THE JOURNAL OF BIOLOGICAL CHEMISTRY

Vol. 259, No. 20, Issue of October 25, pp. 12334-12338, 1984
Printed in U.S.A.

Oxidation of Deoxyhemerythrin to Semi-methemerythrin by Nitrite*

(Received for publication, January 26, 1984)

Judith M. Nocek‡§, Donald M. Kurtz, Jr.‡§, Ruth A. Pickering¶‖, and Michael P. Doyle¶‖

From the ‡Department of Chemistry, Iowa State University, Ames, Iowa 50011 and the ¶Department of Chemistry, Hope College, Holland, Michigan 49423

In anaerobic phosphate buffer, pH 6.3–7.5, deoxyhemerythrin is oxidized to semi-methemerythrin (semi-met) by excess sodium nitrite. This oxidation is quantitative as judged by EPR spectroscopy. Further oxidation to methemerythrin is not detected. The absorbance changes of hemerythrin during the oxidation are biphasic. The rate of the faster first phase is linearly dependent on $[H^+]$ and $[NO_2^-]$ suggesting that the oxidant is nitrous acid rather than nitrite. During the slower second phase, the characteristic EPR spectrum of semi-methemerythrin appears. The first phase can be interpreted by a scheme in which nitrous acid transforms deoxyhemerythrin ($Fe^{II}Fe^{II}$) to the semi-met nitrosyl adduct ($Fe^{II}Fe^{III}NO$) and hydroxide. Independent experiments confirm that the combination of semi-met plus NO produces an EPR-silent adduct. The rates of the absorbance changes for the second phase are nearly independent of nitrite concentration and pH in the range 6.3–7.5. This slower phase involves the transformation of the EPR-silent intermediate to the semi-met nitrite adduct ($Fe^{II}Fe^{III}NO_2^-$) and is consistent with rate-limiting dissociation of nitric oxide followed by rapid attachment of nitrite.

Nitrite appears to be a unique oxidant of deoxyhemerythrin in that when employed in excess, the final, stable product is semi-met- rather than methemerythrin. The lack of reactivity of ethyl nitrite with deoxyhemerythrin suggests that HONO oxidizes deoxyhemerythrin via an "inner-sphere" process in contrast to oxidants such as $Fe(CN)_6^{3-}$. A proposed generalization is that excesses of "inner-sphere" oxidants convert deoxy to $(semi\text{-}met)_R$, which is stabilized with respect to $(semi\text{-}met)_0$ and met because the oxidant and/or a product of the oxidant can bind to the iron site.

Hemerythrin is a non-heme oxygen-carrying protein found in several phyla of marine invertebrates. The protein most often consists of an octamer of M_r = 108,000. Each of the eight identical subunits contains two iron atoms at a binuclear site which reversibly binds one molecule of oxygen (1–3). A great deal of chemical and spectroscopic information has established that upon oxygenation the two Fe^{II} ions in deoxyHr[1] are formally oxidized to Fe^{III} in oxyHr with the bound dioxygen formally reduced to the peroxide oxidation state. MetHr is an oxidized form containing two Fe^{III} ions which does not reversibly bind oxygen but which does bind several other small univalent anions with a 1 anion/2Fe stoichiometry. The met form can be converted back to the oxygen binding form with reducing agents. A mixed $Fe^{II}Fe^{III}$ semi-met oxidation level of Hr can be attained either by reduction of the met form to give so called $(semi\text{-}met)_R$ or by oxidation of the deoxy form with $Fe(CN)_6^{3-}$ or $Co(terpy)_2^{3+}$ to give $(semi\text{-}met)_0$ (3–5). EPR and Mössbauer spectra of Hr indicate that the high spin iron atoms are antiferromagnetically coupled to give an $S = 0$ ground state in oxy- and metHrs (1) and an $S = \frac{1}{2}$ ground state in semi-metHr (6, 7).

Prior to the discovery of semi-metHr, Bradic *et al.* (8) investigated the autooxidation of oxy- to metHr in the presence of anions, nitrite among them. However, either nitrite or nitrous acid is known to function as a redox agent in many reactions (9). Also, unlike most other oxidants whose reactions with Hr have been studied (3), nitrite can potentially function in an "inner sphere" mode by direct binding to iron. Oxidation of the vertebrate oxygen carrier, hemoglobin, by nitrite is well known. Most recently, Doyle *et al.* (10) have found that anaerobic reaction of excess $NaNO_2$ with deoxyhemoglobin yields methemoglobin and nitrosylhemoglobin in a 72:28 mole ratio. For these reasons it seemed worthwhile to investigate the reaction of $NaNO_2$ with deoxyHr.

MATERIALS AND METHODS

OxyHr was isolated and crystallized from the coelomic fluid obtained from live worms of the species *Phascolopsis gouldii* purchased from Marine Biological Laboratories, Woods Hole, MA (11). Solutions of deoxyHr were prepared by overnight dialysis of 1–2 mM oxyHr against ~15 mM $Na_2S_2O_4$ (BDH Laboratories) in deaerated 50 mM sodium phosphate buffer, pH 6–8. Dithionite was then removed by anaerobic dialysis against several changes of buffer. OxyHr was prepared by exposure of deoxyHr to air. MetHr was prepared by $Fe(CN)_6^{3-}$ oxidation of oxyHr (12). Protein concentrations expressed as monomer were determined from visible absorption spectra of freshly prepared oxygenated samples using $\epsilon_{500} = 2200\ M^{-1}\ cm^{-1}$ (12). Reagent grade $NaNO_2$ and recrystallized NaN_3 were added from concentrated stock solutions. Ethyl nitrite was prepared and used as previously described (10). Gaseous NO was passed through a concentrated solution of NaOH before use. All reactions and manipulations were carried out anaerobically under N_2 or Ar transferring solutions or gases via gas-tight syringes. Nitrate levels in the reaction solutions were determined aerobically by high-performance liquid chromatography using a Licrosorb NH_2-reverse phase column.

Kinetic Measurements—Reactions were initiated by injection of concentrated solutions of $NaNO_2$ into 40–120 μM solutions of deoxyHr in sodium phosphate buffer. Rates of oxidation of deoxyHr were determined at 25.0 °C by monitoring the increase in absorbance with time at 380 nm using either a Pye Unicam SP8-200 or Perkin-Elmer 554 recording spectrophotometer. Reactions were carried out under psuedo-first-order conditions with a 10–100-fold molar excess of nitrite over Hr. The resultant time courses were fitted by computer to two exponential processes using the iterative least squares algorithm, NLLSQ. For most fits A_∞ was held fixed at the experimental value while two first-order rate constants and their pre-exponential

* The costs of publication of this article were defrayed in part by the payment of page charges. This article must therefore be hereby marked "*advertisement*" in accordance with 18 U.S.C. Section 1734 solely to indicate this fact.

§ Supported by National Science Foundation Grant PCM-8216447 and United States Public Health Service Grant GM 33157.

‖ Supported by United States Public Health Service Grants ES 01673 and RR07212.

[1] The abbreviations used are: Hr, hemerythrin; met, methemerythrin.

factors were allowed to vary. In a few cases, such as that depicted in Fig. 2, A_∞ was allowed to vary as well. The rate constants reported are the average of 2–6 replicate determinations.

EPR Measurements—All reactions were carried out under Ar in septum-capped vials. Reactions between deoxyHr and nitrite were initiated by injecting a 15–25-fold molar excess of $NaNO_2$ into solutions of 0.75–1.5 mM deoxyHr in phosphate buffer at either pH 6.5 or 7.5. At various times, 0.1-ml aliquots of the reaction solutions were transferred to 4-mm (outer diameter) quartz tubes equipped with rubber septa. Starting at a reaction time of ~2 min, the aliquots were frozen in liquid nitrogen and the tubes were then evacuated and flame-sealed. Samples from other reactions were prepared similarly and details are given in the figure legends. X band EPR spectra were obtained at 4 K on a Bruker ER220D spectrometer equipped with an Oxford ESR 10 helium flow system. Copper sulfate was used as the concentration standard for areas obtained by double integration (13).

RESULTS

Fig. 1 shows a series of visible absorption spectra resulting from addition of $NaNO_2$ to deoxyHr at pH 6.5. Regardless of whether or not the absorbance of $NaNO_2$ (λ_{max} ~ 355 nm) is subtracted, the spectral changes are obviously biphasic. The spectra at all stages are different from those of either deoxy- or metHrs. In particular, the final spectrum is different from that of the met nitrite adduct having a prominent shoulder at ~500 nm compared to a weaker feature at 480 nm and shoulders rather than peaks between 300 and 400 nm (8). Fig. 2 is a plot of the absorbance at 380 nm *versus* time for the reaction at pH 7.05. Excellent agreement is obtained between the observed absorbances and those calculated assuming a biphasic reaction and first-order behavior for both phases. Tables I and II show the concentration dependences of k_{obs} and k'_{obs} for the faster first and slower second phases, respectively. The data in Table I establish the first-order dependence on nitrite concentration for the first phase and also indicate that $k_{obs}/[NO_2^-]$ is pH-dependent. The plot in Fig. 3 demonstrates that this dependence is linear with a unit slope. In contrast to the first phase, k'_{obs} for the slower second phase is nearly independent of pH and nitrite ion concentration, at least in the range of 6.3–7.5 and >25-fold molar excess of $NaNO_2$, respectively (Table II).

Fig. 4 demonstrates that the second phase of the absorbance changes is accompanied by the development of an EPR spectrum. Under the conditions used in Fig. 4, the half-life for the first phase is <10 s while that for the second phase is ~20 min. Except for very weak signals occasionally seen at g ~ 4.0, g ~ 2.7, and g ~ 1.97, the former two only at the earliest reaction times examined (2–4 min), the spectra in Fig. 4

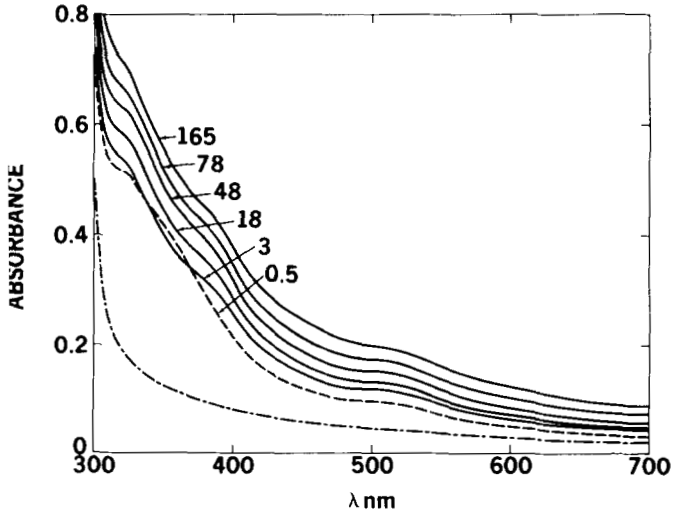

FIG. 1. **Spectral time course for the reaction of deoxyHr with excess $NaNO_2$.** Reaction is in 50 mM sodium phosphate, pH 6.5, 0.128 mM Hr, 2.65 mM $NaNO_2$, 1-cm path.—·—·—, deoxyHr; - - -, deoxyHr + $NaNO_2$, no $NaNO_2$ in reference cell; ——, deoxyHr + $NaNO_2$ with equimolar $NaNO_2$ in the reference cell. Numbers refer to time in minutes after addition of $NaNO_2$.

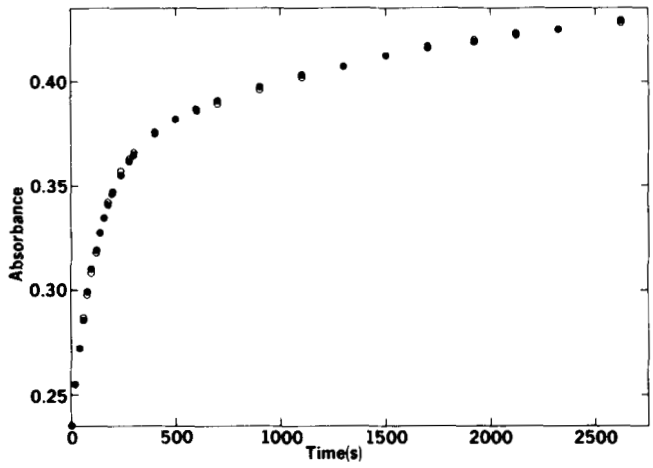

FIG. 2. **Time course of the absorbance changes at 380 nm for reaction of deoxyHr with excess $NaNO_2$.** Reaction is in 50 mM sodium phosphate, pH 7.05, 0.0682 mM Hr, 6.91 mM $NaNO_2$. T = 25.0 °C. *Open circles* are experimental values. *Filled circles* are calculated values from NLLSQ fit. Fitted values of the rate constants were: $k_{obs} = 7.79 \times 10^{-3}$ s^{-1}, $k'_{obs} = 4.99 \times 10^{-4}$ s^{-1}. Calculated A_∞ = 0.453; observed A_∞ = 0.446.

TABLE I

Rate dependence of deoxyHr oxidation on nitrite ion concentration and pH during initial fast phase

Deoxygenated phosphate buffer (0.050 M); 25.0 °C. Rate constants are average values (range ±6%) for two kinetic determinations from NLLSQ fits of the absorbance *versus* time curves.

[Hr] × 10^5 M	[NO_2^-] × 10^5 M	pH	10^3 k_{obs}	$k_{obs}/[NO_2^-]$
			s^{-1}	M^{-1} s^{-1}
6.50	168	6.26	15.40	9.16
7.82	383	6.58	14.4	3.76
7.95	195	6.83	3.88	1.99
6.76	68.2	7.05	0.830	1.22
6.82	173	7.05	2.32	1.34
6.82	346	7.05	4.27	1.23
6.82	691	7.05	7.62	1.10
7.50	697	7.48	2.82	0.405
7.27	725	7.74	1.30	0.180

TABLE II

Rate dependence of deoxyHr oxidation on nitrite ion concentration and pH during slow second phase

Deoxygenated phosphate buffer (0.050 M); 25.0 °C.

[Hr] × 10^5 M	[NO_2^-] × 10^5 M	pH	10^3 k'_{obs}
			s^{-1} [a]
6.50	168	6.26	0.338
7.82	383	6.58	0.532
7.95	195	6.83	0.420
4.91	491	7.00	0.534
6.82	691	7.05	0.449
6.82	346	7.05	0.434
6.82	173	7.05	0.388
6.76	68.2	7.05	0.104
6.97	697	7.48	0.302
7.27	725	7.74	0.180

[a] Average value (range ± 0.050) for 2–4 kinetic determinations from NLLSQ fits of the absorbance *versus* time curves.

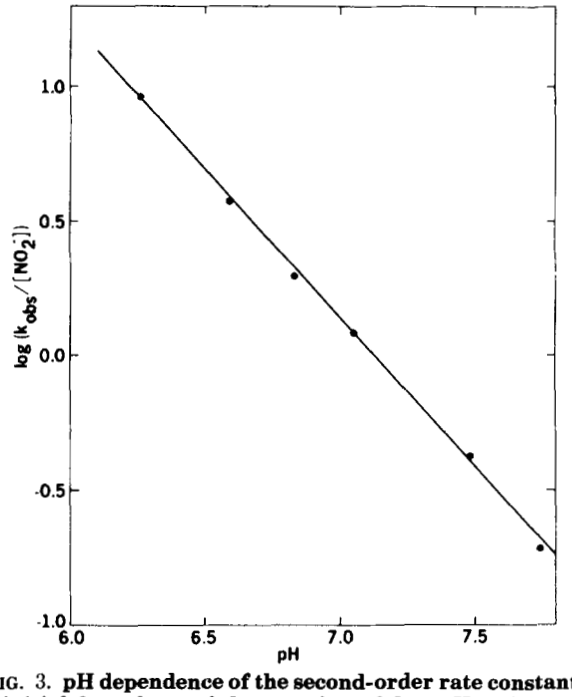

FIG. 3. **pH dependence of the second-order rate constant for the initial fast phase of the reaction of deoxyHr with excess $NaNO_2$.** Data were obtained in 50 mM sodium phosphate buffer at 25.0 °C. $[NaNO_2]/[Hr]$ = 10–100. The plotted values are each the average of from 2–6 kinetic determinations and have a maximum range of ± 6%. The slope of the line is −1.1.

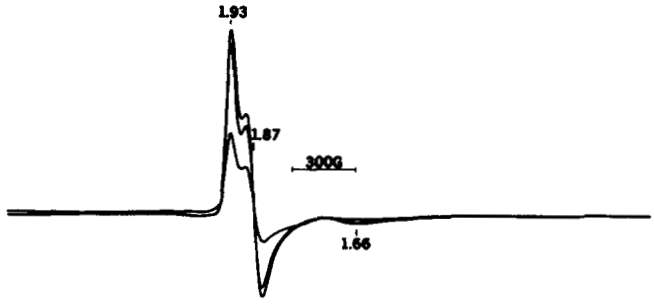

FIG. 4. **Time dependence of the EPR spectrum observed during reaction of deoxyHr with excess $NaNO_2$.** Reaction is in 50 mM sodium phosphate buffer, pH 6.5, 1.3 mM Hr, 24 mM $NaNO_2$. Samples were frozen at 7-, 44-, and 187-min reaction times in order of increasing intensity at g = 1.93. Spectral conditions: temperature, 4 K; frequency, 9.57 GHz; power, 100 microwatts; modulation, 16 G at 100 kHz; field center, 4000 G; field range, 3000 G; time constant, 0.1 s; receiver gain, 3.2 × 10^4. Double integration of the 187-min spectrum yields 1.2 spins/Hr.

represent the only type seen at either pH 6.5 or 7.5 with an excess of nitrite.[2] We observe a strong g = 1.97 signal characteristic of "matrix-bound" NO (14) when NO is added to the pH 6.5 reaction mixture, but the majority of this signal disappears within a few minutes after addition. We have never observed the g = 4.3 signal characteristic of adventitiously bound iron which could be attributed to damaged protein (15). The $(semi\text{-}met)_0$ EPR spectrum, characterized by nearly axial symmetry and g values of 1.94 and 1.71 (3), is produced by addition of stoichiometric amounts of $Fe(CN)_6^{3-}$ to deoxyHr (5). We have never observed this spectrum during nitrite oxidations of deoxyHr even when only one molar equivalent of nitrite is added. However, the EPR spectrum represented by those in Fig. 4 is clearly due to Hr at the semi-met oxidation level. Although the g values, 1.93, 1.87, and 1.66, are close to those reported for $(semi\text{-}met)_R$ in Tris buffers at pH 8 (5, 6), the following evidence indicates that our spectra represent the nitrite adduct of semi-metHr. The EPR spectrum of $(semi\text{-}met)_R$ produced by one electron reduction of metHr by $Na_2S_2O_4$ in pH 6.5 phosphate (Fig. 5*A*) gives g = 1.96, 1.86, and 1.66. The highest g value is clearly distinguishable from that shown in Fig. 4. A mixture of these two EPR spectra is observed when one molar equivalent of nitrite is added to deoxyHr. Also, EPR spectra obtained by addition of excess nitrite to either $(semi\text{-}met)_R$ or $(semi\text{-}met)_0$ are identical with those of Fig. 4. Both the differences from $(semi\text{-}met)_0$ and $(semi\text{-}met)_R$ and the identity when produced from either $(semi\text{-}met)_0$ or $(semi\text{-}met)_R$ are characteristics of the azide adduct of semi-metHr. By analogy, we conclude that the nitrite adduct is the product of the reaction of deoxyHr with excess $NaNO_2$. Double integration of EPR spectra obtained from four separate samples, two at pH 6.5 and two at pH 7.5, at reaction times between 2 and 24 h gave 1.1 ± 0.1 spin/Hr, *i.e.* conversion to the semi-met oxidation level is quantitative.

Fig. 5 demonstrates that the absence of an EPR signal during the first phase is consistent with formation of a species identical with the NO adduct of semi-metHr. An ~5-fold molar excess of gaseous NO added anaerobically to $(semi\text{-}met)_R$ at room temperature causes disappearance of 84% of the EPR intensity within 6 min (Figs. 5, *A* and *B*). This disappearance is accompanied by a change in the absorption spectrum of the solution to one resembling that obtained at 3 min during the reaction between deoxyHr and excess $NaNO_2$ (Fig. 1), *i.e.* semi-met is not reduced to deoxy by NO.[2] The same excess of gaseous NO added to metHr results in no change in the absorption spectrum. That the EPR-silent species of Fig. 5*B* is at the semi-met rather than met oxidation

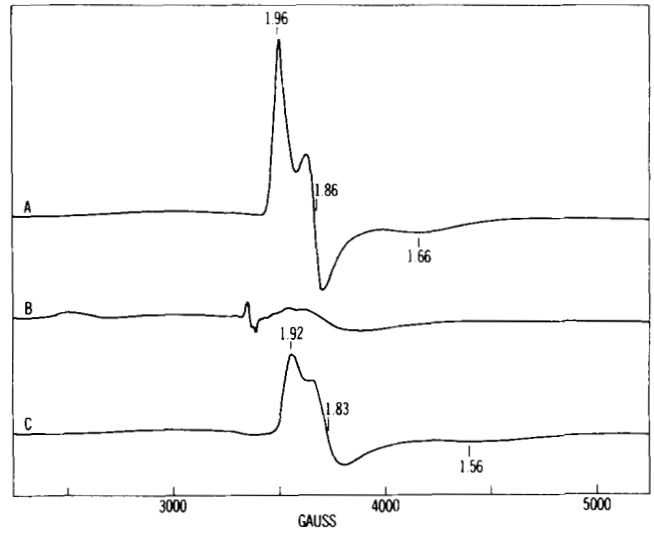

FIG. 5. **EPR silence of the NO adduct of semi-metHr.** EPR spectra are of 0.1-ml aliquots of anaerobic reaction solutions in 50 mM phosphate, pH 6.5, after incubation at room temperature. Spectral conditions are the same as those given in the legend to Fig. 4 except field center, 3750 G; time constant, 0.2 s; receiver gain, 4 × 10^4. A sample of $(semi\text{-}met)_R$ was prepared by addition of one reducing equivalent of $Na_2S_2O_4$ to 1.44 mM metHr. Portions were then treated as indicated. Zero time is that of addition of $Na_2S_2O_4$. *A*, frozen at 5 min, 0.89 spin/Hr; *B*, gaseous NO (~5 mol/mol of Hr) added at 7 min and aliquot-frozen at 13 min, 0.14 spin/Hr; *C*, NaN_3 (22 mol/mol of Hr) added to the remainder of the NO containing portion at 20 min and aliquot-frozen at 5.5 h, $[Hr]_{total}$ = 1.27 mM, 0.86 spin/Hr.

[2] At 4 K, an axial EPR signal with $g_\parallel$ = 2.7 and $g_\perp$ = 1.84 appears upon reaction of NO with deoxyHr (J. M. Nocek and D. M. Kurtz, Jr., unpublished observations).

level can be further demonstrated by addition of excess azide. This anion is not known to engage in any redox chemistry with Hr, but does form stable adducts of both met- and semi-metHr, the latter adduct having $g = 1.91, 1.83$ (5, 6). Within 5.5 h of addition of excess azide to the EPR-silent species, 97% of the semi-met EPR signal is recovered as that of the azide adduct after correction for dilution (Fig. 5*C*). Development of this latter EPR spectrum is accompanied by formation of the characteristic absorption spectrum of semi-metazideHr (not shown) (3, 4).

When azide is added to a reaction mixture of deoxyHr and excess $NaNO_2$ such that $[N_3^-]/[NO_2^-] = 20$, both the absorption and EPR spectra indicate conversion to the semi-metHr azide adduct. As shown in Fig. 6, excess azide added during the first phase does not prevent further reaction as judged by continued increases in A_{470}, the λ_{max} of semi-metazideHr (3, 4). At no stage do the absorption spectra resemble that of metazideHr (12), whereas the final spectrum in Fig. 6, obtained at ~3.5 h reaction time, is in good agreement with that published for semimetazideHr (3). Using $\epsilon_{470} = 2400\ M^{-1}\ cm^{-1}$ (3), the final spectrum in Fig. 6 gives a calculated concentration of semimetazideHr of 0.0925 mM, which is 96% of the starting concentration of deoxyHr.

We observe no reaction of excess ethyl nitrite with deoxyHr ([EtONO]/[Hr] = 2.0 − 100) for about 1 h as judged by absorbance changes in pH 7.8 or 7.0 phosphate. Reaction after 1 h is attributable to nitrite produced by hydrolysis of ethyl nitrite (10, 16). We also observe no EPR signal when excess $NaNO_2$ is added to metHr.

The rates we measure for the decrease in A_{500} of oxyHr in the presence of excess $NaNO_2$ are in good agreement with the rates obtained by Bradić *et al.* for the autooxidation reaction (8). The product as judged spectrophotometrically and by lack of an EPR signal appears to be metHr. However, we do observe weak, transient semi-met EPR signals during this reaction in pH 6.5 phosphate. Addition of excess $NaNO_2$ to solutions of 1–2 mM oxyHr which have been degassed to remove excess dissolved O_2 produces substantial proportions

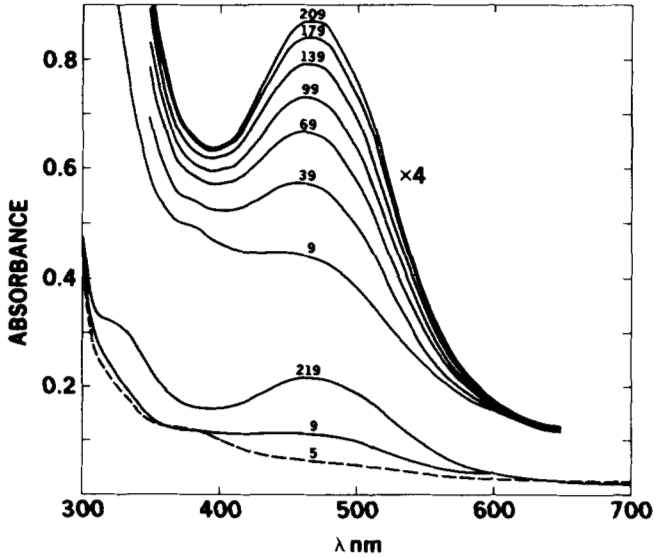

FIG. 6. **Spectral time course for the oxidation of deoxyHr by excess $NaNO_2$ in the presence of NaN_3.** Reaction is in 50 mM sodium phosphate, pH 7.5; 0.0968 mM Hr, 1.94 mM $NaNO_2$, 40 mM NaN_3, 1-cm path. An equimolar concentration of $NaNO_2$ was added to the reference cell.– – –, deoxyHr + $NaNO_2$; ——, preceding solution after addition of NaN_3. *Numbers* near the spectra refer to the time in minutes after addition of $NaNO_2$.

(>20%) of the semi-metHr nitrite adduct and the EPR signal persists for at least 24 h at 4 °C.

As expected from results with deoxyHr, ethyl nitrite caused no detectable change in the spectrum of oxyHr ([EtONO]/[Hr] = 50).

NO_3^- was detected in all reaction mixtures examined by high-performance liquid chromatography analysis. Although variable with pH it was usually found to be ~0.3% of the added $NaNO_2$. NO_3^- was not detected in anaerobic solutions of $NaNO_2$ in the absence of protein.

DISCUSSION

Our results are consistent with the interpretation that the fast first phase of the reaction of deoxyHr with excess $NaNO_2$ consists of conversion of deoxyHr to an EPR-silent state. Although deoxyHr itself is EPR-silent, the increases in absorbance suggest that during this phase at least one of the iron atoms in the binuclear site is oxidized. The linear decrease in rate with decreasing $[H^+]$ suggests that HNO_2 ($pK_a = 3.3$ at 25 °C (9)) is the reactant rather than NO_2^-. The first order dependence on $[NO_2^-]$ is consistent with a mechanism for the first phase similar to that proposed for nitrite oxidation of deoxyhemoglobin:

$$H^+ + NO_2^- \overset{K}{\leftrightarrows} HONO \qquad (1)$$

$$Fe^{II}Fe^{II} + HONO \xrightarrow{k} Fe^{II}Fe^{III}NO + OH^- \qquad (2)$$

The FeFe notation refers to the binuclear site of Hr in deoxy ($Fe^{II}Fe^{II}$) or semi-met ($Fe^{II}Fe^{III}$) oxidation levels. At pH 7.0, the second order rate constant for this first phase is 1.2 ± 0.1 $M^{-1}\ s^{-1}$, about half the value for the similar reaction of deoxyhemoglobin (10). Doyle *et al.* (17) have provided evidence that nitrites oxidize the ferrous iron in deoxyhemoglobin by an "inner-sphere" process. If a similar process occurs with deoxyHr, the lack of reactivity of ethyl nitrite with deoxyHr compared to its relatively facile reaction with deoxyhemoglobin can be explained by the much more severe steric restrictions at the ligand binding site in Hr. Evidence accumulated over several years has shown that the binding site in (at least) metHr is restricted to ligands consisting of three or fewer non-hydrogen atoms (1–3, 8, 12).

Binding rather than the release of NO upon oxidation as depicted in Equation 2 would lead to a mixture with no EPR active species consistent with the time course of development of the EPR signal (Fig. 4) and with the disappearance of the EPR signal when NO is added to semi-met followed by its recovery when azide is added (Fig. 5). Fe^{III} nitrosyls ($[FeNO]^6$ complexes in the notation of Enemark and Feltham (18)) are well known to be diamagnetic (*e.g.* the nitrosyl adducts of metmyoglobin and methemoglobin (19)) and high spin Fe(II) is EPR-silent due to large zero field splitting. The $Fe^{II}Fe^{III}NO$ notation is only a formalism, however. Spin-pairing of the $S = ½$ semi-met state with the unpaired electron on NO would lead to diamagnetism regardless of formal oxidation states of individual iron atoms.

Although metHr ($Fe^{III}Fe^{III}$) is an EPR-silent species and N_2O is known to be a product in oxidations of Fe(II) by $NaNO_2$ (20), we consider an alternative to Equation 2 for the first phase, namely reaction 3

$$2H^+ + Fe^{II}Fe^{II} + HONO \rightarrow Fe^{III}Fe^{III} + 1/2N_2O + 3/2H_2O \qquad (3)$$

less likely for the following reasons. First, since the ultimate Hr product is at the semi-met oxidation level, the second phase would have to involve rereduction to semi-met, a level which it presumably would have had to pass through in

reaction 3. Second, UV-Vis spectra at all stages of the reaction do not resemble those expected for a metHr. Third, based on rates of anion binding and displacement (3, 4, 21), added azide would be expected to trap metHr produced during the first phase as the very stable metazideHr; however none was detected (Fig. 6). Although one could argue that a different metastable form of metHr is produced, the simpler explanation is that deoxyHr never reaches the met oxidation level when exposed anaerobically to excess $NaNO_2$.

Assuming that the Hr product of Equation 2 is the reacting species, the first order kinetic dependence on Hr concentration and near independence of nitrite and hydrogen ion concentrations for the second phase is consistent with rate-limiting unimolecular dissociation of nitric oxide followed by association of semi-metHr with nitrite in a fast subsequent step

$$Fe^{II}Fe^{III}NO \xrightarrow{k'_{obs}} Fe^{II}Fe^{III} + NO \quad (4)$$

$$Fe^{II}Fe^{III} + NO_2^- \rightarrow Fe^{II}Fe^{III}NO_2^- \quad (5)$$

The average value of k'_{obs} over the pH range of 6.3–7.5 is 4.2 ($\pm$1.2) $\times 10^{-4}$ s^{-1}, which is 3–4 times faster than the rate of dissociation of nitric oxide from nitrosylmyoglobin (22). The deviation from this range at $[NaNO_2]/[Hr] \lesssim 25$ and at pH 7.7 could be explained by competing partial disproportionation of uncomplexed semi-met, which would lead to a decrease in A_{380} with time. We have observed decreases in intensities of both the (semi-met)$_0$ and (semi-met)$_R$ EPR signals in pH 6.5 phosphate on time scales similar to that of the second phase of the reaction between deoxyHr and $NaNO_2$.

Nitrite appears to be a unique oxidant of deoxyHr in that, when employed in excess, the final, stable product is semi-met- rather than metHr (3). This stabilization of semi-met indicates that the redox potentials under our conditions for the *P. gouldii* met/(semi-met)$_R$ and (semi-met)$_0$/deoxy couples are different from the previously measured values of 0.11 V and 0.30 V, respectively (23). A possible generalization is that excesses of "outer sphere" oxidants such as $Fe(CN)_6^{3-}$ or $Co(terpy)_2^{3+}$ convert deoxy to (semi-met)$_0$ which is further oxidized to met after conversion to (semi-met)$_R$ (3). On the other hand, excesses of "inner-sphere" oxidants convert deoxy to (semi-met)$_R$, which is stabilized with respect to (semi-met)$_0$ and met because the oxidant and/or a product of the oxidant can bind to the iron site. In this regard, oxidation of deoxyHr by H_2O_2 as well as autooxidation of oxyHr clearly warrant further examinations (8, 24). At least in the presence of nitrite, the latter process is more complicated than previously envisioned.

Acknowledgment—We thank Professor J. H. Espenson for the use of the computer program NLLSQ and for helpful discussions.

REFERENCES

1. Kurtz, D. M., Jr., Shriver, D. F., and Klotz, I. M. (1977) *Coord. Chem. Rev.* **45,** 145–178
2. Klotz, I. M., and Kurtz, D. M., Jr. (1984) *Accts. Chem. Res.* **17,** 16–22
3. Wilkins, R. G., and Harrington, P. C. (1983) *Adv. Inorg. Biochem.* **5,** 51–85
4. Harrington, P. C., deWaal, D. J. A., and Wilkins, R. G. (1978) *Arch. Biochem. Biophys.* **191,** 444–451
5. Babcock, L. M., Bradic, Z., Harrington, P. C., Wilkins, R. G., and Yoneda, G. S. (1980) *J. Am. Chem. Soc.* **102,** 2849–2850
6. Muhoberac, B. B., Wharton, D. C., Babcock, L. M., Harrington, P. C., and Wilkins, R. G. (1980) *Biochim. Biophys. Acta* **626,** 337–345
7. Kurtz, D. M., Jr., Sage, J. T., Hendrich, M., Debrunner, P. G., and Lukat, G. S. (1983) *J. Biol. Chem.* **258,** 2115–2117
8. Bradic, Z., Conrad, R., and Wilkins, R. G. (1977) *J. Biol. Chem.* **252,** 6069–6075
9. Stedman, G. (1979) *Adv. Inorg. Chem. Radiochem.* **22,** 113–170
10. Doyle, M. P., Pickering, R. A., DeWeert, T. M., Hoekstra, J. W., and Pater, D. (1981) *J. Biol. Chem.* **256,** 12393–12398
11. Klotz, I. M., Klotz, T. A., and Fiess, H. A. (1957) *Arch. Biochem. Biophys.* **68,** 284–299
12. Garbett, K., Darnall, D. W., Klotz, I. M., and Williams, R. J. P. (1969) *Arch. Biochem. Biophys.* **135,** 419–434
13. Aasa, R., and Vänngård, T. (1975) *J. Magnetic Res.* **19,** 308–315
14. Martin, C. T., Morse, R. H., Kanne, R. M., Gray, H. B., Malmstrom, B. G., and Chan, S. I. (1981) *Biochemistry* **20,** 5147–5155
15. Bayer, E., Krauss, P., Röder, A., and Schretzmann, P. (1973) in *Oxidases and Related Redox Systems* (King, T. E., Mason, H. S., and Morrison, M., eds) Vol. 1, pp. 227–263, University Park Press, Baltimore
16. Doyle, M. P., Terpstra, J. W., Pickering, R. A., and LePoire, D. M. (1983) *J. Org. Chem.* **48,** 3379–3382
17. Doyle, M. P., Pickering, R. A., and da Conceicão, J. (1984) *J. Biol. Chem.* **259,** 80–87
18. Enemark, J. H., and Feltham, R. D. (1974) *Coord. Chem. Rev.* **13,** 339–406
19. Sharma, V. S., Isaacson, R. A., John, M. E., Waterman, M. R., and Chevion, M. (1983) *Biochemistry* **22,** 3897–3902
20. Bonner, F. T., and Pearsall, K. A. (1982) *Inorg. Chem.* **21,** 1973–1978
21. Meloon, D. R., and Wilkins, R. G. (1976) *Biochemistry* **15,** 1284–1290
22. Moore, E. G., and Gibson, Q. H. (1976) *J. Biol. Chem.* **251,** 2788–2794
23. Harrington, P. C., and Wilkins, R. G. (1983) *J. Inorg. Biochem.* **19,** 339–344
24. Bradic, Z., Harrington, P. C., and Wilkins, R. G. (1979) in *Biochemical and Clinical Aspects of Oxygen* (Caughey, W. S., ed) pp. 459–474, Academic Press, New York