Reduction and autooxidation kinetics of hexacoordinate hemoglobins

by

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A thesis submitted to the graduate faculty

in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Major: Biochemistry

Program of Study Committee:

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Ames, Iowa

2005

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TABLE OF CONTENTS

LIST OF FIGURES

LIST OF TABLES

ABSTRACT

Hexacoordinate hemoglobins (hxHbs) are a recent addition to the hemoglobin family, characterized by a unique coordination state of the heme iron in the ferric and ferrous state. The protoporphyrin IX nitrogen atoms that lie in the heme plane with the central iron occupying four of the coordination sites. The distal and proximal positions (top and bottom, respectively) bind histidine residues from the E and F helix from the surrounding protein structure to stabilize the heme group. This is fundamentally different from pentacoordinate hemoglobins, which have only five endogenous sources of coordination. Although the physiological functions of hexacoordinate hemoglobins remain elusive, some important information can be garnered from the basic chemical reactions of the iron during reduction and autooxidation. Since reduction of heme-based proteins is critical to their function, we have studied this mechanism in hexacoordinate hemoglobins. The results indicate that hexacoordination facilitates reduction kinetics. Although autooxidation has been studied more extensively for pentacoordinate hemoglobins, there has been little conclusive evidence offered for a mechanism of autooxidation in hexacoordinate hemoglobins. Our results show that the plant hexacoordinate hemoglobins have rate constants for oxidation similar to that of pentacoordinate hemoglobins. On the other hand, human hexacoordinate hemoglobins oxidize much faster. Together, these basic chemical reactions indicate that the hexacoordinate heme site provides a common, rapid mechanism for reduction but does not impose a common mechanism for autooxidation.

CHAPTER 1: Introduction

Background

Introduction

Hexacoordinate hemoglobins are significantly different from their pentacoordinate cousins. The name comes from the coordination state of the heme iron in the ferric and ferrous state. The distal histidine of the hxHbs coordinates directly to the heme iron, whereas in pentacoordinate hemoglobins this only occurs under some denaturing conditions. This alteration provides for different basic chemistry in ligand binding, transport, and heme reduction and autooxidation.

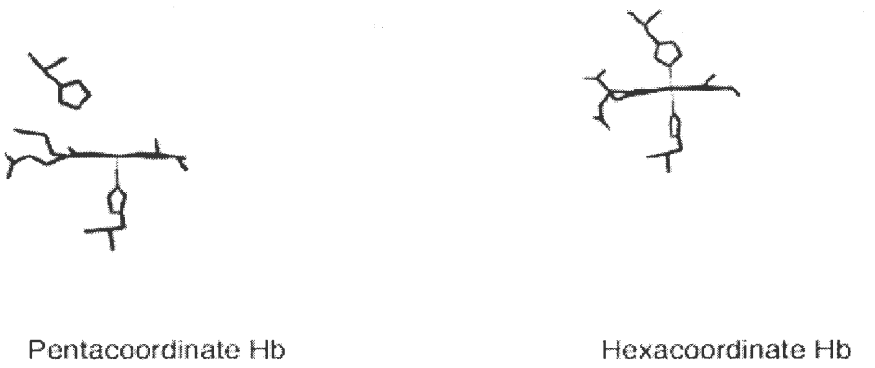

Figure 1. Pentacoordinate Hb vs Hexacoordinate Hb.
The histidine on the distal side of the hemoglobin coordinates the iron compared to pentacoordinate hemoglobins which require a ligand to coordinate. The protein is shown in the background as light-grey ribbons as a reference.

hxHbs are expressed in many different organisms, including cyanobacteria, *Chlamydomonas*, all plants, humans and possibly most animal species (Kundu 2003). hxHbs were first discovered as non-symbiotic plant hemoglobins (Bogusz D 1988), although the coordination state was not determined until the work of Arredondo-Peter and Duff when they characterized rice (Arredondo-Peter R 1997) and barley hemoglobin (Duff SM 1997) respectively, The first structure of a hxHb was published in 2000 (Hargrove 2000), which gave definitive proof of the position of the heme iron in the ferric state, leading to the name of "hexacoordinate hemoglobins." It was at this same time that a hxHb was discovered in humans (Burmester T 2000), primarily in the brain, and was named neuroglobin. A second hxHb was found two years later (Trent 2002) that was expressed in several different tissues, and is now called cytoglobin. Since their first discovery, many studies have been published on the different actions of these proteins, from ligand binding to crystallographic structures (Hargrove 1997; de Sanctis 2004; Hoy J.A. 2004; Pesce 2004). At this time no physiological role of these proteins has been assigned (Trent 2002). Conditions for expression differ, but hypoxia appears to be a common factor for expression of hxHbs(Sun Y. 2001).

Several hypotheses have been formulated for hxHb function: NO sensing (Sun Y. 2001), electron transfer (Dordas 2003; Kundu 2003), oxyradical scavenger (Herold 2004) or oxygen transport (Fago 2004). Other possible roles could include environmental redox indicators or oxygen sensing, though no systems have been designed to test these ideas. Further characterization of hxHbs is necessary to elucidate their function. Four hemoglobins are focused upon in this study: *Synechocystis* hemoglobin (SynHb), cytoglobin (CGb), neuroglobin (NGb), and rice hemoglobin (RHb1).

Electron Transfer Reactions

Knowledge of the basic chemistry of hxHbs is critical to the determination of their physiological function. Reduction kinetics of pentacoordinate proteins have been previously studied (Lambeth 1973; Cox 1977), but analysis of the mechanism has been limited to pentacoordinate hemoglobins. Reduction chemistry is the transfer of (an) electron(s) from one element to another element, which leads to the oxidation of the donating element. One must note that the protein structure of hemoglobins enhances the reduction potential of the hemes, which is unfavorable (Brunori M 1971). This shows the importance of the overall globin structure, as it is designed to facilitate the reduction of the contained iron within the heme, leading to its ability to remain in the ferrous state. This allows certain ligands, like oxygen and carbon monoxide, to bind to the heme iron. Ferric heme proteins bind anionic ligands with but do not react with neutral ligands like oxygen and carbon monoxide for reasons that are not completely understood. Changing parts of the globin structure can change the redox potential, which changes the potential function of the protein by dictating oxidation state (Varadarajan R 1989).

Kinetics of Electron Transfer

The experiments in chapter 2 focus on the speed of formation of the reduced species by addition of excess reducing agent. The excess of reducing agent ensures that reduction of the iron in hemoglobins is thermodynamically favorable, so our experiments focus solely on the rate of the reduction reaction. Reduction has been studied previously (Cox 1977) and two different mechanisms have been considered. The first is called "Outer Sphere" which suggests that reduction occurs before the ligand is released preempting the possibility of

direct binding of the reductant to the ligand-binding site. The second is called "Ligand Off" which assumes that reduction cannot occur until a bound ligand is removed (be it a coordinated solvent or some other exogenous ligand), suggesting direct binding of the reductant to the heme iron. If it is supposed that reduction rates are slower than ligand off-rates, the "Ligand Off" model is used. If observed reduction rates are faster then ligand off-rates, then the "Outer Sphere" which invokes binding of the reductant to a remote site, is more likely. It was in the context of these two models that the following reduction kinetic work was approached, and it was found that hxHbs support the "Outer Sphere" model, as the rates of reduction are faster then the ligand off-rates (Weiland TR 2004).

Oxidation in Hemoglobins

Autooxidation chemistry has often been a focus of hemoglobins. There has been more interest in verifying the autooxidation kinetics of heme proteins, mainly due to the initial discovery of their function as oxygen transporters. The mechanism for autooxidation has been studied extensively. Initial work indicated that the mechanism was unimolecular, with the oxygen leaving the heme and taking the extra electron with it, forming a superoxide molecule and a heme in the ferric state (Weiss 1964). However, the equation derived from this scheme was unable to fit data collected earlier by George and Stratmann (George P 1952). It was several years before a satisfactory mechanism was proposed that could fit the previously collected data. In 1982 (Wallace WJ 1982) Wallace presented a scheme that accounted for the data collected by George and Stratmann. He explained that the mechanism is a bimolecular reaction that involves a weak nucleophile that coordinates with the reduced heme after the release of dioxygen. It then facilitates the transfer of an electron from the

heme to the dioxygen (which still resides in the heme pocket), resulting in a superoxide molecule and a nucleophile bound to the heme. This mechanism works largely because of the weak nucleophiles' ability to bind the ferric heme after oxidation. The Wallace mechanism has a shortcoming, as it cannot completely explain behavior obtained at high oxygen levels. It predicts that the rate of oxidation will approach zero at very high levels of oxygen, because bimolecular rebinding of oxygen will out-compete the nucleophile-oxygen reaction. However that is not what is observed. It was following Wallace's work that Brantley (Brantley 1993) offered a combination of the two mechanisms in order to better explain autooxidation.

Essentially, Brantley brings forth a combination of both the unimolecular and bimolecular schemes. In both cases, the starting point is the stable oxy-ferrous complex with the distal histidine providing the required 6^{th} coordination for stability. The first step is the removal of the hydrogen bond between the histidine and the bound molecular oxygen. In the unimolecular mechanism, the dioxygen is protonated, which is followed by dissociation. The protonation allows for the addition of the electron from the ferrous heme to form the stable HO_2 complex. A stable ferric heme remains bound to water, which is generally associated with ferric myoglobin. The bimolecular mechanism is slightly more complicated. Once the 6^{th} coordination hydrogen bond is broken, the dioxygen dissociates, but does not yet leave the protein completely. Water then acts as a nucleophile, binds to the ferrous heme and facilitates the formation of a superoxide by helping transfer an electron from the heme iron to the free oxygen. The final product is ferric myoglobin bound to water. These "new"

mechanisms provide equations that fit the data seen by George and Stratmann and were used as a basis for the autooxidation research on hxHbs.

Minimal autooxidation work has been done on hxHbs, and results indicate that oxygen transport is quite unlikely for most of them (Hargrove 2000). Plant hxHbs have autooxidation values equivalent to that of myoglobin. Oxygen transport has not been assigned as a physiological role but it may be a distinct possibility. Human hxHbs autooxidize at much higher rates. These rates indicate that oxygen transport is not the main objective.

It is important to understand oxidation in hxHbs, as it could give further insight into physiological function. Understanding reduction kinetics facilitates comparisons between cytochrome *b5* proteins and hxHbs. Cytochrome *b5* proteins function largely as electron transporters, and reduction and oxidation of the central iron plays a critical role in this function. The reduction rate of cytochrome *b5* is 14 s^{-1} at 10.6 mM DT (Davydov DR 1985) and its reduction potential is –7 mV (Rodgers KK 1991). As demonstrated in Chapter 2, this kinetic value is similar to hxHbs but their midpoint reduction potentials are quite a bit lower (on the order of -130 mV) (Lecomte JT 2001; Sawai H 2003; Fago A 2004; Weiland TR 2004). This leads to some interesting conclusions about hxHbs. They are able to bind ligands, like hemoglobin, but have reduction kinetics similar to that of cytochrome *b5*, an electron transporter.

Approach

Several techniques were used to determine the kinetics of reduction and autooxidation of hxHbs. Rapid mixing experiments were used mainly for reduction kinetics and for autooxidation where speeds exceeded 20 hr^{-1}. In fact, some of the reduction speeds exceed that of a stopped flow apparatus, which can measure reactions with half lives up to ~ 300 s^{-1}. Some autooxidation data were unable to be collected as the oxy complex was not stable. Novel methods were developed for determining the reduction and autooxidation kinetics of some of the hxHbs, which had not been previously determined, using a stopped flow apparatus. For slower autooxidation kinetics, a Cary 50 UV/VIS spectrophotometer was utilized to collect data to measure rates. All data were then analyzed with Igor Pro (Wavemetrics).

CHAPTER 2: Reduction Kinetics

Introduction

The heme prosthetic group confers the ability to catalyze reactions ranging from electron and ligand transport to transcriptional regulation in many different proteins (Antonini 1971; Shelver 1997; Rodgers 1999; Delgado-Nixon 2000). 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, while several anions such as azide and cyanide 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 storage respectively, and conditions exist *in vivo* to ensure that the vast majority of these molecules remain in the ferrous form (Jakob 1992).

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 2 (Eich 1996; Brunori 2001; Brunori 2001; Herold 2001). A related observation is that bacterial and

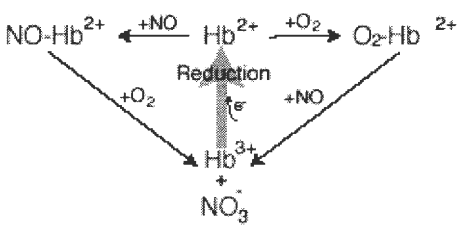

Figure 2. The NO-dioxygenase reaction. Ferrous Hb can bind either O_2 or NO, which can then react with the other ligand to form ferric Hb and NO_3^-. The reaction continues to cycle only if the oxidized Hb is reduced.

yeast flavohemoglobins use this reaction to destroy NO in defense of oxidative bursts (Gardner 1998; Gardner 2000). 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 (Minning 1999).

It is often difficult to assign a mechanism for reduction of many Hbs. Cytochrome *b5* is thought to reduce Myoglobin, and a specific reductase called methemoglobin reductase is reported to work inside red blood cells. However, in most cases these are reports of what can occur *in vitro* rather than support for the reaction in vivo. 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 (Lambeth 1973; Cox 1977). Although DT is widely used as a reactant, it has been used in surprisingly few studies investigating mechanisms of Hb reduction. Lambeth and Palmer (Lambeth 1973) investigated these reactions in detail, and reported reduction kinetics for several 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 (Cox 1977) and Wilkins and coworkers (Olivas 1977; Eaton 1978) 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 1). These proteins, termed "hexacoordinate" hemoglobins (hxHbs), are ubiquitous in plants and animals (Kundu 2003). 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 (Dordas 2003; Kundu 2003; Herold 2004). 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.

Results

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 (Lambeth 1973; Cox 1977; Bellelli 1990), and the following scheme describes this reaction under anaerobic conditions.

Scheme 1

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

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 $S_2O_4^{2-}$(Lambeth 1973).

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 transfer (Lambeth 1973; Cox 1977; Bellelli 1990). As part of their studies, Lambeth and Palmer have provided a detailed description of the behavior of DT as a reductant in aqueous solutions (Lambeth 1973). They found that while K_{SO2} is very small ($0.85x10^{-9}$ 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 $k_R >> k_{on}$, 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 (Espenson 1995).

$$k_{obs} = \frac{k_R k_{on} [SO_2^-]}{k_R + k_{off} + k_{on} [SO_2^-]} \qquad \text{Equation 1}$$

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.

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 (Lambeth 1973). 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 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 Equation 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).

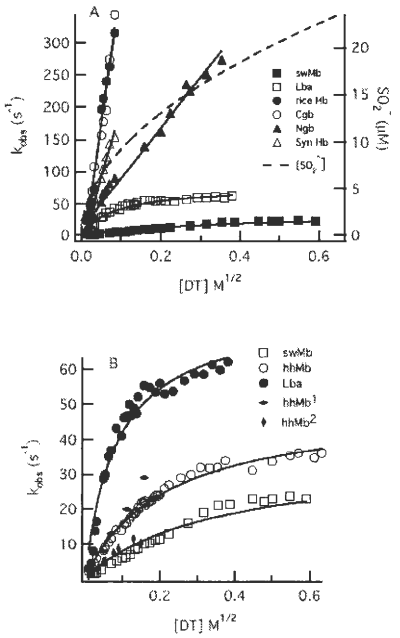

Figure 3. A) Reduction rates of several hxHbs are compared to Mb and Lba. The dotted line shows the $[SO_2^-]$ at each [DT]. B) The y-axis from A) is expanded to allow examination of the pentacoordinate proteins in more detail. hhMb is included in this plot for comparison to Lba and swMb. B references: 1 (Lambeth 1973), 2 (Olivas 1977).

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. 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 (Cox 1977; Eaton 1978). The following experiments were designed to further address this question.

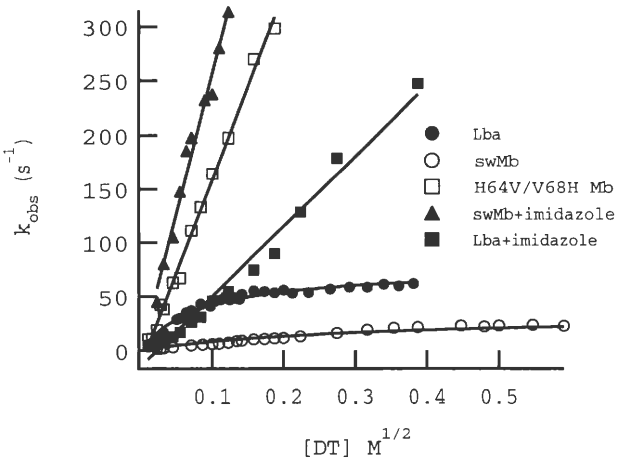

Figure 4. Rates of reduction by DT for imidazole coordinated pentacoordinate Mb and Lba show that the reaction is facilitated regardless of how *bis*-histidyl coordination is achieved.

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 (Dou 1995)), 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 (Hargrove, Singleton et al. 1994; Tang 1994; Kundu and Hargrove 2003; Patel 2003). For the Tyr mutant proteins, values of k_{obs} are much lower

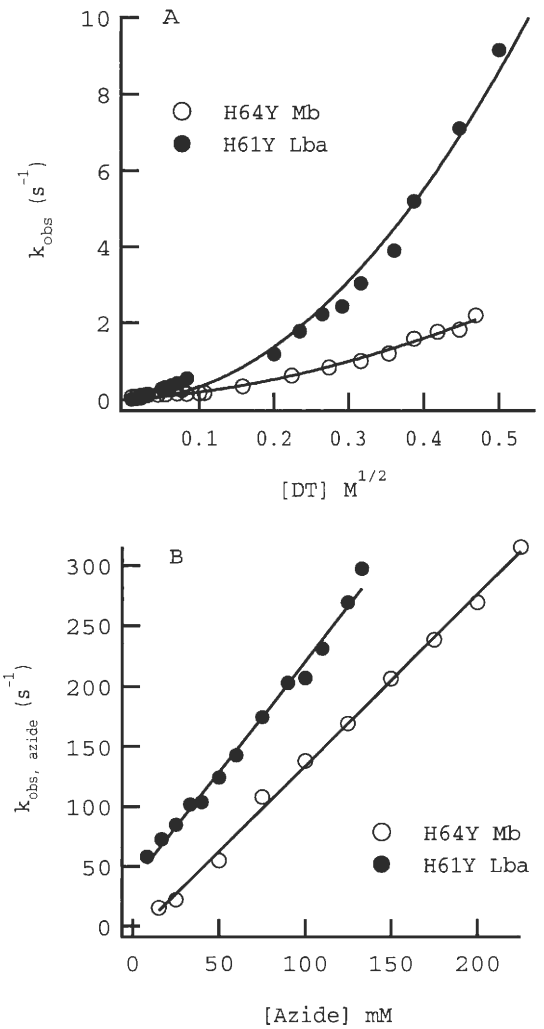

Figure 5. A) Rates of reduction for Tyr-coordinated Mb and Lba mutant proteins. The curved dependence of k_{obs} indicates that $S_2O_4^{2-}$ contributes to the reduction reaction directly. The solid lines are curves fitted to Equation 2. B) Rates of azide binding to ferric H61Y Lba and H64Y Mb demonstrate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

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 (Lambeth 1973), these data were fit to the following expression to extract the relative contributions of each potential reducing species:

Equation 2

$$k_{obs} = a[S_2O_4^{2-}] + b[S_2O_4^{2-}]^2$$

In Equation 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 (Brancaccio 1994)) 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 using equilibrium methods(Patel 2003). 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.

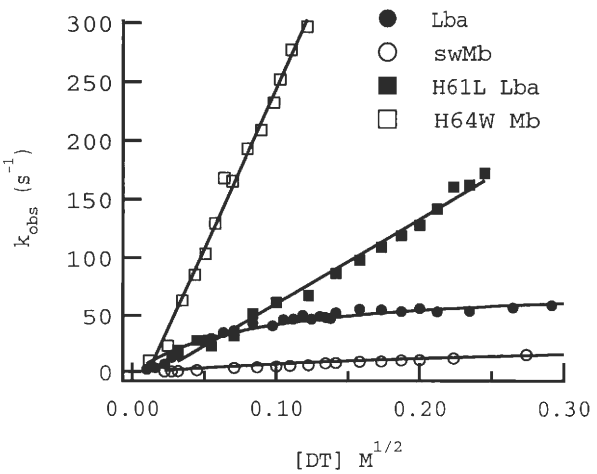

Figure 6. Rates of reduction for H61L Lba and H64W Mb demonstrate that k_{obs} is much faster and unlimited by k_R when water is not coordinated to the ferric heme iron.

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 (Kundu and Hargrove 2003). 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 (Quillin 1993; Kundu and Hargrove 2003). 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 s^{-1}, 49 s^{-1} 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 (Lambeth 1973) and Olivas *et al.*(Olivas 1977) reported the concentration dependence of k_{obs} for hhMb up to ~ 20 mM DT to be 100 $M^{1/2}s^{-1}$, 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 Equation 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.

The role of heme coordination in reduction kinetics - While the neutral imidazole ligand facilitates reduction kinetics, the negatively charged Tyr side chain (Tang 1994; Patel 2003) 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. Changes in pH may have an effect on tyrosine's ability to coordinate by affecting the protonation of the side chain hydroxyl, but was not measured here.

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 (Hargrove 1997). 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.

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}	[a]k_{calc} $[SO_2^-]$ $M^{-1}s^{-1}$	Δ coord.?	K_Δ [b] (s^{-1})
SwMb	435			35		yes	[c]> 1,000
HhMb	435			49		yes	[c]> 1,000
H64V/V68H Mb	428	1,720			$5.8x10^7$	no	
SwMb + imidazole		2,600			$8.9x10^7$	yes	[d]n.d.
HhMb + imidazole		2,800			$9.6x10^7$	yes	[d]n.d.
H64W Mb	435	2,730			$9.4x10^7$	no	
H64Y Mb	435	1.3	6.5		$4.5x10^4$	yes	>300
w.t. Lba	426			77		yes	[c]> 1,000
Lba + imidazole	425	650			$2.2x10^7$	no	
H61L Lba	423	730			$2.5x10^7$	no	
H61Y Lba	431	0	34			yes	> 300
rice Hb	410	4,360			$1.4x10^8$	no	
Cgb	430	4,270			$1.5x10^8$	no	
Ngb	426	780			$2.3x10^7$	no	
SynHb	426	2,150			$3.9x10^7$	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 $K_{SO2} = 0.85x10^{-9}$ M (Lambeth 1973)) 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.*(Brancaccio 1994). [d]The affinity of ferrous Mb is very low (k_D ~ 1.5 M), and the dissociation rate constant for imidazole has not been measured.

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 (Hargrove 1997), 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 (Antonini 1971; Brancaccio 1994). 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 (Cox 1977). 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 (Cox 1977; Bellelli 1990) and nicotinate-bound Lba (Appleby 1973). Reduction of 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.(Dunn 1999), 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, while 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 (Eaton 1978).

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 (Van Dyke 1996). 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 ~ 1,000 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 (Cox 1977; Olivas 1977). In the case of CN-Mb, it is clear that a reduced intermediate is formed (Cox 1977; Olivas 1977; Bellelli 1990). 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 like Mb and Lba. The bimolecular rate constants of ~ 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 (Lambeth 1973; Marcus 1985; Dunn 1999); 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) Autooxidation of the heme iron can occur through two different mechanisms (Brantley 1993). 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 "superoxide 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 "superoxide dissociation" mechanism. This is because the former reaction exhibits water in the distal pocket in both the ferrous and ferric states, while the later employs an anhydrous ferric heme pocket following superoxide dissociation. For this reason, the molecular reorganization energy associated with reduction should resists autooxidation of the ferrous proteins that stabilize water in the distal heme pocket (Van Dyke 1996). Therefore, one would expect rates of autooxidation of hxHbs to be greater than Mb, Lba and other pentacoordinate Hbs that bind water at the ferric heme iron. While this is certainly true for Ngb (Dewilde 2001), these values have not been reported for other hxHbs. If stabilization of coordinated water is favored to minimize autooxidation 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 2. 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 (Hargrove, Krzywda et al. 1994; Hargrove 1996; Liong 2001).

CHAPTER 3: Autooxidation

Introduction

The mechanism used by hemoglobins to stabilize bound oxygen and prevent heme iron oxidation has long been studied. Autooxidation is critical in determining the function of hemoglobins. Mb has a rate of autooxidation around 0.05 per hour (Brantley 1993), indicating that its ability to hold on to oxygen makes it efficient as a short-term oxygen storage protein. Mb is used to a high degree in muscles, where the oxygen facilitates aerobic respiration allowing high ATP useage (Brantley 1993). Other hemoglobins, like leghemoglobin, bind oxygen more tightly, and are thus suitable to act as an oxygen scavenger (Kundu 2003). However, not all proteins have autooxidation rates similar to Mb, and thus their function is believed to be significantly different than those that do. Because hxHbs have remained largely unstudied in this area, it is critical to understand this aspect of these functionally different proteins.

In these reactions sodium dithionite (DT) is used as the reducing agent, and excess DT must be removed because it will scavenge the oxygen necessary to form the oxy-ferrous complex. This is generally accomplished by running the reduced protein sample over a G25 "desalting" column to remove the excess DT. However, some proteins autooxidize quickly, leaving a protein that is already fully oxidized by the time it elutes from the column (~ 5 minutes). Thus, autooxidation occurs before any spectra can be collected. Because of such rapid autooxidation it became imperative to develop a novel method using a stopped-flow apparatus to measure autooxidation rates. It was hoped that the rapid mixing would allow

visualization of a stable oxy-ferrous complex and the subsequent rapid autooxidation. This method was moderately successful, as some proteins can be observed in an oxy-ferrous complex. However, data could not be collected on all proteins tested. Observations indicate that minimal DT solutions can be unstable, probably because of low-level oxygen contamination. However, it may be possible that these proteins are unable to form a stable oxy-ferrous complex, precluding their function as oxygen transporters. This may be an incomplete picture, as other molecules or proteins may associate with these hemoglobins *in vivo* and act as stabilizers and/or reducing agents. The results presented here provide an introductory look at autooxidation of hxHbs.

The four hemoglobins studied are rice non-symbiotic hemoglobin (rhb1), *Synechocystis* hemoglobin (*Syn*Hb), human neuroglobin (Ngb) and human cytoglobin (Cgb). An array of B10 rice mutant hemoglobins were also studied. The B10 residue is a critical influence on the ability of the distal histidine to form the hexacoordinate complex (Kundu 2004).

Autooxidation can occur rapidly, so it became important to be able to quantify the starting point. One has to be able to create a stable oxy-ferrous species from a ferric starting point. To do this, an enzyme system adaptation (Asakura T. 1972) was set up to reduce the ferric hemoglobin. The enzyme system used includes glucose-6-phosphate (G6P), glucose-6-phosphate dehydrogenase (G6PDE), ferredoxin reductase (FDR) and nicotinamide adenine dinucleotide phosphate ($NADP^+$). G6PDE will use G6P to reduce $NADP^+$ to NADPH. NADPH will then reduce FDR, which in turn reduces the heme iron. The available oxygen

in the buffer would provide the oxy-ferrous species. Thus a ratio could be obtained from the difference in ferric peak height and oxy-ferrous peak height, which was then applied to the autooxidation spectra. From there a fit can be calculated through Igor by fixing the amplitude and applying the same single exponential rate equation. It was found that the changes in longer time scale autooxidations are minimal and thus there was no reason to use an extended fit for the data. The difference on rapid time scales is significant, and the extended fit is a better match to the data. The autooxidation values for rates over 0.1 h^{-1} use the extended fit model.

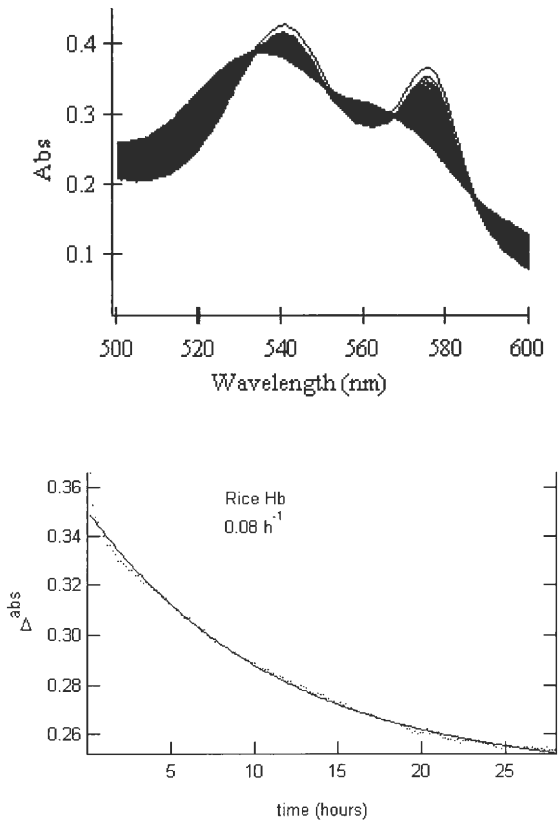

Figure 7. Autooxidation of wt rhb in a Cary 50 UV/Vis spectrophotometer. Protein sample was treated with DT then run over a G25 column to remove excess DT. Absorbance spectra change is measured at 576nm.

Results

Figure 7 shows the typical oxy-ferrous hemoglobin spectra that are oxidizing to ferric. Rice Hb1 is an interesting example, as it is hexacoordinate, yet has an autooxidation rate similar to that of oxygen carrying proteins, like myoglobin. These results indicate that it is not just the initial coordination state of a hexacoordinate protein that determines its ability to bind and retain ligands.

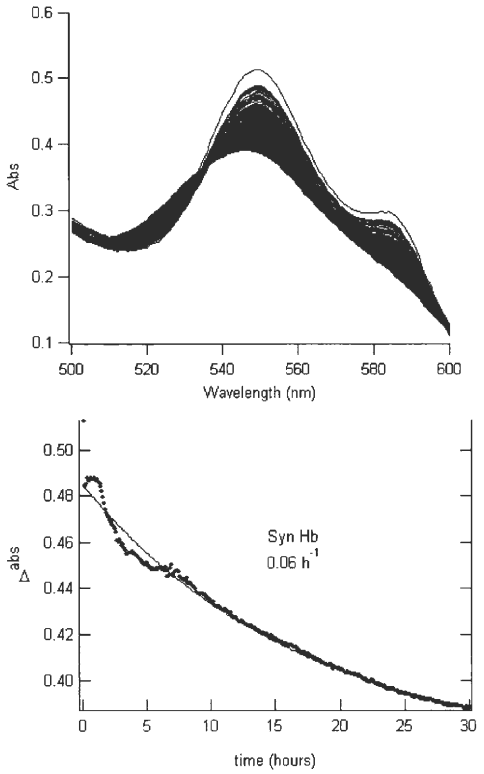

Figure 8. Autooxidation of wt synHb in a Cary 50 UV/Vis spectrophotometer. Protein sample was treated with DT then run over a G25 column to remove excess DT.

only plant hxHbs that autooxidize slowly, other bis-histidyl hemoglobins can release oxygen gradually.

Figure 9 below shows the change in absorbance with wt Ngb in the same conditions as the wt rhb1 above. Wt Ngb's rate of autooxidation is two orders

Wt SynHb is a member of the truncated hemoglobin family, from *Synechocystis* sp. PCC 6803. It's considered truncated because of its structure that is a 2 on 2 fold rather then the 3 on 3 fold found commonly in hemoglobins. It too acts similar to wt rhb1, as seen in figure 8, when it autooxidizes. This demonstrates that it isn't

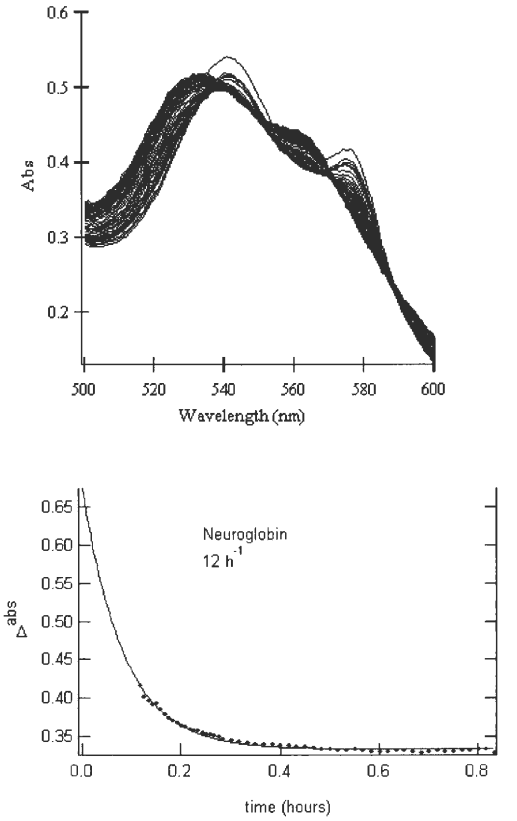

Figure 9. Autooxidation of wt Ngb in a Cary 50 UV/Vis spectrophotometer. Data at right is fitted with an extended exponential fit that accounts for initial autooxidation that occurs while sample is on G25 column. Rates calculated at 576 nm.

of magnitude greater than that of wt rhb1 and thus it became necessary to use an extended fit model in order to get an accurate portrayal of the autooxidation value.

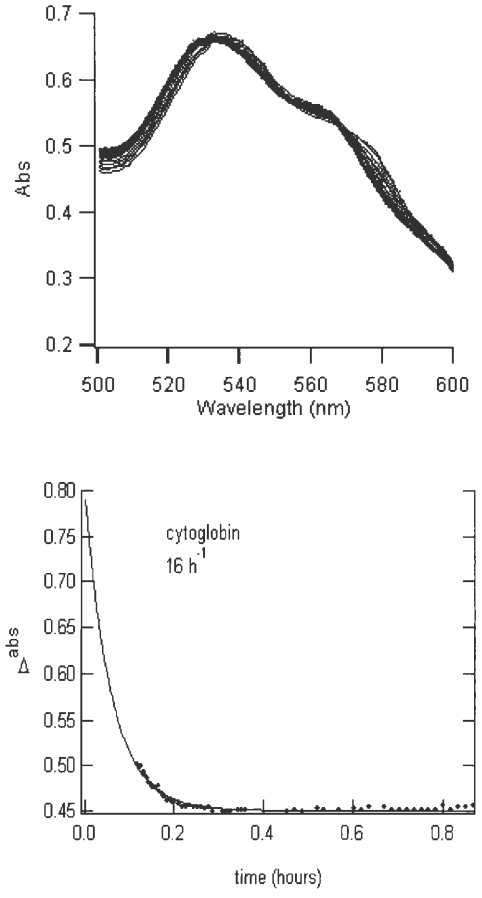

Figure 10. Autooxidation of wt cgb, as collected in the UV/Vis spectrophotometer.

Figure 10 below shows wt Cgb with an even faster rate than that of wt Ngb. Even though all efforts were made to obtain spectra that contain all values of the autoxidizing hemoglobin, it is almost impossible. These results show a definite distinction between human hxHbs versus plant hxHbs. Plant hxHbs have autooxidation rates similar to the pentacoordinate proteins in the animal world, like myoglobin (Brantley 1993). Human hxHbs autooxidize with rates that are two to three orders of magnitude faster than those of plant hxHbs. This indicates that human hexacoordinate proteins cannot act as oxygen carrying molecules. Their autooxidation rates are too fast for oxygen transport to be a practical function.

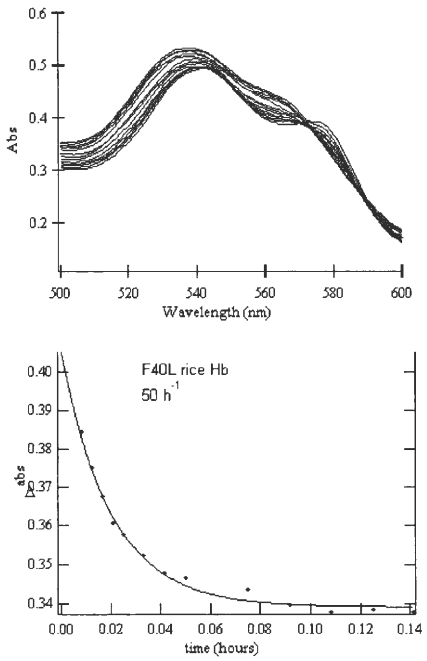

Figure 11. Autooxidation of F40L rhb collected in a rapid mixing stopped-flow apparatus. Data was fit with an extended fit to obtain data that wasn't able to be collected due to the speed of the reaction.

However, not all proteins are measurable in the Cary 50 due to the rapid rate of autooxidation, and thus rapid mixing must be used. Samples are prepared as described in the methods section below. Briefly, a minimal amount of DT is added to make a deoxy-ferrous complex. Oxygen is then injected to not only scavenge what little DT is left after reduction, but to also supply enough oxygen to form a stable oxy-ferrous complex without completely diluting the solution. Figure 11 shows data collected in the stopped-flow apparatus for one of the B10 rhb mutants. The graph shows a clear indication of the shift from oxy-ferrous to ferric. The rate here is one order of magnitude greater then that of wt Ngb, and four orders of magnitude greater then that of wt rhb1. This demonstrates that there is a relationship between the B10 phenylalanine group and the distal histidine as compared to that of the leucine used in Figure 11. The results in Table 2 below indicate that the removal of the wild type phenylalanine influences the ligand stability in most cases.

Table 2. Autooxidation and Oxygen off rates of selected hxHbs.

Protein	Wavelength (nm)	K_{O2} 23C h^{-1}	K_{off} s^{-1}
wt ngb	576	12.0	0.30
wt cgb	577	16.0	0.35
wt syn hb	549	0.06	0.02
wt rhb	576	0.08	0.04
F40G rhb*	575	58.0	2.30
F40W rhb	577	7.5	0.04
F40A rhb	576	12.0	2.60
F40V rhb	576	0.23	0.08
F40L rhb*	578	50.0	3.00
F40Y rhb	576	?	0.06

Rates collected in a UV/Vis spectrophotometer as measured at 23C unless indicated. * rates collected in a stopped-flow apparatus at 20C. K_{off} rates are from Benoit Smagghe (unpublished). All values were calculated with Igor Pro.

Discussion

It is of interest to see that autooxidation values between human and plant hemoglobins differ so radically. However, there is a correlation between the autooxidation rates and oxygen off-rates as determined previously (Hargrove 2000; Trent 2001). If the oxygen off-rates are rapid, this indicates that the protein structure allows the exit of the oxygen molecule. It is likely that the same machinery will react similarly in the presence of the superoxide moiety, thus autooxidation values will correlate directly. This is seen in Table 2, which makes a side-by-side comparison of autoxidation values versus oxygen off-rates.

The B10 residue of rice hemoglobin is important in determining heme function. By simply changing the B10 amino acid to a different residue, the function of the protein is

completely changed. Table 2 summarizes this by showing the relationship between autooxidation rates and O_2 off-rates in wt rhb1 and its mutant proteins. It is interesting that the F40Y autooxidation rate is unable to be collected successfully, although its off rate was comparatively slow. This contradicts against the previously ascribed convention, and further study is needed to elucidate the exact mechanism that explains these actions.

The results in Table 2 indicate that the B10 residue of hxHbs is important in stabilizing the heme pocket during ligand binding and releasing events. By mutating this residue, the heme pocket destabilizes and changes its ability to hold onto the ligand. Further study of CO on/off rates along with other associated ligands will be useful in determining a mechanism for ligand binding and release.

Autooxidation values are not the definitive answer to the function of hxHbs *in vivo*, although they can give insight into how the protein might function. Oxygen off-rates also contribute to the rate of autooxidation by providing a pathway that will generally work with superoxide and diatomic oxygen. By doing further mutagenesis work within the heme pocket, and further studies of autooxidation, one should be able to determine a mechanism for autooxidation. This will also contribute to determining a physiological reason for why hxHbs are expressed, and will help in verifying how cells function on a molecular level.

CHAPTER 4: Methods and Future Directions

Experimental Methods

Expression and purification of proteins

Wild type soybean leghemoglobin (Lba) and its mutant proteins (Kundu 2002), rice nonsymbiotic hemoglobin I (riceHb) (Hargrove 2000), *Synechocystis* hemoglobin (SynHb) (Hvitved 2001), human neuroglobin (Ngb) (Trent, Watts et al. 2001), human cytoglobin (Cgb) (Trent 2002) and wild type sperm whale Mb (swMb) (Kundu 2002) were purified using the methods cited for each. swMb mutant proteins (H64V/V68H (Dou 1995), H64Y (Hargrove, Singleton et al. 1994) 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 reduction

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 gas-tight 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. In our reactions, DT serves both

as reductant and to ensure that any free oxygen is destroyed. The vast excess of DT used, and the fact that most oxygen was removed prior to the experiment, prevented loss of much DT due to its role in oxygen removal. The extinction coefficient used for quantification of DT was 8 mM^{-1} cm^{-1} at 314 nm (Di Iorio 1981). 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 fitted using a single exponential decay expression, and all figures displaying kinetic data were prepared using the program Igor Pro (Wavemetrics).

Autooxidation methods

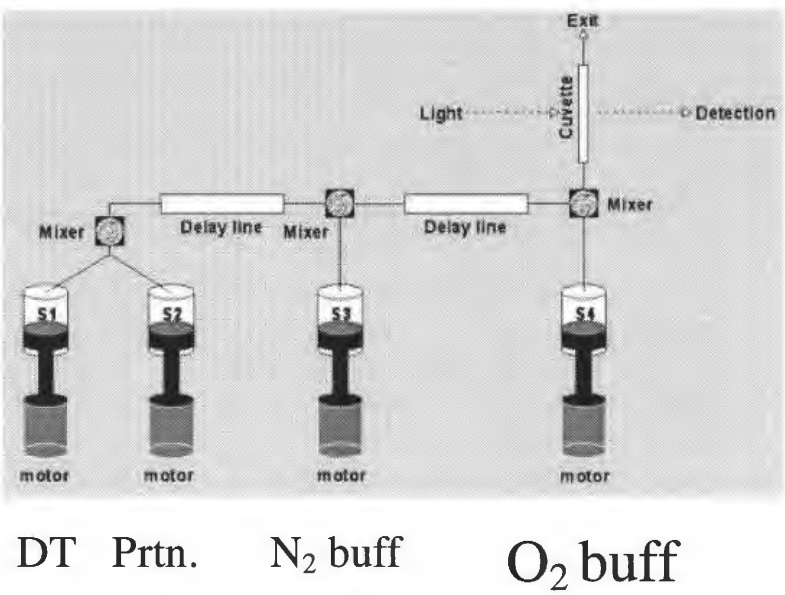

Figure 12. The stopped flow setup for autooxidation. A four syringe independent system is needed for all adjustments needed to control the concentration of DT so that the minimum amount is used for optimum reduction.
Picture from Biologic website.

Methods similar to Brantley (Brantley 1993) and Fago (Fago A 2004) were used. Formation of the ferrous state was completed by adding DT. Proteins were then passed over a G25 column at 4C to remove excess DT. Protein is then placed in a quartz cuvette and spectra are collected until the oxy-ferrous protein is fully oxidized. Observations were made at the visible peaks, between 500 and 600 nanometers. Data were evaluated in Igor Pro where it was fitted with single exponential equation $Y= A_0 + A_T e^{(-k_2 X)}$, where k_2 is equal to the autooxidation rate.

Rapid autooxidiation rates were measured in a BioLogic SFM 400 stopped-flow reactor coupled to a MOS 250 spectrophotometer. The setup can be seen in Figure 11. This system used N_2 sparged buffer in syringes one through three. Syringe four is then saturated with oxygen. Oxygen was injected into the reaction cuvette to remove minimum DT and created an oxy-ferrous complex. The oxy-ferrous complex was then observed to autooxidize. Data were exported into Igor Pro and fitted to a single exponential equation $Y= A_0 + A_T e^{(-k_2 X)}$, where k_2 is equal to the autooxidation rate. DT equilibration samples were collected in a Cary 50 UV/Vis spectrophotometer. 100 mM KH_2PO_4 with 1 mM EDTA buffer was sparged with N_2 gas for twenty minutes prior to use. Cuvette was sealed with latex septum and then sparged with N_2. Buffer, quantitated DT solution in buffer, and protein sample were injected through septum and then scanned in spectrophotometer using scanning kinetics. Data analysis was performed with Igor Pro.

Enzyme Reduction methods

Ferric protein samples were measured in a Cary 50 UV/Vis spectrophotometer. Approximately 5 uM protein in 100mM KH_2PO_4 buffer was incubated with 60 uM NADP, 5ug/ml G6P, 5ug/ml G6PDE, and 0.985uM FDR. Spectra were collected until an oxy-ferrous peak appeared stable. The ratio between the oxy-ferrous peak and the ferric peak was determined and applied to the autooxidation peak changes. Data were exported into Igor Pro and fitted to a single exponential equation $Y= A_0 + A_T e^{(-k_2 X)}$, where k_2 is equal to the autooxidation rate of the given sample and A_T equals the absolute amplitude as determined by the enzyme reductase system.

Discussion of Future Directions

The results presented in this thesis lay the groundwork for the basic chemistry often associated with hemoglobin proteins. Reduction and autooxidation are important mechanisms, and must be understood in order to be able to assign a physiological role. Reduction plays an important role in preparing the protein for specific reactions, such as ligand binding or NO scavenging, as seen in Figure 2. Autooxidation is an important factor for determining whether or not oxygen transport is a viable function of that specific hemoglobin. However, even if oxygen transport is not an integral part of the function of hxHbs, the ability to bind oxygen may play an important role in that protein. A Hypothesis has been presented that neuroglobin may use oxygen binding as a way to signal other molecules in a pathway, specifically in its ability to interact with G proteins (Dahlstrom 2004). This hypothesis has been largely untested, and further studies are required in the basic chemistry of these hemoglobins in order to determine a solid understanding that will direct further research in more feasible areas.

In order to assign a mechanism of autooxidation to hxHbs, more work must be done. An analysis of autooxidation rates under different concentrations of oxygen could be used in accordance with the Brantley model, which should indicate the type of autooxidation that occurs. An in depth mutagenesis study could also be beneficial. One could determine what residues are critical to the function of these expressed proteins. It is also important to find out exactly what hxHbs interact

with, and what conditions up-regulate expression. Basic chemical knowledge with *in vivo* data will help in the assignment of a physiological function to hexacoordinate hemoglobins.

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Acknowledgements

This work was made possible by the National Institutes of Health (R01-GM065948) and the USDA (Award No. 99-35306-7833). We thank John S. Olson for sperm whale myoglobin mutant proteins and helpful discussions regarding this work. I would also like to thank all members of the Hargrove lab for their input, support and patience.