Redox equilibria of liganded forms of methemoglobin

(Hill equation/allosteric transition theory/tetramer-dimer equilibrium)

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Communicated by I. M. Klotz, June 16, 1975

ABSTRACT We have examined the redox equilibria of azidomethemoglobin (low-spin) and fluoromethemoglobin (high-spin). We have derived a modified Hill equation which includes the tetramer-dimer equilibrium of the oxidized form, and also generalized the two-state model to incorporate ligand binding to the ferrirheme. The pH dependence of the redox Hill's constant for fluoromethemoglobin is the same as that for methemoglobin, demonstrating that this dependence and the marked cooperativity achieved (n = 2.2) are not coupled to changes of the ferrirheme spin state. The redox Hill's constant for azidomethemoglobin, however, is as large as the oxygenation Hill's constant (n ≈ 2.7) and is also roughly pH independent.

A chief source of the ongoing interest in ligand binding to hemoglobin (Hb) rests in the allosteric properties which result from its tetrameric nature (1, 2). In constructing a detailed scheme for the ligation process of Hb, the question can be formulated as to the existence and nature of an "allosteric trigger," (3-7), the mechanism by which the ligation (or oxidation) of five-coordinate, ferriheme induces conformational changes within the protein.

The Hb tetramer can be regarded as having essentially two quaternary structures, T (unliganded) and R (liganded) (8-11). The most widely held mechanism for controlling the conformational equilibrium between R and T has been proposed by Perutz (3-7). Following suggestions of Williams (12) and Hoard (13, 14), it includes a proposed coupling between protein conformation and the spin-state of iron: the five-coordinate high-spin (hs) Fe(II) of an unliganded heme appears to lie about 0.7 Å out of the mean porphyrin plane, and is considered to be held in a stressed state by tension exerted through the protein. Upon oxygenation, the Fe atom becomes low-spin (ls) and appears to move into the porphyrin plane. The resultant movement then couples to the protein through motion of the proximal histidine.

The cooperativity of the redox reaction varies markedly with pH and with the presence of organic phosphates (15-17), in contrast to the virtually constant cooperativity of ligation (1, 2). Methemoglobin (met-Hb), however, also exhibits a tertiary structure variability which is spin-state related (3-7): the high-spin Fe(III) in aquometh-Hb is slightly out-of-plane, and thus in an intermediate structure, while the Fe(III) in hydroxymet-Hb is substantially low-spin and should more nearly lie in the porphyrin plane. It has been suggested the pH dependence of the redox cooperativity has two causes, the pH dependence of the met-Hb spin state and of salt bridges between the subunits (3-7, 17).

Although a spin-state coupling contribution is quite appealing in its directness and simplicity, our results for oxygenation of coboglobin (cobalt-substituted Hb) and the redox equilibrium of manganese-substituted hemoglobin nevertheless suggested that Hb cooperativity is not coupled to the spin-state of either unliganded (18-22) or oxidized (23, 24) forms. Evidence against stress in CoHb has also been presented (25-28). Similarly, Edelstein and Gibson (29) conclude that the conformation and spin-state of met-Hb are uncoupled.

We now directly address the question of the coupling of spin-state and cooperative redox behavior by examining the redox equilibria of fluoromet-Hb, and azidomet-Hb, as well as of met-Hb. In this way we examine the influence of pH and organic phosphate binding on a system in which the Fe(III) is either constrained to remain high spin through binding of $F^-$, or low-spin through binding of $N_3^-$.

MATERIALS AND METHODS

Hemoglobin was prepared (30) from pooled human blood samples; carboxy-HbAo was isolated on carboxymethyl cel- lulose (31), concentrated by pressure dialysis, and stored under CO at 4°. Met-Hb was prepared by exchanging O2 for CO, adding 3 equivalents of K3Fe(CN)6, and desalting on a column of Rexyn-I-300 (Fisher). The resultant met-Hb was judged "unperturbed" by the criterion of Cameron (32). Titrations were performed on a known volume of deoxygenated solution (about 15 ml) transferred under Ar to a stirred, thermostatted (24.4°) titration cell mounted in a Beckman Acta III spectrophotometer. Details of the measurements will be published elsewhere.

The degree of protein oxidation was followed in the Soret region, the total heme concentration [h] typically being 11 μM. Because of this low concentration, redox dyes were added to be about 30 μM, thus acting as mediator and redox potential buffer. Mediator concentrations much lower than this resulted in an excessive equilibration time for liganded met-Hb. Monitoring both the absorbance and potential showed that met-Hb reached equilibrium within minutes; about an hour or more was needed in the fluoromet- and azidomet-Hb systems, but near equilibrium the potential was normally stable to better than $10^{-2} \text{mV/min}$. The reductant used in all these titrations was benzyl viologen, reduced with H2 using platinized alumina as catalyst. Performing oxidative titrations on hydroxymet, fluoromet- and azidomet-Hb immediately after a reductive titration proved that the titrations are reversible. A fresh, degassed ferricyanide solution was used as oxidant. These controls were performed at pH 9 where there is no binding of the ferricyanide produced (17).

Mediators employed were thionin, Color Index no. 52000, methylene blue, C.I. no. 52015, and methylene green (nitro-

Abbreviations: [h], heme concentration; hs, high-spin; ls, low-spin; Y, fraction oxidized hemes; IHP, inositol hexaphosphate; $K_{42}$, liganded-Hb tetramer-dimer equilibrium constant; I, ionic strength.

* M. F. Perutz, private communication.
methylene blue, C.I. no. 52020. The latter was purified from residual methylene blue by thin-layer chromatography in 0.01 N HCl. The strong red absorbance of an oxidized mediator was used to calculate corrections for the small absorbance in the Soret region and also to measure the dye redox potential. The presence of the protein was shown to cause no shifts in dye potential, indicating no binding to the protein. The agreement of our redox data for met-Hb with published values further validates the technique (n.f.).

Inert gases used, Matheson ultra high purity nitrogen and argon, were passed across activated Ridox (Fisher Co). Glass-covered magnetic stirrers proved necessary, since the oxygen dissolved in a Teflon stirrer precludes stable potentials.

**RESULTS**

**Met-Hb.** The redox equilibrium of met-Hb has been reexamined. Fig. 1 presents typical plots of log \( Y/(1 - Y) \) versus cell potential, \( E \); \( Y \) is the oxidized fraction of the total heme concentration. The midpoint potentials observed for all experimental conditions employed are listed in Table 1.

A redox \( n \)-value, equivalent to \( n \) of the Hill equation for ligation may be obtained from the slopes of such plots through the formal equivalence (33) of the potential \( E \), and a pressure (fugacity) of "positive electrons" or holes (\( e^+ \)),

\[
\ln[P,+] = EF/RT \tag{1}
\]

Through this relation the analogy between the ligand binding and redox equilibria of Hb is emphasized, and the latter may formally be written as

\[
\text{Hb, } e^+ \leftrightarrow \text{Hb,}^+\]

**FIG. 1.** Log \( \Omega \) versus cell potential, \( E \), in V, for (A) met-Hb and (B) azidomet-Hb redox equilibria; \( \Omega = [Y/(1 - Y)] \), open symbols, or \( \Omega = [Y/(1 - Y)] F(Y,k) \), closed symbols, where \( F(Y,k) \) is given by Eq. 6; pH 9 (\( \Delta \Delta \)); pH 6.65, [IHP] = 0 (\( \bullet \)); pH 6.65, [IHP] = 10^{-3} M (\( \square \)). Buffer concentrations are: pH 6.65, [bis(2-hydroxyethyl)iminoino-tris(hydroxymethyl)methane] = 0.04 M; pH 9, [Tris] = 0.10 M. For azidomet-Hb, (B), sufficient NaCl was added such that [NaN] = 7 \times 10^{-4} M. Sufficient NaCl was added to make ionic strength \( I = 0.1 \). Temperature = 24.4°C.

where Hb, and Hb^+ represent unliganded and oxidized tetrameric hemoglobin, respectively.

However, in the absence of inositol hexaphosphate (IHP) the plots in Fig. 1 are curved in the region \( Y < 0.5 \). This is caused by dissociation of tetrameric met-Hb into dimers. [Dissociation of deoxy-Hb can be neglected (34).] Protein concentrations were low, typically [\( h \)] = 11 \( \mu \)M. During the course of a reduction, the concentration of met-Hb, considered as tetramers, becomes of the order of and finally less than the tetramer-dimer dissociation constant of met-Hb, \( K_{4,2} \). Under these conditions, simple mass-action considerations show that dimerization of the oxidized form will cause curvature as seen in Fig. 1. This interpretation is confirmed by the linear plots in the presence of IHP, for the binding of IHP to met-Hb appreciably reduces \( K_{4,2} \) (3–7, 23, 24, 35).

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**Table 1. Midpoint potentials and redox Hill constants for liganded forms of methemoglobin**

<table>
<thead>
<tr>
<th>Conditions*</th>
<th>Observed†</th>
<th>Dimer corrected‡</th>
</tr>
</thead>
<tbody>
<tr>
<td>pH</td>
<td>IHP (mM)</td>
<td>( E'_o ) (V)</td>
</tr>
<tr>
<td>met-Hb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.65</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>6.65</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>7.95</td>
<td>0.3</td>
<td>1</td>
</tr>
<tr>
<td>8.94</td>
<td>0.1</td>
<td>—</td>
</tr>
<tr>
<td>Fluoromet-Hb</td>
<td></td>
<td></td>
</tr>
<tr>
<td>([F^-] (M))</td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.20</td>
<td>6.83</td>
<td>0.24</td>
</tr>
<tr>
<td>0.20</td>
<td>6.84</td>
<td>0.24</td>
</tr>
<tr>
<td>0.3</td>
<td>8.15</td>
<td>0.32</td>
</tr>
<tr>
<td>0.3</td>
<td>8.05</td>
<td>0.32</td>
</tr>
<tr>
<td>0.21</td>
<td>9.02</td>
<td>0.23</td>
</tr>
<tr>
<td>0.42</td>
<td>9.07</td>
<td>0.44</td>
</tr>
<tr>
<td>Azidomet-Hb§</td>
<td></td>
<td></td>
</tr>
<tr>
<td>6.67</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>6.72</td>
<td>0.1</td>
<td>1</td>
</tr>
<tr>
<td>8.93</td>
<td>0.1</td>
<td>1</td>
</tr>
</tbody>
</table>

* Buffers as in caption to Fig. 1.
† Observed values obtained as described in text; results in the presence of IHP are least-squares values.
‡ Values obtained from least-squares fitting to Eq. 5 with \( K_{4,2} = 1.5 \times 10^{-4} \) M.
§ \([N_3^-] = 7 \times 10^{-4} \) M.
By employing the slopes of the upper, linear portions of the redox curves (Fig. 1) n-values listed in Table 1 are obtained. Results for both observed $E'_0$ and $n$ are in substantial agreement with those of previous workers (15-17).

In order to quantitatively incorporate the tetramer-dimer equilibrium into a description of a redox titration we follow the procedures of Clark (36) to obtain modified expressions which, however, are still within the spirit of the Hill equation. The redox analogue of the Hill equation is (24.4)

$$E = E'_0 + \frac{0.0592}{n} \log \left( \frac{[Hb^+]}{[Hb]} \right)$$  \[2\]

where \([Hb]\) and \([Hb^+]\) are the concentrations of tetrameric deoxy-Hb and met-Hb, respectively. The midpoint potential is $E'_0$ and $n$ is the Hill’s constant, used as an index of cooperativity. This equation typically describes the redox properties of Hb quite well; reported discrepancies are attributable to interference by binding of ferrocyanide to met-Hb (17). When $K_{4,2}$ is neglected, the logarithmic term in Eq. 2 is just $\log (Y/(1-Y))$.

If dimer formation is included, the reaction scheme becomes (37, 38)

$$[Hb] \xrightleftharpoons{[^{+*}_{E'_0}]} [Hb^+] \xleftarrow{K_{4,2}} 2[Hb^+]$$  \[3\]

where \([Hb^+]\) represents the concentration of met-hb dimers. If we set $S_0$ as the total concentration of oxidized hemes, then to apply Eq. 2 we must solve the following equation for \([Hb^+]\):

$$S_0 = 4[Hb^+] + 2[Hb_0^+] = [Hb^+] + \sqrt{K_{4,2}[Hb^+]}$$  \[4\]

Substituting for \([Hb^+]\) in Eq. 2 and rewriting in terms of $Y$ we obtain

$$E = E'_0 + \frac{0.0592}{n} \log \left( \frac{Y}{1-Y} \right)$$  \[5\]

where we define $k = 4K_{4,2}/[h]$ and the correction factor due to dimer formation:

$$F(Y,k) = \left( 1 + \frac{k}{Y} \right)^2 - \sqrt{\frac{k}{Y}} = \frac{4[Hb^+]}{S_0}$$  \[6\]

Fig. 1 also displays the redox equilibria for met-Hb in the absence of IHP, plotted as $\log ([Y/(1-Y)]F(Y,k)$ versus $E$; the value $K_{4,2} = 1.5 \times 10^{-6}$ M was employed (39). Straight lines are obtained, as expected from Eq. 5. The dimer-corrected Hill’s constants, $n$, and midpoint potentials, $E'_0$ obtained from these plots (Table 1) are identical with the results of Kilmartin (17), and very similar to those of Brunori et al. (16). In the presence of IHP, $K_{4,2}$ is about $10^{-9}$ M (29) and inclusion of dimer formation has no appreciable effect, since $F(Y,k) = 1$ for all appropriate values of $Y$ and $[h]$.

In summary, at pH 6.65, where the principal oxidized form is aquomet-Hb, the redox $n$-value is low and is decreased to less than 1 by the binding of IHP. At pH 9, hydroxymet-Hb is the principal oxidized species, and the reaction is markedly cooperative ($n > 2$). IHP does not bind at pH 9 (17). At the PK for the aquomet- to hydroxymet-Hb transition (pK = 8.1) (1, 2), the maximal redox cooperativity has already been obtained. Neither these conclusions nor any presented below depend significantly on the correction for dimer formation (Table 1).

For direct comparison with the results for fluoromet-Hb, experiments were also performed at $I = 0.30$. No significant changes in $n$-value were observed; the midpoint potentials are slightly higher (Table 1).

**Fluoromet-Hb.** The redox equilibrium of fluoromet-Hb ($[F^-] = 0.3$ M) has been measured. Observed midpoint potentials and redox $n$-values obtained directly from plots of $\log ([Y/(1-Y)]$ versus $E$, as described above, are listed in Table 1. As with met-Hb, the redox $n$-value at pH 6.65 is low and is depressed to less than unity by IHP. However, as the pH is increased, we still find marked cooperativity ($n > 2$). The shapes of the plots of $\log ([Y/(1-Y)]$ versus $E$ are quite similar to those for met-Hb (Fig. 1), exhibiting curvature for $Y < 0.5$ in the absence of IHP. Applying Eq. 5 to correct for dimer formation, again using $K_{4,2} = 1.5 \times 10^{-6}$ M, also gives linear plots of $\log ([Y/(1-Y)]F(Y,k)$ versus $E$. The corrected values, $n$, and $E'_0$, are listed in Table 1.

It is possible to examine the relationship between the spin state of the oxidized form of Hb and the observed pH dependence of the redox cooperativity. For met-Hb the ratio of high-spin to low-spin Fe(III) can be calculated using the value $pK = 8.1$ for the aquomet-Hb to hydroxymet-Hb transition (1, 2); around 20°, aquomet-Hb is 100% hs; hydroxymet-Hb is about 50% hs, 50% ls (40, 41,1). At pH 6.65 the fraction of hs oxidized hemes is about 97%; the cooperativity is low ($n = 1.6$ in the absence of IHP). At pH 8.1, where the cooperativity is already high ($n > 2$) there are $\approx 75$% hs hemes, and at pH 9 ($n > 2$) only $\approx 54$% hs.

The redox properties of Hb in the presence of $F^-$ (Table 1) can be considered in this light, using equilibrium constants obtained from Beeston-educ (42, 43); fluoromet-Hb is 100% hs (40, 41,1). At pH 6.65 and $[F^-] = 0.3$ M the composition of oxidized hemoglobin is 93% fluoromet-Hb, 6% aquomet-Hb, and 1% hydroxymet-Hb, or >99% high spin; the low cooperativity ($n = 1.6$) is identical to that in the absence of $F^-$.

At pH 9, the binding constant for $F^-$ is substantially reduced, but formation of fluoromet-Hb still causes a substantial increase in the fraction of hs oxidized hemes. For $[F^-] = 0.4$ M the fractions are 65% fluoromet-Hb, 3% aquomet-Hb, and 32% hydroxymet-Hb, or 84% high spin. This composition is nevertheless associated with marked cooperativity ($n > 2$).

The experiment at pH 8.1 is even more instructive. At this pH the $F^-$ binding constant is sufficiently high that for $[F^-] = 0.3$ M the composition of oxidized Hb is 88% fluoromet-, 6% aquomet-, and 6% hydroxymet-Hb, giving 97% high spin oxidized hemes. From the results at pH 6.65 in the absence of fluoride, a value $n \approx 1.6$ might be expected, whereas $n = 2.2$ (Table 1). Similarly, in the presence of IHP, $n = 1.7$, rather than a value less than unity. Thus, the pH dependence of $n$ in the presence of $F^-$, a high-spin ligand, exactly mimics that for met-Hb, and the change in cooperativity with $F^-$ exhibited by met-Hb cannot be correlated with the parallel change in ferriheme ligation and spin-state.

**Azidomet-Hb.** Fig. 1 also presents some plots, and Table 1 observed midpoint potentials and $n$-values for the redox equilbria of azidometoglobin at saturating (42, 43) values of $[Na^-]$. Correction for dimerization, also using $K_{4,2} = 1.5 \times 10^{-6}$ M, again removes asymmetry in the titration curve (Fig. 2), and the corrected redox parameters also appear in Table 1.

The transition from hemoglobin to azidometoglobin occurs with the full cooperativity of oxygenation: $n = 2.7$. In contrast to the other methemoglobin derivatives studied, the

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1 Note, that by not choosing to discuss the reported $n = 2.5$ for hydroxymet-Hb (16), we present the maximal increase in $n$-value.
n-value is relatively independent of pH. Furthermore, upon addition of IHP to azidometoglobin at pH = 6.65, the cooperativity is lowered only somewhat, n = 2.1. This effect of IHP, similar to that observed (44) in oxygenation equilibria, also differs from the other met-Hb redox reactions studied. Thus, the redox equilibrium between Hb and azidomet-Hb (≥90% is) completely mimics the oxygenation equilibrium of Hb.

Note also that the shift in midpoint potential on addition of IHP is much larger for azidomet-Hb (ΔE0' = 53 mV) than for fluoromet- and aquomet-Hb (ΔE0 = 30 mV). Since this shift is a function of the ratio of the IHP binding constants to deoxy-Hb and to the oxidized form, the binding of IHP to azidomet-Hb is thus seen to be much weaker than to fluoromet- or aquomet-Hb.

Theoretical. The redox equilibrium between Hb and aquomet-Hb can be described by a simple expression for the equilibrium between Hb and azidomet-Hb.

Y = \frac{α_0(1 + α_0) + Lc(H_2O)α_0(1 + c(H_2O)α_0)^3}{(1 + α_0)^4 + L(1 + c(H_2O)α_0)^4} \tag{7}

where α0 = [P+] / [K] is the normalized "ligand" concentration of P+, with the ligand in this case an "electron-hole" plus a water molecule, and K is the intrinsic dissociation constant for the R-state; L is the allosteric equilibrium constant, L = [T] / [R]; and c(H2O) is the ratio of intrinsic "ligand" dissociation constants for the aquomet-Hb R and T states. The same value of L applies to redox and oxygenation.

When the metal-bound water of aquomet-Hb is in equilibrium with the ferriheme ligands the form of Eq. 7 is retained with precisely the same value for L, provided that α0 and c(H2O) are replaced by:

α = \frac{[P^+]}{K_R} \left(1 + \sum \lambda_i\right) \tag{8a}

c = c(H_2O) \left[\frac{1 + \sum c_i^+ \lambda_i}{1 + \sum \lambda_i}\right] \tag{8b}

where λi = [li] / kr is the normalized concentration for the ferriheme-ligand, l = OH-, N3-, F-, etc., and kr is the intrinsic dissociation constant for li from an R-state ferriheme; c_i^+ is the ratio of the intrinsic dissociation constants for li from R and T state ferrihemes, c_i^+ = kr / kc. Chain nonequivalence in Hb redox has been stressed by Edelstein and Gibson (29), and these considerations lead to a straightforward, but unnecessary for purposes of qualitative discussion.

A value of c_i^+ < 1 indicates that li preferentially binds to an R-state ferriheme and correspondingly, that the R-state is stabilized through binding li. Since α merely sets the scale of redox potentials, the binding of ligands to the ferriheme of oxidized-Hb thus alters the observed cooperativity (n) solely through changes in c. In the presence of saturating amounts of li (λi, c_i^+λi ≫ 1), the redox couple between Hb and li-met-Hb, with c(li) = c(H2O)c_i^+. The redox equilibrium between Hb and azidomet-Hb (saturating N3-) completely mimics the Hb oxygenation equilibrium, and thus may be described by Eq. 7 using the values of L versus pH and the value of c determined for oxygenation (29); thus c(N3-) = c(H2O)cN3^+ = 0.01. This result also indicates that chain differences observed in aquomet-Hb are suppressed in azidomet-Hb.

On the other hand, when the decrease in L with pH is taken into consideration (pH 7.0, log L = 5.7; pH 9.0, log L = 3.2) the changes in n with pH for both met-Hb and fluoromet-Hb can be roughly matched (see Fig. 2 of ref. 9) by Eq. 7 assuming c(H2O) = c(F^-) = 0.03-0.05, constant with pH. Thus c_i^+ = 1, and fluoride ion has equal affinity for R and T-state high-spin ferriheme. Using this value of c(H2O), and taking c(N3-) = 0.01, in turn gives cN3_i^+ = 0.3-0.5. Thus azide has roughly a 2- to 3-fold stronger binding to a ferric T-chain than to a ferric T-chain. Such a value of cN3_i^+ < 1 explains (45) the cooperative binding of azide to aquomet-Hb.

Now the above discussion of met-Hb and fluoromet-Hb neglects the contribution to Eq. 8b from the presence of low-spin hydroxymet-Hb. In fact, the above assumption of constant c (29, 45) implicitly assumes that the observed pH dependence of n for hydroxymet-Hb is independent of spin state. It seems plausible from the discussions of Szabo and Karplus (45) and (a) all high spin ligands have similar values: c(he) ≈ c(H2O) and thus c_i^+ = 1; and (b) all low spin ligands have similar values: c(l) ≈ c(N3-), and thus c_i^+ = 0.3-0.5. Thus, increasing the amounts of hydroxymet-Hb (50% is) should act to lower c (Eq. 8b).

We have here shown that the increase in met-Hb redox cooperativity to n = 2.2 at high pH, although paralleled by a change in heme ligation (H2O → OH-) and a partial change to a low-spin form, is independent of these changes and can be observed when they are suppressed: virtually 100% high-spin, fluoromet-Hb exhibits marked cooperativity with n > 2; manganese(III) hemoglobin, which has a high-spin (d^5) Mn(III) ion and no heme-linked ionization, exhibits a pH dependence of n (23, 24) which precisely follows that of met-Hb and of fluoromet-Hb. The increase thus appears to reside in a pH dependence of the allosteric equilibrium and/or a decrease in chain nonequivalence with increasing pH.

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On the other hand, n = 2.2 appears to be the maximum obtainable with the high-spin forms, whereas azidomet-Hb exhibits n = 2.7. Whether this modest increase is due to an effect of spin-state change on the position of the proximal histidine, to other ligand-induced changes in heme conformation (47), or whether it is due to a distal interaction of the N3- ligand and to an abolition of chain differences in the R-state cannot be inferred from our present data. However, results for coboglobin (18-20, 25) and other studies (29) of Hb redox do not support a simple coupling between spin-state, the position of the heme-linked histidine, and cooperativity.

The present study does demonstrate the way in which ferriheme ligands can alter the redox n by changes in c. With c(he) ≈ 0.03-0.05, oxidation of an R-state chain to a high-spin ferriheme is more favorable than a similar oxidation of a T-state chain by RT ln [c(he)] ≈ 2.0 kcal/M per heme (20°). From c(he) = 0.01, oxidizing an R-state chain to a low-
spin ferriheme is \( \approx 2.8 \) kcal/M (20°) more favorable than oxidizing a T-chain. Thus, a change in \( c \) which increases the maximum attainable value of \( n \) from 2.2 to 2.7 corresponds to a free energy per heme of \( RT \ln (\text{cub}^\text{a}) \approx 0.8 \) kcal/M per heme.

We thank Prof. Paul A. Loach for helpful discussions and Prof. Allen S. Husey for the gift of platinized alumina. C.B. is a predoctoral trainee of USPHS, NIH, Training Grant GM00626. This work was supported by the NIH, Grant HL-13351, the donors to the Petroleum Research Fund, administered by the American Chemical Society, and NSF Grant BMS-00478. B.M.H. is an NIH Career Development Awardee.