Binding of Cyanide and Thiocyanate to Manganese Reconstituted Myoglobin and Formation of Peroxide Compound: Optical Spectral, Multinuclear NMR, and Kinetic Studies

Madhu Sudan Mondal, Shyamalava Mazumdar, and Samaresh Mitra*

Chemical Physics Group, Tata Institute of Fundamental Research, Homi Bhabha Road, Bombay 400 005, India

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The interaction of inorganic anions with manganese(III) protoporphyrin IX reconstituted myoglobin ($Mn^{III}Mb$), coordination geometry around the metal ion in the reconstituted protein and the existence of higher oxidation states have been investigated by optical difference, multinuclear NMR and stopped flow techniques. The binding of $SC^{15}N^-$ and $C^{15}N^-$ to $Mn^{III}Mb$ was studied by optical difference spectroscopy and the observation of the spin lattice relaxation rate of the ^{15}N resonance. The values for dissociation constants for SCN^- to $Mn^{III}Mb$ were found to vary from ~5 to ~94 mM in the pH range 5–10. The dissociation constant for the binding of CN^- to $Mn^{III}Mb$ was measured by optical difference spectroscopy in competition with SCN^-, which shows that the binding site for both CN^- and SCN^- is the same. The value of dissociation constant of CN^- to $Mn^{III}Mb$ was found to be ~91 mM at pH = 9.2. These values show that the binding of both CN^- and SCN^- is weak. The dissociation constant values measured from both optical and NMR method agree well. The pH dependence of the line width and the dissociation constant shows that the binding involves a complex pH equilibrium among the amino acid residues in the region of the binding site; *i.e.* in this case more than one acid ⇌ base equilibrium is operative. The distances measured for SCN^- and CN^- from Mn(III) center was found to be 6.7 and 6.2 Å. These observations are comparable to those for the $Mn^{III}HRP$ system, which suggest that the binding site is away from the metal center. The measurements of water proton relaxivity at pH = 4.0 and 9.6 give the distances of the water proton from the Mn(III) center as 12.8 and 13.7 Å, respectively. This indicates that the water molecule is not present in the first coordination sphere of the Mn(III) center. Oxidation of $Mn^{III}Mb$ by excess H_2O_2 shows that it forms a short-lived redox intermediate which has an optical spectrum similar to that of $Mn^{III}HRP$ compound I. The rate measurements by stopped flow kinetics techniques show that the value of the apparent second-order rate constant of the formation of this higher oxidation state is low (2.5 M^{-1} s^{-1}). The results bring out the importance of the protein cavity around the metal in its peroxidase activity.

1. Introduction

The catalytic activity of heme proteins depends on redox properties of the metal ion as well as on the nature of the protein cavity.[1-3] Previous studies on manganese(III) porphyrin reconstituted peroxidases have shown that the catalytic activity of the Mn(III) reconstituted protein is much lower than that of the native protein.[2] Manganese(III) reconstituted horseradish peroxidase ($Mn^{III}HRP$) has in particular been extensively studied in order to understand the effect of metal substitution on the anion binding to the enzyme.[4] These studies show that binding affinity of inorganic anions, such as thiocyanate to $Mn^{III}HRP$ is comparable to that of the native HRP.[4] However, binding of cyanide to $Mn^{III}HRP$ is different and much weaker than cyanide to the native protein. Cyanide binds strongly to the heme iron active center[4] and forms a low-spin cyanide complex of Fe(III) heme in native HRP, causing complete deactivation of the enzyme. It, however, binds to the $Mn^{III}HRP$ at ~6.2 Å away from metal center.[4] Further, native HRP has the five-coordinated Fe(III) heme as the active center; the manganese in $Mn^{III}HRP$ has however been shown[4] to be six-coordinated with one water molecule axially ligated to the manganese ion in the prosthetic group. Binding of inorganic anions to $Mn^{III}(HRP)$ has been very extensively studied by optical difference and NMR spectroscopy.[4] Similar studies on manganese reconstituted myoglobin have been very limited.[5] Manganese apoprotein complexes of HRP and myoglobin have similar prosthetic groups. It was therefore interesting to investigate the binding of inorganic anions and coordination geometry of manganese porphyrin reconstituted apomyoglobin ($Mn^{III}Mb$), which will aid our understanding of the effect of the protein cavity on the electronic and structural properties of these metal-substituted heme proteins.

We report here detailed ^1H and ^{15}N NMR relaxation and optical difference spectroscopic studies carried out to determine the nature of water coordination and binding of thiocyanate and cyanide to $Mn^{III}Mb$. We also report optical spectral and stopped flow studies on peroxide oxidation of $Mn^{III}Mb$. These results have been compared with similar studies on $Mn^{III}HRP$ and native enzymes. The effect of the nature of the protein cavity and coordination geometry of the prosthetic group have been discussed.

2. Experimental Procedure

Protoporphyrin IX dimethyl ester, salt free lyophilized powder of horse heart myoglobin, and deuterium oxide (≥99.96 atom %) were obtained from Sigma Chemical Co. Enriched ^{15}N (% of $^{15}N \geq 99$) sodium thiocyanate and sodium cyanide were obtained from MSD Isotopes. The hydrogen peroxide used was of reagent grade. All other reagents used were of analytical grade.

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Manganese(III) protoporphyrin IX was prepared by the reported method;[2] it was purified on a Celite column following the reported procedure.[6]

The apomyoglobin was prepared from horse heart myoglobin by slight modification of Teale's acid/butanone procedure.[7] An aqueous solution of iron(III) myoglobin was made in 10 mM sodium dihydrogen orthophosphate buffer. Its pH was brought down, at 0 °C, to 2.3–2.4 by addition of dilute ice-cold hydrochloric acid. Immediately the mixture was mixed with equal volume of ice-cold 2-butanone. It was shaken for 30 s and allowed to settle for 5 min. The upper butanone layer was siphoned off and again treated with twice the amount of butanone. The aqueous mixture was dialyzed at 4 °C against 10 mM $NaHCO_3$, then against water, and finally against 0.05 M Tris–HCl buffer, pH = 8.0. The apomyoglobin concentration measured was found to be in the range 0.2–0.3 mM.[8]

Reconstitution of manganese(III) protoporphyrin IX to apomyoglobin was done according to the procedure of Yonetani *et al.*[2] Apomyoglobin in 0.05 M Tris–HCl, pH = 8.2, was mixed with a 1.1-fold excess of manganese(III) protoporphyrin IX. It was shaken thoroughly and was allowed to stand for 20 min. The pH of the mixture was brought down to 7.0 and loaded in a carboxymethyl cellulose column preequilibrated with 10 mM NaH_2PO_4 buffer, pH = 7.0. The column was washed with the same buffer to remove unbound manganese(III) protoporphyrin IX. The bound $Mn^{III}Mb$ was eluted as a strong band by 10 mM NaH_2PO_4 buffer at pH = 8.2. It was lyophilized and stored at −30 °C.

The UV–vis absorption spectra were recorded with a Shimadzu UV-2100 spectrophotometer using a cell with 1-cm path length. Optical difference spectroscopic studies were carried out by taking 30 μM Mn^{III}-Mb in each of the sample and reference cuvettes. Concentration of SCN^- was caried in the sample cell by addition of SCN^- solution. Final concentration of SCN^- in the sample cuvette was in the range 2.0–30 mM. For each addition of SCN^- equal volume of buffer was added to the reference cuvette.

Proton NMR spectra were recorded on Bruker AM 500-MHz FTNMR spectrometer at 25 °C. All the solutions were made in 10 mM NaH_2PO_4 in deuterium oxide solution. The pH values were the meter reading without any correction for a small isotope effect. The ^{15}N NMR measurements were made at 50.68 MHz in a 10-mm NMR tube with D_2O as frequency lock. The spectra were obtained typically with 400–1000 transients at 60K data points. The 180°–τ–90° pulse NMR spectra were used to observe the spin–lattice relaxation time ($T_{1,obs}$). $T_{1,obs}$ was calculated by using a three parameter, nonlinear least-squares fitting to the standard equation[9] for the spin–lattice relaxation time.

The kinetic experiments were performed on a BIOLOGIC (Instruments de Laboratories) made stop flow machine (SFM3) with three machined syringes that were microprocessor (MPS51) controlled. The reaction mixture was thermostated at 25 °C by a temperature controlled water bath. Measurements were done spectrophotometrically by monitoring the formation of higher oxidation state of $Mn^{III}Mb$ at 413 nm. The concentration of H_2O_2 was measured from the absorbance value at 203 nm taking ϵ_{203} as 72.4 M^{-1} cm^{-1}.[10] Concentrations of H_2O_2 were varied in the range 18–75 mM by dilution with buffer from a third syringe just before the reaction was monitored. The concentration of $Mn^{III}Mb$ was kept constant at 30 μM. The reaction under these conditions followed a pseudo-first-order rate law.

3. Results and Discussion

(a) Optical Difference Spectroscopic Studies. Figure 1 shows the optical spectrum of $Mn^{III}Mb$, which matches the reported one.[2] Binding of CN^- and SCN^- to $Mn^{III}Mb$ was first carried out by optical spectroscopy. Addition of cyanide (10 mM) to a solution of $Mn^{III}Mb$ does not show any change at all in the optical spectrum, while addition of thiocyanate (10 mM) shows a definite change in the absorbance (Figure 1). We have, therefore, carried out optical difference spectral studies of binding of thiocyanate to $Mn^{III}Mb$. Figure 2 shows that titration of a $Mn^{III}Mb$ solution by NaSCN (2.0–30 mM) causes a gradual increase in absorbance

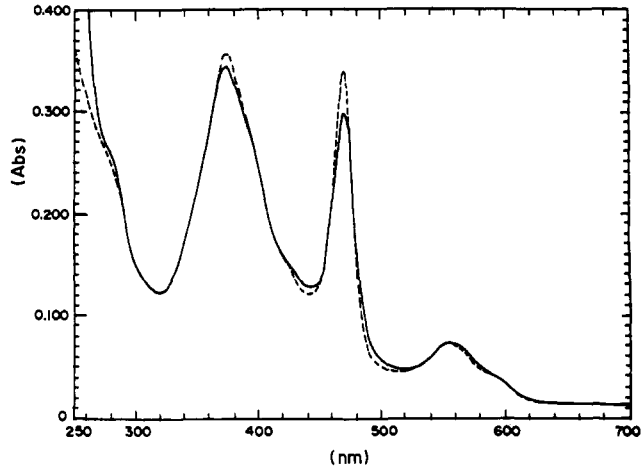

Figure 1. UV–vis spectra: (a) $Mn^{III}Mb$ (14.2 μM); (b) $Mn^{III}Mb$ with SCN^- (10 mM). $Mn^{III}Mb$ with CN^- (10 mM) gives a spectrum overlapping with spectrum a.

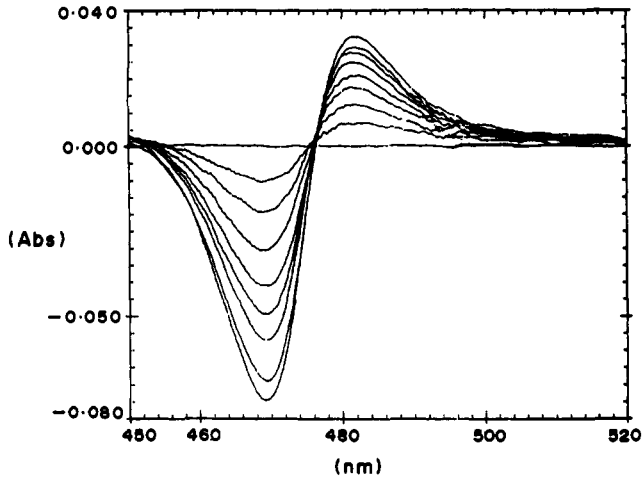

Figure 2. Optical difference spectra for $Mn^{III}Mb$ (30 μM, pH 7.2) at different concentrations of thiocyanate (2.0–30 mM).

at λ = 482 nm and a subsequent decrease in absorbance at λ = 469 nm with an isosbestic point at λ = 476 nm. The plot of the reciprocal of optical difference (ΔA) against $1/[SCN^-]$ follows a straight line (data not shown) for single binding site according to[11]

$$Mn^{III}Mb + SCN^- \rightleftharpoons Mn^{III}Mb - SCN \qquad (1)$$

where

$$\frac{1}{\Delta A} = \frac{1}{\Delta A_\infty} + \frac{K_D}{\Delta A_\infty}\left(\frac{1}{[SCN^-]_0}\right) \qquad (2)$$

where ΔA_∞ is the change in absorbance at saturation concentration of SCN^- and K_D is the dissociation constant for the SCN^- binding to $Mn^{III}Mb$.

The value of K_D for thiocyanate binding was found to vary from 5 to 94 mM between pH 5 and pH 10. The magnitude of K_D supports the observation that the thiocyanate does not bind to the protein at the manganese center, but it binds to it away from the metal center. This is in contrast to the observation that thiocyanate binds to the heme iron in myoglobin. The magnitude of K_D for thiocyanate binding to $Mn^{III}HRP$ at pH 4.2 is 42 ± 5 mM which is comparable to that with native HRP,[12] indicating that SCN^- binds to the $Mn^{III}HRP$ and the native HRP in a similar manner perhaps at the same site.[4] Figure 3 shows a plot of experimental pK_D against pH for thiocyanate binding to Mn^{III}-

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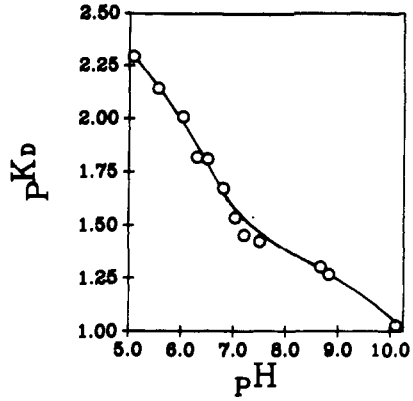

Figure 3. pH dependence of K_D for SCN^- binding to $Mn^{III}Mb$. The measurements were carried out by optical difference spectroscopy.

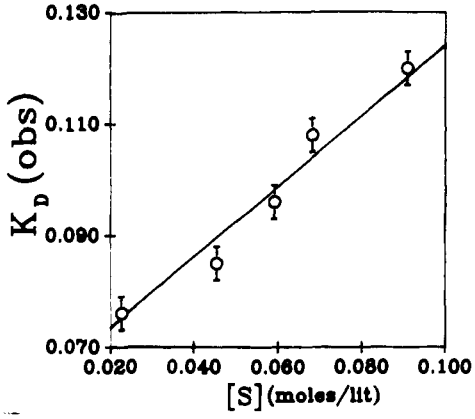

Figure 4. Plot of K_D(obs) for thiocyanate binding to $Mn^{III}Mb$ in the presence of cyanide.

Mb. The variation of K_D of $Mn^{III}HRP$ with pH has been previously[4] fitted to an acid ⇌ base equilibrium model involved an amino acid residue which had a pK_a of 4.0. However, in $Mn^{III}Mb$, the pH variation could not be fitted to any such simple model, indicating that the pH dependence of K_D here may result from the pH equilibrium of more than one amino acid residue. The general trend in the variation of pK_D with pH of SCN^- binding to $Mn^{III}Mb$ is however similar to that found with $Mn^{III}HRP$ and native HRP.

(b) Competitive Binding Studies. The binding of cyanide to $Mn^{III}Mb$ could not be directly studied using optical difference spectroscopy. Competitive binding of thiocyanate in the presence of cyanide was however carried out to study the binding of cyanide to $Mn^{III}Mb$. Figure 4 shows the variation in the observed K_D with cyanide concentration. The magnitude of K_D for thiocyanate binding was found to increase with increasing concentration of cyanide (Figure 4), indicating that both thiocyanate and cyanide might be binding at the same site in $Mn^{III}Mb$. The observed K_D (K_D(obs)) of thiocyanate binding to $Mn^{III}Mb$ in the presence of a competing anion (here CN^-) binding at the same site is given by eq 3,[4] where [I] is the concentration of the CN^- and K_I is the

$$K_D(\text{obs}) = K_D + \frac{K_D[I]}{K_I} \qquad (3)$$

apparent dissociation constant of CN^- in the absence of the SCN^-. The value of K_I (which is the K_D for dissociation of cyanide binding to $Mn^{III}Mb$) can be deduced from the intercept and clope of the plot of K_D(obs) vs [CN^-] in Figure 4. The value of K_D for cyanide binding to $Mn^{III}Mb$ was thus found to be 91 ± 8 mM at pH 9.2. The magnitude of K_D for cyanide binding to $Mn^{III}Mb$ suggests that binding of cyanide to $Mn^{III}Mb$ is weak and that it does not

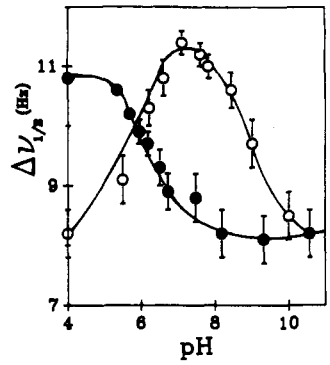

Figure 5. The pH dependence of ^{15}N NMR line width of thiocyanate (371 mM, ●), and cyanide (615 mM, ○) in the presence of 200 and 77 μM $Mn^{III}Mb$, respectively. The height of the vertical bars denotes twice the standard deviation of the Lorentzian line-shape fit.

bind to the metal center.[4,12] Since thiocyanate and cyanide compete for binding, they would bind to $Mn^{III}Mb$ at the same site. Cyanide however binds very strongly to the metal center in native Mb and HRP. The K_D for the cyanide complex of native HRP is 3 μM indicating strong ligation of cyanide with $Fe^{III}HRP$.[12] On the other hand, the binding of cyanide to $Mn^{III}HRP$ is very similar to that to $Mn^{III}Mb$, which suggests that cyanide does not bind to Mn(III) in manganese porphyrin reconstituted proteins.[4]

(c) NMR Line Width. The binding of thiocyanate and cyanide to $Mn^{III}Mb$ has also been investigated using ^{15}N NMR. The ^{15}N NMR spectra of both $C^{15}N^-$ and $SC^{15}N^-$ in presence of $Mn^{III}Mb$ show only one peak due to the labeled anion. Absence of any separate signal from bound cyanide (or thiocyanate) indicates that the anion does not bind to the metal center.[4] No change in the ^{15}N chemical shift is observed. The cyanide and thiocyanate binding to $Mn^{III}HRP$ has previously been shown to give only one ^{15}N resonance centered around the free anion signal, indicating that these anions do not bind to the metal center in $Mn^{III}HRP$.[4] The large values of K_D observed in the optical spectral studies of these systems also support the observation that these anions do not coordinate to the metal atom in $Mn^{III}Mb$.

The line widths of ^{15}N signal of cyanide as well as thiocyanate are found to increase with addition of $Mn^{III}Mb$. The ^{15}N line width in the presence of $Mn^{III}Mb$ was also found to change with pH. Figure 5 shows that the line width of ^{15}N resonance of thiocyanate (371 mM) is 10.8 Hz at pH 4 (200 μM $Mn^{III}Mb$) and that of cyanide (615 mM) is 11.4 Hz at pH 7.1 (77 μM $Mn^{III}Mb$) whereas the ^{15}N line width is 2.3 and 2.8 Hz for free thiocyanate (371 mM) and cyanide (615 mM), respectively. This is in conformity with the optical spectral studies that these anions bind to $Mn^{III}Mb$. The line width of the ^{15}N resonance was found to increase at a given pH with increasing concentration of $Mn^{III}Mb$ indicating presence of fast exchange between the bound and free anions.[13]

The effect of pH on the line width of the ^{15}N resonance of $C^{15}N^-$ and $SC^{15}N^-$ binding to $Mn^{III}Mb$ has been studied (Figure 5). The line width of thiocyanate in the presence of $Mn^{III}Mb$ was found to decrease with increasing pH. A similar observation has been reported for the SCN^- binding to $Mn^{III}HRP$ also.[4] However, in the case of the $Mn^{III}HRP$–SCN system, the line width of the $SC^{15}N^-$ decreases to a constant free thiocyanate value (2.5 Hz) at pH ≥ 7, while in the present case the decrease in line width of $SC^{15}N^-$ is much slower and it reaches an almost constant value of 8 Hz at pH ≥ 8.5. This value is much larger than the free thiocyanate value. The observed line width ($\Delta\nu_{1/2}$) is proportional to the transverse relaxation rate (neglecting the field inhomogeneity contribution),[8] and it can be related to the

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line width of the free ($\Delta\nu_{1/2}^F$) and the bound ($\Delta\nu_{1/2}^B$) anion as follows:

$$\Delta\nu_{1/2} = \frac{E_0(\Delta\nu_{1/2}^B - \Delta\nu_{1/2}^F)}{S_0 + K_D} + \Delta\nu_{1/2}^F \quad (4)$$

The pH dependence of $\Delta\nu_{1/2}$ is due to the variation of the dissociation constant, K_D, with pH. Previous studies of the Mn^{III}-HRP complex system showed that the pH dependence of the ^{15}N line width of thiocyanate binding to $Mn^{III}HRP$ arises from the pH equilibrium of an acid residue (propionic acid of heme).[4] In the present system, the results indicate that the pH dependence of K_D, and hence line width, does not depend on one single acid residue; instead more than one acid–base equilibrium may be operative in the vicinity of the SCN^- binding site, which affects the K_D of thiocyanate binding. However, the line width data could not be used accurately to calculate K_D here.

The pH variation of the ^{15}N NMR line width of the cyanide binding to $Mn^{III}Mb$ shows an interesting feature (Figure 5). The $C^{15}N^-$ line width in the presence of 77 μM $Mn^{III}Mb$ increases initially with increase in pH from 4.0 to 7.0, then it remains nearly unchanged over about one pH unit, and finally it decreases with further increase in pH. At pH $\sim$ 12 the $C^{15}N^-$ line width decreases to 7.8 Hz, which is much higher than that of free cyanide. In case of CN^- binding to $Mn^{III}HRP$ a similar trend in the ^{15}N line width of CN^- has been reported,[4] but the line width was observed to become equal to the line width of free cyanide at very high pH. The possibility of existence of more than one acid residue in the vicinity of the cyanide binding site in $Mn^{III}Mb$ may be responsible for the observed pH dependence of the line width. In the low pH region, Glu (C3), heme propionate, and free histidine (C1) and, at the high pH region, lys (C7), Arg (CD3),[14,15] *etc.*, might be playing some role in the anion binding. It has been shown previously[4] that the cyanide (pK_a = 9.14) as well as thiocyanate ($pK_a = -1.9$) bind in the deprotonated form (*i.e* CN^-, SCN^-) to the protonated form of the $Mn^{III}HRP$.

(d) ^{15}N-T_1 Measurements. Additional structural informations about the anion binding to $Mn^{III}Mb$ can be obtained from nuclear spin–lattice relaxation rate meaasurements.[16] The spin–lattice relaxation time (T_1) measurements were done at different concentration of the anions ($SC^{15}N^-$ and $C^{15}N^-$) with $Mn^{III}Mb$ (4.6 mM). The anion concentrations were in the range 250–600 mM. The observed relaxation rate of $C^{15}N^-$ or $SC^{15}N^-$ is the sum of the rates of relaxation of the free anions and the paramagnetic relaxation enhancement[16]

$$\frac{1}{T_{1\,obs}} = \frac{1}{T_{1f}} + \frac{1}{T_{1p}} \quad (5)$$

where $T_{1\,obs}$ is the observed relaxation time, T_{1f} is the relaxation time in the absence of a paramagnetic protein, and T_{1p} is the paramagnetic contribution to relaxation time. The value of T_{1p} is related to T_{1M}, the relaxation time of the bound species by[16]

$$\frac{1}{T_{1p}} = \frac{1}{T_{1p'}} + \frac{P_m q}{T_{1M} + \tau_M} \quad (6)$$

where $T_{1p'}$ is the paramagnetic relaxation time due to the effect of bulk susceptibility of the paramagnetic solute. $P_m q$ is the total fraction of nuclei at the bound site and $1/\tau_M$ is the rate of chemical exchange between the two sites. In the fast exchange limit, *i.e.*,

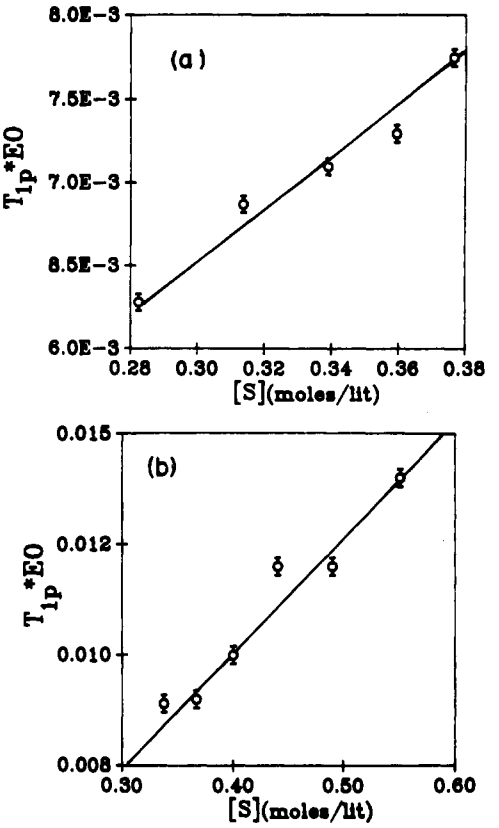

Figure 6. Plot of ^{15}N NMR $T_{1p}E_0$ ($Mn^{III}Mb$ concentration) vs S_0 (anion concentration) for (a) thiocyanate and (b) cyanide binding to $Mn^{III}Mb$.

$1/\tau_M \gg 1/T_{1M}$, for systems with a single binding site, eq 6 can be simplified as

$$\frac{1}{T_{1p}} = \frac{E_0}{K_D + S_0}\frac{1}{T_{1M}} \quad (7)$$

or

$$T_{1p}E_0 = K_D T_{1M} + S_0 T_{1M} \quad (8)$$

where E_0 is the $Mn^{III}Mb$ concentration and S_0 is the total anion concentration. T_{1m} and K_D can be obtained from the intercept and slope of the plot of $T_{1p}E_0$ vs S_0 in eq 8.[16] Plots for thiocyanate with the anion concentration fro 300 to 600 mM at pH 6.4 (E_0 = 4.6 mM) and for cyanide with anion concentration changing from 250 to 400 mM at pH 6.6 (E_0 = 0.62 mM) are shown in Figure 6a,b. The values of K_D obtained from Figure 6 are 15 $\pm$ 5 mM for SCN^- binding to $Mn^{III}Mb$ at pH 6.4 and 120 $\pm$ 5 mM for CN^- binding to $Mn^{III}Mb$ at pH 6.6. These values agree well with those obtained by the optical difference spectroscopic study described in the previous section.

The value of T_{1M} obtained from eq 8 can be used to determine the distance of the anion nucleus from the metal atom using the Solomon and Bloembergen equation[16,17]

$$\frac{1}{T_{1M}} = \frac{2\gamma_I^2 g^2 S(S+1)\beta^2}{15r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) + \frac{2S(S+1)A^2}{3\hbar^2}\left(\frac{\tau_c}{1+\omega_s^2\tau_e^2}\right) \quad (9)$$

where τ_c is the total correlation time, τ_e is isotropic exchange correlation time, A is the electron nuclear hyperfine coupling

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constant, γ_I is the magnetogyric ratio of the nucleus, β is the Bohr magnetron, ω_I and ω_s are the nuclear and electronic Larmor precession frequencies, S is the total electron spin on the paramagnetic center and r is the distance between the nuclear spin I (of the anion nucleus) and the electron spin on the metal ion. In the present case the hyperfine coupling interaction term can be neglected, since no hyperfine shift of the ^{15}N resonance in the NMR spectra was observed. For the manganese(III) ($S = 2$) heme protein with $SC^{15}N^-$ or $C^{15}N^-$, eq 9 can be simplified to[16-18]

$$\frac{1}{T_{1M}} = \frac{C^6}{r^6}\left(\frac{3\tau_c}{1+\omega_I^2\tau_c^2} + \frac{7\tau_c}{1+\omega_s^2\tau_c^2}\right) \qquad (10)$$

where $C = 356$ Å $s^{-1/3}$ for ^{15}N probe nucleus with manganese(III) ($S = 2$) as the paramagnetic metal center.

The value of total correlation time τ_c depends on the metal ion and the size of the protein. τ_c for $Mn^{III}HRP$ has previously been determined[4] to be 6×10^{-10} s. We have used the same value of τ_c in eq 10 to calculate the average distance of the anion from the metal ion in $Mn^{III}Mb$. The value of T_{1M} obtained from Figure 6 for $SC^{15}N$ binding to $Mn^{III}Mb$ was found to be 0.0248 s, and that for the CN^- binding to $Mn^{III}Mb$ was 0.0156 s. Using these values, average distances of the ^{15}N nuclei of the anions (SCN^- and CN^-) from the manganese(III) center were found to be 6.7 Å for $Mn^{III}Mb$–SCN and 6.2 Å for $Mn^{III}Mb$–CN species. The value of τ_c in $Mn^{III}Mb$ may be different from that of MnHRP. This uncertainty in the value of τ_c introduces possibility of some error in the distance parameters. This possible error will not however affect the qualitative nature of the conclusions arrived at here.

(e) Coordination Geometry of the Manganese Ion in $Mn^{III}Mb$. The coordination geometry of the metal ion in metalloproteins has important implication in their biochemical functions.[1,4,19] For example, it has been suggested that the peroxidase activity of HRP requires the sixth coordination site of heme iron to be vacant in the native protein. The stability of redox intermediates and peroxidase catalytic efficiency of metmyoglobin is severalfold less than that of HRP.[20] This may be related to the presence of axial water in metmyoglobin. $Mn^{III}HRP$ has been shown to have much lower activity. Recent NMR studies have shown that the sixth coordination site of manganese(III) in $Mn^{III}HRP$ is occupied by a water molecule.[14] In order to investigate the nature of water coordination and its effect on peroxidase activity in $Mn^{III}Mb$, we have determined water proton relaxation enhancement at two extreme pH values. Paramagnetic enhancement of water proton relaxation can be determined using eq 6. The value of T_{1M} can be determined from the molar relaxivity in the fast exchange condition (*i.e.* $1/\tau_M \gg 1/T_{1M}$) by[4,16]

$$\text{molar relaxivity} = \frac{1}{T_{1p}E_0} = \frac{1}{55.56T_{1M}} \qquad (11)$$

where E_0 is the concentration of $Mn^{III}Mb$. The values of T_{1M} obtained for water protons in the presence of $Mn^{III}Mb$ were 0.0131 and 0.0195 s at pH 4.0 and 9.6, respectively. The average distance of the water proton from the paramagnetic metal center was determined from T_{1M} using eq 10 with $C = 763$ Å $s^{-1/3}$. Distances of the water proton from the metal center were found to be 12.8 and 13.7 Å at pH 4.0 and pH 9.6, respectively. Although the distance parameters determined by NMR relaxation enhancement measurement become less reliable at larger r, these values give a lower limit to the distance of approach of water to the metal ion. The distance of water from metal ion in $Mn^{III}HRP$ have previously been reported to be 2.6 Å, indicating that water is axially coordinated to manganese in $Mn^{III}HRP$.[4] In the present system, the results indicate that water is not likely to be present in the first coordination sphere of manganese in $Mn^{III}Mb$. Optical spectra of $Mn^{III}Mb$ (Figure 1) also do not show any change ove rthe pH range 4–11, indicating absence of any aqua ⇌ hydroxo type equilibrium, which further supports that water is absent in the first coordination sphere of the metal ion in $Mn^{III}Mb$.

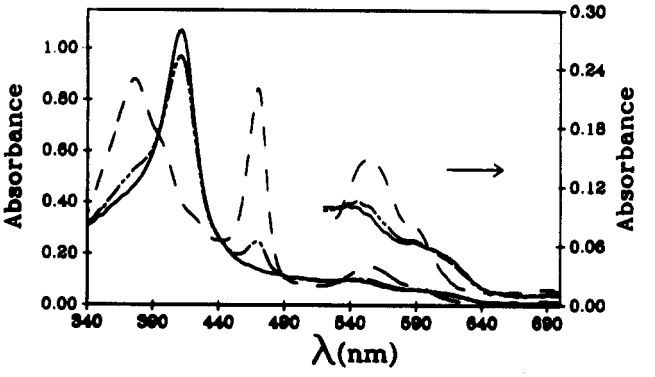

Figure 7. UV–vis spectra: (a) spectrum of $Mn^{III}Mb$ (35.4 μM) at pH 7.2 (– –), (b) spectrum immediately after addition of (~2 mM) H_2O_2 (—), (c) extrapolated spectrum (see text) of intermediate $Mn^{III}Mb$ compound I (– ·· –).

The effect of substitution of iron by manganese in HRP has previously been shown to cause a significant decrease in the activity of the reconstituted protein.[2] However, since the $Mn^{III}HRP$ has an axial water coordination to the metal ion, and water is not coordinated to Fe(III) in $Fe^{III}HRP$, the origin of the decrease in peroxidase activity in $Mn^{III}HRP$ might be 2-fold, *i.e.*, due to metal substitution and presence of axial water in the active site. In Fe(III) met myoglobin also, water is axially coordinated to the metal center, causing an order of magnitude decrease in peroxidase activity of metmyoglobin compared to HRP. Since in $Mn^{III}Mb$ there is no water ligation to the metal ion, the coordination geometry of the metal ion in $Mn^{III}Mb$ is comparable to that of native HRP.

(f) Oxidation of $Mn^{III}Mb$. It has been previously reported[2,21,22] that although the reaction of H_2O_2 with $Mn^{III}HRP$ gives a relatively stable $Mn^{III}HRP$ compound I intermediate, its rate of formation (1.3×10^4 M^{-1} s^{-1}) is much slower compared to that in native HRP (1.8×10^7 M^{-1} s^{-1}). Yonetani *et al.*[2] have investigated the reaction of H_2O_2 with $Mn^{III}Mb$ and reported that even a 10-fold excess of hydrogen peroxide could not oxidize $Mn^{III}Mb$. Initial experiments show that a large excess of H_2O_2 (~20-fold excess) gives a short-lived radical intermediate. We have investigated oxidation of $Mn^{III}Mb$ by H_2O_2 by steady-state absorption and stopped-flow kinetic methods and found that a very large excess (~500-fold excess or more) of H_2O_2 produced the short-lived redox intermediate, which subsequently decomposes to form porphyrin oxidation products. Figure 7 shows the effect of addition of H_2O_2 on the optical spectrum of $Mn^{III}Mb$. The spectrum of $Mn^{III}Mb$ taken immediately after addition of H_2O_2 shows a sharp decrease in absorbance of the 376- and 469-nm bands, which were originally assigned to the native $Mn^{III}Mb$, and a new band at 412 nm appears. The change in the optical spectrum in the visible region is also noticeable (Figure 7). However, this intermediate species subsequently decomposes to porphyrin ring oxidized products.

The extrapolated optical spectrum of the redox intermediate species were obtained by computer simulation of the spectrum of $Mn^{III}Mb$ obtained immediately after addition of H_2O_2 (Figure 7). The digitized absorbance values of the mixed species were fitted at different wavelengths (from 700 to 340 nm; step resolution

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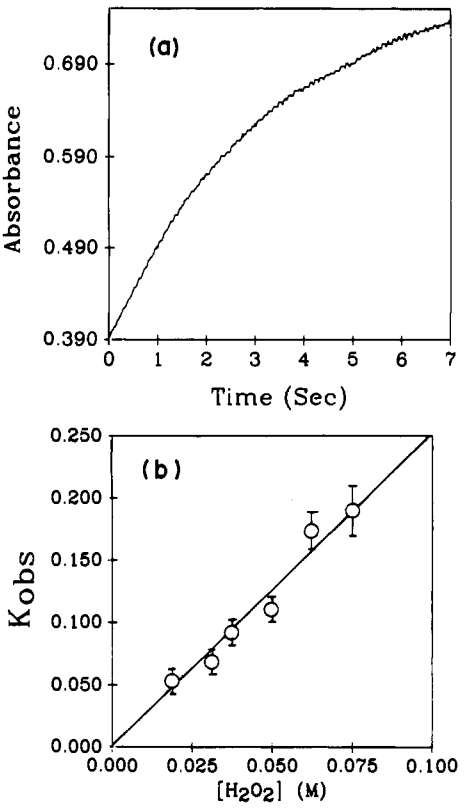

Figure 8. (a) Trace of the stopped flow absorbance response at $\lambda = 413$ with time. $[Mn^{III}Mb] = 30\ \mu M$, and $[H_2O_2] = 18$ mM at 25 °C. (b) Plot of the pseudo-first-order rate constant (K_{obs}) against $[H_2O_2]$ at pH 7.2.

3 nm) to eq 12, where A^i_{obs}, A^i_I and A^i_0 are the observed

$$A^i_{obs} = fA^i_I + (1-f)A^i_0 \qquad (12)$$

absorbance, absorbance of the intermediate $Mn^{III}Mb$ compound and absorbance of native $Mn^{III}Mb$ respectively at wavelength i; f is the mole fraction of the intermediate $Mn^{III}Mb$ compound. The concentration of the decomposed porphyrin ring product, which is likely to be very small (if present) at the time immediately after the addition of H_2O_2, has been neglected in eq 12. The best fit to the observed spectrum was obtained with $f = 0.97$, indicating that about 97% of the redox reaction was over during addition of H_2O_2 (on a steady-state experimental time scale). The extrapolated spectrum (Figure 7) obtained from the fit has close similarity with the optical spectrum of $Mn^{III}HRP$ compound I reported previously.[22] Thus, the species formed on treatment of H_2O_2 on $Mn^{III}Mb$ might be the intermediate compound I with Mn^{IV}=O—protein radical configuration similar to that found in case of $Mn^{III}HRP$.[22]

The rate of formation of this intermediate compound of $Mn^{III}Mb$ has been studied using a stopped-flow technique. Figure 8a shows the trace of the stopped-flow response as a function of time after addition of hydrogen peroxide to $Mn^{III}Mb$ solution. The pseudo-first-order rate constants (K_{obs}) were determined at 25 °C using 30 μM $Mn^{III}Mb$, and the concentration of H_2O_2 varied from 18 to 75 mM at pH 7.2. A plot of K_{obs} vs H_2O_2 concentration (Figure 8b) gave a straight line, and the apparent second-order rate constant (k_{app}) was found to be 2.5 $M^{-1}\ s^{-1}$. The rate of formation of the intermediate compound in $Mn^{III}Mb$ is thus several orders of magnitude slower than that in $Mn^{III}HRP$, and even more slower than that in native HRP.[2,21,22] Thus, although substitution of iron(III) by manganese(III) in metmyoglobin results in an active site coordination geometry as in native HRP, catalytic properties decrease drastically in the $Mn^{III}Mb$ system compared to HRP. It is, however, interesting to note that the ratio of the rate constant for formation of compound I in native HRP ($1.8 \times 10^7\ M^{-1}\ s^{-1}$) to that in iron(III) myoglobin ($1.3 \times 10^3\ M^{-1}\ s^{-1}$)[20] is similar to the ratio of the rate constants of $Mn^{III}HRP$ ($1.3 \times 10^4\ M^{-1}\ s^{-1}$)[2] and $Mn^{III}Mb$ ($2.5\ M^{-1}\ s^{-1}$). Also, the relative decrease in the rate constant on the replacement of Fe(III) by Mn(III) is similar in HRP and myoglobin. Thus, the peroxidase activity decreases (i) upon a change in the nature of the protein cavity from HRP to myoglobin, (ii) upon a change in metal ion from iron(III) to manganese(III) and (iii) upon the axial ligation of water to the metal ion. Our results suggest that the first effect is much more important than the other two effects, while the second factor is more significant than the third one.

4. Conclusion

Binding of inorganic anions such as SCN^- and CN^- to $Mn^{III}Mb$ has been studied by optical difference spectroscopy and ^{15}N NMR relaxation rate measurements. The pH dependence of K_D of the thiocyanate complex indicates that the binding of SCN^- is dependent on acid–base equilibria of more than one acid–base residue in $Mn^{III}Mb$. Competitive binding studies showed that thiocyanate and cyanide bind $Mn^{III}Mb$, probably at the same site, which is away from the metal center. The values of K_D for the thiocyanate and cyanide bindings to $Mn^{III}Mb$ were found to be comparable to those in case of $Mn^{III}HRP$ species. ^{15}N NMR relaxation studies showed that both thiocyanate and cyanide bind to $Mn^{III}Mb$ 6–7 Å from the metal center. Water proton relaxivity measurements show that the water is not coordinated to the manganese in $Mn^{III}Mb$, and the sixth coordination site is perhaps vacant. This makes the coordination geometry of the metal ion in $Mn^{III}Mb$ similar to that of native HRP, but different from both $Mn^{III}HRP$ and native Mb, where water occupies the sixth coordination site. It has been observed that, at very high concentrations of hydrogen peroxide, $Mn^{III}Mb$ can be oxidized to give a short-lived redox intermediate. The optical spectrum of this intermediate species is very similar to that of $Mn^{III}HRP$ compound I, indicating that they have similar electronic structures. The rate of formation of the redox intermediate in $Mn^{III}Mb$ is found to be much slower. The results indicate that the protein cavity around the active center has significant influence on the peroxidase activity of the heme protein. Metal substitution in myoglobin demonstrates that Fe(III) is unique for the catalytic activity of the enzyme, and substitution of the heme iron by manganese(III) causes a drastic decrease in the catalytic activity even though the coordination geometries and oxidation states of the metal centers are similar.

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