with hydroxylamine (eq 14) whose

$$HNO + H_2NOH \rightarrow N_2 + 2H_2O \quad (14)$$

characteristics have been well established through investigations by Bonner and co-workers.[3,39] Furthermore, nitrite reacts with hydroxylamine under these conditions,[3] albeit to a limited extent (<10%), so that the sum of these competing reactions adequately accounts for the difference in yield of NO_2^- and H_2NOH from a quantitative measure according to eq 11.

Reduction of Ferricytochrome *c*. Reductive nitrosylation of Hb^+ or Mb^+ can be described by either inner-sphere or outer-sphere electron-transfer mechanisms. In the inner-sphere pathway, nitroxyl coordinates with iron(III) to form the nitrosylhemoprotein directly. However, if electron transfer is an outer-sphere process, the ferrohemoprotein and nitric oxide are first formed and then combine to produce the observed product. We have employed the unique nitrosyl binding characteristics of cytochrome *c* to distinguish between these two pathways. The nitrosyl adduct of ferricytochrome *c* is readily formed from nitric oxide at pH 7.0.[40] In contrast, although stable at pH 7, nitrosylferrocytochrome *c* cannot be formed by the direct action of nitric oxide at that pH.[40,41] Consequently, ferrocytochrome *c*, but not its nitrosyl adduct, should be produced from the reaction between ferricytochrome *c* and $HN_2O_3^-$ if outer-sphere electron transfer has occurred, but either no reaction or the nitrosyl adduct of ferrocytochrome *c* is predicted if reduction occurs though an inner-sphere electron-transfer mechanism.

Treatment of ferricytochrome *c* (Cyt c^+) with excess $HN_2O_3^-$ in 0.05 M phosphate buffer at pH 7.0 and 25 °C produces only ferrocytochrome *c* (Cyt *c*). Like reactions of $HN_2O_3^-$ with metmyoglobin,[16] the initial rate of formation of Cyt *c* is nearly zero order in [Cyt c^+] but is first order in $[HN_2O_3^-]$. Furthermore, at low $[HN_2O_3^-]_0/[\text{Cyt } c^+]_0$, a plot of $[\text{Cyt } c]_f/[\text{Cyt } c^+]_0$ versus $[HN_2O_3^-]_0/[\text{Cyt } c^+]_0$ is linear (Figure 4) with a slope of 0.5. The $[\text{Cyt } c]_f/[\text{Cyt } c^+]_0$ ratios were determined from individual reactions of Cyt c^+ with $HN_2O_3^-$ rather than by titration of Cyt c^+ with $HN_2O_3^-$. Calculations of concentrations were obtained from absorbance measurements at 550 and 520 nm. Ferrocytochrome *c* is unreactive toward $HN_2O_3^-$ over a wide range of concentrations.

Although not as efficient as Mb^+ or Hb^+ in trapping HNO, ferricytochrome *c* reactivity parallels that of these methemoproteins. The absence of the nitrosyl adduct of ferricytochrome *c* suggests that the reactions of NO^- with these methemoproteins are outer-sphere electron-transfer processes (eq 15) rather than inner-sphere reactions, as has been previously suggested.[16]

$$\text{Cyt } c^+ + NO^- \rightarrow \text{Cyt } c + NO \quad (15)$$

Mechanism of Reactions with Deoxyhemoglobin. Trioxodinitrate decomposition unmistakably occurs by formation of nitroxyl and nitrite. Nitroxyl is responsible for the reduction of ferrihemoproteins and of dioxygen bound to hemoglobin and myoglobin, but this reaction intermediate also causes the oxidation of thiophenol. Only in its reactions with deoxyhemoglobin is a clear explanation for the role of HNO lacking.

A series of reactions between Hb and $HN_2O_3^-$ at constant [Hb] but increasing $[NH_2O_3^-]_0/[Hb]_0$ has been investigated. The time courses for these transformations exhibit an initial increase in $[Hb^+]$ and [HbNO] to a limiting value, and only then is there a decrease in $[Hb^+]$ with concurrent formation of HbNO. The limiting value for $[Hb^+]/[HbNO]$ is 1.0 at $[HN_2O_3^-]_0/[Hb]_0$ equal to 0.5, and this intermediate product ratio does not change significantly as the reactant ratio increases, even though HbNO becomes the sole final hemoprotein product. Figure 5 is typical and, like that reported by Hollocher and co-workers, describes a two-phase reaction in which Hb is converted to Hb^+ and HbNO, and then Hb^+ is transformed to HbNO.

(33) Mansouri, A. *Biochem. Biophys. Res. Commun.* **1979**, *89*, 441.
(34) Doyle, M. P.; LePoire, D. M.; Pickering, R. A. *J. Biol. Chem.* **1981**, *256*, 12399.
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(37) (a) Boustany, K. S.; Sullivan, A. B. *Tetrahedron Lett.* **1970**, 3547. (b) Harpp, D. N.; Ash, D. K.; Back, T. G.; Gleason, J. G.; Orwig, B.; VanHorn, W. F.; Snyder, J. P. *Ibid.* **1970**, 3551.
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Hollocher and co-workers have presented a series of one- and two-electron redox schemes to account for results obtained with deoxyhemoglobin.[12] They suggest that eq 16 (**1*** is an activated

$$Hb + \mathbf{1}^* + H^+ \rightarrow Hb^+ + HNO + NO + OH^- \quad (16)$$

form of $HN_2O_3^-$ or the primary breakdown products) causes the formation of methemoglobin, and either the combination of reductive nitrosylation of Hb^+ and HbNO formation (eq 17 and 18) or HbNO formation and dimerization/dehydration of nitroxyl

$$Hb^+ + HNO \rightarrow HbNO + H^+ \quad (17)$$

$$Hb + NO \rightarrow HbNO \quad (18)$$

in nitrous oxide production (eq 10), which vary in importance depending on the [Hb], are the principal reactions that govern product formation. The nitrite ion is not a product in any of the simplest representations for reduction of **1*** by Hb that were proposed by Hollocher and co-workers, although their discussion of results allowed for the inclusion of nitrite. According to the simplest representations, in reactions with stoichiometric quantities of reactants disporportionation of HNO (eq 10) competes effectively with eq 17 at hemoglobin concentrations of 50–100 μM so that Hb^+ and HbNO are produced in nearly equal amounts. At higher [Hb], eq 17 is dominant, and the hemoprotein reaction product is almost entirely HbNO.

Our concern with these interpretations is not their conclusion regarding reaction stoichiometry but the mechanistic details for the transformation. Since nitrous acid alone is incapable of this oxidation at a rate that is faster than the rate for decomposition of $HN_2O_3^-$,[1,24] and HNO cannot oxidize Hb by itself, either isomerization of $HN_2O_3^-$ to a reactive tautomeric form or the action of HNO in combination with HONO may be the cause of hemoglobin oxidation and subsequent HbNO formation. Schemes V provides a mechanistic rationale for the latter process.

Scheme V

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

$$Hb + HNO \rightarrow Hb(HNO) \quad (19)$$

$$NO_2^- + H^+ + Hb(HNO) \rightarrow Hb^+ + NO + HNO + OH^- \quad (20)$$

$$Hb + NO \rightarrow HbNO \quad (21)$$

$$Hb^+ + HNO \rightarrow HbNO + H^+ \quad (17)$$

Reversible formation of a complex between Hb and HNO, Hb(HNO), is consistent with spectral observation of the coordination of nitrosobenzene to hemoglobin[42] and with kinetic evidence for alkyl nitrite association with this ferrohemoprotein.[32] Although direct oxidation of Hb by HONO is much slower than the rate for hemoglobin oxidation by $HN_2O_3^-$,[24] HNO-mediated oxidation of Hb by HONO is postulated to occur according to Scheme V, and the outcome of this reaction is the production of either NO^- + NO (eq 20) or $N_2O_2^-$. Nitrosyl association with Hb (eq 21) completes the initial reaction sequence whose sum (eq 22: composite of eq 1, 19–21, and 10) is the same net reaction

$$2Hb + HN_2O_3^- + H^+ \rightarrow Hb^+ + HbNO + {}^1/_2N_2O + {}^1/_2H_2O + OH^- \quad (22)$$

that Hollocher reported to explain data obtained with 50–100 μM Hb.[12] By including eq 17, the sum (eq 23: composite of eq 2, 17, 19–21) becomes the same net reaction that Hollocher and co-workers employed to explain data obtained with 1 mM Hb. According to Scheme V, HNO is inhibited from reacting with Hb^+ or with another molecule of nitroxyl until after Hb has been oxidized or converted to HbNO, and this prediction is consistent

$$2Hb + HN_2O_3^- \rightarrow 2HbNO + OH^- \quad (23)$$

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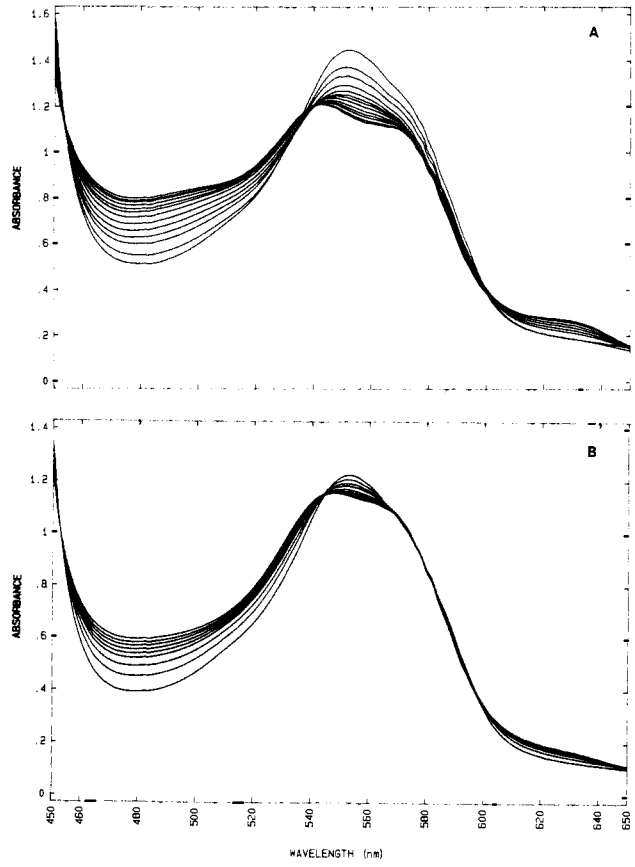

Figure 7. Spectral time courses for the pH-dependent reactions of deoxyhemoglobin with $HN_2O_3^-$ in deoxygenated 0.05 M phosphate buffer at 25.0 °C. (A) pH = 7.0: [Hb] = 10.2×10^{-5} M, $[HN_2O_3^-] = 5.1 \times 10^{-5}$ M; spectral scans were repeated at 4-min intervals. (B) pH = 8.0: [Hb] = 8.8×10^{-5} M, $[HN_2O_3^-] = 4.4 \times 10^{-5}$ M; spectral scans were repeated at 8-min intervals.

with initial methemoglobin formation even when a 10-fold stoichiometric excess of $HN_2O_3^-$ is used (Figure 5).

The sum of eq 19–21 describes a net HNO-catalyzed nitrite ion oxidation of hemoglobin (eq 24) that is amenable to direct evaluation. Figure 6 describes representative time courses for

$$H^+ + NO_2^- + 2Hb \xrightarrow{HNO} Hb^+ + HbNO + OH^- \quad (24)$$

reactions of hemoglobin with limiting amounts of $HN_2O_3^-$ in the absence (Figure 6A) and presence (Figure 6B) of added nitrite. With only 0.1 mol equiv of $HN_2O_3^-$, the conversion of hemoglobin to products is slow and eventually accounts for the transformation of less than 20% of the reactant hemoglobin. In contrast, when this same reaction is performed on the same time scale and under the same reaction conditions, except that 1.0 mol equiv of nitrite ion is employed with 0.1 mol equiv of trioxodinitrate, there is a dramatic increase in the rate for the conversion of Hb to Hb^+ and HbNO. A comparable rate acceleration by nitrite is observed when these reactions are performed with 0.5 mol equiv of trioxodinitrite, but in this case, the time course is complicated by the secondary conversion of Hb^+ to HbNO. Control experiments established that oxidation of Hb by nitrite accounted for less than 30% of the net change observed in Figure 6B, and furthermore, the isosbestic point at 532 nm in Figure 6B is observed at 526 nm in nitrite ion oxidations.[24] Thus nitrite, and not nitroxyl or a tautomer of the nitrogen-protonated monoanion of trioxodinitrate, is the active oxidant of hemoglobin, and nitroxyl is the catalyst for this transformation.

The rate for decomposition of $HN_2O_3^-$ is independent of pH in the range from 4.0 to 8.6,[2,7] but the nitrite ion oxidation of hemoglobin exhibits first-order dependence on the hydrogen ion concentration in this pH range.[24] Consequently, if the nitrite ion is responsible for oxidation, as outlined in eq 20 of Scheme V, we would anticipate that the reaction of Hb with $HN_2O_3^-$ should

also be pH dependent. Figure 7 demonstrates the validity of this interpretation. At pH 7.0 (Figure 7A), Hb is converted to Hb^+ and HbNO and, subsequently, Hb^+ is reduced to HbNO. In the initial stages of this transformation, the characteristic isosbestic point for the conversion of Hb to Hb^+ and HbNO is evident at 536 nm, but as the reaction continues and Hb^+ is reduced to HbNO, a new isosbestic point is observed at 540 nm. The comparable reaction at pH 8.0 (Figure 7B) is distinctly different. The kinetic order with respect to [Hb] and $[HN_2O_3^-]$ at pH 8.0 is the same as that determined at pH 7.0.[1,12] However, the rate of reaction is approximately 10 times slower than the corresponding reaction at pH 7.0, an isosbestic point is observed at 544 nm, and the spectral display of Figure 7B does not match that of Figure 6A at any time during the reaction. Part of this spectral difference may be due to a complex between Hb and nitroxyl, but we do not have independent evidence for this association.

Acknowledgment. This research was funded by grants from the U.S. Public Health Services (ES 03609 and GM 37469). We are grateful to the National Science Foundation for funding provided for the purchase of the GC/mass spectrometer employed for product determinations.

Communications to the Editor

Free Energy Dependence of the Rate of Long-Range Electron Transfer in Proteins. Reorganization Energy in Ruthenium-Modified Myoglobin

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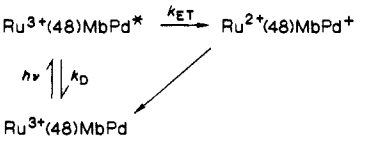
Received June 18, 1987

Long-range electron-transfer (ET) reactions in biological systems are under intense study.[1-8] One way to probe the factors that influence ET is through studies on two-site fixed-distance donor-acceptor systems consisting of a redox-active metal complex attached to the surface of a structurally characterized metalloprotein.[5-8] By using this approach we have demonstrated[6a] that the rate of long-range ET in sperm whale myoglobin (Mb) modified at histidine-48 with pentaammineruthenium (a_5Ru) and *trans*-tetraamminepyridineruthenium ($a_4$pyRu) depends on reaction free energy as predicted by Marcus theory.[2] These low driving force results (ΔG° = 0.020-0.275 V) indicated that the reorganization energy (λ) for ET might be 2 eV or even higher. We now report an investigation covering a wide range of reaction free energies that places the reorganization energy for ruthenium-modified myoglobin between 1.90 and 2.45 eV.

Our strategy involves replacing the heme in a_5Ru(48)Mb and $a_4$pyRu(48)Mb with a photoactive porphyrin that possesses a highly reducing excited state. With this approach we significantly increase the overall ET driving force while maintaining the same well-defined ΔG° step between the a_5Ru and $a_4$pyRu derivatives (0.26 V) as in the heme systems.[6a] The photoactive porphyrin is Pd(mesoporphyrin IX) (PdP), which has an excited-state reduction potential of -0.64 (10) V vs NHE.[9] PdP was inserted into native and ruthenium-modified Mb via reaction with apoprotein (12 h, 5% DMSO/phosphate, pH 7). The native and ruthenium-labeled palladium Mbs (MbPd and Ru(48)MbPd) were purified by gel-filtration chromatography and ultrafiltration.

The MbPd system is particularly attractive because the ET rates can be determined directly by monitoring the quenching of the PdP emission. Electronically excited MbPd (MbPd*) was produced via pulsed laser excitation (10 ns pulse, 532 nm), and the emission intensity was monitored at 670 nm. Kinetic analysis yields an observed first-order rate constant for the decay of the MbPd* emission intensity: $k_{obsd} = k_D + k_{ET}$.[10,11]



The decay in emission intensity for native MbPd closely follows first-order kinetics with a k_D of 1.0 (5) × 10^3 s^{-1}. Both a_5Ru^{3+}(48)- and a_4pyRu^{3+}(48)-modified MbPd exhibit enhanced emission quenching as expected for ET from the PdP excited state; the Ru^{3+}(48)MbPd* → Ru^{2+}(48)$MbPd^+$ driving forces are 0.72 (10) and 0.98 (10) V, respectively. The quenching of a_5Ru(48)MbPd* closely follows first-order kinetics with an ET rate of 9.1 (5) × 10^3 s^{-1} (Figure 1a). The kinetics for $a_4$pyRu(48)MbPd* are biphasic; an ET rate of 9.0 (5) × 10^4 s^{-1} was determined for the major (fast) component (Figure 1b). The minor (slow) component is probably due to residual native MbPd.

The ET results for Ru(48)MbM (M = Fe,[6a] Zn,[12] Pd) are shown in Figure 2.[13] The upper and lower curves represent the

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