MANGANESE HEMOGLOBIN: ALLOSTERIC EFFECTS IN REDOX AND LIGATION EQUILIBRIA

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Summary. Allosteric effects have been observed in the binding of ligands to manganese substituted hemoglobin. Redox measurements indicate a preferred binding of organic phosphates to reduced manganese hemoglobin and the existence of a Bohr effect. The redox n-values, with and without organic phosphate, are similar to those of hemoglobin at all pH values. Differential pH titration of manganese hemoglobin and its nitric oxide derivative demonstrates the existence of both alkaline and acid Bohr effects in the binding of ligands.

Introduction. One of the most specific methods for examining the function of the heme iron in hemoglobin is to replace the iron with another metal. Cobalt-substituted hemoglobin (coboglobin) binds molecular oxygen and recently has been shown to exhibit the hemoglobin allosteric effects. Because the stereochemical properties of cobalt and iron differ, the retention of these effects has important implications with respect to the nature of the allosteric mechanism (1-4).

Manganese hemoglobin does not bind oxygen. Some physical properties of the protein in the divalent and trivalent states have been studied and measurements performed on mixed (Mn^{III}, Fe^{II}) hybrids (5-8). However, no attempt was made to determine whether the conformation of MnHb[2] is equivalent to that of Hb, or whether the conversion of MnHb to one of its derivatives involves a conformational change and allosteric interactions. We now report some equilibrium redox and ligation measurements which demonstrate that manganese hemoglobin retains the allosteric properties of hemoglobin.

Materials and Methods. Hemoglobin was prepared from horse blood (Grand Islands Biological Co., Madison, Wis.) or pooled fresh human blood (9). Globin

(1) Recipient of NIH Career Development Award.
(2) Abbreviations: Hb, ferrohemoglobin; MnHb, manganohemoglobin; Mn^{III}Hb, manganihemoglobin; Bis-Tris, bis-(2-hydroxyethyl)-imino-tris-(hydroxymethyl)methane; DPG; 2,3-diphosphoglycerate; IHP, inositol hexaphosphate.

was prepared by the method of Rossi-Fanelli et al. (10) with the addition of 1mM dithiothreitol to all solutions. $Mn^{III}Hb$ was prepared by the method of Yonetani (6), except that Bis-Tris·HCl replaced inorganic phosphate as buffer for convenience in observing the effects of organic phosphates. MnHb for pH titrations or kinetics measurement (11) was prepared from degassed solutions of $Mn^{III}Hb$ by dithionite reduction with subsequent passage through an anaerobic Sephadex G-25 column.

Optical spectra were recorded on Beckman ACTA III or Cary 14 spectrophotometers. Potentiometric and pH titrations are described elsewhere (unpublished).

Results

Spectral Properties. The Optical spectra of the human and horse manganese proteins are very similar. The "split Soret" peaks of human $Mn^{III}Hb$ occur at 468 and 373nm, $\varepsilon^{III}_{468}/\varepsilon^{III}_{373} = 1.0$. Upon reduction, a single Soret peak appears at 434nm for human MnHb with extinction 2.3 times greater than ε^{III}_{468}. Addition of NO to human MnHb causes a shift to 424 nm; $\varepsilon^{NO}_{424}/\varepsilon^{III}_{373} = 2.34$.

MnHb Redox. DPG binds more strongly to Hb than to Met-Hb because of conformational changes upon oxidation. Because of this differential binding, addition of DPG causes a change in the midpoint potential (E'_o) for Hb oxidation, or, equivalently, a change in the fraction oxidized (Y_{ox}) at constant potential (12,13). The similar effect is here reported for MnHb.

Figure 1a is the Soret spectrum of $Mn^{III}Hb$ ($Y_{ox} = 1.0$) in the presence of an approximately 10-fold excess (per Mn) of the redox dye toluidine blue-O (E'_o (7) = 0.035v) at pH 7.0. Figure 1b is the spectrum after addition of dithionite showing the presence of a small amount of MnHb ($Y_{ox} = 0.82$). At this point the dye is ~ 40% reduced and constitutes a redox buffer. Upon addition of DPG to 0.5 mM at the fixed potential (% reduced dye unchanged) the MnHb is further reduced ($Y_{ox} = 0.50$, Fig. 1c). Thus, at constant potential, the addition of DPG stabilizes MnHb over $Mn^{III}Hb$. From the change in Y_{ox} and the assumption that both MnHb and $Mn^{III}Hb$ can bind a single

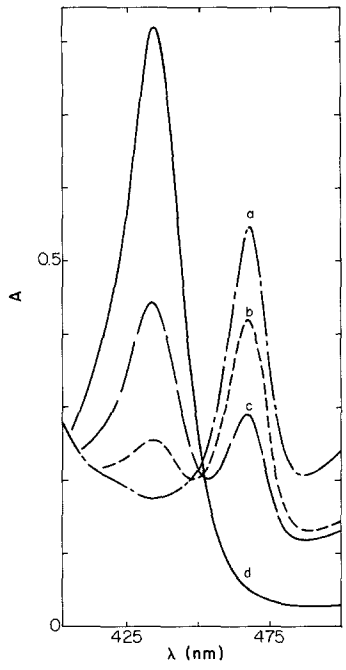

Figure 1. Redox Equilibrium of MnHb in 0.05 M Tris-HCl, pH 7.0 (25°) and in the presence of 10 fold excess toluidine blue-O: a) Fully oxidized (Y_{ox} = 1.0); b) Partially reduced (Y_{ox} = 0.82); c) After addition of DPG to 0.5 mM at constant potential; d) Fully reduced. Isosbestic points are neither seen nor expected because of absorption by toluidine blue-O.

DPG, we may estimate (14) that the ratio of DPG dissociation constants is $K_{DPG}^{Mn^{III}}/K_{DPG}^{Mn^{II}} \approx 4$, quite comparable to the value for Hb itself (12). Addition of DPG to partially reduced manganese myoglobin has no effect.

Potentiometric titrations of Mn^{III}Hb by dithionite extend the above observation. Symmetrical redox curves were obtained, giving linear plots of $\log [Y_{ox}/(1-Y_{ox})]$ vs. E. Values for E_o' and the redox n-value are given in Table I. E_o' for MnHb near neutral pH is more than 0.1 v less positive than that for Hb, as previously observed (5). The ready oxidation of MnHb by oxygen is not primarily due to this fact, since E_o' for MnHb is similar to that for the stable oxygen-carrier myoglobin.

At pH 6.7, the redox n-value for MnHb is the same as that for Hb both in the presence and absence of IHP (Table I). Of particular interest is the partial "cooperativity" (n > 1) in the absence of IHP. The positive shift

Table I

Comparison of Hb and MnHb Redox Properties

	pH = 6.7[b]		pH = 8.5[c]		$\Delta E_o'$
	E_o' (v)	n	E_o' (v)	n	
MnHb, [IHP] = 1 mM	+0.080	(0.93)	+0.030	(1.7)	-0.050
[IHP] = 0	+0.040	(1.40)	-0.002	(1.96)	-0.042
Hb,[a] [IHP] = 1 mM	+0.192	(0.94)	+0.095	(2.0)	-0.097
[IHP] = 0	+0.162	(1.53)	+0.072	(2.1)	-0.090

a) Ref. 10. All Hb in 0.1 M KCl, T = 25°C.

b) MnHb in 0.05 M Bis-Tris · HCl + 0.065 M NaCl, T = 24°C.

c) MnHb in 0.1 M Tris · HCl + 0.04 M NaCl, T = 24°C.

of E_o' caused by IHP (Table I) is the expression of the preferential binding of IHP to MnHb.

Hb demonstrates an oxidation Bohr effect at alkaline pH (decrease of E_o' with pH) which is also found in MnHb, but with a decreased magnitude (Table I). This decrease is predictable from our observation that $Mn^{III}Hb$ does not exhibit a metal-linked ionization in the pH region studied. It is important to note that the increase of the redox n-value for MnHb at elevated pH appears very similar to that for Hb.

Ligand Binding to MnHb. The affinity of Hb for ligands is pH-dependent, and Hb and liganded Hb exhibit different pH titration curves. These two aspects of the Bohr effect are linked by the equation due to Wyman: $\overline{\Delta H^+} = - \frac{d \log p_{1/2}}{d\ pH}$, where $4(\overline{\Delta H^+})$ is the number of titratable protons per deoxy tetramer which are released upon ligation at constant pH and $p_{1/2}$ is the

half-saturation pressure of the (gaseous) ligand (see ref. 9, p. 179-184).

Nitric oxide binds to MnHb, but too tightly for a convenient measurement of the equilibrium. We have therefore examined the Bohr effect in NO binding to MnHb by measuring the change in proton binding to MnHb upon ligation. Figure 2 is a computer-smoothed fit to the pH dependence of $\overline{\Delta H}^{+}$ for NO binding to MnHb. Because substantial corrections are required for the presence of $Mn^{III}Hb$, the results in Fig. 2 must be considered as preliminary, but they are quite comparable to similar titrations for Hb.

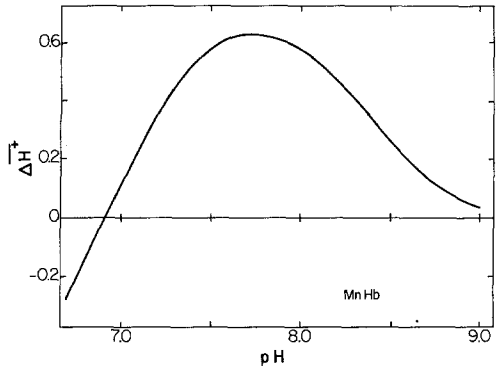

Figure 2. Protons released per manganese porphyrin, $\overline{\Delta H}^{+}$, by MnHb upon binding NO.

By use of Wyman's equation, the positive value of $\overline{\Delta H}^{+}$ at alkaline pH shows that log $p_{1/2}$ decreases with increasing pH, indicating the existence of the alkaline Bohr effect. The negative value of $\overline{\Delta H}^{+}$ at lower pH demonstrates the existence of the acid Bohr effect (9). Integrating the curve in Fig. 2, we find that the pH dependence of the affinity of MnHb for NO follows the change of affinity of Hb for ligands (unpublished).

Discussion. Despite the fact that MnHb cannot reversibly bind molecular oxygen the preliminary results presented here indicate that the equilibrium allosteric properties of Hb are retained when manganese is substituted for iron. These results plus kinetic and sedimentation measurements (11) demonstrate that MnHb undergoes conformational changes upon binding of

ligands equivalent to those of Hb (15), showing once again the flexibility of the allosteric mechanism for Hb.

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