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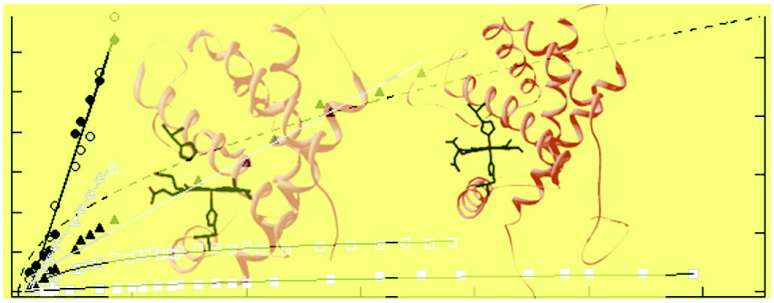

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Bis-Histidyl Hexacoordination in Hemoglobins Facilitates Heme Reduction Kinetics

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Abstract: Hexacoordinate hemoglobins are a class of proteins that exhibit reversible bis-histidyl coordination of the heme iron while retaining the ability to bind exogenous ligands. One hypothesis for their physiological function is that they scavenge nitric oxide, a reaction that oxidizes the protein and requires reduction of the heme iron to continue. Reduction kinetics of hexacoordinate hemoglobins, including human neuroglobin and cytoglobin, and those from *Synechocystis* and rice, are compared to myoglobin, soybean leghemoglobin, and several relevant mutant proteins. In all cases, bis-histidyl coordination greatly increases the rate of reduction by sodium dithionite when compared to pentacoordinate hemoglobins. In myoglobin and leghemoglobin, reduction is limited by the rate constant for electron transfer, whereas in the hexacoordinate hemoglobins reduction is limited only by bimolecular binding of the reductant. These results can be explained by differences in the reorganization energy for reduction between hexacoordinate and pentacoordinate hemoglobins.

Introduction

The heme prosthetic group confers the ability to catalyze reactions ranging from electron and ligand transport to transcriptional regulation in many different proteins.[1−4] The iron in most heme proteins exists predominately in the ferrous (Fe^{2+}) or ferric (Fe^{3+}) oxidation state, although a ferryl (Fe^{4+}) intermediate is observed in some reaction cycles. Oxygen and carbon monoxide bind hemoglobins (Hbs) only in the ferrous state, whereas several anions bind only the ferric protein. Nitric oxide, cyanide, imidazole, and other ligands can bind both the ferrous and ferric forms with varying affinities. The major role of red blood cell Hb as well as myoglobin (Mb) is oxygen transport, and conditions exist in vivo to ensure that the vast majority of these molecules remain in the ferrous form.[5]

In addition to oxygen transport, another central role for Hbs is nitric oxide regulation. This is due to the fact that red blood cell Hb and Mb scavenge this chemical messenger through a common reaction shown in Figure 1.[6−9] A related observation is that bacterial and yeast flavohemoglobins use this reaction to destroy NO in defense of oxidative bursts.[10,11] This reaction cycle is completed in the flavohemoglobins by a reductase domain that rapidly transfers electrons from NADPH to the ferric heme iron. In addition, it has been suggested that *Ascaris* Hb deoxygenates its surroundings by producing NO specifically to catalyze oxygen destruction, although no specific reductase or reduction mechanism has yet been identified in this system.[12]

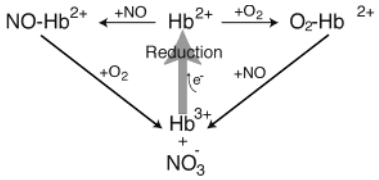

Figure 1. NO-dioxygenase reaction. Ferrous Hb can bind either O_2 or NO, which cna then react with the other ligand to form ferric Hb and NO_3-. The reaction continues to cycle only if the oxidized Hb is reduced.

It is often difficult to assign a mechanism for reduction of many Hbs. Researchers have therefore used chemical reductants, such as sodium dithionite (DT, $S_2O_4^{2-}$), to investigate reduction mechanisms and generate reduced Hbs for use in other reactions.[13,14] Although DT is widely used as a reductant, it has been used in surprisingly few studies investigating mechanisms of Hb reduction. Lambeth and Palmer[14] investigated these reactions in detail and reported reduction kinetics for several

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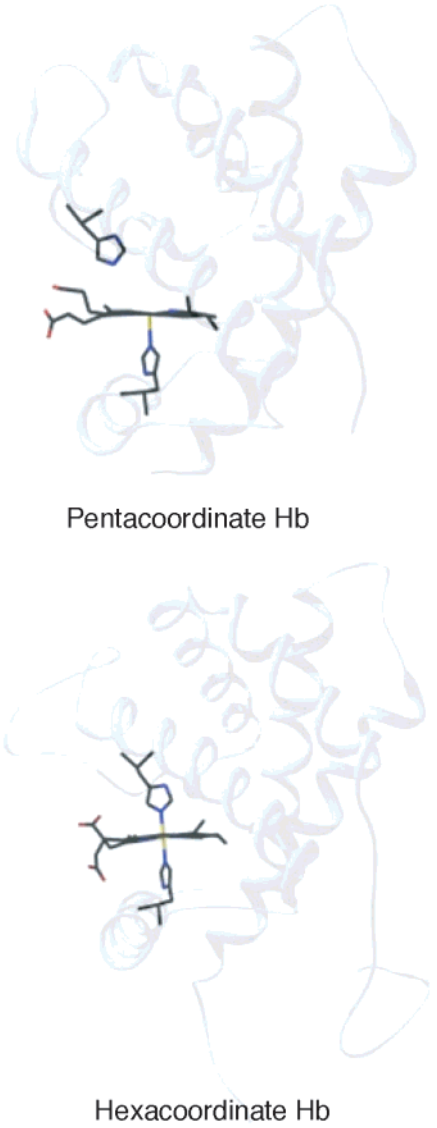

Figure 2. Structures of a pentacoordinte (Lba; PDB 1bin) and hexacoordinate (riceHb; PDB 1du8) Hb are compared to illustrate the similarities in protein fold, and the difference in heme coordination.

heme proteins and redox proteins. This work demonstrated that (in most cases) SO_2^- serves as the reductant and that heme proteins can exhibit very different rates of reduction with DT. Cox and Hollaway[13] and Wilkins and co-workers[15,16] reported reduction rates for several different ligation states of horse Mb, establishing the fact that bound ligands have pronounced effects on reduction rates.

A number of recently discovered Hbs display intramolecular coordination of the ligand binding site by an endogenous histidine side-chain (Figure 2). These proteins, termed "hexacoordinate" hemoglobins (hxHbs), are ubiquitous in plants and animals.[17] A specific physiological function has yet to be attributed to hxHbs, but it has been hypothesized that they may play a role in scavenging NO or other reactive oxygen species.[17−19] In this capacity, a mechanism for reduction is required to achieve catalytic activity. Given that the ligand state of the heme can affect reduction kinetics, we investigated the role of endogenous hexacoordination on reduction kinetics by comparing the reactions of several hxHbs, pentacoordinate Hbs, and pertinent mutant proteins with DT. Our results indicate that bis-histidyl hexacoordination greatly facilitates reduction rates by lowering the degree of heme-pocket reorganization required due to the presence of solvent in most pentacoordinate Hbs. This work suggests a possible role for the unusual heme pocket chemistry present in hxHbs.

Methods

Expression and Purification of Proteins. Wild-type soybean leghemoglobin (Lba) and its mutant proteins,[20] rice nonsymbiotic hemoglobin I (riceHb),[21] *Synechocystis* hemoglobin (SynHb),[22] human neuroglobin (Ngb),[23] human cytoglobin (Cgb),[24] and wild-type sperm whale Mb (swMb)[20] were purified using the methods cited for each. swMb mutant proteins (H64V/V68H,[25] H64Y,[26] and H64W) were given to us by Professor John S. Olson. Horse heart Mb (hhMb) was purchased from Sigma and further purified using size-exclusion chromatography. All the proteins were oxidized by potassium ferricyanide, desalted on Sephadex G-25 and stored at −80 °C in the ferric form in 10mM phosphate buffer, pH 7.0.

Stopped Flow Reactions. All reactions were conducted using a BioLogic SFM 400 stopped-flow reactor coupled to a MOS 250 spectrophotometer. The starting buffer for each reaction was deoxygenated 0.1 M potassium phosphate, pH 7.0 prepared in gastight luer tip glass syringes by sparging with N_2. DT solutions were freshly made just prior to the experiments by adding solid reagent to deoxygenated buffer. Solutions of DT were quantitated using a Varian Cary 50 Bio spectrophotometer or (for appropriate concentrations) a direct absorbance scan of the solution in the stopped-flow reactor. The extinction coefficient used for quantification of DT was 8 mM^{-1} cm^{-1} at 314 nm.[27] All protein concentrations were ∼4 μM (in heme). Reactions in the presence of imidazole were carried out by adding imidazole to all syringes (prior to N_2 sparging) at a concentration of 5 mM for Lba, or 0.5 M for Mb. The total reaction volume of each experiment was 300 μL, and the temperature was maintained at 20 °C. The specific wavelengths used for observation of each time course are listed in Table 1. Time courses were fit using a single-exponential decay expression, and all figures displaying kinetic data were prepared using the program Igor Pro (Wavemetrics).

Kinetic Considerations. Both DT and its derivative, SO_2^-, can serve as a reductant. DT reacts directly with some proteins, but in most cases SO_2^- is the reducing species,[13,14,28] and Scheme 1 describes this reaction. If the rate constants for reduction are linearly dependent on the square-root of [DT], as predicted by Scheme 1, SO_2^- is assigned as the reductant. Alternatively, a linear dependence on [DT] is indicative of a direct reaction between protein and $S_2O_4^{2-}$.[14]

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Table 1. Rate Constants for Reduction by Sodium Dithionite

protein	λ (nm)	reductant = SO_2^- k_{obs} [$S_2O_4^{2-}$] ($M^{-1/2}$ s^{-1})	reductant = $S_2O_4^{2-}$ k_{obs} [$S_2O_4^{2-}$] (M^{-1} s^{-1})	k_R (s^{-1})	k_{calc}[a] [SO_2^-] (M^{-1} s^{-1})	Δ coord.?	k_Δ[b] (s^{-1})
swMb	435			35		yes	>1000[c]
hhMb	435			49		yes	>1000[c]
H64V/V68H Mb	428	1720			5.8×10^7	no	
swMb + imidazole		2600			8.9×10^7	yes	n.d.[d]
hhMb + imidazole		2800			9.6×10^7	yes	n.d.[d]
H64W Mb	435	2730			9.4×10^7	no	
H64Y Mb	435	1.3	6.5		4.5×10^4	yes	>300
w.t. Lba	426			77		yes	>1000[c]
Lba + imidazole	425	650			2.2×10^7	no	
H61L Lba	423	730			2.5×10^7	no	
H61Y Lba	431	0	34			yes	>300
rice Hb	410	4360			1.4×10^8	no	
Cgb	430	4,270			1.5×10^8	no	
Ngb	426	780			2.3×10^7	no	
SynHb	426	2150			3.9×10^7	no	

[a] For comparison to other electron-transfer proteins, the values in column three (reflecting reduction by SO_2^-) were divided by $[K_{SO2}]^{1/2}$ (with $K_{SO2} = 0.85 \times 10^{-9}$ M[14]) to convert them to units of $[SO_2^-]$. [b] This is the rate constant for dissociation of the pertinent ligand if one dissociates following reduction. [c] These values are taken from Brancaccio et al.[33] [d] The affinity of ferrous Mb is very low ($k_D \sim 1.5$ M), and the dissociation rate constant for imidazole has not been measured.

Scheme 1. Oxidation of Hb^+ by DT via SO_2^-

$$1/2S_2O_4^{2-} \overset{K_{SO2}}{\rightleftharpoons} SO_2^- + Hb^+ \underset{k_{off}}{\overset{k_{on}}{\rightleftharpoons}} \{SO_2^-:Hb^+\} \xrightarrow{k_R} SO_2 + Hb^O$$

Previous work with Mb has suggested that SO_2^- serves as the reductant and does so by forming an outer-sphere complex which is followed by electron transport.[13,14,28] As part of their studies, Lambeth and Palmer have provided a detailed description of the behavior of DT as a reductant in aqueous solutions.[14] They found that while K_{SO2} is very small (0.85×10^{-9} M under our conditions), if the total DT concentration is greater than 5x[Hb] then the reaction does not significantly perturb the $S_2O_4^{2-}/SO_2^-$ equilibrium and reduction is not limited by the kinetics of DT dissociation to form SO_2^-. Under these conditions, and when $k_R \gg k_{on}$, one expects the observed rate constant (k_{obs}) to be linearly dependent on the square root of DT concentration.

Under conditions where k_R is not extremely fast compared to SO_2^- binding, the following equation (derived from Scheme 1) can be used to calculate the dependence of k_{obs} on SO_2^- concentration[29]

$$k_{obs} = \frac{k_R k_{on}[SO_2^-]}{k_R + k_{off} + k_{on}[SO_2^-]} \quad (1)$$

Equation 1 predicts that as k_R decreases with respect to k_{on}, the dependence of k_{obs} on SO_2^- concentration will be nonlinear and approach an asymptote equal to k_R.

Results

Reduction of Hexacoordinate Hbs Compared to Pentacoordinate Hbs. Figure 3A shows a plot of k_{obs} values as a function of the square-root of DT concentration for four bis-histidyl hxHbs, (Cgb, Ngb, riceHb, and SynHb) compared with two pentacoordinate Hbs (swMb and Lba). Also included is the initial $[SO_2^-]$ calculated from $[SO_2^-] = K_{SO2}[S_2O_4^{2-}]^{1/2}$ with $K_{SO2} = 0.85 \times 10^{-9}$ M.[14] Figure 3 presents two important points. The first is that under our experimental conditions $[SO_2^-]$ is not limiting the reaction. The second is that hxHbs exhibit linear dependences of k_{obs} on $[DT]^{1/2}$ instead of approaching asymptotic [DT]-independent rates of reduction like the pentacoordinate Hbs swMb and Lba. Figure 3B shows the dependencies of

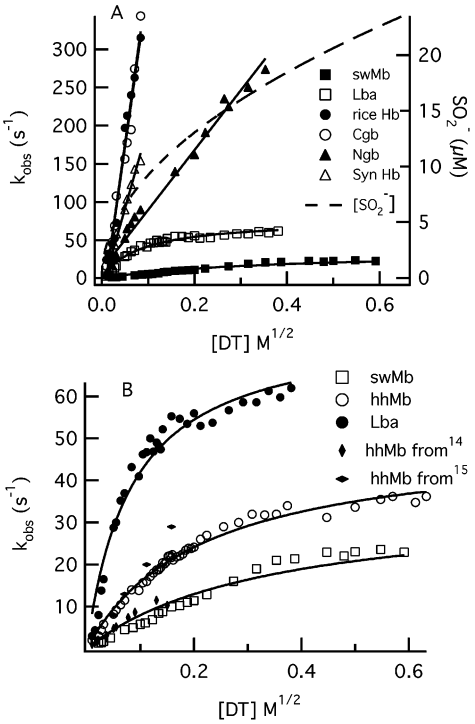

Figure 3. (A) Reduction rates of several hxHbs are compared to Mb and Lba. The dotted line shows the $[SO_2^-]$ at each [DT]. (B) The *y* axis from (A) is expanded to allow examination of the pentacoordinate proteins in more detail. hhMb is included in this plot for comparison to Lba and swMb.

k_{obs} on $[DT]^{1/2}$ for swMb and Lba with the *y* axis adjusted to provide a more detailed view of these data. Values for hhMb are also included for comparison. To estimate values for the asymptote of each protein, K_{SO2} was substituted for k_{on}/k_{off} in eq 1 and the data were fitted for this ratio and the value of k_R. This calculation yielded values of k_R for swMb, hhMb, and Lba of 35, 49, and 77 s^{-1}, respectively (Table 1).

The data presented in Figure 3 for Mb and Lba resemble what is expected when k_R is limiting the reaction. This suggests that the asymptotes of these data are equivalent to the value of k_R. On the contrary, for hxHbs, k_{obs} depends only on $[DT]^{1/2}$ and reaches values at the limit of detection by rapid mixing (Table 1). There are two possible explanations for these results.

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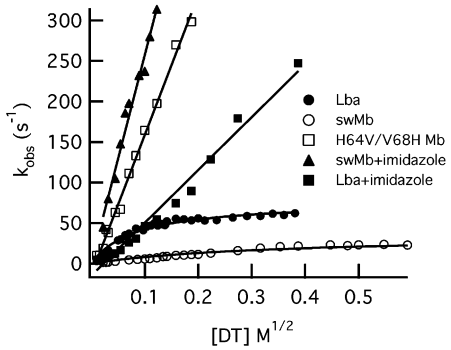

Figure 4. Rates of reduction by DT for imidazole coordinated pentacoordinate Mb and Lba show that the reaction is facilitated regardless of how bis-histidyl coordination is achieved.

(1) The primary sequences and tertiary structures of the hxHbs facilitate the electron-transfer reaction to a much greater extent than those of Mb and Lba, or (2) bis-histidyl hexacoordination of the heme iron increases k_R to the extent that it is no longer a kinetic limit to reduction. Previous work with imidazole-coordinated hhMb, which report increased rates of reduction, supports the latter possibility.[13,16] The following experiments were designed to further address this question.

Reduction Rates Are Dependent on Ligation State, Not Protein Structure. The experiments presented in Figure 4 were designed to test the possibility that different protein primary sequences are the origin of the more rapid rates of reduction in the hxHbs, as opposed to the nature of coordination. Rate constants for DT reduction of the bis-histidyl swMb mutant protein (H64V/V68H Mb[25]), imidazole-bound Lba, and imidazole-bound swMb were compared to those of the wild-type proteins. In each case, the bis-histidyl forms have k_{obs} values that are linearly dependent on $[DT]^{1/2}$ (Table 1) and reach values far exceeding those of the wild-type proteins. These results indicate that the nature of heme coordination is the principal determinant of reduction kinetics, rather than other structural features associated with sequence differences between the proteins.

To find out whether the chemical nature of the intramolecular coordinating ligand affects this phenomenon, rates of reduction were measured for swMb and Lba mutant proteins where the distal His was replaced by Tyr (Figure 5). Unlike the distal His in the wild-type proteins (which does not coordinate the heme iron), the Tyr side chain coordinates the heme iron in the ferric (but not the ferrous) form of each protein.[26,30−32] For the Tyr mutant proteins, values of k_{obs} are much lower than those of the respective wild-type Hbs. Furthermore, plots of k_{obs} versus $[DT]^{1/2}$ show a pronounced upward curvature that indicates a dependence on [DT] rather than just $[DT]^{1/2}$, which suggests that the $S_2O_4^{2-}$ serves as a reductant directly (Table 1). In accordance with Lambeth and Palmer,[14] these data were fit to the following expression to extract the relative contributions of each potential reducing species:

$$k_{obs} = a[S_2O_4^{2-}] + b[S_2O_4^{2-}]^2 \qquad (2)$$

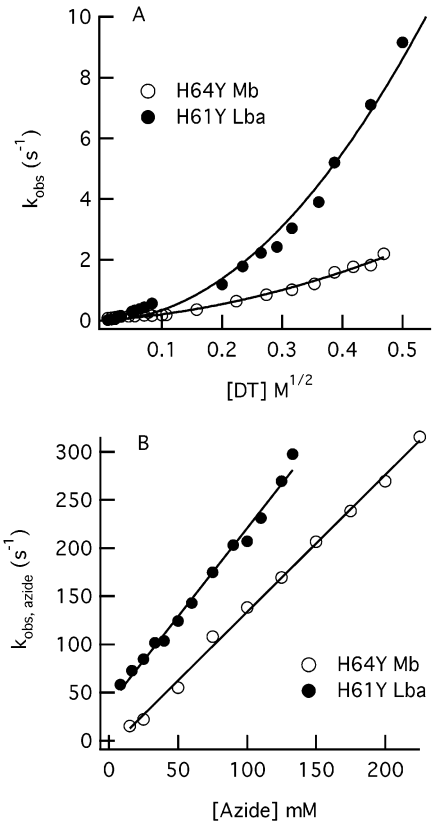

Figure 5. (A) Rates of reduction for Tyr-coordinated Mb and Lba mutant proteins. The curved dependence of k_{obs} indicates that $S_2O_4^{2-}$ contributes to the reduction reaction directly. The solid lines are curves fitted to eq 2. (B) Rates of azide binding to ferric H61Y Lba and H64Y Mb demonstrate that Tyr dissociation does not limit the reduction reactions in A.

In eq 2, a fit to *a* and *b* provides the respective rate constants for reduction by SO_2^- and $S_2O_4^{2-}$, when the data are plotted as k_{obs} versus $[DT]^{1/2}$. Values of *a* and *b* for Mb H64Y are 1.3 $M^{-1/2}$ $s^{-1}$and 6.5 M^{-1} s^{-1}, respectively, suggesting significant contributions from both species. In Lba H61Y, k_{obs} depends only on $S_2O_4^{2-}$ with a rate constant of 34 M^{-1} s^{-1} (Table 1).

A possible explanation for the slower rates of reduction in the Tyr mutant proteins is that dissociation of the Tyr side-chain from the ferric heme iron is the rate-limiting step. If this is true, then the kinetics for binding of an exogenous ligand to the ferric protein should also be limited by this value. In Figure 5B, azide binding to ferric H64Y Mb and H61Y Lba was measured with the understanding that if Tyr dissociation is the rate-limiting step in the reaction, one would expect $k_{obs,azide}$ to be nonlinear as a function of azide concentration and approach the rate constant for Tyr dissociation. The bimolecular rate constant for azide binding to H64Y Mb is 1.4 mM^{-1} s^{-1} (very similar to that reported previously[33]) and the data are linear to the limit of detection (~300 s^{-1}). In the case of Lba H61Y, the bimolecular rate constant is 1.8 mM^{-1} s^{-1} and the data are also linear to values >300 s^{-1}. The azide dissociation rate constant for this protein (37 s^{-1}) is evident from the *y* intercept of the fit in Figure 5B. Dividing this by the association rate constant of 1.8 mM^{-1} s^{-1} provides a dissociation equilibrium constant of 21 mM which is very similar to the value measured by others

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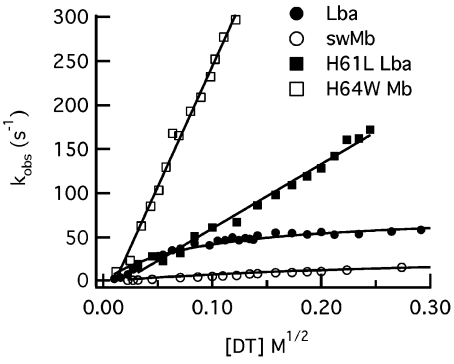

Figure 6. Rates of reduction for H61L Lba and H64W Mb demonstrate that k_{obs} is much faster and unlimited by k_R when water is not coordinated to the ferric heme iron.

using equilibrium methods.[31] For both proteins, rate constants for azide binding reach values much larger than those for reduction by DT. Therefore, the rate of Tyr dissociation must be $>300\ s^{-1}$ for each protein, and not responsible for limiting reduction by DT as observed in Figure 5A.

Coordinated Water Limits Reduction Rates in Wild-Type Lba and Mb. The ferric forms of wild-type Mb and Lba have water molecules loosely coordinating their heme irons.[32] To explore the possibility that water coordination is correlated with limiting values of k_{obs} for these proteins, reduction rates were measured for H61L Lba and H64W Mb, both of which lack coordinated water molecules.[32,34] Figure 6 shows that k_{obs} is linearly dependent on $[DT]^{1/2}$ and much more rapid in these mutant proteins than in the respective wild-type forms. Therefore, the slow values of k_{obs} in wild type-Lba and swMb are linked to water coordination in the ferric forms of each protein.

Discussion

For the wild-type pentacoordinate proteins swMb, hhMb, and Lba, the values of k_{obs} approach asymptotes equal to 35, 49, and 77 s^{-1}, respectively. hhMb has been investigated previously, but the limiting value was not observed because k_{obs} was measured using much lower concentrations of DT. Lambeth and Palmer[14] and Olivas et al.[15] reported the concentration dependence of k_{obs} for hhMb up to ~20 mM DT to be 100 and 140 $M^{1/2}s^{-1}$, respectively. Their data are overlaid with our hhMb values in Figure 3B, showing that all three are consistent at these low [DT] values. It is only at [DT] greater than twice those previously examined that the data become noticeably nonlinear with $[DT]^{1/2}$. When our data are analyzed only up to 20 mM DT, they appear linear with $[DT]^{1/2}$ and yield a slope of 144 $M^{1/2}\ s^{-1}$, in agreement with both other groups.

A [DT]-independent rate of reduction is predicted from eq 1 when k_R is the limiting step in the reaction, so it can be concluded that Mb and Lba have much lower k_R values than the hxHbs, or when Mb or Lba is bound to exogenous imidazole. The data in Figure 3 demonstrate that the coordination state of the heme iron has a profound effect on reduction kinetics. Imidazole facilitates the reaction regardless of whether it is provided as a His side chain from within the heme pocket, or as an exogenous ligand. An explanation for these data is that bis-histidyl coordination increases k_R, removing electron transport as the rate-limiting step in reduction.

Role of Heme Coordination in Reduction Kinetics. Although the neutral imidazole ligand facilitates reduction kinetics, the negatively charged Tyr side chain[30,31] inhibits rates of reduction. It does not appear to do this solely by lowering k_R, as k_{obs} is very much dependent on the DT concentration even at high values in the H64Y Mb and H61Y Lba mutant proteins (Figure 5). This suggests that Tyr coordination slows reduction by decreasing the affinity of the protein for the reductant. This hypothesis is supported by the fact that k_{obs} for these proteins is linearly dependent on [DT] rather than $[DT]^{1/2}$, indicating a shift from SO_2^- to $S_2O_4^{2-}$ as the principal reductant. In this scenario, we cannot be certain of the effect of Tyr coordination on k_R because we cannot overcome the low affinity for $S_2O_4^{2-}$ to achieve k_{obs} values that are even as large as those observed for the wild-type proteins.

In swMb, hhMb, and Lba, water coordinated in the ferric states dissociates following reduction. Likewise, the Tyr side chains in the respective Tyr mutant proteins dissociate in the ferrous state (Table 1). Solvent and other heme pocket amino acids must reorganize their structures to accommodate ligand dissociation and the altered charge of the heme iron.[39] A likely cause for the slower rates of reduction in Mb and Lba (and probably an additional cause in the Tyr mutant proteins) is the reorganization energy associated with this transition. In contrast, the bis-histidyl Hbs are hexacoordinate in both the ferrous and ferric states, so the reorganization energy associated with these complexes will be lower than in Lba and Mb.

The relative values of k_R for swMb, hhMb, and Lba support a correlation between water coordination and slower electron transfer. Ferric Lba has the most loosely coordinated distal pocket water molecule of the three, evidenced by the optical absorbance spectrum and ligand binding kinetics,[35] and shows the largest value of k_R (Table 1). The kinetics of azide binding to ferric Mb can be used to estimate water affinity, showing that swMb has a higher affinity than most other Mbs, including pig, human, and hhMb.[1,33] The slightly larger value of k_R for hhMb compared to swMb is consistent with a lower water affinity in the ferric horse protein.

The only exception to the observation that slower reduction rates are associated with a change in coordination state is for Mb bound with exogenous imidazole. Ferric Mb binds imidazole with a dissociation equilibrium constant of 16 mM, and the value for ferrous Mb has been estimated to be 1.5 M.[13] Our experiments were carried out at 0.5 M imidazole in which ferrous Mb would be ~30% saturated following reduction. There are two possible explanations for the rapid reduction rates reported in Table 1 for imidazole-bound Mb. (1) The observed time courses are associated with the fraction of protein that remains coordinated after reduction, or (2) the reaction forms a transient, ferrous imidazole-bound intermediate that allows reduction to occur prior to structural reorganization. The second explanation would correspond to the reaction observed for cyanoMb[13,28] and nicotinate-bound Lba.[36] Reduction of

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cyanoMb proceeds via a reduced CN-bound intermediate from which CN^- dissociates to form deoxyferrous Mb. The reduction reaction is much faster than the CN^- dissociation rate constants of both the ferrous and ferric complexes.

The results from Figure 6 demonstrate that the reorganization energy encumbering reduction in wild-type Lba and Mb is absent in mutant proteins lacking coordinated water in the ferric states. Corroborating results have been reported by Dunn et al.,[37] who showed that the oxidation of ferrous (deoxygenated) Mb is much faster in mutant proteins lacking coordinated water than in the wild-type protein. Interestingly, although the bimolecular rate constant for oxidation is lower in the wild-type protein, no limiting value is observed, and rates much greater than 35 $s^{-1}$are achieved. This suggests that the oxidation reaction might generate a ferric intermediate of the wild type protein in which water is not yet coordinated. Alternatively, the kinetics of structural reorganization could be slower in the reduction reaction than with oxidation. Regardless of these differences, our results indicate that heme coordination affects reduction kinetics by its influence on reorganization energy, not by altering the pathway for electron transfer as has been suggested previously.[16]

Our results are also consistent with equilibrium reduction reactions with Mb that show that mutant proteins lacking coordinated water are reversible in direct voltammetry experiments.[38] Reorganization of solvent and heme pocket amino acids resulting from the change in water coordination precludes accurate cyclic voltammetry measurements with wild-type Mb presumably because the scan rate is impeded by kinetic events associated with the redox reaction. Our experiments predict that hxHbs should be amenable to cyclic voltammetry studies because reorganization energy is lower due to the stable ferrous bis-histidyl heme iron.

Rates of Ligand Dissociation Are Not the Kinetic Limit to Molecular Reorganization. When oxidation or reduction involves a change in coordination state, the resulting reorganization energy can limit the kinetics of the reaction. However, the kinetic limit is not simply the rate constant for dissociation (or association) of the ligand. For example, the limiting values (k_R) for Mb and Lba are 35 and 77 s^{-1}, yet the rate constants for water dissociation from the ferric proteins are $\sim$1000 s^{-1}. Similarly, Tyr dissociation from H64Y Mb and H64Y Lba is at least $>$300 s^{-1}, which is much faster than the reduction reaction. This can be explained by arguing that ligand dissociation triggers the more global molecular reorganization associated with the change in redox state and should therefore not be the rate-limiting step. Alternatively, others have observed rates of reduction of azide and CN-bound Mb that are on-par or faster than the dissociation rate constants for these ligands.[13,15] In the case of CN-Mb, it is clear that a reduced intermediate is formed.[13,15,28] For other ligands, like azide, it is possible that ligand dissociation is the rate-limiting step or that a reduced intermediate is formed that has yet to be detected.

Biological Implications. bis-histidyl coordination greatly enhances DT reduction rate constants compared to pentacoordinate Hbs such as Mb and Lba. The bimolecular rate constants of $\sim 10^7$ M^{-1} s^{-1} (for the reaction with SO_2^-; Table 1) are as fast as those achieved by many electron transport proteins with their cognate redox partners;[14,37,39] however, the limiting values of k_R for Mb and Lba make these proteins much poorer candidates for electron transfer.

In light of these results, several important conclusions can be drawn regarding potential biological roles of hxHbs. (1) Autoxidation of the heme iron can occur through two different mechanisms.[40] In the "bimolecular" mechanism, which is predominant under low oxygen concentrations, O_2 dissociation initiates the reaction and the resulting Mb(II):H_2O (where ":" indicates a water in the distal heme pocket, but not coordinated to the heme iron) complex reacts with free oxygen to form superoxide anion and Mb(III)H_2O. In the "supoeroxide dissociation" mechanism, bound oxygen is protonated forming superoxide, which dissociates leaving Mb(III)H_2O. The relevance of these two mechanisms to our reduction reactions comes from the fact that the "bimolecular mechanism" should require less reorganization energy that the "speroxide dissociation" mechanism. This is because the former reaction exhibits water in the distal pocket in both the ferrous and ferric states, whereas the later employs an anhydrous ferric heme pocket following superoxide dissociation. For this reason, the molecular reorganization energy associated with reduction should resist autoxidation of the ferrous proteins that stabilize water in the distal heme pocket.[38] Therefore, one would expect rates of autoxidation of hxHbs to be greater than Mb, Lba, and other pentacoordinate Hbs that bind water at the ferric heme iron. Although this is certainly true for Ngb,[41] these values have not been reported for other hxHbs. If stabilization of coordinated water is favored to minimize autoxidation in oxygen transport proteins, our results argue against an oxygen transport role for hxHbs.

(2) Enhanced reduction kinetics would favor a role in electron transport or NO scavenging using a mechanism similar to that described in Figure 1. However, if hxHbs simply serve as electron transporters (such as cytochrome *b5*), there would be no need for the ability to bind exogenous ligands. The NO scavenging hypothesis requires the capability for rapid reduction *and* exogenous ligand binding (either NO, O_2, or both) and, therefore, fits with the biochemical properties of hxHbs.

It is clear from Figure 6 that rapid reduction can be achieved in the absence of intramolecular hexacoordination by removing the ability of the ferric protein to bind water. It is possible that intramolecular hexacoordination evolved because a stable (both in folding and heme retention), reversible bis-histidyl heme center is more easily achieved structurally than a water-proofed pentacoordinate heme pocket.[42−44]

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