Nitrite reduction by Co\textsuperscript{II} and Mn\textsuperscript{II} substituted myoglobins
Towards understanding necessary components of Mb nitrite reductase activity

Julie L. Heinecke\textsuperscript{a}, Jun Yi\textsuperscript{b}, Jose Clayston Melo Pereira\textsuperscript{a}, George B. Richter-Addo\textsuperscript{b,*}, Peter C. Ford\textsuperscript{b,**}

\textsuperscript{a} Department of Chemistry and Biochemistry, University of California at Santa Barbara, Santa Barbara, CA 93106-9510, United States
\textsuperscript{b} Department of Chemistry and Biochemistry, University of Oklahoma, 101 Stephenson Parkway, Norman, OK 73019, United States

A B S T R A C T

Nitrite reduction to nitric oxide by heme proteins is drawing increasing attention as a protective mechanism to hypoxic injury in mammalian physiology. Here we probe the nitrite reductase (NiR) activities of manganese(II)- and cobalt(II)-substituted myoglobins, and compare with data obtained previously for the iron(II)-analog wt Mb\textsuperscript{II}. Both Mn\textsuperscript{III}Mb and Co\textsuperscript{III}Mb displayed NiR activity, and it was shown that the kinetics are first order each in [protein], [nitrite], and [H\textsuperscript{+}], as previously determined for the Fe\textsuperscript{II} analog wt Mb\textsuperscript{II}. The second order rate constants (k\textsubscript{2}) at pH 7.4 and T\textsubscript{eq}=25 °C were 0.0066 and 0.015 M\textsuperscript{-1} s\textsuperscript{-1} for Co\textsuperscript{III}Mb and Mn\textsuperscript{III}Mb, respectively, both orders of magnitude slower than the k\textsubscript{2} (6 M\textsuperscript{-1} s\textsuperscript{-1}) for wt Mb\textsuperscript{II}. The final reaction products for Mn\textsuperscript{III}Mb consisted of a mixture of the nitrosyl Mn\textsuperscript{III}Mb(NO) and Mn\textsuperscript{III}Mb, similar to the products from the analogous NiR reaction by wt Mb. In contrast, the products of NiR by Co\textsuperscript{III}Mb were found to be the nitrito complex Co\textsuperscript{III}Mb(NO\textsubscript{2})\textsuperscript{-} plus roughly an equivalent of free NO. The differences can be attributed in part to the stronger coordination of inorganic nitrite to Co\textsuperscript{III}Mb as reflected in the respective M\textsuperscript{II}Mb(NO\textsubscript{2})\textsuperscript{-} formation constants K\textsubscript{form} (2100 M\textsuperscript{-1} (Co\textsuperscript{III}) and \textasciitilde0.4 M\textsuperscript{-1} (Mn\textsuperscript{III}). We also report the formation constants (3.7 and 30 M\textsuperscript{-1}, respectively) for the nitrite complexes of the mutant metmyoglobins H64V Mb\textsuperscript{III}(ONO\textsubscript{2})\textsuperscript{-} and H64V/V67R Mb\textsuperscript{III}(ONO\textsubscript{2})\textsuperscript{-} and a K\textsubscript{nitrite} revised value (120 M\textsuperscript{-1}) for the nitrite complex of wt metMb. The respective K\textsubscript{nitrite} Values for the three ferric proteins emphasize the importance of a H-bonding residue, such as His64 in the Mb\textsuperscript{III} distal pocket or the Arg67 in H64V/V67R Mb\textsuperscript{III}, in stabilizing nitrite coordination. Notably, the NiR activities of the corresponding ferrous Mbs follow a similar sequence suggesting that nitrite binding to these centers are analogously affected by the H-bonding residues.

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1. Introduction

Nitric oxide (NO) in mammals is produced during aerobic respiration from the enzyme nitric oxide synthase and has many biological roles including vasodilation, signaling, and regulation of cellular function [1–4]. It has been recently established that under conditions of low oxygen tension, such as organ ischemia, nitrite-derived NO is responsible for similar signaling responses. Although enzyme-independent pathways exist for the nitrite-to-NO conversion, the reaction is greatly enhanced by various metalloproteins [5,6]. This in turn has led to increased attention to mammalian proteins that may display nitrite reductase (NiR) activity (Eq. (1)) [7–15].

\[
\text{NO}_2^- + e^- + 2H^+ \rightarrow \text{NO} + \text{H}_2\text{O}
\]  

(1)

The potential physiological roles of mammalian NiRs add imperative to understanding the specific protein components and traits that dictate such activity. For example, accessibility of nitrite to the ferrous site of cross-linked hemoglobin-based oxygen carriers has been argued to limit the nitrite reduction rate [16]. In another example, sickle cell hemoglobin (HbS) has higher NiR activity than hemoglobin A (HbA), and the difference correlates with the more favorable Fe\textsuperscript{II} to Fe\textsuperscript{III} half-cell potential of HbS [17]. In these laboratories, we have shown that modification of distal pocket residues regulates NiR activities of ferrous myoglobin (Mb\textsuperscript{II}) as well as the mode of nitrite coordination in ferric myoglobins (Mb\textsuperscript{III}) [18,19]. Thus, while wild type (wt) Mb\textsuperscript{III} binds NO\textsubscript{2} in the distal site as the O-nitrito (i.e., O-bound nitrite) isomer, the N-nitrito isomer is seen in the Mb\textsuperscript{III} mutant HbS4, where the H-bonding His64 residue is replaced by a non-polar valine. Furthermore, nitrite reduction by wt Mb\textsuperscript{III} is significantly faster
than by H64V MbIII, indicating that His64 is crucial for the NiR activity [18].

Perhaps an even greater perturbation to the properties of a protein such as Mb would be expected from substituting another metal center for the heme iron. In this context, the present study compares the NiR reactivity of wt MbIII to those of the metal-substituted myoglobins MnIII M and CoIII M, where the FeIII of wt MbIII has been replaced by the metal centers MnIII or CoIII. Various properties of the protein change as a result of such metal substitution, including the binding mode and affinity of nitrite, the redox potential of the protein, and the d-electron configuration. Synthetic MnIII porphyrin complexes bind nitrite via the O-nitrito mode [20,21], whereas the CoIII porphyrins [22–25] and most of the FeIII and FeII bind nitrite as the N-nitro isomers [25]. Nonetheless, the nitrite complexes of the proteins MnIII M and CoIII M are in the O-nitrito form [26] and thus in the same structural class as wt MbIII [27], although the strength of nitrite binding would be expected to differ among these species.

Described here are studies to assess the formation constants for the nitrite complexes of MnIII M and CoIII M and wt MbIII as well as those for the metMb distal pocket mutants H64V MbIII and H64V/ V67R MbIII [18]. We have determined the kinetics for nitrite reduction by the reduced proteins MnIII M and CoIII M, and compare these data with that obtained for wt MbIII.

2. Materials and methods

2.1. Materials

Horse heart myoglobin (hh-Mb) was obtained from Sigma. Manganese(II) protoporphyrin IX chloride and cobalt(III) protoporphyrin IX chloride were purchased from Frontier Scientific.

Apo-myoglobin (apo-Mb) was prepared using the method of Yonetani [28] and Teale [29]. The apo-Mb was reconstituted with MnIII (PPIX) and CoIII (PPIX) to give MnIII M or CoIII M, respectively, using the method of Yonetani and Asakura [30]. A 1.2-fold of CoIII (PPIX) was slowly added into a solution of ~2 mM apo-Mb in 10 mM sodium phosphate buffer at pH 6.8. The protein solution was stirred on ice for 30 min and then was centrifuged at 5000 g for 10 min. The supernatant was then loaded on a CM-52 column equilibrated with 10 mM sodium phosphate buffer at pH 6.8. After washing the column with a two-fold volume equivalent of buffer, the protein was eluted with a pH gradient developed by using an equal volume of 10 mM sodium phosphate buffer (pH 6.8 at 4 °C) and 10 mM sodium dibasic phosphate. The fractions displaying a change in the optical spectra. Unless otherwise noted, reaction solutions were maintained at pH 7.4 (100 mM phosphate buffer) and at 25 °C under deaerated conditions in a quartz cell adapted for sample preparation on a Schlenk vacuum line. High grade argon (4.8), which was first passed through a commercial grade O2 scrubber (OxiClear®) to give dioxygen levels below 50 ppb, was used for all Schlenk line manipulations. Additions to these closed systems were accomplished using gas tight syringes previously purged with high grade argon by injecting through a Thermogreen LB-2 septa (Sigma) sealed electrode adaptor.

Solutions of the reduced myoglobin derivatives MnIII M and CoIII M were prepared as follows. A concentrated sample of the respective MIII M protein (5–6 μM) and protocatechuate 3,4-dioxygenase (0.2 units, Sigma) in 1 mL phosphate buffer solution was deaerated by entraining with argon for 20 min. To this solution, solid sodium dithionite (1.0 mg, 5.7 μmol) was added to the reaction mixture and bubbled with argon for 5 more minutes. The color of the protein solution changed to yellow (Mn) or pink (Co), indicating that reduction occurred. This solution was then passed through a G-25 Sephadex column (to remove the excess dithionite and byproducts) on a Schlenk line using deoxygenated pH 7.4 phosphate buffer (100 mM) as the eluent, and the colored fraction was collected into a gas tight UV/Vis cell equipped with an injection port. This cell was previously deaerated and contained 3.4 dihydroxybenzoic acid (~1.0 mg, ~6.5 μmol) as a substrate for the dioxygenase, which is present to deplete spurious O2. The final solution volume ranged between 1.9 and 2.4 mL, and this was used to calculate the amount of NaNO2 needed to obtain the desired nitrite concentrations.

3. Results and discussion

3.1. Chemical reduction of MnIII M and CoIII M

It was previously reported from these laboratories that cysteine is a mild and effective reducing agent for the ferriheme proteins wt MbIII and the mutants H64V MbIII and H64V/V67R MbIII [18]. However, cysteine (1 mM) is not effective in reducing MnIII M, a difference we attribute to the less favorable reduction potential of MnIII M (E' = −170 and −10 mV vs. NHE [32]) compared to wt metMb (E' = 61 mV) [33]. As a consequence, it was necessary to use dithionite as the reducing agent. Fig. 1 shows the absorption spectrum of MnIII M (2.5 μM, split Soret band at 378 and 470 nm [28]) and that of MnIII M (440 nm) formed by reduction with excess dithionite (0.1 mM) under anaerobic conditions. Although two distinct reduction potentials have been reported for MnIII M [32], the temporal spectral changes appear to be first order in protein with clean isosbestic points (400 and 459 nm) (Supporting Information Fig. S-1). The reduction of

[Image 320x81 to 535x236]
MnIII\textit{Mb} by dithionite was previously attributed to the SO\textit{d}− anion radical formed by the reversible dissociation of S\textit{O}2\textit{d}− (Eq. (2)) due to a square root dependence of the rate on dithionite concentration (\(k_{\text{obs}} = k_{\text{Mn(III)}} \cdot [\text{S} \text{O}_2\text{d}^2]^{-1/2}\)) [34]. Under our conditions (2.5 μM MnIII\textit{Mb} and 0.10 mM S\textit{O}_2\textit{d}−), the \(k_{\text{obs}}\) for MnIII\textit{Mb} reduction was found to be 7.6 x 10^{-3} s\textsuperscript{-1}. Dividing by [S\textit{O}_2\textit{d}^2]^{-1/2} gives a \(k_{\text{Mn(III)}}\) value of 0.76 M\textsuperscript{-1/2}s\textsuperscript{-1}, consistent with the reported value of 0.54 M\textsuperscript{-1/2}s\textsuperscript{-1}.

\[
\text{S}_2\text{O}_4^{2−} \rightleftharpoons \text{2SO}_2^{−}.
\] (2)

Similar reduction of CoIII\textit{Mb} (4 μM; \(E^\circ = 100\) mV vs. NHE [35]) with dithionite (0.3 mM) led to loss of the Soret band for CoIII\textit{Mb} (\(\lambda_{\text{max}} = 424\) nm) and appearance of a new band at 404 nm indicating formation of CoII\textit{Mb} (Fig. 2). The temporal changes were biphasic (SI Fig. S-2), giving \(k_{\text{obs}}\) values of 4.9 x 10^{-3} and 5.0 x 10^{-4} s\textsuperscript{-1}, consistent with a previous report [36]. Hambright et al. attributed this biphasic behavior to two forms of CoIII\textit{Mb} in solution, which after reduction generates a single species [36]. The nature of the two forms of CoIII\textit{Mb} has not been conclusively established, although contributions from a hemichrome-like species were suggested [37]. The two rate constants, \(k_{\text{Co(III)}}\) and \(k_{\text{Co(II)}}\) (\(k_{\text{obs}}/\text{[S} \text{O}_2\text{d}^2]^{-1/2}\)), were calculated to be 0.28 and 0.029 M\textsuperscript{-1/2}s\textsuperscript{-1}, roughly consistent with those reported (0.17 and 0.012 M\textsuperscript{-1/2}s\textsuperscript{-1}).

3.2. Nitrite binding to MnIII\textit{Mb} and CoIII\textit{Mb}

Titration of nitrite (1 mM to 2.5 M) into a solution of MnIII\textit{Mb} (3 μM) led to minimal changes in the electronic absorbance spectrum (SI Fig. S-3). Thus, we conclude that the equilibrium constant for formation of the nitrite complex MnIII\textit{Mb}(ONO\textit{d}) must be very small (\(K_{\text{Mn(III)}} < 0.4\) M\textsuperscript{-1}) (Eq. (3)), \(M = \text{Mn}\).

\[
\text{M}^{\text{III}}\text{Mb} + \text{NO}_2^− \rightleftharpoons \text{M}^{\text{II}}\text{Mb}(\text{ONO}^−)
\] (3)

A likely explanation is that the axial Jahn–Teller distortion for this high-spin d\textsuperscript{5} system [38] would favor a five-coordinate structure. Consistent with this explanation is the observation that the crystallographically characterized MnIII\textit{Mb}(ONO\textit{d}) displaying a long Mn–O (nitrite) bond (2.3 Å) was only obtained after excess nitrite was added to MnIII\textit{Mb}, oxidizing the metal center to MnIII by the NiR activity described below [26].

Nitrite coordination to CoIII\textit{Mb} proved to be slow, so the temporal spectroscopic changes were recorded to determine the kinetics of the NO\textit{d} substitution into the Co(III) coordination sphere. Addition of various NaN\textit{O}_2 concentrations to buffered CoIII\textit{Mb} solutions (pH 7.4) resulted in a small, unexplained initial shift in the Soret band, followed by larger changes indicating a very slow binding event. The reason for the initial shift is unclear, but loss of CoIII\textit{Mb} (424 nm) coincided with formation of the new Soret absorption at 429 nm, presumed to be the result of CoIII\textit{Mb}(ONO\textit{d}) formation (SI Fig. S-4). This slow change was fit to a single exponential, indicating it was first order in CoIII\textit{Mb}. The plot of \(k_{\text{obs}}\) vs. [NO\textit{2}−] (0.5–24 mM) was linear with a non-zero intercept; the slope (2.30 ± 0.03) x 10\textsuperscript{-2} M\textsuperscript{-1}s\textsuperscript{-1} corresponds to \(k_{\text{on}}\) and the intercept (1.04 ± 0.30) x 10\textsuperscript{-3}s\textsuperscript{-1} is attributed to \(k_{\text{off}}\) (Fig. 3). The equilibrium constant, \(K_{\text{on}}\), for nitrite binding determined from the ratio \(k_{\text{on}}/k_{\text{off}}\) was 2100 ± 600 M\textsuperscript{-1}, a value much higher than that reported previously for wt metMb (60 M\textsuperscript{-1}) [39] and that indicated above for the manganese analog MnIII\textit{Mb} (<0.4 M\textsuperscript{-1}). The crystal structure of the nitrite complex CoIII\textit{Mb}(ONO\textit{d}) shows the nitrite ligand to be O-coordinated which is stabilized by H-bonding with the distal His64 residue [26]. In contrast, synthetic CoIII porphyrins not having such H-bonding interactions display N-nitro coordination [22–25,40].

3.3. Nitrite binding to wt MbIII and the metMb mutants H64V MbIII and H64V/V67R MbIII

To place the nitrite binding events in proper perspective, we also determined \(K_{\text{on}}\) for the metMb mutants H64V MbIII and H64V/V67R MbIII and re-determined \(K_{\text{on}}\) for wt MbIII using a spectroscopic titration technique (Fig. 4, and SI Figs. S-5 and S-6). The respective \(K_{\text{on}}\) values determined thus are 3.7, 30 and 120 M\textsuperscript{-1} for H64V, H64V/V67R and wt MbIII, respectively. These values reinforce the importance of a H-bonding residue such as His64 in the MbIII distal pocket (or the Arg67 in H64V/V67R MbIII) in stabilizing nitrite coordination.

Fig. 3. A plot of \(k_{\text{obs}}\) vs. [NO\textit{2}−] for the reaction of CoIII\textit{Mb} with NO\textit{2}− is linear giving a slope \(k_{\text{on}}\) of 0.023 M\textsuperscript{-1}s\textsuperscript{-1} and y-intercept \(k_{\text{off}}\) of 1.04 x 10\textsuperscript{-3}s\textsuperscript{-1}. The equilibrium constant \(K_{\text{on}}\), for the formation of CoIII\textit{Mb}(ONO\textit{d}) was determined from \(k_{\text{on}}/k_{\text{off}}\) to be 2100 M\textsuperscript{-1}.

Fig. 4. Spectral changes for titration of nitrite (0.3 mM to 0.1 M) into a solution of wt MbIII (2.0 μM, pH 7.4) showing loss of MbIII (500 nm) and formation of MbIII(ONO\textit{d}) (540 and 574 nm), \(K_{\text{on}}\) was calculated by plotting the log (Abs 574 nm) vs. log [NO\textit{2}−] and fitting to a sigmoidal curve (inset).
should be a stronger reductant than wt MbII (see above), the slower MnIIMb(NO) (471 nm) and MnIIMb(NO) (427 nm).

3.4. Reduction of nitrite by MnIII Mb and CoIII Mb

Solutions of MnIII Mb (2 μM) were prepared by dithionite reduction of MnIII Mb followed by passage through a G-25 Sephadex column to remove excess dithionite and its oxidation byproducts. Addition of NaNO2 (1 mM) to the resulting solution led to little spectral change over a 10 min period, indicating that the reaction is much slower than that observed previously for the wt ferrous Mb (k2 = 6 M⁻¹ s⁻¹) [41,42]. Fig. 5 illustrates the spectral changes observed for a deaerated solution containing nitrite (3.9 mM) and MnIII Mb (2.1 μM). The spectrum observed after completion of the reaction (SI Fig. S-7) analyzed as a 1:1 mixture of MnIII Mb and MnIII Mb(NO) as expected if the NO formed in the NiR reaction (Eq. (4)) is trapped by the remaining MnIII Mb (Eq. (5)) owing to the large equilibrium constant for MnIII Mb(NO) formation [43]. The behavior of the MnIII Mb/nitrite system in this regard is quite similar to that seen in solution studies with wt Mb, where the NO is trapped by the ferrous Mb, so the NiR reaction only proceeds to 50% completion.

\[
\text{Mn}^{II} \text{Mb} + \text{NO}_2^- + 2\text{H}^+ \rightarrow \text{Mn}^{III} \text{Mb} + \text{NO} + \text{H}_2\text{O} \quad (4)
\]

\[
\text{Mn}^{II} \text{Mb} + \text{NO} = \text{Mn}^{III} \text{Mb(NO)} \quad (5)
\]

The temporal absorbance changes at 440 nm (λmax for MnIII Mb) deviated from exponential behavior (Fig. 5, inset), and for this reason the initial rates method was used to analyze the kinetics of the spectral changes. Plots of the initial rates (M/s) vs. [MnIII Mb] (1.5−3.7 μM), [NO2⁻] (4.3−8.1 mM), or [H⁺] (pH range 6.8−7.4) were each linear (Fig. 6) consistent with the rate law presented in Eq. (6). From these data the second order rate constant for nitrite reduction by MnIII Mb at pH 7.4 (k_Mn = k'_Mn [H⁺]) was calculated to be 0.015±0.002 M⁻¹ s⁻¹, which is a factor of 400 smaller than the analogous k2 for wt Mb (6 M⁻¹ s⁻¹). Since MnIII Mb

\[
d(MnIII Mb)/dt = -k_{Mn}[NO_2^-][H^+]/[MnIII Mb]k_Mn = 3.8 \times 10^{-4} M^{-2} s^{-1} \quad (6)
\]

should be a stronger reductant than wt MbII (see above), the slower rate for the former clearly indicates that other mechanistic factors must be playing an important role in defining the dynamics of these systems.

The NO2⁻/MnIII Mb kinetics were also studied without removing the excess dithionite from the solutions, a procedure that eliminates concern about spurious contamination with trace oxygen, since dithionite reacts readily with O2 [44]. The concentration of excess dithionite (0.1 mM) was calculated from its absorption band at 314 nm (8000 M⁻¹ cm⁻¹). The second rate constant (k'Mn) determined by the initial rates method was 0.019±0.003 M⁻¹ s⁻¹ at pH 7.4, close to the k'Mn value in the absence of dithionite. Thus we conclude that the excess reductant does not have a large impact on the reaction rate. Furthermore, the initial rates were found to be independent of [S2O4²⁻] below 0.1 mM. However, one key difference is that the final product is not a mixture, but only MnIII Mb(NO), which is formed with clean isosbestic points (SI Fig. S-8). This observation is readily explained by the dithionite reduction of the MnIII Mb formed (Eq. (4)) back to MnIII Mb, which is trapped by NO. The net reaction

Fig. 5. Spectral changes and kinetic trace (inset) for reduction of nitrite (3.9 mM) by MnIII Mb (2.1 μM) showing loss of MnIII Mb (440 nm) and formation of MnIII Mb (471 nm) and MnIII Mb(NO) (427 nm).

Fig. 6. The linear dependences of the initial rates of NiR reaction by MnIII Mb on [MnIII Mb] (1.5−3.7 μM MnIII Mb, 8.1 mM NO2⁻, pH 7.4) (A), [nitrite] (4.3−8.1 mM NO2⁻, 2.7 μM MnIII Mb, pH 7.4) (B), and [H+] (pH range 6.8−7.4, 1.5 μM MnIII Mb, 4.3 mM NO2⁻) (C).
under these conditions is represented by Eq. (7), where the extra electron is provided by the dithionite.

\[
\text{Mn}^{11}\text{Mb} + \text{NO}_2^- + 2\text{H}^+ + e^- \rightarrow \text{Mn}^{11}\text{Mb(NO)} + \text{H}_2\text{O} \quad (7)
\]

Fig. 7 illustrates the temporal spectral changes when nitrite (18 mM) was added to a solution of CoIII Mb (2.5 μM). Notably, the final spectrum was not consistent with formation of either CoIII Mb (λmax 424 nm) or CoIII Mb(NO) (420 nm) or of a mixture of these two species as the final products. Instead the spectrum indicated formation of the O-nitrito complex CoIII Mb(NO⁻) (429 nm) (see also Fig. 2) as the end product. Clean isosbestic points were not the case, with the spectral intersection trailing from 412 nm to 418 nm as the reaction proceeded, indicating an A→B→C type process. There was no clear spectral demonstration that CoIII Mb(NO) is formed, although the formation constant from NO and CoIII Mb should be large [45]. One complication is that the Soret λmax for CoIII Mb(NO) (420 nm) is in the same region as the trailing isosbestic point and the other expected product CoIII Mb has a similar λmax (424 nm). Thus, while these species may be formed transiently, it appears that the system evolves during the course of the experiment to give CoIII Mb(NO⁻). If so, then NO must be liberated as the other product as indicated by Eq. (8).

\[
\text{Co}^{II}\text{Mb} + 2\text{NO}_2^- + 2\text{H}^+ \rightarrow \text{Co}^{III}\text{Mb(NO)}^- + \text{NO} + \text{H}_2\text{O} \quad (8)
\]

In order to test this premise, a buffered solution of CoIII Mb (1.2 μM, 3.2 nmol) was generated in the presence of protocatechuate 3,4-dioxygenase and 3,4 dihydroxybenzoic acid (to maintain an anaerobic environment). Nitrite (18 mM) was then added, and the headspace above the solution was sampled using a gas tight syringe and analyzed using the Seivers NOA. A sample taken shortly after the reaction was initiated (~2 min) showed very little NO in the headspace, as might be expected since excess CoIII Mb is present at the beginning of the reaction. However, another sample taken at the conclusion of the reaction (90 min) showed that, accounting for the partitioning between the gas and liquid phase, 2.8 ± 1.1 nmol (0.9 ± 0.3 equivalents) of free NO were formed (SI Fig. 5-5), thus confirming the stoichiometry of Eq. (8). When the analogous experiment was carried out with wt Mb, the amount of free NO present in the gas and solution phases was dramatically smaller (~0.01 equiv.) confirming NO capture by the ferrous protein present.

The initial rates method showed that the kinetics for CoIII Mb disappearance were linearly dependent on [CoIII Mb] (1.5–6.5 μM, [NO₂⁻] (3–20 mM), and [H⁺] (pH 6.4–7.4) (Fig. 8, Eq. (9)), consistent with the NiR kinetics seen for MnIII Mb and for wt MbII. At pH 7.4, the second order rate constant for CoIII Mb (k_{Co}) was determined to be 0.0066 ± 0.0009 M⁻¹ s⁻¹ three orders of magnitude slower than wt MbII and ~2-fold slower than MnIII Mb.

\[
d(\text{Co}^{III}\text{Mb})/dt = -k_{Co}^\text{NO}^2^-([\text{H}^+]) |\text{Co}^{III}\text{Mb}| k_C^\text{NO}^2 = 1.7 \times 10^5 \text{M}^-2 \text{s}^{-1} \quad (9)
\]

In the presence of excess dithionite (20 μM), addition of nitrite (12 mM) to a solution of CoIII Mb (2.5 μM) gave only CoIII Mb(NO) \( \lambda \) in nm (ε M⁻¹ cm⁻¹), 420 (1.4×10⁵), 540 (1.8×10⁴), 576 (1.8×10³), Fig. 2 suggesting that any CoIII Mb formed is rapidly intercepted by this reductant, and that the resulting CoIII Mb is trapped by the NO generated. However, the reaction rate is much faster than in the absence of dithionite, and temporal absorbance changes were quite complex. Further details are presented in the Supporting Information (Figs. S-10 to S-12). Catalyzed dithionite reduction of nitrite has been observed with the iron and cobalt phthalocyanine complexes in alkaline solutions [46,47], so it is likely that a similar process is occurring in the present case as well.
3.5. Comparing the relative rates of nitrite reduction

The first order dependence of the initial rates on the three concentration parameters [MIIIMb], [NO\textsubscript{2}^\textsuperscript{-}] and [H\textsuperscript{+}], shown here for MnIIIMb and CoIIIMb and previously for wt Mb\textsuperscript{[13]}, suggests a common sequence of steps such as illustrated by Eqs. (10–12). The first would be the irreversible formation of the Mn\textsuperscript{III} O-nitrito complex (Eq. (10)) followed by irreversible reaction in an H\textsuperscript{+} dependent rate limiting step (Eq. (11)) to give the nitrosyl complex of MnIIIMb, which readily dissociates NO (Eq. (12)). A second H\textsuperscript{+} is necessary in the second step(s) in order to balance the reaction, although the actual transformation of coordinated nitrite to NO may be assisted by a proton source within the distal cavity of the protein. This sequence of reactions would be consistent with the rate laws indicated by Eqs. (6) and (9) with $k_5 = k^{-1}_4$, where $k_5$ is the metal-center dependent equilibrium constant for forming the Mn\textsuperscript{III} nitrite complex (Eq. (10)) and $k_4$ the rate constant for the rate limiting step (Eq. (11)).

\[
\text{MnIIIMb} + \text{NO}_2^\text{-} \rightleftharpoons \text{MnIIIMb(ONO)}^\text{\textsuperscript{2+}} \tag{10}
\]

\[
\text{MnIIIMb(ONO)}^\text{\textsuperscript{2+}} + \text{H}^+ \rightarrow \text{MnIIIMb(NO)} + \text{H}_2\text{O} \tag{11}
\]

\[
\text{MnIIIMb(NO)} \rightarrow \text{MnIIIMb} + \text{NO} \tag{12}
\]

We were not able to observe spectrally the formation of nitrite complexes of MnIIIMb and therefore can only speculate regarding what are the contributions of these two terms may be to the overall rate. For wt Mb\textsuperscript{II} and the mutant myoglobins H64V Mb\textsuperscript{II} and H64V/V67R Mb\textsuperscript{II},\text{ et al.} analog\textsuperscript{h.s.} d\textsuperscript{6} wt Mb, although, if this is accompanied by a h.s. to l.s. conversion, the higher ligand field stabilization of the putative Mn\textsuperscript{III} nitrosyls, which, unlike Fe\textsuperscript{IIIMb} \textsuperscript{[51]}, were not observed for Mn\textsuperscript{IIIMb} or Co\textsuperscript{IIIMb} upon addition of NO. Nitrite reduction by h.s. Mn\textsuperscript{IIIMb} \textsuperscript{[49]} could also be limited by the large reorganizational energy associated with oxidation of Mn\textsuperscript{II} to Mn\textsuperscript{IIIMb}. We note that the crystal structures of Mn\textsuperscript{IIIMb} and Mn\textsuperscript{IIIMb} show a change in metal displacement out of the porphyrin plane from 0.34 to 0.18 Å ($\Delta = 0.16$ Å) \textsuperscript{[26]}.

With regard to CoMb species, it is notable that the final species detected in solutions containing CoMb and NO or nitrite was always CoMb(ONO\textsuperscript{\textsuperscript{-}}), the thermodynamic sink for this system.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at doi:10.1016/j.jinorgbio.2011.10.006.

References
