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RICE UNIVERSITY

The Structural Determinants of Myoglobin Stability

by

Mark S. Hargrove

A THESIS SUBMITTED IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE DEGREE Doctor of Philosophy

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Abstract

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A quantitative understanding of the stability and expression of hemoglobins and myoglobins must take into account all of the factors which govern heme affinity and the folding of the globin portions of the molecules. In order to evaluate these factors, a new assay for measuring the rate of hemin loss was developed, and fluorescence techniques were adapted to monitor apoglobin unfolding.

Native apomyoglobin has roughly the same size and shape as the holoprotein, including a hydrophobic crevice for heme binding, but its overall structure is less helical and more solvated. Addition of small amounts of denaturant to apomyoglobin produces an intermediate which lacks a well-defined heme pocket but still contains a hydrophobic core made up of three $\alpha$ helices. Further addition of denaturants produces a completely unfolded state. This three state unfolding reaction has traditionally been monitored by changes in circular dichroism but is more easily visualized by changes in tryptophan fluorescence. The heme pocket in native apomyoglobin is stabilized by the presence of hydrophobic amino acids which exclude solvent from the binding site. Introduction of polar amino acids into this region greatly destabilizes native apomyoglobin causing
formation of the molten globular intermediate due to solvation of the heme pocket.

The binding of hemin to apomyoglobin is very favorable and results in the release of $\sim -77$ kJ/mol of Gibbs' free energy under physiological conditions. The association rate constant is $\sim 1 \times 10^8$ M$^{-1}$s$^{-1}$ and little affected by the structure of the apoglobin or reaction conditions. As a result, the equilibrium constant for hemin binding is determined primarily by its dissociation rate constant which is markedly dependent on apoprotein structure and ranges from $<0.01$ h$^{-1}$ to $>100$ h$^{-1}$ at pH 7, 37°C. The principle factors determining hemin affinity are: (1) non-specific hydrophobic interactions, which account for $\sim -36$ kJ/mol of the free energy change produced by the formation of holomyoglobin from hemin and apoglobin; (2) specific van der Waals and electrostatic interactions between the porphyrin and heme-pocket amino acid residues, which account for another $-18$ kJ/mol; and (3) the covalent bond between His$^{93}$ (F8) and the iron atom, which accounts for the remaining $\sim -23$ kJ/mol.
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I would like to thank Dr. Graham Palmer, Dr. Michael Quillin, Tod Romo, and Jennifer Barry for invaluable help with my work and leisure time. Dr. Palmer has offered much help and friendship since my first summer in the department. Mike has taken many of my “wild ideas” and helped me turn them into functional hypotheses. Tod can make a computer behave, and Jennifer knows what will and won’t work in the laboratory and has sent me in the right direction more times than wrong. I also appreciate the selfless laboratory organization and hard work of Eileen Singleton, without whom I would have taken much more time to finish much less work. Thanks also to Dolores Schwartz for her personal commitment to the success of each graduate student in the department.

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<td>circular dichroism.</td>
</tr>
<tr>
<td>DMEH</td>
<td>dimethylester hemin.</td>
</tr>
<tr>
<td>GdmCl</td>
<td>guanidinium chloride.</td>
</tr>
<tr>
<td>Hb</td>
<td>hemoglobin.</td>
</tr>
<tr>
<td>IP6</td>
<td>inositol hexaphosphate.</td>
</tr>
<tr>
<td>k_H</td>
<td>rate constant for hemin dissociation.</td>
</tr>
<tr>
<td>K_H</td>
<td>equilibrium association constant for heme binding to apomyoglobin to form holomyoglobin.</td>
</tr>
<tr>
<td>K_{I,U}</td>
<td>equilibrium constant for the transition from the intermediate to the unfolded state of apomyoglobin.</td>
</tr>
<tr>
<td>K_{Mb,D}</td>
<td>equilibrium constant for the observed, GdmCl induced unfolding of holomyoglobin.</td>
</tr>
<tr>
<td>K_{N,I}</td>
<td>equilibrium constant for the transition from the native to the intermediate state of apomyoglobin.</td>
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<tr>
<td>NMR</td>
<td>nuclear magnetic resonance.</td>
</tr>
<tr>
<td>rHb0.0</td>
<td>recombinant human hemoglobin.</td>
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<tr>
<td>rHb0.1</td>
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Chapter 1

Introduction to studies of the stability and folding of myoglobin.

Myoglobin is a 17 kD, globular, monomeric heme-protein which is found in muscle tissue where it stores and facilitates the movement of oxygen. It is comprised of eight α helices (designated A-H) tightly packed around the iron protoporphyrnin IX prosthetic group, and is similar in structure to the α and β subunits of hemoglobin. The interior consists of predominately hydrophobic residues and a shell of polar residues coat the outside of the protein, interacting with solvent molecules. Heme binds in a hydrophobic crevice formed by the B, E, and F helices, and the heme propionates interact with polar surface residues and solvent. One F helix residue, His$^{93}$ (the proximal histidine), coordinates to the iron atom and plays a large role in securing the heme group to the protein matrix. The heme iron also coordinates to the four pyrrole nitrogens of the porphyrin macrocycle, leaving one site open for external ligand binding.

On brief examination, the chemistry of ligand binding to myoglobin is very similar to that of free heme. However, free heme is very insoluble under physiological conditions and rapidly oxidizes, losing its ability to bind oxygen. The resulting heme aggregates are toxic and difficult to remove from tissues where they accumulate.

Thus, a key function of globin is to sequester heme and prevent aggregation by isolating it from solvent. Globin also surrounds heme with specific amino acid side chains which modify the reactivity of the heme group to allow greater discrimination among the gaseous ligands O$_2$, CO,
and NO and to facilitate or inhibit oxidase and peroxidase reactions (Springer et al. 1994). These differences in environments imposed through apoprotein allow the wide variety of functions found among heme proteins.

Hemoglobin is a tetrameric protein consisting of two α and two β subunits and is the oxygen binding component in red blood cells. Hemoglobin binds oxygen cooperatively with an overall affinity which is significantly lower than that of myoglobin. Cooperativity allows efficient O2 uptake in the lungs where oxygen partial pressure is high and release in muscle capillaries where the partial pressure is much lower. The higher affinity of myoglobin facilitates O2 transfer from red blood cells into aerobic muscle tissue. Cooperative O2 binding to hemoglobin is a result of allosteric interactions between the α and β subunits. When isolated and purified separately, the α and β monomers are structurally and functionally similar to myoglobin showing high O2 affinity and hyperbolic binding. However, when these subunits aggregate to form α2β2 tetramers, allosteric interactions across the α1β2 interface cause a reduction in heme reactivity which ultimately leads to cooperative oxygen binding.

When free in solution, ferrous (Fe2+) heme rapidly oxidizes to the ferric (Fe3+) state (hemin) in the presence of air. Hemoglobin and myoglobin can only bind oxygen when the heme iron is in the ferrous oxidation state. In addition, the ferric His93-iron bond is weaker and results in rapid dissociation of the prosthetic group which ultimately leads to denaturation of the protein. Thus, another key role of the protein structure in hemoglobin and myoglobin is to slow oxidation of the heme iron. Under normal circumstances in vivo, oxidation and subsequent hemin loss can be
reversed by intracellular enzymatic reduction systems. However, oxidation does occur in vivo and a significant fraction of ferric hemoglobin and myoglobin is present at equilibrium (e.g. ~ 3% of the hemoglobin in human red blood cells is present in the ferric state (Bunn and Forget 1986).

Interest in the structure, function, and stability of hemoglobins and myoglobins has increased over the past ten years because of the possibility of engineering these molecules to serve as extracellular blood substitutes. A protein-based blood substitute produced synthetically in microorganisms could alleviate the need for donation of large quantities of whole human blood which is expensive, difficult to store for long periods of time, and can carry blood-borne pathogens. A major problem in the development of extracellular blood substitutes has been the instability of hemoglobin outside of the red blood cell. This instability is a problem both for oxygen transport in plasma and for expression of the recombinant protein in bacteria.

The breakdown of myoglobin and the subunits of hemoglobin can be visualized in the following scheme (adapted from Winterbourn and Carrel (1977) and Bunn and Forget (1986)):

\[
\begin{align*}
2\text{MbO}_2 & \rightarrow 2\text{Mb}^+\text{(H}_2\text{O}) \rightarrow \text{hemin + apoglobin} \rightarrow \text{precipitates (red cell lysis)} \\
2\text{H}^+ + 2\text{O}_2^- & \rightarrow \text{O}_2 + \text{H}_2\text{O}_2 \quad \text{irreversible hemichromes (7.1)}
\end{align*}
\]

Autooxidation produces aquometmyoglobin, Mb^+(H_2O), in which the proximal His^93(F8)-iron bond is weakened allowing hemin dissociation. In vivo, the oxidative step can be reversed by enzymatic reduction systems, and reactions with peroxide are inhibited by the presence of catalase. The molecular mechanisms involved in autooxidation have been determined in
great detail (Brantley et al., 1993). Hydrogen bonding between Ne-H of the distal histidine and bound oxygen inhibits dissociation of superoxide, and the apolar nature of the surrounding distal pocket limits contact of the bound ligand with H2O molecules which accelerate the oxidation reaction. Until very recently, much less was known about factors governing the rate of hemin dissociation from myoglobin and hemoglobin, due primarily to the lack of a convenient assay for measuring this process.

Apomyoglobin and apohemoglobin are much less stable than the corresponding holoproteins. Large concentrations of free globin enhance formation of Heinz Bodies in red blood cells. These bodies consist of large aggregates of precipitated apoprotein and heme and are associated with many blood disorders resulting from unstable hemoglobins. These types of globin aggregates are also found in inclusion bodies when hemoglobins are expressed in *E. coli*, both in the presence and absence of exogenously added hemin. Apohemoglobin is much less stable than apomyoglobin and heme dissociation from hemoglobin is nearly irreversible under physiological conditions due to denaturation and aggregation of the apoglobin. Thus, the molecular mechanisms involved in folding and stabilization of apoglobin are important both for understanding certain hemoglobinopathies and for the expression of extracellular blood substitutes in microorganisms.

The overall stabilities of myoglobin and hemoglobin depend on the oxidation state of the iron, the presence and nature of bound ligands, and the structure of the globin molecule which surrounds the prosthetic group. All of these factors complicate traditional measurements of folding and stability. Historically, the denaturation of myoglobin has been analyzed without
regard to the individual processes which contribute to its overall stability. The goal of my thesis research was to evaluate quantitatively the factors which govern the stability of myoglobin. First, a convenient assay for hemin loss was developed which allowed, for the first time, a systematic investigation of heme affinity in myoglobin and hemoglobin. Second, mutagenesis of distal pocket residues was used to obtain correlations between apoglobin folding, stability, and expression levels in bacteria. Third, the ideas developed in the heme affinity and apoprotein folding studies have been integrated to obtain a comprehensive and quantitative interpretation of the structural basis of holomyoglobin stability.
Chapter 2

Development of an Assay for Measuring Hemin Dissociation.

INTRODUCTION

All natural heme proteins contain Fe-protoporphyrin-IX as their prosthetic group. The term “heme” usually refers to ferrous or Fe-protoporphyrin-IX whereas “hemin” refers specifically to the ferric oxidation state. As shown in Fig. 2.1, the organic part of heme is made up of four pyrrole rings which are connected by methene groups. The optical absorption of heme results from $\pi \rightarrow \pi^*$ transitions within the porphyrin ring, charge transfer reactions between the porphyrin and the iron atom, and $d \rightarrow d$ transitions of the iron atom. The predominant Soret absorbance band is found between 380nm (free ferric heme) and 434nm (deoxy-myoglobin). Charge transfer and $d \rightarrow d$ transitions occur at lower energy ($\geq 560$nm) and are much less intense than the Soret band.

The intensity and energy of all of these absorbances are influenced by the oxidation and liganded state of the heme-iron. When heme binds to myoglobin or hemoglobin, the proximal histidine coordinates the fifth position of the heme-iron resulting in a much more intense, red-shifted Soret band than is present in free heme. More subtle changes in the heme spectrum are due to effects of other protein residues which define the heme pocket, but are not covalently bound to the heme. Heme dissociation from myoglobin results in a large change in absorbance which should, in principle, make measurements of heme dissociation convenient. However, myoglobin has a very high affinity for heme so that even in a very dilute
Figure 2.1: The structure of heme and its optical absorbance. A, Iron protoporphyrin IX contains four pyrrole groups, labeled A through C. B, The absorbance spectrum of CO heme showing the Soret, and a and b bands.
solution containing an equal ratio of apomyoglobin and heme, the steady state concentration of free heme is virtually zero \( (i.e. \ 10^{-12} \text{ to } 10^{-13} \text{ M}) \). One way to overcome this problem is to use a heme-sink to remove dissociated heme from the system before it re-associates with apomyoglobin. This would require the heme-sink to have an equivalent or higher affinity for heme or be present in higher concentrations.

Banerjee (1962 a,b,c) attempted to measure rates of hemin dissociation from myoglobin by mixing holoprotein with extremely high concentrations of alkyl imidazoles. Unfortunately, these experiments were not very successful and only partial removal of hemin from the protein was achieved. However, he did show that apomyoglobin can completely extract hemin from hemoglobin, demonstrating that the affinity of hemoglobin for heme is much lower than that of myoglobin. Smith et al. (1991) took advantage of the high affinity of myoglobin for hemin. They used excess apomyoglobin to measure the rates of hemin loss from a variety of peroxidases and other heme proteins with Soret absorption maxima quite different from that of ferric myoglobin.

Rates of heme dissociation from myoglobin can also be estimated by following time courses for heme re-orientation in newly re-constituted myoglobin. This measurement takes advantage of the fact that heme is present in only one of two possible stereochemical conformations in native myoglobin. When ferric heme is mixed with apomyoglobin, it binds equally in both conformations but then re-orient to give the native orientation. For heme to re-orient it must first dissociate from the protein matrix. Therefore, rates of re-orientation should correspond to rates of hemin dissociation.
La Mar and coworkers have estimated the rates of hemin loss from mammalian myoglobins by following the NMR changes associated with the loss of heme orientational disorder in newly reconstituted holoproteins (La Mar et al., 1984). This process has also been followed by changes in the CD spectrum of the heme group (Aojula et al., 1986; Light et al., 1987). In both cases the process exhibits a half-time of roughly one hour at pH 5.0, 25°C and was much slower at higher pH or in the presence of bound ligands.

Measurement of hemin dissociation from hemoglobin has been more successful because of its lower affinity for heme (which allows the use of lower affinity or lower concentrations of heme-sinks in heme loss experiments). Application of our hemin loss assay to this protein will be discussed in Chapter 3. However, the previous work on hemoglobin provided a number of key ideas. Bunn and Jandel (1968) measured time courses for the transfer of Fe$^{59}$ labeled hemin between chromatographically separable hemoglobins. In these experiments, fetal hemoglobin containing normal Fe$^{57}$-heme was incubated with adult hemoglobin containing Fe$^{59}$-heme. Because heme association is random, any dissociation would result in a re-distribution of the Fe$^{57}$ and Fe$^{59}$ hemes. After various incubation times, the proteins were resolved chromatographically and analyzed for radiolabeled iron. It was found that adding low spin ligands (i.e. CN$^-$, N$_3^-$) to aquomethemoglobin or reducing the iron to the ferrous state, in the presence or absence of O$_2$, effectively blocked heme exchange for times up to at least 3 to 4 days. Although successful, the Fe$^{59}$ exchange assay is expensive, tedious, and not well-suited for screening large numbers of different proteins and reaction conditions.
The rates of hemin exchange between methemoglobin and human serum albumin were re-examined by Benesch and Kwong (1990) by who monitored visible absorbance changes in the pH range 8 to 9. At lower pH, the spectrum of hemin bound to albumin is too similar to that of aquomet-hemoglobin to allow accurate time course measurements. Unfortunately, human serum albumin has a fairly low affinity for heme. As a result, even very high (millimolar) concentrations of albumin are not sufficient to completely extract hemin from adult human hemoglobin and no uptake from myoglobin can be measured.

In view of the difficulties with these existing methods, we constructed a mutant apomyoglobin which exhibits abnormal spectral properties but still retains a high affinity for hemin. This mutant apoprotein can be used for complete extraction of hemin from both hemoglobins and myoglobins, and yields absorbance changes large enough to allow reactions at low hemin concentrations. Based on previous work by Egeberg et al. (1990a), His$^{64}$ was replaced by Tyr. This substitution results in a metmyoglobin with a green color due to an intense absorbance band at 600 nm. This band has been attributed to direct binding of the tyrosine phenol side chain to the iron atom. We then characterized apomyoglobins containing the Tyr$^{64}$ mutation as potential reagents for measuring hemin loss. The crystal structure of the single Tyr$^{64}$ mutant was determined to 1.8 Å resolution. Additional substitutions were made at positions 68 and 29 in an effort to optimize the stability of the Tyr$^{64}$ apoglobin. Then rates of hemin loss from native and wild type myoglobin and selected mutants were measured at several temperatures and pH values to test the reliability of the assay.
METHODS

 Preparation of myoglobins and hemoglobins - Native sperm whale myoglobin was obtained from Sigma prior to the ban on whale products. H64Y, H64A, and H64Y/V68F mutants were constructed at Rice University using the cassette mutagenesis method described by Egeberg et al. (1990a). L29F/H64Y and L29F/H64Y/V68F were also constructed by cassette mutagenesis using a pEMBL19 vector containing the gene for L29F myoglobin (Carver et al., 1992). The R45E sperm whale myoglobin mutant was constructed by oligonucleotide-directed mutagenesis of the wild-type gene in pEMBL19. V68A and V68F myoglobins were expressed from pUC19 vectors constructed by Egeberg et al. (1990b) at the University of Illinois. All mutant myoglobins were purified as described by Springer and Sligar (1987) and Carver et al. (1992). Extinction coefficients of the Tyr$^{64}$ holoproteins were measured using the pyridine hemochromogen method described by Riggs (1981).

Apomyoglobins were prepared using the methylethyl ketone method described by Ascoli et al., (1981). After hemin extraction with methylethyl ketone at low pH, globin samples were dialyzed extensively against 10 mM potassium phosphate, pH 7.0. The apoprotein was then centrifuged to remove precipitate, concentrated to approximately 0.8 mM using $\varepsilon_{280} = 15.2$ mM$^{-1}$ cm$^{-1}$ (Light, 1987), and stored in liquid nitrogen. After rapid thawing, samples were centrifuged, used within 8 hours, and never refrozen.

Crystal structure of H64Y - Crystals of the ferric form of Tyr$^{64}$ were grown in 2.6 M ammonium sulfate at pH 9.0 as described by Phillips et al.
(1990) and belonged to the P6 space group. Diffraction data were collected on a Siemens X-1000 area detector as described by Quillin et al. (1993). The data were 91.1% complete at the limiting resolution of 1.8 Å and had an \( R_{\text{sym}} \) of 5.1% for all reflections. The starting model for refinement was generated from wild-type metmyoglobin coordinates (Protein Data Bank entry 1mgn, Brookhaven national Laboratory). All refinement and map calculations were performed with X-PLOR using the PROLSQ parameter set (Brunger et al., 1989). After several cycles of refinement, manual refitting, and solvent placement, the crystallographic R-factor converged to 15.3 %, with RMS deviations of 0.02 Å in bond lengths, 2.6° in bond angles, 21° in dihedral, and 4.3° in improper angles. The atomic coordinates have been submitted to the Brookhaven Protein Data Bank (1mgn).

**Heme loss assay parameters** - All hemin dissociation experiments were carried out in 1 cm path length, 1 ml volume cuvettes, containing a 800 µl reaction mixture. For most experiments this mixture consisted of 600 µl of 0.2M buffer in 0.6M sucrose, 150µl of 10 mM potassium phosphate pH 7.0 containing the appropriate amount of apoglobin, and 50 µl of a stock holoprotein solution. The pH of these reaction mixtures never deviated more than 0.02 pH units from the pH of the 0.2M buffer used.

The reactions were started by adding holoprotein to a buffer-apoglobin mixture that had been pre-equilibrated in the six cell holder of a Shimadzu 2101 UV-VIS spectrophotometer equipped with a thermoelectric temperature controller (CPS-260). When multiple reactions were carried out, absorbance time courses were collected at a single wavelength, either 410 or 600 nm depending on the holoprotein concentration. In selected
cases, full spectra were collected as a function of time. In practice, most
time courses were monitored at 410 nm using 1-5 µM holoprotein. Data
were stored as sets of time and absorbance points and analyzed in terms of
exponential decays using the data analysis program IGOR™. The 0.2 M
reaction buffers were sodium acetate at pH 5.0 and 5.5, MES (2-[N-
morpholino]ethane sulfonic acid) at pH 6.0, and potassium phosphate at pH
7.0 and 8.0. The rate of hemin dissociation from myoglobin is very pH
dependent and great care must be taken to ensure that the final reaction
mixture is at the desired pH.

In most of the assays, the ratio of apoglobin to holoprotein was kept
high enough for complete hemin exchange so that the observed rate would
equal that of hemin dissociation from the original intact heme protein
(Equations 2.1 and 2.2). For myoglobins, the observed time courses were
fitted to a single exponential expression with either an offset to represent the
absorbance value at the end of the reaction and/or a second exponential term
constrained to represent slow absorbance drifts due to denaturation.
Hemoglobin data were fitted to a two exponential expression with an offset.

At high apomyoglobin concentrations and temperatures, increases in
absorbance due to apoprotein denaturation were observed for incubation
times ≥ 3 hours. To address this problem, sucrose was used to stabilize
apoglobin at 37°C for the long periods of time necessary to carry out many
of the hemin loss experiments. To determine the assay conditions which
would minimize absorbance drift, the assay was conducted in the presence of
different concentrations of sucrose and glycerol varying from 0.1 to 2 M.
Glycerol had little effect at low concentrations but increased the amount of
absorbance drift and lowered the rate at higher concentrations (0.5 to 1.0 M). The rates of hemin loss begin to decrease significantly (≥30%) at sucrose concentrations approaching 1M whereas at concentrations less than 0.3 M little stabilization of the apoprotein was observed. As a compromise, most of our kinetic experiments were carried out in 0.45M sucrose, which significantly inhibits globin precipitation but has little effect on rates of hemin loss. However, even this solvent perturbation is not completely successful at alleviating slow drifts in absorbance traces recorded for longer than 10 hours.

RESULTS AND DISCUSSION

Spectral and structural characteristics of the His\textsuperscript{64}→Tyr mutation-
Substitution of Tyr for His\textsuperscript{64} results in a mutant protein with spectral characteristics distinct from native metmyoglobin (Fig. 2.2A). These changes include a decrease in intensity and slight red-shift of the Soret maximum and the appearance of an intense band at 600 nm which gives Tyr\textsuperscript{64} mutants their unique green color. Egeberg \textit{et al.} (1990a) suggested that these spectral changes are a result of direct coordination of Tyr\textsuperscript{64} to the ferric iron atom. We have verified this interpretation directly by X-ray crystallography.

A |2F\textsubscript{o}-F\textsubscript{c}| electron density map of the distal pocket in the single His\textsuperscript{64}→Tyr mutant is shown in Fig. 2.3. As observed for most other position 64 mutants (Quillin \textit{et al.}, 1993), the Tyr\textsuperscript{64} substitution does not cause significant perturbations of the overall myoglobin structure. Two major changes do occur in the immediate vicinity of the iron atom. First,
**Figure 2.2:** Spectral characteristics of H64Y/V68F metmyoglobin as a reagent for measuring hemin loss. A) Absorbance spectra of native (solid line) and H64Y (dashed line) myoglobin. The inset enlarges the visible wavelength region. B) Time courses for hemin dissociation from native sperm whale myoglobin in 0.15 M buffer and 0.45 M sucrose at pH 5.0, 5.5, and 7.0, 37°C. The buffers were sodium acetate at pH 5.0 and pH 5.5, and potassium phosphate at pH 7.0. These reactions were carried out by mixing 3 μM native myoglobin with 30 μM H64Y/V68F apoprotein, and time courses were measured at 410 nm. These differences between the spectra of native and H64Y metmyoglobin allow measurement of hemin transfer from one protein to the other.
Figure 2.3 Stereoscopic view of the crystal structure of H64Y. A $|2F_o-F_c|$ density map of the heme pocket is overlaid with the H64Y model backbone structure. This view shows the heme pocket looking from position CD1 (Phe$^{43}$) toward E11 (Val$^{68}$) which is behind the page and not shown. The covalent bonding of the phenoxide to the heme iron atom is clearly shown.
there is continuous electron density between the iron atom and the Tyr$^{64}$ side chain. The phenol O-Fe distance is very short, 1.9 Å, and the C-O-Fe angle is 143°. Both of these values are very similar to those reported for the tyrosine-iron complex in catalase (Murphy et al., 1985). Second, the average proximal His$^{93}$-Fe bond length increases from 2.2Å in wild-type to 2.6Å in the Tyr$^{64}$ mutant which indicates significant weakening of this bond. Maurus et al. (1994) obtained a similar but lower resolution structure for H64Y horse heart myoglobin several months after the results in Fig. 2.3 were published.

*Attempts to engineer a more stable apomyoglobin with high hemin affinity* - Time courses for hemin exchange between native metmyoglobin and excess Tyr$^{64}$ apomyoglobin are shown in Fig. 2.2B. At pH 5.0, 37°C, the hemin dissociation reaction is relatively fast ($t_{1/2} \approx 40$ min at 37°C) and easily measured. In contrast, the reaction at pH 7.0 is extremely slow ($t_{1/2} \approx 100$ hr). These rates are consistent with the rates of self-exchange estimated by La Mar et al. (1984), Aojula et al. (1986), and Light et al. (1987) for the loss of heme orientational disorder in newly reconstituted myoglobins.

The rate of hemin dissociation from the Tyr$^{64}$ single mutant is \( \sim 20 \text{ h}^{-1} \) at pH 5.0, whereas that for wild-type protein is \( \sim 1 \text{ h}^{-1} \). Assuming similar association rate constants, the differences in dissociation rate constants suggests that native myoglobin has a 20-fold higher affinity for hemin than the single mutant. Thus, a 50 to 100-fold excess of Tyr$^{64}$ apoprotein has to be added for complete extraction of hemin from the native holoprotein at pH 5.0. Such high concentrations of apoprotein enhance spurious absorbance
changes due to globin denaturation, which is fairly rapid for the single Tyr64 apoglobin at 37°C.

Two double mutants, H64Y/V68F and L29F/H64Y, and one triple mutant, L29F/H64Y/V68F, were constructed in an attempt to find a more stable apoprotein with higher affinity for hemin. Previous work with single mutants had shown that the single Leu29→Phe and Val68→Phe substitutions produce proteins with enhanced expression levels and resistance to denaturation. However, the benzyl side chain of Phe29 appears to prevent tyrosine coordination with the iron in the double mutant H64Y/L29F. As shown in Fig. 2.4A and Table 2.1, the Soret maximum of Phe29Tyr64 myoglobin is 390 nm at both pH 5.0 and 7.0, which is indicative of a five-coordinate, high spin ferric iron-porphyrin complex. This interpretation is supported by the high rates of hemin dissociation observed when Phe29Tyr64 myoglobin is mixed with native apomyoglobin (Fig. 2.4B). In the triple mutant L29F/H64Y/V68F, Tyr64 is coordinated to the iron atom at pH 7.0, but not at pH 5.0. However, even at neutral pH, the rate of hemin dissociation from the triple mutant is much greater than that of the single Tyr64 mutant (Tables 2.1 and 2.2).

The Val68→Phe mutation was more successful at stabilizing the Tyr64 apoprotein. At pH 7.0, 37°C the rate of denaturation of Tyr64Phe68 apoglobin was much slower than that of the Tyr64 single mutant as judged by absorbance increases due to turbidity. In addition, the rate of hemin dissociation from Tyr64Phe68 holoprotein is ~2-fold slower than that from the single mutant at pH 5.0 (Fig. 2.4B, Table 2.2). At pH 7.0, the rates of hemin dissociation from Tyr64 and Tyr64Phe68 myoglobins are very slow
Figure 2.4: Spectral and functional characteristics of other Tyr$^{64}$ containing mutants. A, absorbance spectra of the double and triple mutants at pH 7.0. The Soret maximum at pH 7.0 for the L29F/H64Y/V68F and H64Y/V68F mutants is 411 nm; that for L29F/H64Y is 390 nm. B, rates of hemin loss from Tyr$^{64}$ containing mutants in 0.15 M sodium acetate and 0.45 M sucrose at pH 5.0, 37° C. The reaction mixture consisted of 3 mM ferric Tyr$^{64}$ holoprotein and 11 mM native apomyoglobin in 0.15 M sodium acetate, pH 5.0, and 0.45 M sucrose. The time courses were measured at 410 nm. H64Y/V68F was chosen as the best Tyr$^{64}$ mutant for use as a hemin loss reagent because of its spectral characteristics and high hemin affinity.
Table 2.1: Millimolar extinction coefficients and absorption maxima of the Tyr^{64} sperm whale myoglobin mutants.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>pH 5.0</th>
<th></th>
<th>pH 7.0</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>λ_{max}</td>
<td>ε_{max}</td>
<td>λ_{max}</td>
<td>ε_{max}</td>
</tr>
<tr>
<td></td>
<td>(nm)</td>
<td>(mM^{-1}cm^{-1})</td>
<td>(nm)</td>
<td>(mM^{-1}cm^{-1})</td>
</tr>
<tr>
<td>wild type</td>
<td>409</td>
<td>157</td>
<td>409</td>
<td>157</td>
</tr>
<tr>
<td>H64Y</td>
<td>411</td>
<td>88</td>
<td>411</td>
<td>88</td>
</tr>
<tr>
<td>H64Y/V68F</td>
<td>411</td>
<td>90</td>
<td>411</td>
<td>90</td>
</tr>
<tr>
<td>L29F/ H64Y</td>
<td>390</td>
<td>61</td>
<td>390</td>
<td>61</td>
</tr>
<tr>
<td>L29F/H64Y/V68F</td>
<td>390</td>
<td>68</td>
<td>411</td>
<td>79</td>
</tr>
</tbody>
</table>
Table 2.2: Rates of hemin dissociation from Tyr64 myoglobin mutants at 37°C. Excess native apomyoglobin was mixed with 2 to 6 μM holoprotein. The buffer conditions were 0.15 M sodium acetate, 0.45 M sucrose pH 5.0, and 0.15 M potassium phosphate, 0.45 M sucrose pH 7.0. Most experiments were carried out in triplicate and the average value of the rate constants are shown. The errors represent the standard deviation from the mean of at least three completely independent experiments carried out on separate days.

<table>
<thead>
<tr>
<th>Mutant</th>
<th>k-γ at pH 5.0 (h⁻¹)</th>
<th>k-γ at pH 7.0 (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>1±0.5</td>
<td>0.01±0.004</td>
</tr>
<tr>
<td>H64Y</td>
<td>16</td>
<td>0.04</td>
</tr>
<tr>
<td>H64Y/V68F</td>
<td>6 ± 2</td>
<td>0.04</td>
</tr>
<tr>
<td>L29F/H64Y</td>
<td>110</td>
<td>25</td>
</tr>
<tr>
<td>L29F/H64Y/V68F</td>
<td>55</td>
<td>11</td>
</tr>
</tbody>
</table>
and more comparable to those of the native holoprotein. In view of its enhanced stability and affinity for hemin, Tyr$^{64}$Phe$^{68}$ apoprotein was chosen as the best reagent for routine measurements of hemin dissociation.

*The hemin displacement mechanism* - Hemin exchange between holoproteins and Tyr$^{64}$ apoglobin can be described by:

\[
\begin{align*}
\text{PH} & \underset{k_H}{\overset{k_{H'}}{\rightleftharpoons}} P + \text{H} + \text{Y} \underset{k_Y}{\overset{k_{Y'}}{\rightleftharpoons}} \text{YH}
\end{align*}
\]  

(2.1)

where PH is the holoprotein originally containing hemin; P, apoprotein; H, free hemin; Y, the displacing apoglobin (usually H$^{64}$Y/V$^{68}$F); and YH, holoprotein of the displacing apoglobin. A simple expression for the rate of change of [PH] can be derived by assuming that $d[H]/dt = 0$ and that the concentration of free hemin can be neglected compared to the total present, (*i. e.* $[H] \ll [PH] + [YH] = [H]_{\text{total}}$):

\[
\frac{d[PH]}{dt} = -\left(k_{H'}k_Y[Y] + k_{Y'}k_H[P]\right)[PH] + \frac{k_H[P]k_{Y}[H]}{k_H[P] + k_Y[Y]}
\]

so that

\[
k_{\text{obs}} = \frac{k_{-H} + k_{-Y}\left(\frac{k_H[P]}{k_Y[Y]}\right)}{1 + \left(\frac{k_H[P]}{k_Y[Y]}\right)}
\]  

(2.2)

$k_{\text{obs}}$ is the observed rate constant at a given ratio of the apoproteins $[P]/[Y]$.

In the presence of excess Tyr$^{64}$ apoglobin, the time course will exhibit simple first order kinetics since $[P]/[Y] \approx 0$ throughout the reaction.
and $k_{\text{obs}} = k_{-H}$, the rate constant for hemin dissociation from the original holoprotein. At lower initial Tyr$^{64}$ apoprotein to holoprotein ratios, the initial rate will still be $k_{-H}$ since $[P] = 0$ at the beginning of the reaction. Under these conditions, the observed rate will change as the ratio of $[P]/[Y]$ increases, and the reaction will accelerate if $k_{-Y} > k_{-H}$, decelerate if $k_{-Y} < k_{-H}$, or exhibit simple exponential behavior if $k_{-Y} = k_{-H}$. These effects are small and difficult to measure experimentally because they can only be observed when the equilibrium hemin displacement is less than ~70% complete. For example, if native myoglobin were mixed with a very small amount of Tyr$^{64}$Phe$^{68}$ apoglobin at pH 5, the observed rate would increase from 1 h$^{-1}$ at the beginning of the reaction to 6.0 h$^{-1}$ at the end, and a fit of the time course to a single exponential expression would give $k_{\text{obs}} \sim 3$ h$^{-1}$. This would be difficult to measure because the observed absorbance change would be diminishingly small. The reverse reaction, Tyr$^{64}$Phe$^{68}$ holoprotein plus a small amount of native apoglobin, would show the opposite effect; $k_{\text{obs}}$ would decrease from 6.0 h$^{-1}$ to 1 h$^{-1}$. We have not been able to observe these effects experimentally due to slow drifts in the final absorbance values as a result of hemin precipitation and/or globin denaturation. In practice, the fitted values of $k_{\text{obs}}$ were similar with changing apoglobin (Y) to holoprotein (PH) ratios, and the initial rates were clearly the same.

An example of this type of experiment is shown in Fig. 2.5 for the reaction of Tyr$^{64}$Phe$^{68}$ metmyoglobin with increasing amounts of native apomyoglobin at pH 5.0, 37°C. In this case, the displacing apoprotein contains His$^{64}$ and the original holoprotein contains Tyr$^{64}$ so that an
Figure 2.5: Time courses for hemin dissociation from H64Y/V68F myoglobin as a function of the ratio of apoglobin to holoprotein. Hemin loss from H64Y/V68F myoglobin was measured at 410 nm in 0.15 M sodium acetate, pH 5.0, and 0.45 M sucrose 37°C, pH 5.0. Holoprotein concentration was 1.3 µM; native apomyoglobin concentrations are given beside each trace. The absorbance changes were defined as $(A_t - A_0)$, where $A_t$ is the absorbance at any time $t$ and $A_0$ is the initial absorbance. The initial velocities are the same for all ratios of apoglobin to holoprotein.
absorbance increase at 410 nm occurs (Fig. 2.1). The initial velocities are roughly the same in each trace and the fitted rate constants varied from 10 to 5 h⁻¹. At roughly equimolar apoprotein to hemin concentration, ~75% of the hemin was extracted from Tyr⁶⁴Phe⁶⁸ holoprotein. This shows that native apoprotein has a greater affinity for hemin than Tyr⁶⁴Phe⁶⁸ apoprotein, as predicted by the rates of dissociation shown in Tables 2.2 and 2.3.

Rate constants for hemin association were obtained from stopped flow experiments in which the apoglobins were reacted with either free hemin or reduced CO-heme in 0.2 M potassium phosphate, pH 7.0, 20°C. A detailed description of this reaction is given in Chapter 5. The lower temperature was chosen both to slow down the reaction and to allow comparison with previous experiments. As observed by Gibson and Antonini (1960, 1963), the reactions with free hemin are very heterogeneous and only a small, fast bimolecular phase is observed. The apparent bimolecular rate constants for the most rapid phase were 18, 14, and 17 μM⁻¹s⁻¹ for native, Tyr⁶⁴, and Tyr⁶⁴Phe⁶⁸ apoglobin, respectively. Rates of CO-heme binding were also measured because this form of the prosthetic group is mono-disperse and large bimolecular phases are observed. The association rate constants for CO-heme binding were 25, 40, and 30 μM⁻¹s⁻¹, respectively for the same set of proteins. Thus, the Tyr⁶⁴ mutation has little effect on the rate of hemin association; the ratio of k_H/k_Y in Equations 2 and 3 is ~1, meaning hemin affinity is governed primarily by the dissociation rate constant.

**Hemin dissociation from myoglobin mutants** - Rates of hemin dissociation from native, wild-type, and several mutant sperm whale
Table 2.3: Rates of hemin dissociation from selected myoglobin mutants at pH 5.0 and pH 7.0, 37°C. Excess H64Y/V68F apomyoglobin was mixed with 2 to 6 μM holoprotein. The buffer conditions were 0.15 M sodium acetate, 0.45 M sucrose pH 5.0, and 0.15 M potassium phosphate, 0.45 M sucrose pH 7.0. Most experiments were carried out in triplicate and the average value of the rate constants are shown. The limits shown for the native and H64A parameters represent the standard deviation from the mean of at least three completely independent experiments carried out on separate days.

<table>
<thead>
<tr>
<th>Sperm Whale Myoglobin</th>
<th>$k_{-H}$ at pH 5.0 (h⁻¹)</th>
<th>$k_{-H}$ at pH 7.0 (h⁻¹)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>1 ± 0.5</td>
<td>0.007 ± 0.004</td>
</tr>
<tr>
<td>Wild-type (E. coli)</td>
<td>0.7</td>
<td>0.01</td>
</tr>
<tr>
<td>H64A</td>
<td>17±2</td>
<td>0.40</td>
</tr>
<tr>
<td>V68A</td>
<td>5.8</td>
<td>0.10</td>
</tr>
<tr>
<td>V68F</td>
<td>2.0</td>
<td>0.01</td>
</tr>
<tr>
<td>R45E</td>
<td>5.5</td>
<td>0.20</td>
</tr>
</tbody>
</table>
Figure 2.6: Time courses of hemin loss from selected myoglobin mutants at pH 5.0, 37°C. Reaction conditions were 1.3 μM holoprotein, 30 μM H64Y/V68F apoglobin, in 0.15 M sodium acetate and 0.45 M sucrose. The absorbance changes are defined as $(A_t - A_0)$, where $A_0$ is the end point at infinite time, and have been normalized for comparison. Identical time courses were observed in the absence of sucrose, except for slow absorbance increases observed toward the end of the reaction due to apoprotein denaturation (see Methods). It is clear that the structure of the distal pocket plays a key role in hemin affinity.
**Table 2.4:** pH Dependence of the rates of hemin dissociation from native and selected myoglobin mutants at 37°C. Reactions were carried out using the buffer conditions described in Methods. The errors represent the standard deviation from the mean of three or more completely independent experiments.

<table>
<thead>
<tr>
<th>Myoglobin</th>
<th>pH = 5.0</th>
<th>pH = 5.5</th>
<th>pH = 6.0</th>
<th>pH = 7.0</th>
<th>pH = 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>1±0.5</td>
<td>0.1</td>
<td>0.005</td>
<td>0.007±0.004</td>
<td>0.03</td>
</tr>
<tr>
<td>H64A</td>
<td>17 ± 2</td>
<td>1.4</td>
<td>0.3</td>
<td>0.4</td>
<td>1.7</td>
</tr>
<tr>
<td>H64Y/V68F</td>
<td>6.5 ± 2</td>
<td>1.3</td>
<td>0.08</td>
<td>0.04</td>
<td>0.02</td>
</tr>
</tbody>
</table>
Table 2.5: Temperature dependence of the rate of hemin dissociation from native and selected myoglobin mutants at pH 5.0. Buffer conditions are described in the Methods. The errors represent the standard deviation from the mean of at least three independent experiments.

<table>
<thead>
<tr>
<th>Myoglobin</th>
<th>T = 15°C</th>
<th>T = 20°C</th>
<th>T = 25°C</th>
<th>T = 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>Native</td>
<td>0.3</td>
<td>0.4</td>
<td>0.5</td>
<td>1 ± 0.5</td>
</tr>
<tr>
<td>H64A</td>
<td>2.6</td>
<td>4.6</td>
<td>7.2</td>
<td>17 ± 2</td>
</tr>
<tr>
<td>H64Y/V68F</td>
<td>1.5</td>
<td>1.8 ± 0.2</td>
<td>3 ± 1</td>
<td>6.5 ± 2</td>
</tr>
</tbody>
</table>
myoglobin were measured in the presence of excess Tyr64Phe68 apoglobin (Fig. 2.6 and Tables 2.3-2.5). These mutants were selected to test the reliability of the assay system. Decreasing the size of the His64(E7) and Val68(E11) residues by changing them to alanines was expected to increase the rate of hemin dissociation (H64A and V68A in Table 2.3). Similarly, Arg45 has been predicted to hold hemin in place by forming a salt linkage with the heme-6-propionate. Thus the Arg45→Glu (R45E) substitution was expected to increase the rate of hemin loss (Table 2.3). The Val68→Phe (V68F) mutant has a rate of hemin dissociation which is effectively the same as that of native myoglobin, but this replacement does decrease the rate of hemin loss almost 2-fold at pH 5.0 when introduced into Tyr64 myoglobin (Table 2.2).

The rate of hemin dissociation from myoglobin increases 100-fold in going from pH 6.0 to 5.0. This effect is most easily measured for the H64A mutant which still exhibits a moderately fast rate of hemin loss at pH 7.0. In the case of wild-type or native myoglobin, the observed rate at pH 7.0 is very slow (~0.01 h⁻¹) and difficult to measure experimentally because of significant apoglobin denaturation during the 1 to 2 day incubation time required to define the complete time course. The strong pH dependence in the acid range is a potential source of variability at pH 5.0 because even an inadvertent change of 0.1 unit in this pH region will produce 50 to 100% changes in rate.

The dramatic increase in the rate of hemin loss at low pH is most likely due to protonation of the proximal His93 imidazole side chain, resulting in disruption of the Fe³⁺-His⁹³ bond. Giacometti et al. (1977)
and Coletta et al. (1985) have studied the effects of protonation of His\textsuperscript{93} on CO binding and estimated a pK\textsubscript{a} of about 3.5 for ferrous deoxy myoglobin. The higher rate of hemin dissociation from the Tyr\textsuperscript{64} mutants is due to a similar phenomenon. In this case, chelation to the Tyr\textsuperscript{64} side chain elongates the Fe\textsuperscript{3+}-His\textsuperscript{93} bond, weakening it significantly even at pH 7.0 (Table 2.2), and protonation of both the phenoxide and imidazole side chains occurs at low pH.

The temperature dependencies of hemin loss from native and H64A metmyoglobin at pH 5.0 are shown in Table 2.5. Studies were not attempted at pH 7.0 because hemin loss from native myoglobin at this pH is already very slow at 37°C.

As shown in Fig. 2.7, this assay can also be used to measure rates of hemin loss from methemoglobin. This time course clearly shows a biphasic process having a fast phase with a rate of about 8 h\textsuperscript{-1} and a slow phase with a rate of about 1 h\textsuperscript{-1} at pH 7.0, 37°C. A detailed study of hemin dissociation from hemoglobins is given in Chapter 3.

The results presented here show that Tyr\textsuperscript{64} apoproteins can be used as reagents for measuring hemin loss. The large differences between the absorption spectra of the ferric Tyr\textsuperscript{64} mutants and those of native metmyoglobins and methemoglobins allow measurement at relatively low (μM) protein concentrations which facilitates surveying a wide variety of mutants and reaction conditions. To our knowledge, this is the first convenient and quantitative assay of hemin dissociation from mammalian myoglobins at physiological temperatures and pH values.
Figure 2.7: Time courses for hemin loss from native hemoglobin at pH 7.0, 37°C in 0.15 M sodium acetate, pH 5.0, and 0.45 M sucrose. The symbols represent observed data, and the solid line represents a fit to a two exponential equation with equal amplitudes and an offset representing the absorbance at the end of the reaction. The reaction conditions were 2-4 mM hemoglobin and 10-30 μM H64Y/V68F apomyoglobin. These results show that H64Y/V68F apoprotein can be used also to measure rates of hemin dissociation from hemoglobin.
The temperature dependence results point out the major dilemma in studying hemin dissociation (Table 2.5). Prolonged incubation at physiological temperature leads to artifacts as a result of apoglobin denaturation. However, cooling the solution slows the reaction markedly, requiring much longer incubation times. The net result is about the same in terms of absorbance artifacts. This problem is partially alleviated by adding sucrose to inhibit protein denaturation and aggregation. The double mutant, H64Y/V68F, represents our first successful attempt to engineer a more stable Tyr$^{64}$ myoglobin which still retains high hemin affinity. Although considerably better than the single mutant, Tyr$^{64}$Phe$^{68}$ apomyoglobin still requires the use of 0.45M sucrose for reaction times $\geq$ 10-20 hours at 37°C. Fortunately, this concentration of sucrose does not appear to significantly affect the rates of hemin loss or the tertiary structure of the proteins measured here (Bellelli et al., 1993). Even in the presence of sucrose, the practical limit of the current double mutant assay system is 30-40 hours, which makes it difficult to quantitate the effects of mutations that inhibit hemin loss from myoglobin.

**Factors stabilizing heme binding in myoglobin** - In ferrous myoglobin and hemoglobin, the Fe$^{2+}$-proximal His$^{93}$ bond is effectively covalent and prevents heme loss from taking place on ordinary time scales at pH 7.0 ($\leq$ 3 or 4 days). After oxidation, this bond is considerably weakened which accounts for the more rapid rates of hemin dissociation from the ferric forms of these proteins. The marked increase in the rate of hemin dissociation at acid pH appears to be due primarily to protonation of the proximal histidine since a large pH dependence was observed for all distal
pocket mutants that were examined (Tables 2.2-2.4; Giacometti et al., 1977; Coletta et al., 1985).

Fig. 2.6 and Table 2.3 show that steric contacts between the side chains of His$^{64}$ and Val$^{68}$ and the porphyrin ring also inhibit hemin dissociation. Replacement of these amino acids with smaller alanine residues results in 5 to 10-fold increases in the rate of hemin loss from myoglobin, regardless of pH or temperature. Electrostatic interactions with the porphyrin propionates also play a role in lowering the rate of hemin dissociation. The interaction between Arg$^{45}$ (CD3) in sperm whale myoglobin (or Lys$^{45}$ in pig myoglobin) and the heme-6-propionate appears to inhibit hemin dissociation. When Arg$^{45}$ was mutated to glutamate, the rate of hemin loss from sperm whale metmyoglobin increased greater than 2-fold (Fig. 2.4, Table 2.3) and a similar result was observed for the Lys$^{45}$→Glu mutation in pig myoglobin. A more extensive study with a larger set of mutants at these and other positions in the heme pocket is presented in Chapter 6.

The only mutation in Table 2.3 that doesn't increase the rate of hemin loss is the Val$^{68}$→Phe replacement. In this case, the side chain of Phe$^{68}$ makes new contacts with the porphyrin ring (Quillinet et al., 1995). These interactions must be either neutral or favorable, because this mutation causes a 2-fold decrease in the rate of hemin dissociation from Tyr$^{64}$ myoglobin at pH 5.0 and has little effect on hemin loss from native metmyoglobin (Tables 2.2 and 2.3). In contrast, the Leu$^{29}$→Phe substitution promoted hemin dissociation from Tyr$^{64}$ myoglobin by preventing tyrosine coordination to the iron atom (Tables 2.1 and 2.2).
The results in Tables 2.3 and 2.5 show that H64Y/V68F apomyoglobin can be used to assay rates of hemin dissociation rapidly and accurately at micromolar concentrations. The remaining chapters in this thesis describe the use of the assay in determining the factors governing hemin dissociation from hemoglobins and myoglobins, determining the importance of heme affinity for holoprotein stability, and evaluating the importance of heme affinity in myoglobin expression.
Chapter 3
Hemin Loss from Human Hemoglobin

INTRODUCTION

Human hemoglobin is a tetrameric molecule made up of two different myoglobin-like subunits. These subunits, the $\alpha$ and $\beta$ chains, form $\alpha\beta$ dimers which themselves associate to form hemoglobin tetramers. Hemoglobin subunits assemble as shown in the following scheme:

\[
\begin{align*}
\alpha_1 + \beta_1 & \rightleftharpoons \alpha_1 \beta_1 \\
& \rightleftharpoons \alpha_1 \beta_1 \rightleftharpoons \alpha_1 \beta_1 \alpha_1 \\
& \approx 10^{-14} \text{M} \\
& \approx 10^{-6} \text{M}
\end{align*}
\] (3.1)

The $\alpha_1\beta_1$ dimer is predominantly held together through strong hydrophobic interactions between the two subunits. Formation of the hemoglobin tetramer results from relatively weaker electrostatic interactions between two $\alpha_1\beta_1$ dimers, resulting in formation of a tetramer with two new subunit interfaces called $\alpha_1\beta_2$ and $\alpha_2\beta_1$.

The hemoglobin tetramer can exist in either the T (low oxygen affinity) or the R (high oxygen affinity), quaternary conformation. In the absence of oxygen, hemoglobin is held in the T state by a lattice of electrostatic interactions at the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces. Interconversion between the T and R states is accomplished by rotating the $\alpha_1\beta_1$ dimer $15^\circ$ with respect to the $\alpha_2\beta_2$ dimer, or vice versa. The $\alpha_1\beta_1$ and $\alpha_2\beta_2$ interfaces are not affected by T to R interconversion, but formation of the R
state requires disruption of a significant number of the electrostatic bonds in the T-state $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces.

Like myoglobin, each hemoglobin monomer contains a heme group which reversibly binds oxygen. However, as described briefly in Chapter 1, aggregation of these subunits into tetramers results in cooperative ligand binding (Fig. 3.1). The first oxygen molecules bind to hemoglobin with low affinity and the last with high affinity. This cooperativity in oxygen can be explained by structural changes in the tertiary structure of each subunit which result from the T to R quaternary transition.

The difference in oxygen affinity between T and R state hemoglobin is determined by the reactivity of the heme iron. The iron in the heme groups of deoxyhemoglobin is five coordinate. The four pyrrole nitrogens of the porphyrin ring each bond to the iron and the proximal histidine bonds to the 5th axial position. Because there is no sixth ligand bound, the bond to the proximal His pulls the iron $\sim 0.4$ Å out of the plane of the porphyrin ring (Dickerson and Geis 1983). In the T quaternary state, the F-helix is constrained by interactions at the $\alpha_1\beta_2$ interface which inhibit in-plane movement of the iron atom. O$_2$ binding requires that the iron move into the plane of the porphyrin ring and, in the T-state, this movement puts strain on the proximal His and the F helix. One way for the tetramer to relieve this strain is to convert from the T to the R quaternary state. For this to happen, the strain on the proximal histidine resulting from oxygen bound to T state subunits must be sufficient to overcome the electrostatic interactions preventing the $\alpha_1\beta_2$ and $\alpha_2\beta_1$ interfaces from rotating to their positions in the R-state. In human hemoglobin, this much force is established sometime
Figure 3.1: Fractional saturation of sperm whale myoglobin and human hemoglobin as a function of oxygen partial pressure. Myoglobin binds oxygen hyperbolically with higher affinity than hemoglobin. Hemoglobin binds oxygen cooperatively showing a sigmoidal curve. At an oxygen pressure of 20 torr (present in muscle capillaries) the affinity of oxygen is relatively low. At 100 torr (present in the lungs) affinity is relatively high.
between the binding of the second and third oxygen molecules. After conversion to the R state, the last oxygen binds to a high affinity heme.

The binding of several small molecules affects the interconversion between the T and R quaternary states. These molecules include organic phosphates, H\(^+\), and CO\(_2\). Organic phosphates bind to a positively charged cleft which is present between the β subunits in the T, but not the R, quaternary states. Protons also bind preferentially to T state deoxyhemoglobin. This process, referred to as the Bohr effect, increases the likelihood of oxygen release by forcing hemoglobin into the state in which oxygen affinity is lower. Deoxyhemoglobin with bound H\(^+\) travels to the lungs where oxygen is in high concentration. The ligand binding reaction results in a conversion back to the R state, causing release of H\(^+\) which reacts with plasma HCO\(_3^-\) to give H\(_2\)O and CO\(_2\), both of which are expired in the alveolar capillaries.

Hemoglobin exists in vivo as either tetramers or dimers. The fraction of dimeric hemoglobin in a red blood cell or free in solution is a function of protein concentration and can be calculated by the following equation:

\[
\gamma_D = \frac{-K_{4,2}^{4,2} + \sqrt{\left(\frac{K_{4,2}^{4,2}}{H_o}\right)^2 + \left(\frac{4K_{4,2}^{4,2}}{H_o}\right)}}{2H_o} \quad (3.2)
\]

\(K_{4,2}^{4,2} = \) tetramer to dimer equilibrium constant
\(\gamma_D = \) heme fraction of dimers
\(H_o = \) total heme concentration
Since this equation shows that $\gamma_D$ depends on the ratio of $K_{4,2}$ to protein concentration, at high [protein], $\gamma_D \to 0$; at low, $\gamma_D \to 1.0$. Fig. 3.2 shows a plot of $\gamma_D$ vs. concentration with $K_{4,2}$ set at $1.5 \times 10^{-6}$ M. It is apparent that hemoglobin is predominantly in the tetrameric state at concentrations above 1 mM in heme. Hemoglobin is estimated to be about 20 mM in heme in the red blood cell. At this concentration the fraction of hemoglobin in the tetrameric state is about 99%. However when hemoglobin is released from red blood cells it is diluted significantly, and unless massive lysis has occurred, a large fraction of the extracellular protein will exist as a dimer. Dimerization of hemoglobin prevents cooperative oxygen binding and is the first step in the removal of hemoglobin from the blood stream (Bunn and Forget 1986).

Heme is incorporated into the $\alpha$ and $\beta$ chains of hemoglobin in much the same way that it is bound to myoglobin. The proximal histidine of each of these subunits forms a covalent bond to the iron atom and the other heme pocket residues form steric and electrostatic interactions with the porphyrin ring that are analogous to those seen in myoglobin. However, as described in the previous chapter, the overall heme affinity of hemoglobin is much lower than that of myoglobin.

Bunn and Jandel (1968) showed that time courses for hemin exchange between fetal and adult methemoglobins were biphasic leading to the proposal that $\alpha$ and $\beta$ chains lose hemin at different rates. They tested this interpretation with two types of exchange experiments. First, hybrids containing $\alpha$ subunits selectively labeled with Fe$^{59}$ were found to exchange
Figure 3.2: Fraction of dimeric hemoglobin as a function of subunit concentration. The curve was calculated using Equation 2 and a $K_{4,2}$ of $1.5 \times 10^{-6} \text{M}$. In red blood cells where the concentration of hemoglobin is $\approx 20 \text{ mM}$, hemoglobin exists as $\approx 99\%$ tetramer. A significant proportion of hemoglobin is dimeric at the concentrations used in most of the heme loss experiments (1 to 35 $\mu\text{M}$).
the isotope much more slowly than uniformly labeled hemoglobin. Second, when uniformly labeled fetal hemoglobin was incubated for 100 to 120 minutes with adult hemoglobin and the subunits of the adult protein were separated, the label was found almost exclusively in β subunits. Quantitative analysis of both experiments indicated that the rate of hemin exchange of β subunits was ~ 8-fold greater than that of α subunits.

Bunn and Jandel (1968) suggested that the cause of these differences in hemin loss probably involves structural differences at the CD corner in the region near the proximal histidine. In β subunits, residue CD3 is Ser$_{44}$ and there does not appear to be any stabilizing interactions between the side chain hydroxyl of this residue and the β heme-6-propionate, which is disordered in the oxy and deoxy structures of human hemoglobin (Shaanan, 1983). In α subunits, the CD3 residue is His$_{45}$ which forms a hydrogen bond with the α heme-6-propionate. In all mammalian myoglobins, residue CD3 is either Lys$_{45}$ or Arg$_{45}$ and is part of an extended hydrogen bonding lattice with the heme-6-propionate, His$_{64}$(E7), distal pocket water molecules, and Thr$_{67}$(E10) (Quillin et al., 1993).

This chapter describes the use of the H64Y/V68F apoglobin reagent developed in Chapter 2 to measure hemin loss from human hemoglobin. The first objective was to determine the relative rates of heme loss from the α and β subunits of hemoglobin and to confirm or refute the previous interpretations of Bunn and Jandel. The second objective was to determine the effects of protein concentration on the rates of hemin loss in an effort to understand the role of quaternary structure in heme affinity. The third objective was to determine the effects of allosteric effectors on rates of
hemin loss. Organic phosphates such as 2,3 diphosphoglycerate and inositol hexaphosphate hold hemoglobin preferentially in the T state and reduce $K_{4,2}$ significantly (Hensley et al., 1974). Therefore, it was expected that organic phosphates should have the same effect as high protein concentration on the rates of heme loss from hemoglobin.

**METHODS**

*Preparation of proteins-* Native and recombinant human hemoglobins were prepared as described by Mathews et al. (1990) using genes and expression vectors constructed by Nagai et al. (1987) and Tame et al. (1991). Recombinant hemoglobins contain the V1M mutation in both subunits to allow expression in E. coli. $\alpha$ and $\beta$ subunits were purified by the method of Bucci (1981). Recombinant hemoglobin genetically crosslinked with a glycine linker (rHb0.1) between the C-terminus of one $\alpha$ subunit and the N-terminus of another was made by Antony. J. Mathews at Somatogen Inc., and prepared as described in Looker et al. (1992).

Hemoglobin valence hybrids were also constructed by Antony. J. Matthews at Somatogen, Inc. Ferrous [(O2)$\alpha$(O2)]2 hemoglobin (72 mg/ml) in 10 mM sodium phosphate pH 6.9 was oxidized by slow addition (1 ml/min.) of 0.5 heme-equivalents of 1 mM potassium ferricyanide. After 30 minutes, the protein solution was equilibrated with one volume CO, diluted into an equal volume of ice cold buffer, and then loaded on to a CM-sepharose column (1.5 cm x 15 cm) equilibrated with the same pH 6.9 buffer at 4°C. From this point on, all buffers were saturated with CO gas. The sample was washed with one column volume of equilibration buffer and
then eluted with a linear sodium phosphate gradient from 10 mM pH 6.9 to 20 mM, pH 8.0 in 10 column volumes. The first peak (fully oxidized Hb4⁰⁺) eluted as a single band and was followed by two poorly resolved peaks (valence hybrids) and a final well resolved fourth peak (fully reduced Hb4(CO)⁴). The two valence hybrid peaks were fully resolved at room temperature on a Mono-S FPLC column (1.5 cm x 10 cm) equilibrated with 10 mM sodium phosphate pH 6.8. Proteins were eluted with a linear gradient from 10 mM pH 6.8 to 20 mM sodium phosphate pH 7.5. Assignment of peaks two and three to [α(CO)β⁺]₂ and [α⁺β(CO)]₂, respectively, were made with reference to Tomoda et al., (1978).

**Measurements of hemin loss** - The general procedures for measurement of hemin loss are described in Chapter 2. Unless otherwise noted, hemin loss from hemoglobin was measured in 0.15 M potassium phosphate, pH 7.0, in the presence of 0.45 M sucrose and at 37°C. The effects of pH and temperature on rates of hemin loss from hemoglobin were carried out as described in Chapter 2 for myoglobin. For the measurement of hemin loss as a function of hemoglobin concentration, the amount of apo-H64Y/V68F was always twice that of the hemoglobin subunit concentration. Time courses for hemin loss from samples at concentrations below 12 μM were monitored at 410 nm. Above 12 μM, time courses were monitored at 600 nm. Useful data could not be easily obtained above 35 μM using the simple spectrophotometric assay due to apohemoglobin precipitation which resulted in slow increases in absorbance at all wavelengths.

Hemin loss at high (600 μM) hemoglobin concentration was measured as follows. A mixture of 600 μM hemoglobin and 1200 μM apo
H64Y/V68F was divided into ~ 20 different 150 µl portions and incubated at 37°C. For each time point, one 150 µl sample was removed, brought to 0°C on ice, and centrifuged at 4°C to remove precipitate. 10 µl of this supernatant was diluted into 1 ml in a cuvette and an absorbance spectrum was measured. The change in absorbance at 410 nm was used to compute the ratio of holohemoglobin to holoH64Y/V68F myoglobin. The time course of loss of holohemoglobin was used to determine rates of hemin dissociation.

The rate of hemin loss from β subunits depends strongly on hemoglobin concentration. This dependence was analyzed in terms of differences in β k_-H for dimers and tetramers using Equation 3.2. The rate constant of hemin loss from tetrameric α and β chains was fixed to the values observed at high hemoglobin concentrations (600 µM) and that observed for genetically cross linked recombinant hemoglobin (rHb0.1). The values of k_-H for dimeric α and β chains and K4,2 were varied to obtain the best fit to the observed data using Equation 3:

\[ k_{-H(\text{observed})} = k_{-H(T)} + \gamma_D (k_{-H(D)} - k_{-H(T)}) \]  \hspace{1cm} (3.3)

where k_-H(T) = rate of heme loss from tetramers, k_-H(D) = rate of heme loss from dimers and \( \gamma_D = \) fraction of dimeric hemoglobin as given in Equation 3.2.

Rates of hemin loss from isolated α and β chains were measured in a Gibson-Dionex stopped flow apparatus at 37°C. Potassium ferricyanide was added to a syringe containing 20 µM of the oxygenated forms of either α or β chains in 0.15 M potassium phosphate, pH 7.0, 0.45 M sucrose to generate
the ferric forms in the apparatus. The contents of this syringe were quickly reacted with 40 μM apo-H64Y/V68F in the same buffer. Time courses of hemin dissociation were measured at 600 nm due to the absorbance of ferricyanide in the Soret region. Both chains showed large, very slow absorbance drifts toward the end of the reaction due to precipitation of the newly generated apoglobin chains. Rate constants for hemin loss were estimated from points at the beginning of the reaction by fitting to exponential expressions with an offset. Time courses for hemin dissociation from isolated β subunits were biphasic and showed an increase in the amount of slow phase with increasing protein concentration. These results suggest that hemin loss from monomeric β chains is greater than that from tetramers. The fraction of β monomers as a function of concentration was estimated from the following equations:

\[
\begin{align*}
4\beta & \xrightarrow{K_{4,1}} \beta_4 \\
\gamma_m 4 + \frac{K_{4,1}}{4H_0^3} \gamma_m - \frac{K_{4,1}}{4H_0^3} &= 0
\end{align*}
\]  

(3.4)  

(3.5)

where \( \gamma_m \) = fraction of β monomer and \( H_0 \) = total heme concentration. \( K_{4,1} \) was estimated by McGovern et al. (1976) to be \( = 1.25 \times 10^{-12} \) M³. Roots of Equation 3.4 were solved for each value of \( H_0 \) over the concentration range shown in Fig. 3.5.

Hemin loss from hemoglobin was also measured in the presence of inositol hexaphosphate (IP6). In this case, the reaction buffer contained
1mM IP6. Experiments involving IP6 were performed in 0.2 M Bis/Tris pH 7.0 instead of potassium phosphate to observe the maximum effect.

RESULTS

_Hemin dissociation from human hemoglobin_ - As shown in Fig. 3.3 A, hemin loss from methemoglobin is clearly a biphasic process having a fast phase with a rate of about 8 hr\(^{-1}\) and a slow phase with a rate of 0.5 to 1 hr\(^{-1}\) at 10μM heme and pH 7.0, 37°C. Bunn and Jandel (1968) observed a similar time course using isotope exchange techniques and assigned the fast phase to hemin loss from β subunits and the slow phase to hemin loss from α subunits.

These assignments have been verified in two different experiments. Valence hybrids were constructed in which one subunit pair was oxidized and the other was kept in the reduced state with bound carbon monoxide. These proteins were reacted with excess H64Y/V68F apoglobin, and time courses for hemin dissociation from the ferric subunits were monitored at 410 nm. As shown in Fig. 3.3B, the rate of hemin dissociation from the \([α^+(H_2O)β(CO)]_2\) hybrid was ~0.3 hr\(^{-1}\) which corresponds to the rate of the slow phase observed for native methemoglobin. Similarly, the rate for the major initial phase of hemin loss from the \([α(CO)β^+(H_2O)]_2\) hybrid was ~7 hr\(^{-1}\) which corresponds to the rate observed for the fast phase with native methemoglobin. A very slow absorbance decrease was observed for the β ferric hybrid and appears to correlate with slow oxidation and subsequent hemin loss from the α subunits which originally had CO bound to them (middle trace, Fig. 3.3B).
Figure 3.3: Time courses for hemin loss from native hemoglobin, valence hybrids, and Gly-E7 mutant hybrids at pH 7.0, 37°C. A) Time course for native methemoglobin in 0.15 M sodium acetate, pH 5.0, and 0.45 M sucrose. The symbols represent observed data, and the solid line represents a fit to a two exponential equation with equal amplitudes and an offset representing the absorbance at the end of the reaction. These data were collected at long times to define the slow α subunit phase. Other time courses were collected with more initial time points to define the rate of the β subunit phase, and controls in the absence of sucrose gave the same rate constants when slow absorbance drifts were removed from the data. B) Time courses for native methemoglobin, \([α^+(H_2O)β(CO)]_2\), and \([β^+(H_2O)α(CO)]_2\) hybrids. C) Time courses for native methemoglobin, \([α^+(Gly-E7)β^+(native)]_2\), and \([α^+(native)β^+(Gly-E7)]_2\) hybrids. The reaction conditions were 2-4 μM hemoglobin and 10-30 μM Tyr64Phe68 apomyoglobin.
A. Methemoglobin, pH 7.0, 37°C.

B. Valence Hybrids

C. Mutant Hybrids

Absorbance

Time (minutes)
Mutant hybrid methemoglobin were constructed with one native subunit and one subunit in which His(E7) had been replaced with Gly. These hybrid hemoglobins had been prepared previously in the ferrous state for ligand binding studies (Mathews et al., 1991). The samples were oxidized with a 3-fold excess of ferricyanide and then passed though a small Sephadex G25 column to remove the oxidizing agent. As expected from the previous work with myoglobin mutants (Table 2.3), hemin loss from the subunits containing the His(E7)→Gly mutation was extremely rapid. Some hemin was lost on the Sephadex column and the remainder was lost during the time interval between mixing the hybrids with excess H64Y/V68F apoglobin and initiation of recording in the spectrophotometer. As a result, the conventional time courses for these mutant hemoglobins were monophasic and represent hemin loss from the native subunits (Fig. 3.3C). The rate of hemin loss observed for the \([\alpha^+(\text{native})\beta^+(\text{Gly-E7})]_2\) hybrid was 1.3 hr\(^{-1}\), corresponding to the smaller rate observed for native hemoglobin. The rate for the \([\alpha^+(\text{Gly-E7})\beta^+(\text{native})]_2\) was 16 hr\(^{-1}\), corresponding with the larger rate observed for native hemoglobin (Table 3.1). Thus, both our experiments and those of Bunn and Jandl (1968) show that hemin dissociates much more rapidly from \(\beta\) subunits than from \(\alpha\) subunits in native human methemoglobin.

Rates of hemin loss from the subunits containing His\(^{64}\)(E7)→Gly mutations were estimated in rapid mixing experiments. Mutant hybrids were oxidized with ferricyanide and then reacted quickly with H64Y/V68F apoprotein in the stopped-flow apparatus at pH 7.0, 37°C. Rapid absorbance increases were observed at 600 nm due to hemin dissociation from the Gly\(^{64}\)
mutants and subsequent uptake by the Tyr64 containing apoglobin. The observed rate constants were \(~360\) hr\(^{-1}\) and \(~252\) hr\(^{-1}\) for the \(\beta\)(Gly-E7) and \(\alpha\)(Gly-E7) hybrids, respectively. These results show that removal of the E7 imidazole side chain causes 50 to 500-fold increases in the rate of hemin dissociation from hemoglobin. A similar 40-fold increase at pH 7.0 was observed for the His\(^{64}\)→Ala mutation in sperm whale myoglobin (Table 2.3).

Rates of hemin loss from methemoglobin were also measured as a function of pH and temperature (Table 3.1). A much smaller dependence on pH was observed for hemoglobin than for myoglobin. For example, the \(\beta\) and \(\alpha\) rate constants increase only 2 to 3 fold in going from pH 6.0 to 5.0, whereas a 100-fold increase in rate is observed for native myoglobin. The temperature dependence of the rates of hemin loss from \(\alpha\) and \(\beta\) chains at pH 5.0 is comparable to that of myoglobin (Table 2.5; Table 3.1). However, a more thorough analysis of both will require resolution of these effects on the \(k_H\) values of tetramers and dimers.

**Hemin loss from \(\alpha\) and \(\beta\) monomers** - Time courses for hemin loss from isolated ferric \(\alpha\) and \(\beta\) chains are shown in Fig. 3.4. Absorbance drifts due to precipitation occur almost immediately after heme loss is complete. Hemin loss from \(\alpha\) chains is monophasic with a rate constant equal to \(\approx 12\) hr\(^{-1}\) at 37°C, pH 7.

Hemin loss from \(\beta\) chains is multi-phasic and exhibits a significant slow phase (Fig. 3.4). The larger rate constant for hemin loss from \(\beta\) chains is \(\approx 40\) hr\(^{-1}\). It is likely that the slow phase is associated with hemin loss from tetrameric \(\beta\) chains. The concentration dependence of \(\beta\) chain
**Figure 3.4:** Hemin loss from isolated α and β subunits. Hemin loss was measured from isolated α and β chains at pH 7, 37°C. Because isolated ferric α and β chains are very unstable, oxidation of the ferrous O2 complexes was carried out in the stopped-flow syringes by addition of 1 mM potassium ferricyanide just prior to initiation of the experiment. The resultant ferric chains were then mixed with apo-H64Y/V68F in the stopped flow apparatus, and the time course of hemin loss was monitored at 600 nm. Hemin loss from 1 μM methemoglobin under these condition shows that ferricyanide does not affect rates of hemin loss, and the rates observed for the α and β subunits at this low protein concentration were 1.4 h⁻¹ and 15.4 h⁻¹, respectively, presumably representing the values for hemoglobin dimers. The time course for hemin loss from isolated α chains gave a k-H value of ≈ 12 h⁻¹. Hemin loss from isolated β chains showed a fast phase (k-H ≈ 40 h⁻¹), presumably associated with β monomers, and a slower phase associated with β tetramers.
Table 3.1: Rates of hemin dissociation from adult human methemoglobin as a function of pH and temperature. Reactions were carried out using the buffer conditions described in Methods. Protein concentration was 6 μM, at which concentration hemoglobin is primarily dimeric. The errors represent the standard deviation from the mean of three or more completely independent experiments. Compared to myoglobin (Table 2.4), rate constants for hemin loss from hemoglobin show little dependence on pH.

### A. Rate of Hemin loss at 37°C (h⁻¹)

<table>
<thead>
<tr>
<th>Subunit</th>
<th>pH = 5.0</th>
<th>pH = 5.5</th>
<th>pH = 6.0</th>
<th>pH = 7.0</th>
<th>pH = 8.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>0.3±0.2</td>
<td>1.0</td>
<td>0.48</td>
<td>0.6±0.4</td>
<td>1.2</td>
</tr>
<tr>
<td>β</td>
<td>20±5</td>
<td>14</td>
<td>6.6</td>
<td>7.8±2.0</td>
<td>12</td>
</tr>
</tbody>
</table>

### B. Rate of Hemin loss at pH 5.0 (h⁻¹)

<table>
<thead>
<tr>
<th>Subunit</th>
<th>T = 15°C</th>
<th>T = 20°C</th>
<th>T = 25°C</th>
<th>T = 30°C</th>
<th>T = 37°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>α</td>
<td>0.048</td>
<td>0.054</td>
<td>0.11</td>
<td>0.22</td>
<td>0.3±0.2</td>
</tr>
<tr>
<td>β</td>
<td>5.4</td>
<td>5.7</td>
<td>10</td>
<td>15</td>
<td>20±5</td>
</tr>
</tbody>
</table>
aggregation is shown in Fig. 3.5 and indicates that tetramers should be present at the concentrations used in our experiment based on the $K_{4,1}$ value obtained for $\beta$ chains by McGovern et al. (1976). The rate constant for the slower phase was estimated to be $\sim 2.0 \text{ h}^{-1}$ which is similar to that observed for $\beta$ subunits within hemoglobin tetramers.

**Hemin loss as a function of hemoglobin concentration** - The plot of the fraction of dimeric hemoglobin versus concentration (Fig. 3.2) shows that the rates of hemin loss from hemoglobin measured conventionally with the apo-H64Y/V68F assay at 1-35 $\mu$M will show significant contributions from both tetramers and dimers. At these concentrations the rates of hemin loss from the $\alpha$ and $\beta$ subunits are different enough to see clearly the biphasic nature of the time courses. At higher concentrations (Fig. 3.6) where hemoglobin is mostly tetrameric, time courses appear more monophasic because the difference between rates of hemin loss from $\alpha$ and $\beta$ subunits is only 3 to 4 fold.

Rates of hemin loss were measured as a function of heme concentration to determine the rates of hemin loss from hemoglobin tetramers and dimers. Time courses were measured at protein concentrations ranging from 1 to 600 $\mu$M heme and fitted to a two exponential expression to determine the rates of hemin loss from $\alpha$ and $\beta$ subunits (e.g Fig. 3.6). The $\alpha$ subunit rate constant was 0.3 $\text{h}^{-1}$ at high concentration, compared to 0.6 $\text{h}^{-1}$ measured at 1 $\mu$M heme (Table 3.2). In contrast, the $\beta$ subunit rate increased 10-fold over the same range, from 1.5 $\text{h}^{-1}$ at high hemoglobin concentration to 15 $\text{h}^{-1}$ at 1 $\mu$M. These data are shown in Fig. 3.7. Under these conditions, $K_{4,2}$ was estimated to be about
**Figure 3.5:** Fraction of $\beta$ chain monomer as a function of protein concentration. Aggregation of $\beta$ chains increases sharply around 50 to 100 $\mu$M in heme. This figure shows that $\beta$ tetramers could be responsible for the slow phase seen in the time courses of hemin loss from isolated $\beta$ chains.
Figure 3.6: Hemin loss from 1 and 600 μM native hemoglobin. The open circles are the time course at high concentration and the small dots are the time course at 1 μM hemoglobin. Each curve was fitted to a two exponential expression which was forced to have equal amplitudes. It is clear that hemoglobin concentration has an effect on the faster, $\beta$ rate constant for hemin dissociation.
**Figure 3.7:** Dependence of rate constants for heme loss from native and recombinant hemoglobins on protein concentration. Hemin loss was measured from human hemoglobin at concentrations ranging from 1 to 600 μM in heme. Each time course was fitted to a two-exponential expression with equal amplitudes. The slow and fast phases were assigned to hemin loss from α and β subunits, respectively. The protein concentration dependence of the rate constants for hemin loss from α and β subunits was then fitted to Equation 3.3. Data for native α and β subunits are shown as closed and open circles, respectively; data for wild type recombinant (rHb0.0) α and β subunits are shown as closed and open squares; and data for genetically cross linked hemoglobin (rHb0.1) are shown as closed and open triangles for the α and β subunits, respectively.
1.5 × 10^{-6} \text{M}^{-1} for native hemoglobin. The fitted rate constants for hemin loss from native α and β subunits at high hemoglobin concentrations were similar to those of Bunn and Jandel (1968), who estimated the α and β k_{H} values to be 0.13 h^{-1} and 1.0 h^{-1}, respectively, at pH 7, 37°C.

The rate constants for hemin loss from recombinant human hemoglobin (rHb0.0) were similar to native hemoglobin. The rate of hemin loss from recombinant β subunits was faster than that from the β subunits of native hemoglobin, particularly at low concentrations, whereas the rate constant for hemin loss from α subunits in rHb0.0 and native hemoglobin are nearly identical under all conditions (Table 3.2). rHb0.0 exhibits a concentration dependence analogous to that of native hemoglobin, and the fitted value of K_{4,2} for rHb0.0 is also ~ 2 μM.

Looker et al. (1992) constructed a recombinant hemoglobin (rHb0.1) with a glycine linker between the α subunits to create a (α-α)β2 tetramer that does not appear to dissociate under physiological conditions. As expected, hemin loss from this genetically cross linked hemoglobin shows no concentration dependence. The rate constants of hemin dissociation from the α and β subunits in rHb0.1, 0.5 and 1.5 h^{-1}, respectively, are identical to the values estimated for tetrameric native and recombinant hemoglobins (Table 3.2). These results show unambiguously that the protein concentration dependence observed for native and recombinant hemoglobins represents interconversion between tetramers and dimers.

**Effects of inositol hexaphosphate on hemin dissociation** - Rates of hemin loss from native hemoglobin were measured in the presence and absence of IP6. IP6 promotes tetramer formation since it binds to a single
Figure 3.8: Effects of inositol hexaphosphate (IP6) on rates of hemin dissociation from hemoglobin. Hemin loss from 1 μM hemoglobin was measured in 0.2 M Bis/Tris, 0.45 M sucrose, pH 7.0, ± 0.2 mM IP6. The rate of hemin loss from β subunits was 16 h⁻¹ in the absence of IP6 and 3 h⁻¹ in the presence of 0.2 mM IP6. The decrease in k-H for β subunits is due to IP6 induced aggregation to tetramers.
Table 3.2: Rates of hemin dissociation form hemoglobin monomers, dimers, and tetrayers as a function of concentration.

<table>
<thead>
<tr>
<th>Hemoglobin</th>
<th>K-H monomer h⁻¹</th>
<th>K-H dimer h⁻¹</th>
<th>K-H tetramer h⁻¹</th>
</tr>
</thead>
<tbody>
<tr>
<td>α chains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>α Native</td>
<td>12</td>
<td>0.6</td>
<td>0.25</td>
</tr>
<tr>
<td>α 0.0</td>
<td></td>
<td>0.5</td>
<td>0.50</td>
</tr>
<tr>
<td>α 0.1</td>
<td></td>
<td></td>
<td>0.42</td>
</tr>
<tr>
<td>β chains</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>β Native</td>
<td>40</td>
<td>15</td>
<td>1.5</td>
</tr>
<tr>
<td>β 0.0</td>
<td></td>
<td>33</td>
<td>1.7</td>
</tr>
<tr>
<td>β 0.1</td>
<td></td>
<td></td>
<td>1.6</td>
</tr>
</tbody>
</table>
site between the β subunits of tetrameric hemoglobin (Hensley et al. 1974). As shown in Fig. 3.8, IP₆ does decrease the rate of hemin loss at 1 µM hemoglobin in Bis/Tirs buffer at pH 7. As expected there is little effect on the α chain rate constant for hemin dissociation. The β kₗH value in the presence of 0.2 mM IP₆ is close to the rate constant expected for β subunits in hemoglobin tetramers (Table 3.2).

**DISCUSSION**

The association rate constant for the binding of heme to apohemoglobin is ~ 2 to 10 x 10⁷ M⁻¹ regardless of the structure of the protein. Apoglobins of the isolated subunits bind heme at the same rate as apohemoglobin dimers. Therefore, the affinity of hemoglobin for hemin is determined by the rate constant for dissociation, kₗH. Irrespective of quaternary structure, α subunits have a higher affinity for hemin than β subunits. This is probably due in part to two structural features of β chains. The hydrogen bond between His(E7) and coordinated water is weaker in the ferric form of β subunits than in α subunits. Additionally, the Ser(CD3) side chain in β subunits is too short to form a stabilizing hydrogen bond with the heme-6-propionate, whereas in α subunits this residue is a histidine which does interact electrostatically with the heme propionate. The loss of these favorable interactions in β subunits provides a plausible explanation for the higher rate of hemin dissociation of this protein.

The quaternary structure of hemoglobin also has a profound effect on heme affinity (Figs. 3.4 - 3.8). For both α and β subunits, the relative affinity for heme is a function of quaternary structure. In α subunits, the rate
of hemin loss is in the order: tetrarmers = dimers << monomers whereas in β subunits the order of k-H is tetrarmers << dimers < monomers.

α and β subunits in tetrameric hemoglobin have stabilizing contacts between the C and N termini, the C helices, and the FG corner which are associated with the α1β2 interfaces. The stronger α1β1 interfaces result from contacts between the A, G, and H helices. The α1β2 contacts are lost in going to dimers, and all subunit interface contacts are gone in isolated subunits. Loss of these contacts results in exposure of hydrophobic side chains to solvent which undoubtedly destabilizes the tertiary structure of the monomers.

The high rate of hemin loss and subsequent precipitation of isolated chains explains why α and β subunits of human hemoglobin must be expressed simultaneously to obtain native hemoglobin (Looker et al 1992). If native α and β chains are expressed individually they are unable to fold properly as monomers, have poor affinity for heme, and precipitate irreversibly in the cytoplasm of E. coli. The effects of poor folding and hemin retention on denaturation and expression yields have been quantitated for several unstable myoglobin mutants in the next Chapter.

Human hemoglobin appears to have evolved to break down rapidly when released during lysis of red blood cells. Dilution of hemoglobin into plasma initiates dimer formation, subsequent oxidation, and hemin loss. Loss of heme results in destabilization and unfolding of the α and β globins. All of these processes enhance the rate at which hemoglobin is cleared from the circulatory system once the red blood cell has lysed. Rapid removal of extracellular hemoglobin is necessary to prevent extravasation of
hemoglobin and deposition of free hemin in endothelial cells and surrounding tissues. Such deposits are almost always associated with oxidative damage (Bunn and Forget 1986).

Under normal physiological conditions, the quantity of free hemin and apohemoglobin present in the blood stream is very low. The presence in the blood serum of several proteins capable of binding heme, including hemopexin and albumin, prevent free hemin from aggregating in vivo, and low levels of apoglobin are easily removed by haptoglobins (Bunn and Forget 1986). Administration of extracellular blood substitutes results in much higher levels of plasma hemoglobin. If these proteins lost heme rapidly, the non-specific heme binding proteins in serum would quickly become saturated and deposition in and on endothelial cells would occur. Pathological effects of free heme were observed in clinical studies with protein-based blood substitute prototypes (Vandigriff 1995).

The first attempts to solve this problem involved stabilizing hemoglobin tetramers by chemical and genetic cross linking to prevent clearance by haptoglobin. As shown in Fig. 3.7, genetically cross linking hemoglobin to prevent dimerization markedly inhibits hemin loss from β subunits at low protein concentrations. Zhang et al. (1991) have shown that autooxidation also increases markedly when hemoglobin dissociates. Thus, dimerization appears to be a key process in the denaturation and removal of native hemoglobin from plasma.
Chapter 4

Stability of Apomyoglobin: A Model for the Folding of Heme Proteins

INTRODUCTION

Studies of myoglobin stability are complex. Functionally, it would be more meaningful to study the effects of a denaturant on the ability of ferrous myoglobin to bind oxygen but structurally, it would be better to study secondary and tertiary interactions as the apoprotein unfolds. In vivo, the stability of myoglobin is probably more a function of the rate at which the heme iron oxidizes than either of the former processes. However, since oxidation is reversible, it could also be argued that hemin affinity is the most important determinant of myoglobin stability.

Myoglobin unfolding has been measured by a variety of techniques. In most previous studies, myoglobin was titrated with a denaturant while observing changes in the Soret absorbance band (Puett, 1973; Flanagan et al., 1983; Hughson and Baldwin 1989). As will be shown in Chapter 7, this type of experiment measures heme loss but gives no indication of the structure or stability of the globin portion of the molecule. A good example of the complexity in interpreting myoglobin denaturation comes from the work of Pinker et al., (1993) and Lin et al., (1994a,b). These authors were interested in the effects of alanine substitutions on the secondary and tertiary structure of myoglobin. Unfolding was measured by following the decrease in Soret absorbance and CD in the far UV region as the protein was titrated with acid. After observing that CD changes at 222 nm and Soret signals do not occur simultaneously when metmyoglobin is acidified, they decided to
examine pH titrations of cyano-metmyoglobin. In this case, the CD222 and the Soret absorbance peak decreased simultaneously as pH was decreased. However, even though cyanometmyoglobin was used instead of metmyoglobin, Pinker et al. (1993) were measuring the effects of alanine substitutions on heme affinity and not apoglobin stability. Not surprisingly, they found no clear relationship between alanine substitution and protein stability. Until direct unfolding experiments in the absence of heme are carried out, little can be said about the effects of their alanine substitutions on protein folding.

The stability and folding of apomyoglobin has also been studied extensively. When heme dissociates from myoglobin, 15-20% of its helicity is lost and the resulting protein is not as tightly packed as when heme is bound. Kirby and Steiner (1970) characterized three changes in tryptophan fluorescence resulting from acidification of apomyoglobin. Upon titration from pH 8.3 to 5.5 they observed a slight decrease in quantum yield (attributed to histidine protonation and subsequent quenching) and no shift in $\lambda_{\text{max}}$ of tryptophan emission, indicating no change in the polarity of the environments of the tryptophans. Combined with CD measurements, it was determined that no major conformational changes occurred over this pH range. Between pH 5.5 and 4.0, a large increase in quantum yield and relatively small red shift of $\lambda_{\text{max}}$ was observed. These changes were accompanied by a 40% loss in helicity as measured by CD. A decrease in fluorescence intensity and a larger red shift of $\lambda_{\text{max}}$ was observed below pH 4.0.
Based on additional fluorescence measurements, Balestrieri et al., (1976) suggested that apomyoglobin unfolding is a two step process. During titration with GdmCl, the inability of apomyoglobin to bind ANS specifically to the heme pocket occurs earlier than the red shift of tryptophan fluorescence emission which is characteristic of complete unfolding. Increases in the quantum yield of tryptophan fluorescence similar to those described by Kirby and Steiner were also observed during GdmCl unfolding. Balestrieri et al. (1976) suggest that one of the two unfolding domains contains the heme pocket and that this domain unfolds before the rest of the molecule.

Irace et al., (1981) used bluefin tuna myoglobin to identify the individual contributions of myoglobin tryptophans to the fluorescence changes occurring during acid and GdmCl unfolding. Almost all chordate myoglobins contain Trp residues at the 7 and 14 positions. Bluefin tuna is an exception and contains only a single Trp residue at position 14. Based on comparisons between the tuna and sperm whale apomyoglobins, Irace et al. (1981) suggested that Trp\(^7\) and Trp\(^{14}\) have \(\lambda_{max}\) values of 333 nm and 321 nm, respectively, under non-denaturing conditions. Under native conditions, the fluorescence of Trp\(^7\) appears to be quenched by Lys\(^{79}\)(EF3), resulting in a relatively small contribution from Trp\(^7\) to total emission. Upon acidification from pH 5.5 to 4.0 or increase in [GdmCl] from 0 to 1.2M, quenching of Trp\(^7\) is removed due to unfolding of the EF corner, resulting in a large increase in emission. Further acidification or GdmCl addition exposes both Trp\(^7\) and Trp\(^{14}\) to solvent causing quenching of both fluorophores.
Colonna et al., (1982) used differential binding of ANS and 1,5 AEDANS to human apomyoglobin to provide further evidence of a two state unfolding model. 1,5 AEDANS specifically alkylates Cys$^{13}$(A12), a residue far removed from the heme binding site, whereas 1,8 ANS binds specifically in the heme pocket. The fluorescence of 1,5 AEDANS is a measure of the local environment of Trp$^{14}$ as well as the A helix. Colonna et al. (1982) showed that loss of ANS fluorescence emission intensity due to denaturation of the heme pocket occurred at GdmCl concentrations well below those required to change the fluorescence of the apomyoglobin-1,5 AEDANS complex. These results support the earlier conclusions of Balestrieri et al (1976) and Irace et al (1981) that acid and GdmCl induced unfolding of apomyoglobin is a two step process; the first step involves disappearance of the heme pocket and the second, unfolding of the region containing the two tryptophans.

Hughson and Baldwin (1989) and Griko et al. (1988) observed the apomyoglobin folding intermediate (I) while monitoring CD changes for the acid induced unfolding of human apomyoglobin. They estimated that the I state has a helix content of 35%. Native apomyoglobin (N) and acid unfolded apomyoglobin were estimated to be 60% and 10-15% helical, respectively. Hughson and Baldwin (1989) used site directed mutagenesis to study the effects of changes in the G-B helix packing region on the stabilities of the N and I states. They found that substitutions in this region, particularly residue 110, were able to affect the stability of the N state, but had little effect on the I state.
Hughson et al., (1990) used H₁/D exchange and H₁ NMR to investigate the structure of the apomyoglobin folding intermediate (Fig. 4.1). It was found that all amides studied exchanged much more freely in I than in N, and that amides in the B and E helices of I exchanged more rapidly than those in the A, G and H helices. Amides in the D and F helix regions exchanged freely in N as well as I, suggesting that these helices unfold when heme is removed. Unfolding of these helices accounts for some the difference in helicity between holo- and apomyoglobin. Based on these results, a model of I was proposed in which the B, C, D, F, and E helices which make up the distal heme pocket, are unfolded, but the A, G, and H helices remain in a separate folded domain. This proposed structure supports earlier models of apomyoglobin unfolding (Balestrieri et al., 1976; Irace et al., 1981; Colonna et al., 1982). It also suggests that the G-B helix packing mutations would be unlikely to affect the stability of I because the B helix is no longer intact and in contact with the G helix (Hughson et al., 1990). Jennings and Wright (1994) have shown that this intermediate also occurs kinetically during the folding of apomyoglobin.

Hughson et al., (1991) constructed sperm whale Mb mutants at the A-G and G-H interfaces in a effort to disrupt helix packing and affect the stability of I. Mutations at the A-G interface destabilized N but did not effect I. Mutations resulting in differences in polarity at the G-H interface were successful at influencing the mid-point of the I to U transition. In the same study, they showed that G and H peptide fragments do not pair when free in solution. These results suggest that charge interactions may play a more significant role in determining the stability of folding intermediates
**Figure 4.1:** Structural model for myoglobin denaturation. The model was adapted from Barrick and Baldwin (1993) and Hughson *et al.*, (1990). Ferric myoglobin oxidizes and loses heme, yielding apomyoglobin in the N state. The B, C, and E helices which make up the heme pocket then unfold to give the molten globule I state in which the A, G and H helices are still intact. The last step is conversion of the intermediate to the completely unfolded U state. The wide ribbons indicate intact helical secondary structures. The narrow lines indicate unfolded strands that have much more random positions than implied in the MOLSCRIPT (Kraulis, 1991) drawings which keep the atoms in the original locations in the P6 wild-type metmyoglobin structure (Phillips *et al.*, 1990). Trp^7^ and Trp^14^ are shown on the right side of each globin, along the A helix, and residues Leu^29^, Phe^43^, Val^68^, and Ile^107^ are seen in the hemin binding pocket.
than close interactions, like helix packing, might not be as significant a factor (Hughson et al., 1991). Barrick and Baldwin reviewed the evidence supporting a molten globule folding intermediate and described a detailed three state analysis of the CD changes accompanying apomyoglobin unfolding as a function of pH and urea concentration (Barrick and Baldwin 1993; Barrick and Baldwin 1993a).

The best way to measure myoglobin stability is to examine the denaturing processes individually. In this Chapter rates of hemin loss and equilibrium apoglobin unfolding parameters were determined for 22 mutants with substitutions in the distal portion of the heme pocket. Plots of tryptophan fluorescence emission intensity versus [GdmCl] facilitate quantitative analysis in terms of the 3-state N, I, U scheme because the intermediate is observed as a peak in titration data (Figs. 4.1, 4.2). Attention was focused on residues located adjacent to the bound ligand and the heme group in an effort to understand the evolutionary pressures which strike a compromise between efficient oxygen binding, resistance to autooxidation, high heme affinity, and apoglobin stability. Spectacular and often compensating changes are observed in resistance to hemin loss and apoglobin stability. These results demonstrate that the stability of heme proteins must be considered in terms of both folding of the apoprotein and binding of the prosthetic group. They also confirm independently that the E and B helices are probably disrupted in the molten globule intermediate of apomyoglobin and suggest protein engineering strategies for improving both the overall expression yield and extracellular half-lives of heme proteins that may serve as blood substitute prototypes.
METHODS

Preparation of myoglobins and hemin loss assay - Native sperm whale myoglobin was obtained by special permit from Sigma. Sperm whale myoglobin mutants at positions 68 and 64 were constructed at Rice University using cassette mutagenesis or had been prepared by Barry A. Springer and Karen D. Egeberg (H64L, H64F, H64Q, V68A, and V68F; Rohlfs et al., 1990; Egeberg et al., 1990). Sperm whale myoglobin mutants at positions 29, 43, and 107 were constructed from a pEMBL19 vector containing the gene for wild type myoglobin (Carver et al., 1992). All recombinant myoglobins were expressed and purified at Rice University following the procedures described by Springer and Sligar (1987) and Carver et al., (1992). Expression yields of recombinant myoglobins were estimated as milligrams of purified protein produced per 100 g of cell paste. These levels were estimated from routine fermentation runs and protein preparations. For the unstable mutants, the protein was kept in the reduced, CO form during and after cell lysis in an effort to prevent hemin loss and denaturation during purification. Some losses still occurred during purification of the L29N and V68N mutants, but the lack of color in the cell pastes confirmed that the major cause of the low yield was poor expression. Pig and human myoglobin mutants were constructed and expressed as fusion proteins following the procedures described by Varadarjan et al. (1985), Smerdon et al. (1991), and Ikeda-Saito et al. (1991). Apomyoglobins were prepared using the methyl ethyl ketone method described by Ascoli et al. (1981) and Hargrove et al. (1994a).
Hemin dissociation was measured by following the absorbance changes (usually at 409 nm) associated with transfer of hemin from holoprotein to excess H64Y/V68F apomyoglobin (Chapter 2). Resultant time courses were fitted to a single exponential expression to obtain the first order dissociation rate constant for hemin loss, $k_H$. These experiments were carried out in 0.15 M buffer/0.45 M sucrose, 37°C at either pH 7.0 (potassium phosphate) or pH 5.0 (sodium acetate).

*Fluorescence measurements* - Frozen apomyoglobin samples were quickly thawed, centrifuged at 4°C to remove any precipitate, and stored on ice. Desired GdmCl concentrations were achieved by mixing a stock GdmCl solution (prepared as described in Pace *et al.*, 1990) with an appropriate volume of 0.2M potassium phosphate, pH 7.0 to give a total volume of 1 ml in the cuvette. 10 μl of a stock (100 to 500 μM) apoprotein solution was added to give a final protein concentration between 1 and 5 μM. Each spectrum in Fig. 4.2 and point in Figs. 4.3 and 4.4 represents an independently prepared solution, with great care taken to ensure that the same protein concentration was present during a titration. All denaturation studies were carried out at 25°C. Fluorescence intensity and emission spectra were measured with an SLM 8100 spectrofluorimeter. One photomultiplier was used to measure emission spectra between 300 and 400 nm. At the same time, a 320 nm cutoff filter was placed in front of another photomultiplier to measure total intensity during the emission scan. Excitation was normally at 285 nm, but the results are independent of excitation wavelength between 285 to 295 nm. To ensure that equilibrium unfolding was achieved, emission spectra were recorded successively until
no changes in total intensity were observed during the scan. Most samples (including native and wild type sperm whale apomyoglobin) reached equilibrium before the first scan. The V68T, V68S, and V107T mutants often required 2 to 5 minutes to reach equilibrium.

Estimates of the unfolding equilibrium constants, $K_{N,I}$ and $K_{I,U}$, were obtained by fitting the fluorescence titration data to the two step scheme described by Barrick and Baldwin (1993). Total fluorescence intensity, $F_{obs}$, is given by:

$$F_{obs} = \frac{F_N + F_I K_{N,I} e^{(m_{N,I} x)}}{1 + K_{N,I} e^{(m_{N,I} x)}} + \frac{F_U K_{N,I} e^{(m_{N,I} x)} K_{I,U} e^{(m_{I,U} x)}}{K_{N,I} e^{(m_{N,I} x)} K_{I,U} e^{(m_{I,U} x)}}$$ (4.1)

where $F_N$, $F_I$, and $F_U$ are the fluorescence intensities of pure N state, I state, and U state, respectively; $X$ is the molarity of GdmCl, and $m_{N,I}$ and $m_{I,U}$ are the differential denaturant binding parameters (see Equation 15 of Barrick and Baldwin, 1993). The unfolding equilibrium constants are assumed to vary exponentially with denaturant concentration (i.e. a linear dependence of $\Delta G_{\text{transition}}$ on [GdmCl]; Pace et al., 1990). Initially, the titration data for all proteins in Table 4.2 were fitted to Equation 4.1 allowing all seven parameters to vary. Then the average of the $m$ values was calculated and fixed in succeeding analyses to allow more direct comparisons between the mutants. The average values were 4.1 M$^{-1}$ and 2.8 M$^{-1}$ for $m_{N,I}$ and $m_{I,U}$, respectively. Fluorescence intensity was normalized to the maximum observed at intermediate GdmCl concentrations and then fitted to Equation 1 allowing $F_N$, $F_I$, $F_U$, $K_{N,I}$, and $K_{I,U}$ to vary. For most mutants, relative values of $F_N$, $F_I$, and $F_U$ were 0.6, 1.0, and 0.7, respectively. Because L29N and
V68N apomyoglobin are almost entirely in the I state under non-denaturing conditions, $F_N$ was fixed at 0.6 for these mutants during fitting. The overall stability of the native apoglobin was estimated as $1/ K_{N,I}K_{I,U}$ or $K_{U,N}$. A compilation of the fitted equilibrium constants is given in Table 4.2 along with rates of hemin loss at pH 5 and 7 for 25 different recombinant myoglobins.

RESULTS

Expression levels of holomyoglobin and rates of hemin loss—A large number of thermal, pH, urea, and guanidinium chloride unfolding studies have focused on holomyoglobin, primarily because large absorbance changes accompany denaturation and the loss of hemin. However, interpretation of these results is ambiguous because in most cases resistance to hemin loss, rather than stability of the apoprotein structure, is being measured. It is often assumed that the two properties are directly correlated, but as will be shown, this is generally not true. Constitutive expression of myoglobins and hemoglobins in *Escherichia coli* is related to the efficiency of the promoter, codon usage, the rate of hemin synthesis, and the intrinsic stability of the apo- and holoproteins (Springer and Sligar, 1987). In Table 4.1, the yield of recombinant sperm whale myoglobin is compared to the rate of hemin dissociation from the corresponding purified metmyoglobin. Because the same expression vector and host strain were used in each case, the differences in expression among the mutants must be due to changes in overall stability of the protein. The results show that the yield is not readily predicted from the rate of hemin loss. For example, the rate of hemin
dissociation from V68N metmyoglobin at pH 7.0 is approximately equal to that of the wild-type holoprotein, whereas the yield of the mutant protein is 25 to 100-fold less than that of wild-type myoglobin. The opposite situation occurs for H64A myoglobin; this mutant loses hemin ~40 times more rapidly than native metmyoglobin but is expressed at a level which is only 30% lower than that of the wild-type protein. The simplest explanation is that H64A apoprotein is very stable, compensating for rapid loss of hemin, whereas V68N apoprotein denatures readily in the bacterial cytoplasm, presumably before hemin can bind. In order to test this interpretation, the resistance of the corresponding apoproteins to denaturation in GdmCl was measured.

**Fluorescence titrations and analyses** - The fluorescence changes associated with apomyoglobin unfolding are shown in Figures 4.2-4.5. Holomyoglobin has little tryptophan fluorescence due to quenching by the heme group. Fluorescence from the N state of apomyoglobin is due to Trp7, which is significantly quenched by Lys79, and Trp14, which emits strongly because it is buried in an apolar region between the A, G, and H helices (Irace et al., 1981 and Fig. 4.1). There is an increase in fluorescence and small red shift in the emission maximum as [GdmCl] is increased from 0 to 2 M. These changes appear to be due to movement of Lys79 away from Trp7 when the E-helix unfolds during formation of the molten globule intermediate. The increase in fluorescence is not due to aggregation because the shapes of the titration curves are independent of apoprotein concentration, at least in the range 1-10 μM (Figures 4.2A and 4.2B). Complete denaturation at [GdmCl] > 2 M exposes both tryptophans to
Table 4.1: Hemin Loss Rates and Constitutive Expression Yields of Sperm Whale Myoglobin Mutants. $k_H$ is the rate of hemin dissociation at pH 7, 37°C. Myoglobins were prepared and expression yields estimated as described in Experimental Procedures.

<table>
<thead>
<tr>
<th>Myoglobin</th>
<th>$k_H$ pH 7, 37°C (h⁻¹)</th>
<th>Yield of Pure Protein (mg MbCO / 100 g cells)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type(D122N)</td>
<td>0.007±0.005</td>
<td>~120</td>
</tr>
<tr>
<td>L29N</td>
<td>0.10</td>
<td>~1-5</td>
</tr>
<tr>
<td>L29F</td>
<td>&lt;0.01</td>
<td>~100</td>
</tr>
<tr>
<td>F43V</td>
<td>2</td>
<td>~6</td>
</tr>
<tr>
<td>F43I</td>
<td>2</td>
<td>~50</td>
</tr>
<tr>
<td>H64A</td>
<td>0.40</td>
<td>~80</td>
</tr>
<tr>
<td>H64F</td>
<td>0.08</td>
<td>~120</td>
</tr>
<tr>
<td>V68N</td>
<td>~0.01</td>
<td>~1-5</td>
</tr>
<tr>
<td>V68F</td>
<td>≤0.01</td>
<td>~110</td>
</tr>
</tbody>
</table>
solvent causing quenching and a red shift in the emission maximum to \( \sim 350 \) nm (Irace et al., 1981 and Fig. 4.2). When the same GdmCl titration is carried out with V68N or L29N sperm whale apomyoglobin, the initial fluorescence is much greater than that of the native or wild-type protein (Figs. 4.2C, 4.3A, and 4.3B). These mutant apoproteins are partially or completely in the I state even before denaturant is added. As shown in Figs. 4.2C and 4.3A, the L29N apoprotein shows only a single transition associated with conversion of I to U.

**Effects of mutagenesis at positions 29(B10) and 68(E11)** - Replacing aliphatic side chains with amides or alcohols at these positions produces a more polar heme pocket and, in general, destabilizes the native apoglobin conformation (N) with respect to the molten globule intermediate (I) and the completely unfolded state (U). These changes are observed as increases in the unfolding equilibrium constants, \( K_{N,I} \) and \( K_{I,U} \), and a decrease in overall stability, \( 1/K_{N,I}K_{I,U} \) (Table 4.2). Replacing smaller residues with larger aliphatic or aromatic amino acids creates a more hydrophobic distal heme pocket and stabilizes the N state, with the major effect being a decrease in \( K_{N,I} \).

The L29N mutation causes complete loss of the native apoprotein conformation and, as a result, the mutant apoglobin exists in the molten globular I state in the absence of GdmCl at pH 7, 25°C (Figs. 4.2, 4.3A, Table 4.2). The instability of L29N apomyoglobin results in extremely poor constitutive expression in *E. coli* TB-1 cells (Table 4.1). Presumably the molten globule intermediate is incapable of binding heme efficiently and is readily degraded by intracellular proteases. However, once the prosthetic
**Figure 4.2:** Fluorescence measurements. A) and B), 3D plots of fluorescence intensity, wavelength, and [GdmCl] for titrations of wild type apomyoglobin at 1 and 10 μM, respectively. C, The same titration carried out with L29N sperm whale apomyoglobin at 1 μM.
group is bound, the L29N mutant folds into a relatively stable holoprotein whose rate of hemin loss is only 8-10 times greater than that of wild type myoglobin at both pH 5.0 and 7.0.

Val68 was replaced by six different residues of varying size and polarity (Figs. 4.3 B, Table 4.2). Again, increasing polarity increases $K_{N,I}$ but does not have a large effect on $K_{I,U}$. The asparagine substitution produces the most unstable apoprotein, explaining the poor expression yield of the V68N mutant (Table 4.1). The V68T and V68S mutations also produce large increases in $K_{N,I}$, whereas the V68F substitution causes an ~5-fold decrease in $K_{N,I}$ and a 2-fold decrease in $K_{I,U}$.

The generality of these results for sperm whale myoglobin was tested by measuring unfolding of V68N, V68S, and V68T mutants of pig myoglobin. Surprisingly, wild-type pig apomyoglobin appears to be ~100-fold less stable than sperm whale apoprotein (Fig. 4.4). As a result, it is difficult to quantitate the effects of mutagenesis in the pig system. However, qualitatively similar effects are observed when Val68 is replaced with Asn, Ser, and Thr. All three mutations cause extensive unfolding. The fluorescence emission spectrum of the pig V68T apoprotein at 0 M GdmCl appears to be a mixture of contributions from the molten globular intermediate and the completely unfolded state with no evidence for the presence of the N state (Fig. 4.5B). V68N and V68S pig apoproteins have almost identical spectra at 0 and 4M GdmCl, implying that these proteins are completely unfolded under non-denaturing conditions (Fig. 4.5C).

In both pig and sperm whale myoglobin, the isosteric V68T replacement uncouples the rate of hemin loss from apoglobin stability.
Figure 4.3: Analysis of fluorescence titrations. A) Total fluorescence intensity of Leu$_{29}$ mutants are plotted as a function of [GdmCl]. B), C), and D), The same plots for Val$_{68}$, Ile$_{107}$, and His$_{64}$ mutants, respectively. The symbols represent observed data points and the solid lines fits to Equation 4.1 using the equilibrium parameters listed in Table 4.2. Total fluorescence intensity was measured directly using a 320 nm cutoff filter or computed by numerically integrating the observed emission spectrum. The same fitted parameters were obtained by either method.
Table 4.2: Hemin loss, Folding, and Stability Parameters for apoMb. Hemin dissociation experiments were carried out at 37°C. Unfolding equilibrium constants were measured at 25°C. The errors (~±20-30%) estimated for the wild-type sperm whale apoglobin (N122) equilibrium constants represent the standard deviation from the mean of three completely independent titrations and analyses carried out for this purpose. Normally, wild-type sperm whale myoglobin is expressed with an Asn122 residue to facilitate crystallization in the P6 form (Phillips et al., 1990). The unfolding of "corrected" wild-type (D122) was measured to examine the effect of the Asn substitution. All the recombinant sperm whale proteins have an extra N-terminal Met residue which is designated as residue 0.
<table>
<thead>
<tr>
<th>Protein</th>
<th>kₜ (h⁻¹)</th>
<th>kₜ (h⁻¹)</th>
<th>Kₜ,I (nmol L⁻¹)</th>
<th>Kₜ,U (nmol L⁻¹)</th>
<th>1/Kₜ,U (stability)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>pH 5.0</td>
<td>pH 7.0</td>
<td>pH 7.0</td>
<td>pH 7.0</td>
<td></td>
</tr>
<tr>
<td>A. Sperm whale proteins</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Native</td>
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<td>-0.01</td>
<td>0.002</td>
<td>0.004</td>
<td>125,000</td>
</tr>
<tr>
<td>Wild-type(N122)</td>
<td>1.0</td>
<td>-0.01</td>
<td>0.011</td>
<td>0.002</td>
<td>45,000</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>(±0.003)</td>
<td>(±0.0002)</td>
<td>(±18,000)</td>
</tr>
<tr>
<td>Wild-type(D122)</td>
<td>1.0</td>
<td>-0.01</td>
<td>0.009</td>
<td>0.002</td>
<td>55,000</td>
</tr>
<tr>
<td>L29F</td>
<td>2.5</td>
<td>&lt;0.01</td>
<td>0.01</td>
<td>0.001</td>
<td>100,000</td>
</tr>
<tr>
<td>L29N</td>
<td>8.3</td>
<td>0.10</td>
<td>~50</td>
<td>0.01</td>
<td>&lt;10</td>
</tr>
<tr>
<td>F43V</td>
<td>54</td>
<td>2</td>
<td>0.7</td>
<td>0.001</td>
<td>1,400</td>
</tr>
<tr>
<td>F43I</td>
<td>38</td>
<td>2</td>
<td>0.018</td>
<td>0.002</td>
<td>28,000</td>
</tr>
<tr>
<td>H64Q</td>
<td>3.5</td>
<td>0.12</td>
<td>0.01</td>
<td>0.002</td>
<td>50,000</td>
</tr>
<tr>
<td>H64A</td>
<td>17</td>
<td>0.40</td>
<td>0.003</td>
<td>0.001</td>
<td>330,000</td>
</tr>
<tr>
<td>H64L</td>
<td>11</td>
<td>0.20</td>
<td>0.0027</td>
<td>0.0005</td>
<td>740,000</td>
</tr>
<tr>
<td>H64F</td>
<td>4.8</td>
<td>0.08</td>
<td>0.0016</td>
<td>0.0004</td>
<td>1,600,000</td>
</tr>
<tr>
<td>V68F</td>
<td>2.0</td>
<td>-0.01</td>
<td>0.002</td>
<td>0.001</td>
<td>500,000</td>
</tr>
<tr>
<td>V68A</td>
<td>5.8</td>
<td>0.10</td>
<td>0.019</td>
<td>0.002</td>
<td>26,000</td>
</tr>
<tr>
<td>V68Q</td>
<td>2.4</td>
<td>0.03</td>
<td>0.16</td>
<td>0.003</td>
<td>2,100</td>
</tr>
<tr>
<td>V68N</td>
<td>5.2</td>
<td>-0.01</td>
<td>1</td>
<td>0.005</td>
<td>200</td>
</tr>
<tr>
<td>V68S</td>
<td>0.9</td>
<td>0.10</td>
<td>0.60</td>
<td>0.002</td>
<td>800</td>
</tr>
<tr>
<td>V68T</td>
<td>-0.1</td>
<td>&lt;0.01</td>
<td>0.068</td>
<td>0.002</td>
<td>7,400</td>
</tr>
<tr>
<td>I107F</td>
<td>1.1</td>
<td>-0.01</td>
<td>0.003</td>
<td>0.001</td>
<td>330,000</td>
</tr>
<tr>
<td>I107V</td>
<td>1.3</td>
<td>-0.01</td>
<td>0.01</td>
<td>0.005</td>
<td>20,000</td>
</tr>
<tr>
<td>I107T</td>
<td>1.4</td>
<td>0.15</td>
<td>0.038</td>
<td>0.009</td>
<td>2,900</td>
</tr>
<tr>
<td>B. Pig myoglobin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>1.0</td>
<td>&lt;0.01</td>
<td>0.057</td>
<td>0.029</td>
<td>600</td>
</tr>
<tr>
<td>V68N</td>
<td>1.6</td>
<td>0.07</td>
<td></td>
<td></td>
<td>&lt;&lt;1</td>
</tr>
<tr>
<td>V68S</td>
<td>8.7</td>
<td>≤0.01</td>
<td></td>
<td></td>
<td>&lt;&lt;1</td>
</tr>
<tr>
<td>V68T</td>
<td>0.5</td>
<td>≤0.01</td>
<td></td>
<td></td>
<td>&lt;&lt;1</td>
</tr>
<tr>
<td>C. Human myoglobin</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Wild-type</td>
<td>3</td>
<td>-0.01</td>
<td>0.019</td>
<td>0.026</td>
<td>2,000</td>
</tr>
<tr>
<td>H64V/V68H</td>
<td>&lt;0.1</td>
<td>&lt;&lt;0.01</td>
<td></td>
<td></td>
<td>&lt;&lt;1</td>
</tr>
</tbody>
</table>
Sperm whale Thr$^{68}$ apoprotein is 8 times less stable than wild-type apoglobin but loses hemin at least 10 times more slowly at both pH 5 and 7. The extremely slow rate of hemin dissociation is due to the "extra" hydrogen bond between the coordinated water molecule and the β-hydroxyl group of the Thr$^{68}$ side chain (Smerdon et al., 1991; Chapters 6,7). This interaction increases hemin affinity, compensates for apoglobin instability, and facilitates higher yields of holoprotein in both the pig and sperm whale myoglobin expression systems. The most extreme example of compensation for low globin stability was observed for the H64V/V68H double mutation in human myoglobin. Removal of hemin produces a completely unfolded apoglobin (Fig. 4.5 D). The reason for success in producing holoprotein is that His$^{68}$ coordinates the heme iron, creating a cytochrome b-like structure (Qin et al., 1994; Dou et al. 1995; Chapter 6). As a result, hemin binding is effectively irreversible under physiological conditions, and hemin loss from the H64V/V68H mutant could not be detected at either pH 5 or 7 (Table 4.2).

**Mutagenesis at positions 107(G8) and 43(CD1)** - Unfolding of I107V, I107T, and I107F sperm whale apoglobins was examined to test whether the effects of mutagenesis at positions 29 and 68 are due to specific changes in polarity of residues in the B and E helices or a result of more global changes in polarity of the distal portion of the heme pocket. The latter interpretation appears valid since the overall stabilities of the G8 mutants are Phe$^{107} >$ Ile$^{107}$(wild-type)$ ≥ $Val$^{107} >$ Thr$^{107}$ apoglobin (Table 4.2, Fig. 4.3 C). Again, the greatest effect was on the N,I equilibrium
Figure 4.4: Comparisons of sperm whale, pig, and human apomyoglobins. The total fluorescence intensities of sperm whale, pig, and human apomyoglobins are plotted versus [GdmCl] and the lines represent fits to Equation 4.1.
Figure 4.5: Stabilities of pig and human apomyoglobin mutants. A), Fluorescence emission spectra of pig (solid lines) and human (dashed lines) apomyoglobins at 0, 1, and 4 M GdmCl. B), C), and D), spectra of pig V68T, Pig V68S, and human H64V/V68H myoglobins, respectively, at 0 (solid lines) and 4 M GdmCl (dashed lines). Spectra were recorded as described in the Methods section.
constant. Only the I107T mutation had a significant effect on the rate of hemin loss, causing $k_{\text{H}}$ to increase from $\sim 0.01$ to $0.15 \text{ h}^{-1}$ at pH 7.

Phe$^{43}$ and the proximal histidine, His$^{93}$, are conserved in all globins (Dickerson and Geis, 1983). Naturally occurring point mutations at the position 43 (CD1) in the $\alpha$ and $\beta$ subunits of human hemoglobin produce unstable proteins which readily lose heme and cause the formation of Heinz bodies inside red cells (Bunn and Forget, 1986). These observations led Honig et al. (1990) and others to suggest that Phe-CD1 is required for high heme affinity. This interpretation is verified quantitatively in Table 4.2. Both the F43V and F43I mutations cause 200-fold increases in the rate of hemin dissociation at pH 7. Phe$^{43}$ also plays an important role in stabilizing the native apoglobin structure because there is a 40-fold decrease in apoprotein stability when Val replaces this residue (Table 4.2). As with the other substitutions in the distal pocket, this appears to be a general hydrophobic effect since the F43I mutation produces only a small, $\sim 2$-fold decrease in the stability of the N state and in expression yield (Tables 4.1 and 4.2).

**Replacement of the distal histidine** - Mutations at position 64 exert the same general effects on apomyoglobin stability as those at other positions in the heme pocket. Replacing the polar distal histidine with Leu or Phe stabilizes the apoprotein 20 to 30-fold (Fig. 4.3 D and Table 4.2) whereas the Gln substitution has little effect. Even the H64A mutation stabilizes the apoprotein 6-fold with respect to wild-type apoprotein. As in the case of the position 68 mutations, there is little correlation between rates of hemin loss and apoglobin stability. His$^{64}$ (wild-type) metmyoglobin has
a low rate of hemin loss because the imidazole side chain stabilizes coordinated water by hydrogen bonding interactions which are lost or weakened in the aliphatic mutants at this position (Quillin et al., 1993).

DISCUSSION

Relative importance of hemin affinity and apoglobin stability - The resistance of holomyoglobin to denaturation is a function of both the intrinsic stability of the apoprotein tertiary structure and the strength of interactions with the prosthetic group. Because the affinity of apomyoglobin for hemin is extremely high (≈ $10^{13}$ M$^{-1}$), the holoprotein is much more stable to denaturation than the apoprotein (Griko et al., 1988; Hughson and Baldwin 1989). Thermal, urea, and GdmCl induced "melting" of intact myoglobin is usually determined by the extent of hemin dissociation, which is followed immediately by rapid globin unfolding at the elevated temperatures and denaturant concentrations required to induce loss of the prosthetic group (see Chapter 7). Because the bimolecular rate of hemin binding is approximately the same for all apoproteins, $k'_H \approx 10 \times 10^7$ M$^{-1}$s$^{-1}$, affinity is governed primarily by the rate of hemin dissociation, $k_H$, which varies over several orders of magnitude (see Chapter 5 and 6).

Polar residues adjacent to the iron atom can inhibit hemin loss by forming hydrogen bonds with coordinated water but, after hemin dissociation, destabilize the native apoglobin structure by causing solvation of the heme pocket and subsequent unfolding of the E and B helices. Aliphatic and aromatic substitutions at the B10, E11, and G8 positions have a greater favorable effect on $K_{N,I}$ than on $k_H$ (Table 4.2). Thus, the apolar
residues found naturally at these locations are more important for stabilizing the native apoglobin structure than for enhancing hemin binding. These results also support the two step unfolding model shown in Fig. 4.1, which predicts that the introduction of polar side chains in the middle of the E and B helices should induce unfolding of these secondary structures and formation of the molten globule intermediate.

**His 64(E7): functionality at the expense of stability** - Hydrogen bonding interactions with the distal histidine are required for high O2 affinity, discrimination against CO binding, and inhibition of autooxidation (Brantley *et al.*, 1993; Springer *et al.*, 1994). Many of these functions can also be carried out by the amide side chain of Gln. Consequently, His or Gln are found at the E7 helical position in almost all myoglobins and hemoglobins, and high rates of autooxidation and poor discrimination against CO binding are observed for the exceptions which have Val or Leu at the E7 position (Dickerson and Geis, 1983; Rohlfs *et al.*, 1990; Brantley *et al.*, 1993). As shown in Tables 4.1 and 4.2, conservation of the distal histidine is at the expense of globin stability. All aliphatic or aromatic substitutions at position 64 produce apoglobins that are more stable than wild-type apoprotein.

The results in Table 4.2 also provide a plausible explanation for why peroxidase and oxidase activities have not evolved in proteins with a myoglobin fold and why it will be difficult to engineer these activities into myoglobin. The oxidative reactions require multiple polar or charged residues in the heme pocket. Introduction of polar groups at the B10, E11, or G8 positions inhibits globin folding and expression markedly, particularly
in pig and human myoglobin, where complete denaturation appears to occur when hemin is removed from these mutants (Fig. 4.5). This unfavorable effect on holoprotein expression can be overcome by putting His at the E11 position to facilitate direct coordination to the iron atom (Table 4.2). However, the resultant protein is hexacoordinate in both the ferrous and ferric states and relatively unreactive toward exogenous ligands (Qin et al., 1994; Dou et al. 1995).

**Species differences** - As shown in Fig. 4.4, both pig and human apomyoglobins are much more readily denatured than the sperm whale apoprotein. Both $K_{N,I}$ and $K_{I,U}$ are larger for the pig and human apoproteins, and their overall stabilities are decreased 30 and 100-fold, respectively, compared to sperm whale apomyoglobin. In contrast, the rates of hemin loss from all three holoproteins are the same at pH 7.0. Because most previous comparative studies involved denaturation of the holoproteins, the marked differences between these apoglobins have gone relatively unnoticed (Flanagan et al., 1983; Puett, 1973). However, Baldwin and coworkers have reported differences between the pH denaturation profiles of sperm whale and human apoglobins. The human protein forms the I state at somewhat higher pH values than the whale globin (Hughson and Baldwin, 1989; Hughson et al., 1991).

The physiological significance of enhanced stability of sperm whale apomyoglobin is probably related to the harsh conditions that occur in whale muscle tissue during diving. Prolonged exposure to anaerobic conditions causes acidification of muscle cell cytoplasm, and denaturation may also be enhanced by the high pressures associated with deep dives (Schmidt-
Nielsen, 1990). A molecular explanation of the differences in stability of pig, human, and sperm whale proteins is less clear. Sequence differences among the three proteins are listed in Table 4.3. Our working hypothesis is that the Val/Ala vs. Gly differences at positions 1, 15, and 74 and the Ser vs. Gly difference at position 35 cause the differences in stability to GdmCl denaturation. The V1G and A15G replacements may serve to destabilize the A helix causing an increase in $K_{i,j}$ and the A74G and S35G substitutions could destabilize the E and C helices, respectively, causing an increase in $K_{N,I}$. Finally, native sperm whale myoglobin is ~3 times more stable than the recombinant protein which contains an "extra" N-terminal Met residue. This is the first large difference that has been noted between the properties of native and wild-type myoglobin and also indicates that the N-terminal region of the A helix plays a role in stabilizing the native globin structure relative to the molten globule intermediate.

**Protein engineering strategies** - The results in Tables 4.1 and 4.2 provide background data for rationally designing more efficient expression systems and more stable, functional proteins. For example, large quantities of V68N and V29N myoglobin can not be made using the simple constitutive expression system developed by Springer and Sligar (1987) for sperm whale myoglobin. The apoproteins are too unstable and hemin synthesis is too slow to prevent denaturation. However, holoproteins containing these mutations can be made in fairly large quantities with the fusion-protein system used to express pig and human myoglobins as inclusion bodies (Nagai and Thørgensen, 1987). Refolding of the denatured
Table 4.3: Comparison of the sequences of sperm whale, human and pig myoglobin. A listing of known mammalian myoglobin sequences was obtained using the EuGene & SAM software package developed by the Molecular Biology Information Resource, Department of Cell Biology, Baylor College of Medicine, Houston, TX and the Protein Sequence Data Bank from the National Biomedical Research Foundation, Washington, D. C. Key differences are marked in bold.

|      | 1  | 4  | 4  | 1  | 2  | 2  | 2  | 2  | 2  | 3  | 4  | 5  | 5  | 6  | 7  | 8  | 8  | 8  | 8  | 1  | 1  | 1  | 1  | 1  | 1  | 1  | 1  |
|------|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|----|
| Mb   | 2  | 5  | 1  | 2  | 7  | 8  | 5  | 5  | 1  | 3  | 6  | 4  | 6  | 7  | 0  | 1  | 1  | 1  | 1  | 3  | 4  | 4  | 4  | 4  | 5  | 1  |
| Sperm Whale | V E H A V A D I S R T A V A L K I A H H R N K I A K Y |
| pig   | G D N G V A E V G K S D N G L T V A Q Q K S N M A K F |
apoglobin is induced *in vitro* with excess hemin, and once formed, the mutant holoproteins are almost as resistant to hemin loss as wild-type myoglobin.

The results in Tables 4.1 and 4.2 indicate that expression yields can be enhanced by stabilizing the native apoglobin structure, and provide examples of distal pocket mutations which decrease $K_{N,I}$. The $V68F$ substitution was combined with the distinctive spectral properties of the $Tyr^{64}$ mutant to engineer a more stable reagent for measuring hemin loss (e.g. apoH64Y/V68F; Chapter 2). This substitution has also been incorporated into recombinant hemoglobin in an attempt to increase apohemoglobin stability without increasing rates of autooxidation or hemin loss. The differences between pig, human, and sperm whale myoglobin suggest other approaches to stabilizing apohemoglobin subunits. Increasing resistance to hemin loss and globin denaturation is of great clinical importance for maximizing the safety, effectiveness, and retention time of extracellular hemoglobin-based blood substitutes.
Chapter 5
The Bimolecular Reaction of Heme with Apomyoglobin

INTRODUCTION

Under most conditions the bimolecular reaction of heme with globin is complex, showing at least two phases which vary greatly in rate and amplitude as a function of reaction conditions. Gibson and Antonini (1960) provided the first comprehensive kinetic model for the reaction of heme with globin. They described the problems associated with hemin aggregation and reactivity and demonstrated the necessity of using a monomeric heme to interpret the observed reaction. The following model was proposed for the reaction of heme with globin:

\[
\text{Heme + Globin} \xrightarrow{k_1} \text{complex} \xrightarrow{k_2} \text{holo-protein} \quad (5.1)
\]

The initial bimolecular process results in the formation of a reversible heme/globin complex with \(k_1\) and \(k_{-1} \approx 3 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}\) and 2000 \(\text{s}^{-1}\), respectively. First order formation of the final holoprotein complex appears to occur at a rate of \(\approx 400 \text{ s}^{-1}\) at pH 7.0, 20°C. Their experiments with apomyoglobin and CO-heme gave very similar results.

In contrast to CO-heme, Gibson and Antonini (1960) showed that the reaction of dicyanohemin with globin is first order, monophasic, and very slow. The reaction of hemin with globin shows multiple phases and depends markedly on hemin concentration. The complex behavior observed for hemin binding to globin has been attributed to multiple aggregation states
of the prosthetic group whereas the slow reaction of dicyanohemin was attributed to the interconversion between di- and monocyanohein prior to reaction with globin (Gibson and Antonini, 1960; Brown et al., 1970).

Adams (1977) and Kawamura-Konishi and Suzuki (1985) re-investigated the reaction of hemin with apomyoglobin and apohemoglobin, respectively, and demonstrated that an initial fast bimolecular reaction does occur, presumably involving the small fraction of monomeric hemin present in solution. In both studies the reaction conditions were adjusted to minimize hemin aggregation. Adams kept hemin at pH 13 until the instant of mixing with globin and Kawamura-Konishi and Suzuki used caffeine, which intercalates between porphyrin rings to solubilize hemin aggregates. Adams carried out reactions with equimolar concentrations of hemin and apomyoglobin and measured a single bimolecular association rate of 3.3 x 10^5 M^{-1}s^{-1} at pH 7.2. Kawamura-Konishi and Suzuki, working with excess hemin/caffeine, measured a bimolecular association rate of 2.9 x 10^6 M^{-1}s^{-1} at pH 7.2 for the reaction of hemin/caffeine with globin but this phase accounted for only a fraction of the total absorbance change observed. The bimolecular phases observed by Gibson and Antonini (1960) for hemin binding were much faster (∼10^7 M^{-1}s^{-1}) than those reported by Adams and Kawamura-Konishi and Suzuki.

Leutzinger and Beychok (1981) measured the rate of dicyanohemin binding to isolated human α chains as part of a study of α globin refolding. A large fraction of the reaction occurred with a rate of ∼ 0.02 s^{-1} when measured by stopped-flow absorbance or far-UV circular dichroism. However, when measured by quenching of tryptophan fluorescence, the
rate of dicyanohemin binding was very fast with a bimolecular rate of $3.3 \times 10^7$ M$^{-1}$ s$^{-1}$. The fast rate measured by fluorescence was interpreted as the bimolecular binding of cyanohemin to the protein. The slow changes seen in absorbance and CD measurements were attributed to global folding events resulting in the final holo-α chain structure.

Rose and Olson (1983) measured rates of CO-heme binding to globin under many different conditions in an effort to understand the kinetic mechanisms behind the model proposed by Gibson and Antonini (Equation 5.1). Their data were fitted to this model by assigning one variable, $K_D$, to account for the ratio of $k_{-1}/k_1$ which describes the equilibrium dissociation constant for the formation of the initial heme/globin complex. The initial bimolecular rate constant, $k_1$, was estimated to be $8 \times 10^7$ M$^{-1}$ s$^{-1}$ and interpreted as the binding of CO-heme to hydrophobic surfaces on globin. The fitted value of $k_2$ was $\sim 500$ s$^{-1}$ and interpreted as the rate constant for formation of the heme-proximal histidine bond. However, this rate constant decreased markedly with increasing glycerol concentration which is not expected if $k_2$ represents only the internal bond-forming event.

Gibson and Antonini (1963), Chu and Bucci (1979), and Rose and Olson (1983) also measured the rates of reaction of modified hemes with apohemoglobin. Gibson and Antonini showed that the CO forms of meso-, deuto-, and hematohemes react with globin at rates 2 to 4 times smaller than that for CO proto heme binding at pH 9.1, and Rose and Olson reported similar results at pH 7.2. Chu and Bucci showed that CO-dimethylester heme reacts with apohemoglobin at the same rate as CO protoheme. These results indicate that specific heme-globin interactions
and pH probably do not play a large role in the bimolecular binding reaction of heme with globin.

Hargrove et al. (1994 b) showed that the stability of apomyoglobin can be significantly decreased by introduction of polar residues in the heme pocket (Chapter 4). In this Chapter, the association rate constants for heme binding to a large number of apomyoglobins which differ widely in apoglobin stability have been measured. The goal was to determine the effect of apoglobin structure on the kinetics of heme binding. Surprisingly, most of the mutations have little effect on the observed association rate constant for heme binding. These results show more definitively that heme affinity is governed almost exclusively by the dissociation rate constant. Rates of binding of different heme derivatives to apomyoglobin and bovine serum albumin (BSA) were also measured using absorbance and fluorescence techniques in order to test the kinetic mechanism proposed by Rose and Olson (1983).

MATERIALS AND METHODS

Production of Proteins- Sperm whale, horse, and sheep native myoglobins were purchased from Sigma. The whale protein was obtained by special permit. Wild type sperm whale myoglobin and mutants at positions 29, 43, 64, 68, and 107, and pig V68T myoglobin were made as described in Chapter 4. Mutants at positions 22, 32, 45, 66, 89, 97, and 110 were made using oligonucleotide-directed mutagenesis starting with the
synthetic sperm whale myoglobin gene developed by Springer and Sligar (1987).

Native leghemoglobin a (Lba) was provided by Gautam Sarath at the University of Nebraska-Lincoln. H93G myoglobin containing non-covalently bound imidazole was prepared by Barrick (1994). Human $\alpha$ and $\beta$ chains were prepared by A. J. Mathews using the method of Bucci (1981). All recombinant myoglobins were expressed and purified at Rice University as described by Springer and Sligar (1987) and Carver et al. (1992). Human H64V/V68H was expressed and purified at Case Western Reserve University following the method of Ikeda-Saito et al. (1991). Apomyoglobins were prepared using the methyl ethyl ketone method described by Ascoli et al. (1981) and Hargrove et al. (1994a). Bovine serum albumin was purchased from Sigma and used without further purification.

**Preparation of reaction solutions** - The buffer used in all of the heme association reactions was 50 mM Tris, 50 mM NaCl, pH 8.0 (Light et al., 1990). Under these conditions heme aggregation and non-specific binding are at a minimum. Reactions at lower pH showed more amplitude associated with slow phases due to these processes. All heme containing solutions were prepared and used within 3 hours to minimize any long term aggregation or oxidation. A stock hemin (Sigma) solution was prepared by dissolving a small amount ($\sim 10$ mg) in $\sim 1$ ml of 0.1 M NaOH. This solution was then passed through a 0.2 $\mu$m filter into an eppendorf tube.

For the production of CO-heme, the buffer was pre-equilibrated by bubbling with 100 % CO gas for 20 minutes. 1 ml of this buffer was
drawn into a 5 ml Hamilton Gas-Tight syringe and several granules of dry sodium dithionite (DT) were added. Approximately 200 µl of the filtered hemin solution was added to the syringe containing CO buffer and sodium dithionite. The concentration of CO-heme was measured spectrophotometrically using $\varepsilon_{407} = 147 \text{ mM}^{-1} \text{ cm}^{-1}$ (Light 1987). This solution (usually 100 to 200 µM) was used as a stock for dilution to appropriate concentration in the reaction syringe of the stopped flow apparatus. Small amounts of sodium dithionite were added to all reaction syringes prior to addition of heme to prevent oxidation.

Due to the effects of hemin aggregation on absorbance and the relatively weak hemin Soret peak, the production of a stock hemin solution of known concentration was carried out as follows. Approximately 200 µl of the filtered stock hemin was added to 1 ml of air equilibrated buffer. A small aliquot of this solution was added to a cuvette containing CO equilibrated buffer and dithionite and the concentration of CO-heme produced was used to compute the concentration of the original stock hemin solution. Stock deoxyheme solutions were prepared in a similar fashion, except that all buffers were bubbled with 100% nitrogen for 20 minutes and sodium dithionite was added to reduce the hemin.

Due to the low affinity of hemin for CN$^-$ as compared to myoglobin, a relatively large CN$^-$ concentration must be added to ensure that free hemin is in the dicyano form. In the experiments involving dicyanohemin, all reaction solutions contained 10 mM KCN. Higher concentrations of KCN had no effect on the absorbance changes or reaction kinetics. The concentration of stock dicyanohemin solutions were prepared by
determining the concentration of a hemin solution as described above followed by appropriate dilution into buffer containing 10 mM KCN.

The concentration of most apomyoglobin solutions, apoleghemoglobin, and human β apoglobin was determined spectrophotometrically using $\varepsilon_{280} = 15.2$ mM$^{-1}$ cm$^{-1}$ (Light 1987). $\varepsilon_{280} = 22.8$ mM$^{-1}$ cm$^{-1}$ was used for V68W and L89W apomyoglobins to account for the additional tryptophan and $\varepsilon_{280} = 7.6$ mM$^{-1}$ cm$^{-1}$ was used for human α apoglobin which contains only one tryptophan. BSA solutions were prepared by weight.

**Stopped flow measurements** - Stopped flow absorbance measurements were made with a Gibson-Dionex stopped flow apparatus equipped with an OLIS, Inc. data acquisition system. The reactions with CO-heme, hemin, dicyanohemin, and deoxyheme were monitored at 423, 409, 421, and 432 nm, respectively. Fluorescence measurements were made with a SLM 8100 spectrofluorimeter equipped with a Milliflow$^\text{TM}$ stopped flow attachment. Reactions with CO and deoxyheme were not measured by fluorescence due to the strong UV absorbance of dithionite, which was a necessary component in these reaction syringes. Excitation was at 285 nm, and total fluorescence emission was measured using a 320 nm cutoff filter. All reactions except for those in Fig. 5.2B were carried out with excess apoglobin. At apoglobin concentrations greater than 5 μM, a significant portion of the reaction is lost in the dead time of the apparatus, which is 3 ms. Data were fitted using the data analysis program IGOR$^\text{TM}$.

**Heme dissociation experiments** - Heme loss from H93G myoglobin and bovine serum albumin (BSA) was measured by stopped flow techniques
using the H64Y/V68F apomyoglobin assay described by in Chapter 2. For the reactions involving the H93G metmyoglobin-imidazole complex, 12 μM holoprotein was mixed with 60 μM H64Y/V68F apoglobin and the reaction was monitored as a decrease in absorbance at 410 nm. Hemin loss from BSA was difficult to analyze, presumably due to hemin aggregation and the resulting heterogeneous binding to BSA. Instead, the rate of CO-heme loss from BSA was measured. In this case, one reaction syringe contained 60 μM BSA and 12 μM CO-heme and the other 60 μM apo-H64Y/V68F. This reaction was measured as an increase in absorbance at 423 nm. Heme loss from H93G was measured at 37°C for comparison with other myoglobins (Hargrove et al. 1994 a,b) and heme loss from BSA was measured at 20°C for direct comparison with heme association rates.

RESULTS

The reaction of heme with apomyoglobin- We repeated the studies of Gibson and Antonini (1960) and measured rates of hemin, dicynohemin, and CO-heme binding to apomyoglobin. The observed time courses for these reactions (Fig. 5.1 A,B,C) are very similar to the results obtained by Gibson and Antonini. In their work the slow rate of hemin binding was attributed to hemin aggregates and that of dicynohemin binding was attributed to CN⁻ dissociation prior to heme binding. Fig. 5.1A shows the changes in absorbance and fluorescence associated with hemin binding to apomyoglobin. The fluorescence of apomyoglobin is quenched nearly 50 fold upon hemin binding. The normalized absorbance and fluorescence time courses overlay fairly well and show that the majority
**Figure 5.1:** Time courses of heme binding. A) Ferric hemin binding to wild type apomyoglobin. The traces labeled Soret represent absorbance measurements of 0.25 μM hemin reacting with 0.5, 1, and 2 μM apomyoglobin. The time courses are nearly identical and are biphasic with a slow rate of 0.9 s^{-1}. The trace labeled Fluorescence is the same reaction at 0.5 μM apomyoglobin monitored by fluorescence. The inset is an expanded view of the early time points showing that there is only a very small bimolecular fast phase associated with this reaction. B) Dicyanohemin binding to apomyoglobin as monitored by absorbance changes. 0.5, 1, and 2.5 μM apomyoglobin were reacted with 0.25 μM dicyanohemin. In each case the reaction was monophasic with a rate of 0.019 s^{-1}. The inset is an expanded view of the early time points showing that no fast reaction is observed. C) Dicyanohemin binding to apomyoglobin as monitored by changes in fluorescence. The reactions of 0.25, 0.5, and 1 μM apomyoglobin with 0.25 μM dicyanohemin were fitted to a single exponential. A plot of k_{obs} vs. [globin] gave a bimolecular association rate constant of 5 \times 10^{7} M^{-1} s^{-1}. No fluorescence changes were observed over a 5 minute period following these reactions. D) CO-heme binding to apomyoglobin. The reactions of 0.5, 1, and 2.5 μM apomyoglobin with 0.25 μM CO-heme were fitted to a two exponential expression. The apparent bimolecular association rate constant for the larger fast phase is 7 \times 10^{7} M^{-1} s^{-1}. 
of the reaction is very slow. Presumably, slow dissociation of hemin aggregates is required before the prosthetic group can interact with apoprotein.

Fig. 5.1B shows that dicyanohemin binding to apomyoglobin is a slow first order process with a rate constant of $\sim 0.02 \text{ s}^{-1}$ when measured by Soret absorbance changes. However the fluorescence time course for the same reaction shows a rapid bimolecular process with a rate constant of $5 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$ (Fig. 5.1 C). Similar results were obtained by Leutzinger and Beychok (1981) for the reaction of dicyanohemin with isolated human hemoglobin $\alpha$ chains. These results show that dicyanohemin enters in the apomyoglobin heme pocket as rapidly as monomeric CO-heme (Fig. 5.1 D), quenching most of the endogenous tryptophan fluorescence. In contrast, there is little or no absorbance change associated with the binding of dicyanohemin to the apoglobin since no change in coordination occurs. The large slow absorbance changes observed in Fig. 5.1B are due to displacement of the proximal cyanide ligand by the imidazole side chain of His$^{93}$(F8). The rate of this displacement reaction should be governed by the rate at which cyanide dissociates from dicyanohemin, which is expected to be slow (Antonini and Brunori 1971).

Fig. 5.1D shows the reaction of CO-heme with apomyoglobin. Each trace fits to two phases: one large bimolecular phase with a second order rate constant $= 7 \times 10^7 \text{ M}^{-1} \text{ s}^{-1}$, and one small first order phase with a rate $\approx 9 \text{ s}^{-1}$. The fast phase represents the combination of heme with apomyoglobin to form holo CO-myoglobin. Figs. 5.2A and 5.2B demonstrate that the slow process represents the non-specific binding of
**Figure 5.2:** Non-specific interactions account for the slow phase of CO-heme binding. A) CO-heme binding to myoglobin and bovine serum albumin (BSA). The reaction of 0.5 μM apomyoglobin and 0.25 μM heme is almost monophasic with a pseudo first order rate of 35 s⁻¹. The slow, non-specific binding phase is increased by the addition of 5 μM BSA to the reaction mixture. The reaction of CO-heme with CO-myoglobin or GdmCl unfolded apomyoglobin shows only a small, slow phase with a rate ~ 9 s⁻¹.

B) Binding reactions in the presence of excess CO-heme. In this experiment 0.5 μM apomyoglobin was reacted with increasing concentrations of CO-heme in excess of 0.5 μM. The ratio of heme/globin is shown next to the corresponding time course. The amplitude of slow phase increases in proportion to heme concentration, suggesting that the non-specific interaction of CO-heme with apomyoglobin results in absorbance changes similar to the formation of CO myoglobin.
heme to globin. In Fig. 5.2A, 0.25 µM CO-heme was reacted with 0.5 µM apomyoglobin resulting in absorbance changes like those in Fig. 5.1 D. However, when the same reaction was carried out in the presence of 5 µM BSA the observed rate of reaction slows dramatically due to rapid non-specific binding to BSA. This latter process probably occurs in the dead time of the apparatus and is followed by slow dissociation from this less stable complex and subsequent rapid, high affinity binding to apomyoglobin.

Fig. 5.2B shows that increasing CO-heme concentration results in slow absorbance changes which are also due to non-specific binding. As the concentration of CO-heme reacting with apomyoglobin is increased from 0.5 to 5 µM, the rate of the observed reaction slows and the amplitude of the slow phase increases linearly with heme.

All of these results suggest that the slow phases observed in heme binding experiments represent CO-heme binding to and exchange from non-specific sites which compete with the heme pocket. It is clear that the reaction of CO-heme with apomyoglobin is best carried out with the lowest possible concentrations of heme and with the protein in excess. The absorbance change associated with the reaction of 0.25 µM CO-heme is sufficient for analyses and the use of 0.5 µM apomyoglobin results in a half time of about 25 ms which is easily measured in most stopped-flow apparatus.

Effects of the heme pocket and the proximal histidine on heme affinity- The affinity of BSA for heme is due primarily to non-specific hydrophobic interactions (Marden et al. 1989). The increased heme affinity
of myoglobin is due to the specificity of the heme pocket and the strength of the proximal histidine-heme iron bond. To estimate the relative importance of each of these two factors, heme loss and CO-heme binding to BSA and H93G myoglobin were measured. The affinity of H93G apomyoglobin for heme is determined solely by non-covalent interactions with the heme pocket residues since the proximal His-iron bond cannot be formed (Barrick 1994). As shown in Table 5.1 each of these proteins has about the same CO-heme association rate constant $k'_{H} \left(7 \times 10^{7} \text{ M}^{-1} \text{ s}^{-1}\right)$, and their heme affinities are governed almost exclusively by dissociation rate constants ($k_{H}$).

H93G myoglobin forms a stable complex with hemin in the presence of exogenously added imidazole (Barrick 1994). The spectral and functional properties of this complex are similar to those of native aquometmyoglobin, and hemin loss can be measured using the H64Y/V68F apomyoglobin assay. The observed rate constant for the later process is $\sim 0.04 \text{ s}^{-1}$ in the presence of a stoichiometric amount of imidazole. Assuming that $k'_{H}$ is the same for both CO-heme and monomeric hemin, the affinity of H93G myoglobin for hemin is $\sim 6 \times 10^{9} \text{ M}^{-1}$.

The rate of hemin dissociation from bovine serum albumin is $\sim 0.01 \text{ s}^{-1}$ and most likely represents the dissociation of hemin bound in a hydrophobic pocket, resulting in a rate of hemin loss much like that of H93G myoglobin. Assuming an association rate constant of $\sim 5 \times 10^{7} \text{ M}^{-1} \text{ s}^{-1}$, the affinity of BSA for coordinated hemin is also $\sim 5 \times 10^{9} \text{ M}^{-1}$. CO-heme dissociation from albumin involves the dissociation of CO-heme from the surface of the protein and from hydrophobic pockets containing
coordinating bases. An initial, very rapid process is observed when BSA containing CO-heme is mixed with apomyoglobin and causes an increase in absorbance at 423 nm. The rate of this phase is ~ 8 s\(^{-1}\), similar to the slow phase seen in the reaction of apomyoglobin and CO-heme which is attributed to non-specific binding and/or exchange into the heme pocket. It is likely that CO-heme coordinated to bases in albumin dissociates much more slowly, and is not readily observed. The rate of dissociation of non-specifically bound (and presumable 5 coordinated) CO-heme indicates an association equilibrium constant of ~ 6 x 10\(^6\) M\(^{-1}\) for BSA.

**The effects of apomyoglobin structure on rates of CO-heme binding** - CO-heme binding to apomyoglobin was measured as a function of GdmCl in order to examine the effects of apoprotein structure on this reaction. GdmCl induced unfolding of apomyoglobin proceeds in two steps (Barrick and Baldwin (1993); Chapter 4):

\[
\begin{array}{c}
N \rightleftharpoons^{K_{N,I}} I \rightleftharpoons^{K_{I,U}} U \\
=1.8 M \text{ GdmCl}
\end{array}
\]  

The structure of native apomyoglobin (N) is less compact and has ~ 15 % less helicity than that of holo-myoglobin. The intermediate (I) state lacks a defined heme pocket due to complete unfolding of the B, C and E helices. The unfolded (U) state contains very little secondary structure (Hughson et al. 1991). As shown in the previous chapter, formation and decay of the I state can be visualized by monitoring tryptophan fluorescence during titration of apomyoglobin with GdmCl. Fig. 5.3A shows fluorescence
**Table 5.1**: Kinetic and equilibrium parameters for hemin and CO-heme binding to bovine serum albumin and wild type and H93G myoglobin. All association rate constants were measured in 50 mM Tris, 50 mM NaCl, pH 8.0 at 20°C using CO-heme. These rate constants are assumed to apply to all monomeric forms of iron-porphyrin including hemin. Values of $k'_{H}$ for monomeric hemin binding to myoglobin and bovine serum albumin can not be measured easily due to aggregation (Fig. 1A). Hemin dissociation from H93G myoglobin was measured in 0.15 M potassium phosphate, 0.45 M sucrose, pH 7.0 at 37°C. This $k_{H}$ values were extrapolated to 20°C using $E_a = 12$ kcal/mol and are assumed to apply at both pH 7 and 8 (Chapter 2). Hemin and CO-heme dissociation from bovine serum albumin was measured at pH 7.0, 20°C. $K_H$ was computed as the ratio $k'_{H}/k_{H}$ and is an estimate of hemin affinity for the apomyoglobin samples.

<table>
<thead>
<tr>
<th>Myoglobin</th>
<th>$k'_{H}$ ($\text{M}^{-1} \text{s}^{-1}$)</th>
<th>$k_{H}$ ($\text{s}^{-1}$)</th>
<th>$K_H$ ($\text{M}^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>wild type</td>
<td>$7 \times 10^7$</td>
<td>$8.4 \times 10^{-7}$</td>
<td>$8.3 \times 10^{13}$</td>
</tr>
<tr>
<td>H93G</td>
<td>$7 \times 10^7$</td>
<td>$1.2 \times 10^{-2}$</td>
<td>$5.8 \times 10^9$</td>
</tr>
<tr>
<td>BSA/Hemin</td>
<td>$\sim 5 \times 10^7$</td>
<td>$1.1 \times 10^{-2}$</td>
<td>$4.5 \times 10^9$</td>
</tr>
<tr>
<td>BSA/CO-heme (Weakly bound)</td>
<td>$5 \times 10^7$</td>
<td>$7.7$</td>
<td>$6.8 \times 10^6$</td>
</tr>
</tbody>
</table>

\(^a\) The value of $k'_{H}$ for monomeric hemin is assumed to be the same as that for CO-heme.
**Figure 5.3:** Effects of unfolding on rates of CO-heme binding to apomyoglobin.  
A) Fluorescence emission spectra of apomyoglobin prior to CO-heme binding. Fluorescence emission spectra of the apomyoglobin reaction syringe were measured just prior to reaction with CO-heme. These spectra show apomyoglobin in various stages of unfolding just before mixing with CO-heme.  
B) Time courses for CO-heme binding to apomyoglobin in different concentrations of GdmCl. Each reaction contained 0.5 μM apomyoglobin and 0.25 μM CO-heme after mixing. As apomyoglobin is unfolded it loses its ability to bind heme specifically. Nearly all specific heme binding is lost in 2M GdmCl. At this denaturant concentration, apomyoglobin is in the I state and the heme pocket is unfolded. At 5M GdmCl, apomyoglobin is completely unfolded, and time courses obtained under these conditions fit to a single exponential expression with a rate constant = 9 s\(^{-1}\).  
C) Absorbance spectra of CO-heme and apomyoglobin at various GdmCl concentrations. CO myoglobin with an absorbance peak at 423 nm is formed rapidly when CO-heme is mixed with apomyoglobin at 0M GdmCl. As [GdmCl] is increased, progressively less CO-myoglobin is formed. At 5M GdmCl, CO-heme remains monomeric in solution with a peak at 411 nm. These spectra show that CO-heme cannot bind specifically to apomyoglobin under denaturing conditions. A partially formed heme pocket, or the ability to fold rapidly around the heme, is required for specific heme association. This capacity is lost during formation of the molten globule intermediate, or I state in the presence of GdmCl.
emission spectra of apomyoglobin under various denaturing conditions. The increase in fluorescence at 1 M GdmCl is associated with unfolding to the I state. Further titration with GdmCl results in a decrease in fluorescence as the protein unfolds to the U state (5 M GdmCl). Fig. 5.3B shows time courses for the reaction of CO-heme with apomyoglobins at the various GdmCl concentrations in Fig. 5.3A. The amplitude of the fast bimolecular reaction decreases markedly at concentrations of 1 and 2 M GdmCl, and only slow non-specific processes occur for U state apomyoglobin in 5 M GdmCl. Fig. 5.3C shows the equilibrium absorbance spectra of these reaction mixtures. CO-heme is no longer able to bind apomyoglobin specifically when the protein is completely unfolded. The spectra with peaks at 411 nm seen in 4 and 5 M GdmCl are identical to that of free CO-heme in GdmCl.

These results demonstrate that the ability to form a folded heme pocket is necessary for specific CO-heme binding to apomyoglobin. It was shown in Chapter 4 that point mutations which place a polar residue in the predominately apolar heme pocket destabilize the native N state of apomyoglobin. To determine if the structural changes incurred by these mutations affect the association reaction, the rate constants for CO-heme binding to a number of these myoglobin mutants were measured. The values of \( k' \) for CO-heme binding to several other native myoglobins, soybean leghemoglobin, and isolated \( \alpha \) and \( \beta \) chains of human hemoglobin were also determined. The results for all of these proteins are shown in Table 5.2.
Table 5.2: Association rate constants for CO-heme binding to native myoglobins, myoglobin mutants, and other heme proteins. In most cases, the reactants were 0.50 μM apoglobin and 0.25 μM CO-heme after mixing. The reactions were carried out in 50 mM Tris, 50 mM NaCl at pH 8.0, 20°C. This buffer was equilibrated with 1 atm CO, and sodium dithionite was added to prevent oxidation. Each time course was fitted to a two exponential expression. The rate constants listed apply to the large, bimolecular phase which accounts for 70-90% of the total absorbance change observed in each experiment.

<table>
<thead>
<tr>
<th>Protein</th>
<th>(k'_H) (M(^{-1}) s(^{-1})) pH 8.0, 20°C</th>
<th>Protein</th>
<th>(k'_H) (M(^{-1}) s(^{-1})) pH 8.0, 20°C</th>
</tr>
</thead>
<tbody>
<tr>
<td>A. native heme proteins</td>
<td></td>
<td>F46V</td>
<td>11 \times 10^7</td>
</tr>
<tr>
<td>Sperm whale Mb</td>
<td>5 \pm 3 \times 10^7</td>
<td>H64L</td>
<td>11 \times 10^7</td>
</tr>
<tr>
<td>Horse Mb</td>
<td>14 \times 10^7</td>
<td>H64Y</td>
<td>6 \times 10^7</td>
</tr>
<tr>
<td>Sheep Mb</td>
<td>14 \times 10^7</td>
<td>G65V</td>
<td>6 \times 10^7</td>
</tr>
<tr>
<td>Soybean LbA</td>
<td>5 \times 10^7</td>
<td>V68A</td>
<td>16 \times 10^7</td>
</tr>
<tr>
<td>Hb α chains</td>
<td>4 \times 10^7</td>
<td>V68F</td>
<td>7 \times 10^7</td>
</tr>
<tr>
<td>Hb β chains</td>
<td>7 \times 10^7</td>
<td>V68W</td>
<td>12 \times 10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V68Q</td>
<td>4 \times 10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V68T</td>
<td>13 \times 10^7</td>
</tr>
<tr>
<td>B. Recombinant Proteins</td>
<td></td>
<td>L89G</td>
<td>12 \times 10^7</td>
</tr>
<tr>
<td>Sperm whale Mb</td>
<td>7 \times 10^7</td>
<td>L89A</td>
<td>17 \times 10^7</td>
</tr>
<tr>
<td>L29F</td>
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<td>L89S</td>
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</tr>
<tr>
<td>R45H</td>
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<td>L89F</td>
<td>4 \times 10^7</td>
</tr>
<tr>
<td>R45K</td>
<td>9 \times 10^7</td>
<td>L89W</td>
<td>12 \times 10^7</td>
</tr>
<tr>
<td>R45T</td>
<td>18 \times 10^7</td>
<td>H97A</td>
<td>10 \times 10^7</td>
</tr>
<tr>
<td>R45E</td>
<td>4 \times 10^7</td>
<td>H97V</td>
<td>5 \times 10^7</td>
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<tr>
<td>R45A</td>
<td>16 \times 10^7</td>
<td>H97D</td>
<td>6 \times 10^7</td>
</tr>
<tr>
<td>R45S</td>
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<td>I107F</td>
<td>6 \times 10^7</td>
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<tr>
<td>R45Y</td>
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<td>Human Mb w.t.</td>
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</tr>
<tr>
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<td>5 \times 10^7</td>
<td>H64V/V68H(^a)</td>
<td>14 \times 10^7</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V68T(^b)</td>
<td>22 \times 10^7</td>
</tr>
</tbody>
</table>

\(^a\)Human myoglobin

\(^b\)Pig myoglobin
None of the proteins listed in Table 5.2 have a bimolecular rate constant of heme binding deviating by more than 4 fold from that of wild type apomyoglobin. To a first approximation, each apoglobin binds heme with a second order rate constant $\approx 10 \times 10^7$ M$^{-1}$ s$^{-1}$. Given the varied effects of these mutants on the structure and function of each myoglobin, and the differences in structure between myoglobins, leghemoglobin, and hemoglobin chains, it seems probable that specific interactions between heme and apoglobin have little effect on the rate determining step of bimolecular association. It is more likely that the initial reaction is driven by hydrophobic interactions between heme and globin. These experiments complement those of Gibson and Antonini (1963), Chu and Bucci (1979), and Rose and Olson (1983) in which modified CO-hemes reacted with globin at nearly the same rate as CO protoheme. All of these studies suggest little or no role for specific heme-globin interactions in determining heme association rate constants. Specificity is manifested primarily by differences in heme dissociation rate constants.

**DISCUSSION**

*The kinetic mechanism of heme binding-* On the whole, our new data support the model proposed by Gibson and Antonini (1960) for describing the reaction of heme with apoglobins. Some modification is required to account for the slow first order phases that are observed even when monomeric CO-heme used:
The super- and subscripts NS refer to non-specific binding. In this scheme, heme is rapidly absorbed into apolar regions of the protein. The primary site of binding in apomyoglobin is the heme pocket and rapid formation of the final holoprotein complex occurs when this site is occupied (i.e. $k_2 \geq 500 \text{ s}^{-1}$). However, some absorption of heme at non-specific sites ($[\text{Globin}^{\text{heme}}]_{\text{NS}}$) appears to occur. In the presence of excess apoglobin, non-specifically bound heme must dissociate before final incorporation into the heme pocket. When excess heme is present, heme binds non-specifically to the holoprotein ($[\text{Mb}^{\text{heme}}]_{\text{NS}}$).

This mechanism is supported by four observations: 1) The rate of the slow phase for CO-heme binding is independent of protein concentration when protein is in excess; 2) reactions with excess CO-heme are complex and the amplitudes of the slow phases increase as CO-heme concentration is raised; 3) the initial rates of dissociation of 5 coordinate CO-heme from bovine serum albumin are similar to the rates of the slow phases observed when CO-heme is mixed with excess apomyoglobin; 4) the estimated equilibrium constants for the initial formation of both the specific ($k_1/k_{-1}$)
and non-specific ($k_1^{NS}/k_{-1}^{NS}$) heme-globin complexes are on the order of that observed for CO-heme binding to albumin, i.e. $10^5$ to $10^7$ M$^{-1}$ (Table 5.1, Equation 5.3).

The final formation of holomyoglobin involves coordination of His$^{93}$ to the iron atom, but this process appears to be governed by larger scale protein conformational transitions (Equation 5.3). In studies with apohemoglobin, Rose and Olson (1983) observed a large dependence of $k_2$ on exogenous glycerol concentrations suggesting a substantial dependence on solvent viscosity. Their observations, combined with the results of Leutzinger and Beychok (1981) for heme-induced refolding of $\alpha$ globin, suggest that $k_2$ is associated with the final folding of the apoglobin around the prosthetic group. This conformational event occurs in a few milliseconds, is slower than the early U to I events in apomyoglobin folding, but is similar to or faster than the I to N events measured by Jennings & Wright (1993).

When globin is in excess and in the absence of non-specific binding, the overall pseudo first order rate constant is given by $k_2[globin]/(K_d+g[l]obin)$ where $K_d = k_{-1}/k'_{1}$. At the low protein concentrations required to measure the heme binding reaction by rapid mixing, the observed rate depends linearly on $[g[lobin]]$, and the apparent second order rate constant is given by $k'H = k_2/K_d$. This observation suggests that $K_d$ is significantly greater than the protein concentration used, i.e. $K_d \geq 1 \times 10^{-5}$ M.

In the case of dicyanohemin binding, the rate limiting step for holoprotein formation is no longer folding around the heme group. Instead,
a CN⁻ ligand must dissociate before the proximal histidine can bind the heme. The rapid fluorescence quenching observed in Fig. 5.1C shows that dicyanohemin is binding rapidly to the heme pocket at a rate similar to that observed for CO-heme. In contrast, the change in Soret absorbance only occurs when the proximal histidine displaces coordinated CN⁻. This conclusion contradicts the mechanism proposed by Gibson and Antonini (1960) in which dicyanohemin was thought to inter-convert to monocyanohemin prior to entering the heme pocket. In that case, one would expect the fluorescence and absorbance data to overlap much like that seen with hemin binding. In the latter case, the limiting step is dissociation of hemin polymers which occurs outside of the heme pocket in the solvent phase.

**Factors governing heme affinity** - The overall association equilibrium constant for hemin binding to apomyoglobin to form metmyoglobin is roughly 10¹⁴ M⁻¹. The equilibrium constant for the initial heme/apoglobin complex is estimated to be 10⁵-10⁷ M⁻¹ based on the affinity of bovine serum albumin for weakly bound CO-heme, and the values of k₁/k₋₁ estimated by Gibson and Antonini (1963) for both apohemoglobin and apomyoglobin, and Rose and Olson (1983) for apohemoglobin. The driving force for this process appears to be non-specific, involving partitioning of the amphipathic prosthetic group into the apolar heme pocket. The equilibrium constant for folding around the prosthetic group and Fe³⁺ coordination, k₂/k₋₂, must be ~ 10⁸ to account for the overall affinity for hemin.
When the proximal histidine is replaced with Gly, the affinity of the resultant apomyoglobin for hemin decreases from $8 \times 10^{13}$ to $6 \times 10^9$ M$^{-1}$, suggesting that direct coordination of His$^{93}$ to Fe$^{3+}$ contributes a factor of $10^4$ to the overall affinity of wild type apomyoglobin for hemin. Barrick (personal communication) estimated a similar value of $\sim 10^5$ M$^{-1}$ for the equilibrium constant describing imidazole binding to hemin embedded non-covalently in H93G myoglobin. Thus, under physiological conditions, the individual free energy contributions to hemin binding are $\sim -30$ to $-40$ kJ/mol for non-specific partitioning into the apolar heme pocket, $\sim -20$ kJ/mol for formation of the proximal histidine-Fe$^{3+}$ bond, and $\sim -20$ kJ/mol for specific interactions with amino acid residues surrounding the porphyrin ring. Formation of the Fe-His$^{93}$ bond is also governed by the protein through restrictions in coordination geometry, the electrostatic environment of the proximal histidine, and interactions with the other axial ligand which is normally a water molecule in ferric myoglobin (Chapter 7). It is striking that the majority of the free energy released by hemin binding is due to simple hydrophobic partitioning. This result accounts for the tendency of heme groups to adsorb non-specifically to almost all proteins.
Chapter 6

Factors Governing Hemin Dissociation from Myoglobin

INTRODUCTION

As shown in the previous chapter, the affinity of myoglobin for hemin is approximately $10^{13}$ M$^{-1}$ at neutral pH and is determined by a complex set of hydrophobic, ionic, and covalent interactions (Antonini and Brunori 1971). The tetrapyrrrole ring makes multiple contacts with residues in the heme pocket, and the propionate carboxyl groups are involved in electrostatic contacts with polar amino acid residues at the surface of the protein. The heme iron atom is coordinated to the four equatorial pyrrole nitrogen atoms and to the protein by a bond to the proximal histidine (His$^{93}$ (F8)). Depending on the oxidation state of the iron, the sixth coordination site can either remain unoccupied or contain a reversibly bound ligand including O$_2$, CO, NO and isocyanides in the ferrous state and CN$^-$, N$_3^-$, F$^-$, SCN$^-$, NO$_2^-$, and NO in the ferric state (Antonini and Brunori 1971; Dickerson and Geis 1983).

The heme pocket is composed of the F helix, to which heme is covalently bound on the proximal side, and the B, C, and E helices which form the top and sides of the porphyrin binding site (Fig. 6.1A). The heme pocket of apomyoglobin is thought to be more loosely packed than in the holoprotein and the C, D, and F helices are thought to be partially unfolded (Hughson et al. 1990). Heme binding causes the pocket to collapse tightly around the porphyrin ring, resulting in a more compact protein with a $\sim$ 20% increase in helicity (Griko et al. 1988). Three sides of the porphyrin are
Figure 6.1: Structure of the sperm whale metmyoglobin heme pocket. A) Sperm whale metmyoglobin. This ribbon diagram shows each of the eight helices (labeled A through H) comprising sperm whale myoglobin. The heme group is shown in red and the proximal His$^{93}$ (F8), to which the heme iron is covalently bound, is shown in pink. B) Heme contacts (the residues in Fig. 6.1) are blue and other protein residues are yellow. The heme group is shown in red. Three sides of the heme are buried in the protein and the fourth, containing the heme propionates, is exposed to solvent.
buried in the protein interior and are protected from solvent and the fourth, containing the heme-6 and heme-7-propionates, interacts with solvent and several surface residues.

Although a majority of the residues lining the heme pocket are apolar, four key polar residues are present: Thr$^{39}$, His$^{64}$, His$^{93}$, and Tyr$^{103}$. His$^{93}$ is required for direct coordination to the heme iron atom. His$^{64}$ is highly conserved for its role in discriminating in favor of O$_2$ and against CO binding and in inhibiting autooxidation (Springer et al. 1994). However, the polarity of this residue causes significant destabilization of apomyoglobin as judged by the enhanced resistance of H64A, L, and F apomyoglobin mutants to unfolding in GdmCl when compared to wild type apoprotein (Chapter 4). Thus apoglobin stability is sacrificed for more efficient O$_2$ binding and resistance to autooxidation (Hargrove et al. 1994 b). Thr$^{39}$ is also a highly conserved residue in both myoglobins and the $\alpha$ and $\beta$ subunits of hemoglobin, although its role in ligand binding and stability is unknown. Tyr$^{103}$ is less conserved and again its function is also unclear.

La Mar and others have shown that heme often initially binds to apomyoglobin and other heme proteins in two orientations (La Mar et al., 1978, 1983, 1984; Aojula et al. 1986; Light et al., 1987) whereas at equilibrium and in nature it normally exists (>90%) in only one of these conformers. The native heme orientation in sperm whale myoglobin is shown in Fig. 6.2. The non-native orientation results from rotation of heme about the vertical axis so that the C and D pyrroles replace the B and A pyrroles and vice versa. The presence of the disoriented conformation
Figure 6.2: Heme-contact residues. Each residue within 4 Å of the heme group in sperm whale metmyoglobin is shown as a circle. Each contact is shown as an arrow to the appropriate porphyrin atom.
can be detected by multiple NMR signals for the heme methyl groups and by circular dichroism in the Soret region, but is not seen in crystal structures which presumably represent the final equilibrium state.

The His$^{93}$-Fe bond is a key force holding heme in myoglobin, and the strength of this bond is determined primarily by the oxidation state of the iron atom. Ferrous iron forms a relatively strong covalent bond, whereas His$^{93}$ coordination with ferric iron is more ionic and weaker. The distal ligand can have a significant effect on the strength of the His$^{93}$-iron bond. Strongly $\pi$-accepting ligands such as CO and CN$^-$ increase the heme affinity of myoglobin by increasing the covalent nature of the iron-His$^{93}$ bond (Bunn and Jandel 1968; Sage et al. 1991). Consequently, ferrous CO and cyanometmyoglobin are much more stable than the corresponding ferrous deoxy or aquometmyoglobins (Antonini and Brunori 1971; Chapter 7).

Hydrophobic interactions between heme and globin have also been implicated in heme binding. In an aqueous medium, heme interacts non-specifically with many hydrophobic surfaces including other proteins, lipid micelles, and lipid bilayers (Bunn and Jandel 1968; Light and Olson 1990). At room temperature the non-specific interaction of heme with protein accounts for roughly half of the free energy released during heme binding to myoglobin (i.e. $K_{\text{nonspecific}} \approx 10^6$ M$^{-1}$ vs. $K_{\text{specific}} = 10^{13}$ M$^{-1}$; Chapter 5). The remaining free energy is released due to formation of the iron-His$^{93}$ bond and specific interactions between the porphyrin ring and amino acid side chains lining the heme pocket. The strength of the latter interactions can be estimated from the affinity constant for H93G
myoglobin which lacks the proximal histidine residue. The affinity of H93G apoglobin for hemin is \( \approx 10^9 \, \text{M}^{-1} \) suggesting that specific porphyrin/amine acid interactions account for an additional factor of \( 10^3 \) and that the iron-His\textsuperscript{93} bond accounts for the remaining factor of \( 10^4 \) for overall hemin binding. This interpretation is, of course, a simplified view. Many of the residues in the heme pocket are polar and can affect heme affinity by influencing the chemistry of the His\textsuperscript{93}-iron bond as well as through specific interactions with the tetrapyrrole ring (Momenteau and Reed, 1994).

Hydrogen bonding and salt bridges between the two heme propionates and residues at the surface of the heme pocket are thought to be some of the specific interactions which enhance heme affinity. In sperm whale myoglobin, Arg\textsuperscript{45} forms a hydrogen bond with the heme-6-propionate, and His\textsuperscript{97} and Ser\textsuperscript{92} hydrogen bond with the heme-7-propionate (Fig. 6.3B; Smerdon et al. 1993). Internally, the side chain of His\textsuperscript{93} hydrogen bonds to the carboxyl group of Leu\textsuperscript{89} and the side chain of Ser\textsuperscript{92}, and the distal histidine (His\textsuperscript{64}(E7)) forms a hydrogen bond to bound oxygen or water (Quillin et al. 1993). All of the latter interactions are thought to enhance heme affinity.

Gibson and Antonini (1963), Rose and Olson (1983), and the results in the previous Chapter have shown that heme affinity is governed primarily by the dissociation rate constant. The overall association rate constant for heme binding to apomyoglobin is \( \sim 1 \times 10^8 \, \text{M}^{-1}\text{s}^{-1} \), regardless of oxidation state and the nature of the globin molecule. Until recently direct measurement of heme dissociation from myoglobin was difficult due
**Figure 6.3:** Key residues in the heme pocket of myoglobin. A) This view of the heme pocket shows the outline of each heme-pocket residue mutated in this study. At least one mutation of the pink residues (His^{93} and Leu^{89}) results in a >100-fold increase in the rate of hemin loss at pH 5.0, 37°C. Mutation of the blue residues can result in >10 fold increases in hemin loss, whereas mutation of those residues in white have little or no effect on hemin affinity. B) This view is the same as that in panel A, but the background amino acids have been removed. Each mutated residue has been labeled. Electrostatic interactions between the heme propionates and protein residues are indicated with a dashed red line.
to its unusually high affinity for the prosthetic group and the small spectral
colors associated with transfer of heme from myoglobin to other heme
binding proteins (e.g. bovine and human serum albumin). Methods of
measuring hemin loss from hemoglobin, which has a lower affinity for
heme than myoglobin, have been developed and have resulted in some
understanding of the interaction of heme with globin (Banerjee 1962 a,b,c;
Bunn and Jandel 1968; Benesch and Kwong 1990). Bunn and Jandel (1968)
showed that α subunits lose hemin faster than β subunits in intact
hemoglobin and first measured the effects of a mutation on the affinity of
hemoglobin for heme. They showed that Hb M (Boston), in which the α
His(E7) is replaced by Tyr, loses heme much more rapidly than hemoglobin
A.

In Chapter 2 a relatively fast and convenient assay was developed to
measure hemin dissociation from almost any heme protein, including
myoglobin. This assay has been used in combination with site-directed
mutagenesis to test some of the factors presumed to regulate heme affinity.
The results of Bunn and Jandel for hemin loss from Hb (Boston) were
simulated by examining hemin loss from H64Y myoglobin. This mutant
loses heme 16 times faster than wild type myoglobin at pH 5.0, 37°C. The
phenoxide side chain of Tyr(E7) coordinates the ligand binding site of the
heme and destabilizes the His93- iron bond. It was shown in the same
Chapter that H64A and V68A myoglobin mutants lose heme more rapidly
than wild type metmyoglobin. Furthermore, increased rates of hemin loss
from Ser(F7) and Arg(45)(CD3) mutants suggested that electrostatic
interactions between the porphyrin propionates and globin are important for
maintaining a high affinity for hemin (Smerdon et al. 1993; Hargrove et al. 1994 a).

In Chapter 4, hemin loss rates ranging from $< 0.1$ h$^{-1}$ (V68T) to 1.0 h$^{-1}$ (wild type) to 54 h$^{-1}$ (F43V) were measured for 21 myoglobin mutants at five different positions. Some of these mutations were predicted to cause increased rates of hemin loss based on pathologies associated with abnormal hemoglobins (Antonini and Brunori 1971; Dickerson and Geis 1981). However, to date there has been no comprehensive description of the factors regulating heme affinity nor of the roles of all the amino acid residues in the active site. In this Chapter, we have systematically mutated most of the amino acids within 4 Å of bound heme in sperm whale myoglobin and looked at the effects on hemin dissociation. The rates of hemin loss from 104 different myoglobins have been determined often at both pH 5 and 7. The results have been used to evaluate the chemical mechanisms governing overall heme affinity and to suggest possible mutations for enhancing heme retention in recombinant myoglobins and hemoglobins.

METHODS

Preparation of proteins - Native sperm whale myoglobin was obtained by special permit from Sigma. Initial sperm whale myoglobin single mutants at positions 68 and 64 were constructed using cassette mutagenesis by Barry A. Springer and Karen D. Egeberg at the University of Illinois (H64L, H64F, H64Q, V68A, and V68F; Rohlfs et al., 1990; Egeberg et al., 1990). The remaining mutants at these positions and those at
residues 65, 66, and 67 were prepared at Rice University. Sperm whale myoglobin mutants at positions 22, 29, 43, 45, 46, 89, 71, 97, 99, 107, and 111 were constructed using oligonucleotide directed mutagenesis with a pEMBL19 vector containing the gene for wild type myoglobin (see Carver et al., 1992 for a description of Leu29 mutants). All recombinant myoglobins were expressed and purified at Rice University following the procedures described by Springer and Sligar (1987) and Carver et al., (1992). Sperm whale H93G was constructed and purified by Doug Barrick as described in Barrick (1994). Pig and human myoglobin mutants were constructed and expressed as fusion proteins following the procedures described by Varadarajan et al., (1985), Smerdon et al., (1991), and Ikeda-Saito et al., (1991). Apomyoglobins were prepared using the methylethyl ketone method described by Ascoli et al., (1981) and Hargrove et al. (1994a).

Reconstitution of apomyoglobin with hemin (Sigma) was carried out at pH 7 in 10 mM potassium phosphate at 0°C. The concentration of free hemin was measured by diluting a sample into a cuvette containing buffer equilibrated in one atmosphere carbon monoxide and excess sodium dithionite. In contrast to ferric heme, CO heme has a sharp Soret peak at 407 nm which is convenient for concentration determination (ε407nm=147mM⁻¹cm⁻¹ (Light, 1987)). Excess apomyoglobin (≈ 0.5mM) was mixed with hemin (≈ 0.45mM) to yield reconstituted holomyoglobin. The extent of apomyoglobin reconstitution was estimated by the presence of the ferric myoglobin peak at 410 nm and absence of the free ferric heme peak at ≈390 nm (Fig. 6.6A). Reconstitution of myoglobin with hemin
dimethylester was performed as described by La Mar et al., (1986). Hemin
dimethylester was obtained from Porphyrin Products.

**Measurement of hemin loss rates**—Hemin dissociation was measured
by following the absorbance changes (usually at 409 nm) associated with
transfer of hemin from holoprotein to excess H64Y/V68F apomyoglobin as
described in Chapter 2. Resultant time courses were fitted to a single
exponential expression to obtain the first order dissociation rate constant for
hemin loss, $k_{-H}$. These experiments were carried out with 0.15 M
buffer/0.45 M sucrose, at 37°C in either potassium phosphate at pH 7.0 or
sodium acetate at pH 5.0. Most hemin loss reactions were measured in a
Shimadzu 6100 spectrophotometer; hemin loss from H93G, and L89A, G,
and S myoglobins was measured using a Gibson-Dionex stopped flow
apparatus. Reconstituted wild type sperm whale myoglobin was prepared,
stored on ice, and used within 30 minutes of preparation. Under these
conditions the hemin does not have time to re-orient prior to measurement.
Myoglobin reconstituted with dimethylesterhemin was allowed time for re-
orientation prior to measurement of hemin loss.

In general, hemin dissociation data were collected for 200 minutes at
pH 5, and for over 1,000 minutes at pH 7. It was not always possible to
collect endpoints for experiments at pH 7. In these cases, rates were
estimated from initial velocities, and the expected absorbance change of the
reaction. Time courses for hemin loss from several myoglobin mutants
showed slow absorbance increases due to denaturation at pH 5.0. These
rates were fitted to a two exponential expression and the larger, fast rate
was attributed to hemin loss. Two rates of hemin loss from G65I at pH 7.0
were reported because time courses were clearly biphasic and precipitation was not observed.

RESULTS AND DISCUSSION

_Hemin loss from myoglobin_- Table 6.1 lists rates of hemin loss from native sperm whale, horse, tuna, and cow myoglobins, three different recombinant wild type sperm whale myoglobins and recombinant human and pig myoglobins, 73 sperm whale myoglobin mutants at 21 different sites, 13 pig myoglobin mutants at 5 different sites, and 6 different human myoglobin mutants. Amino acid positions where a mutation results in a $\geq 10$ fold increase in the rate of hemin loss are shown in bold and those which clearly decrease hemin loss are underlined. Rates of hemin loss from all myoglobins were measured at pH 5.0, and rates were measured from many myoglobins at pH 7.0. Rates of hemin loss at pH 5.0 varied from $700 \text{ hr}^{-1}(L89G)$ to $<0.1 \text{ hr}^{-1} (V68T)$.

Native and recombinant sperm whale myoglobins lose hemin at rates of 1.0 and 0.01 hr$^{-1}$ at pH 5.0 and 7.0, respectively. Recombinant pig myoglobin loses hemin at the same rate, and myoglobins from tuna, human, and horse lose hemin at only slightly greater rates. Since the amino acid sequences, structures, and functions of these myoglobins are very similar, it is reasonable to expect similar resistances to hemin loss. In addition, homologous mutations in sperm whale and pig myoglobins have roughly the same effects (Table 6.1).

Roughly two thirds of the mutants listed in Table 6.1 were originally created for studies of ligand binding (e.g. His$^{64}$, Val$^{68}$, Leu$^{29}$ mutants);
Table 6.1. Survey of the rates of hemin loss from myoglobin mutants. The hemin loss properties of all the proteins were surveyed in 0.15M acetate, 0.45M sucrose, pH 5, 37°C. Selected mutants were examined in 0.15 M phosphate, 0.45 M sucrose, pH 7.0, 37°C. Mb mutants that cause > 10-fold changes in k-H are marked in bold. Old wild type contains Glu122, new wild type (corrected) contains Asn122 and Real wild type is new wild type without the n-formyl methionine. Those mutants which decrease k-H are underlined. Reconstituted myoglobin results from the addition of hemin to apomyoglobin and DME-hemin is myoglobin reconstituted with hemindimethyl ester.
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the remainder were made to examine interactions between residues in the heme pocket and the prosthetic group (e.g. His\textsuperscript{97}, Ile\textsuperscript{99}, Leu\textsuperscript{89} mutants). A schematic drawing of contacts in the heme pocket of sperm whale metmyoglobin is shown in Fig. 6.2. Residues which make contact within 4 Å of the heme are connected to heme atom(s) they contact. The view is down onto the ligand binding site with His\textsuperscript{93} below the page and not shown. Fig. 6.1B shows these residues in relation to the entire protein and demonstrates that the heme pocket is enclosed on all sides but the one in which the heme-propionates are in contact with surface residues and solvent.

Fig. 6.3A presents expanded views of the heme pocket looking in from solvent. The side chain of each heme-pocket residue in Table 6.1 is shown as well as the space filling atoms of the three heme contact residues which were not mutated in this study (Lys\textsuperscript{42}, Leu\textsuperscript{104}, Phe\textsuperscript{138}). Fig. 6.3B presents the same view in which the contact side chains have been labeled appropriately and all other protein residues have been deleted. At least one mutation of the residues shown in blue results in a ≥ 10 fold increase in the rate of hemin loss, and at least one mutation of the red residues increases hemin loss ≥ 100 fold. Mutations of the white residues have smaller effects on hemin loss.

**Mutations causing a > 100 fold increases in $k_{-H^-}$**: His\textsuperscript{93} (F8): It is no surprise that removal of His\textsuperscript{93} results in a myoglobin with a very poor affinity for heme and a high rate of hemin dissociation. Early attempts at making proximal histidine mutants were unsuccessful due to lack of expression as a result of poor heme affinity. Barrick (1994) was able to
express and purify H93G in the presence of imidazole. The heme-imidazole complex binds in the heme pocket and results in a myoglobin with a structure and absorption spectra very much like that of native and wild type protein. However, the lack of a covalent bond to the globin increases the rate of hemin loss nearly 1000 fold with respect to that of wild type metmyoglobin, regardless of pH.

It is unclear whether hemin dissociation from H93G myoglobin occurs by direct dissociation of the imidazole-heme complex from the protein, or requires two steps: first disruption of the heme-imidazole bond and then the loss of both free hemin and imidazole. The rate of coordinated imidazole dissociation from metmyoglobin is $\approx 1 \times 10^4 \text{ h}^{-1}$ (Antonini and Brunori 1971) which is only 10-fold greater than the rate of hemin loss from H93G myoglobin. In addition, the rate of hemin loss from H93G myoglobin becomes slower at high concentrations of exogenous imidazole at both pH 5.0 and pH 7.0. This result can be explained by increased binding of the proximal imidazole to the bound heme group which would lower the observed value of $k_{\text{H}}$ if the imidazole-iron bond has to break prior to hemin dissociation.

The structural differences between the His$^{93}$-heme geometry in wild type metmyoglobin and the proximal imidazole-iron coordination in the H93G mutant have been reported by Barrick (1994). Because it is not constrained by linkage to the globin, the proximal imidazole in the H93G mutant adopts a more favored bonding conformation with the heme iron, and the aromatic ring forms an additional hydrogen bond with the heme-7-propionate. In contrast, His$^{93}$(F8) is held in place by direct covalent
linkage to the F helix and is forced into a fixed orientation with respect to the heme iron. Thus, it is possible that the iron-imidazole bond is actually stronger in the H93G mutant even though the rate of hemin loss is $10^6$ times greater due to the lack of covalent attachment of the base to the polypeptide backbone.

**Leu$^\text{89(F4)}$:** The abnormally high rates of hemin loss from L89G and L89S mutants can be explained by effects on the proximal imidazole-iron bond and solvation of this portion of the heme pocket. Leu$^\text{89(F4)}$ sits at the base of the proximal heme pocket next to an internal cavity referred to as the xenon binding site, and contributes to the non-polar environment around His$^\text{93}$. Hemoglobin (Boras), in which $\beta$ F4 Leu is changed to Arg results in a pathology which is associated with hemin loss and formation of semihemoglobins. This effect has been attributed to solvation of the proximal pocket (Antonini and Brunori 1971; Dickerson and Geis 1983). It is likely that L89G and L89S have the same effect on the proximal pocket in myoglobin and that the resulting polarity around His$^\text{93}$ destabilizes the bond with the heme-iron presumably by solvating the imidazole group and facilitating its protonation. This interpretation is supported by the smaller effect of the L89A mutation which produces only a 50-fold increase in $k_H$ at pH 5 as compared to the 500-fold effect observed for the more polar L89S substitution. In agreement with this idea, the L89F mutation has little effect on hemin dissociation because the size and polarity of the phenyl side chain are similar to those of the naturally occurring isobutyl group. In contrast, the large indole side chain of the L89W mutant appears to
sterically hinder the porphyrin resulting in moderate increases in the rate of hemin loss at both pH 5 and 7.

**Mutations resulting in a > 10 fold increases in \(k_{H^-}\)**  
\(\text{Phe}^{43}(\text{CD1})\):

Several hemoglobin pathologies result from mutation of the conserved Phe(CD1) residue: Torino: \(\alpha\) Val (CD1), Louisville: \(\beta\) Leu (CD1), and Hammersmith: \(\beta\) Ser(CD1). All of these mutations are associated with heme dissociation and unstable hemoglobins. The most severe, Hammersmith, results in the presence of extra waters in the heme pocket and causes disruption of other regions of the protein. Mutations of the corresponding residue in myoglobin produce similar effects. The respective rates of hemin loss from the F43V and F43I mutants are \(\sim 50\) and \(\sim 40\) fold greater than wild type metmyoglobin at pH 5.0, and the effects of these substitutions are even greater at pH 7.0. The decrease in hemin affinity caused by the F43V mutation is enhanced by apoglobin unfolding. The net result is a markedly destabilized holoprotein (Hargrove et al. 1994 b). Although the F43V mutation does not result in additional water in the heme pocket it does disrupt the structure of the CD corner and associated regions of the protein (Whitaker 1995).

It is interesting that placing leucine at position 43 does not increase hemin loss as much as isoleucine. This is probably because the two \(\delta\) methyl groups of Leu are better able to interact hydrophobically with the heme group than the single \(\delta\) carbon of Ile. The \(\gamma\) methyl group of isoleucine is probably too far away to interact favorably with the heme group. Tryptophan at position 43 is too big to fit well in the distal heme
pocket in the presence of the prosthetic group and causes unfavorable steric contacts with the porphyrin ring and other surrounding amino acid residues (Whitaker 1995).

**His<sup>64</sup>(E7):** The distal histidine regulates ligand binding in myoglobin and hemoglobin by stabilizing bound oxygen through a hydrogen bond which enhances O<sub>2</sub> affinity and prevents autooxidation of the heme iron (Springer et al., 1994; Brantley et al., 1993). In metmyoglobin, His<sup>64</sup> also forms a hydrogen bond to the coordinated water and provides important steric contacts on the distal side of the heme (Fig. 6.1; Quillin et al., 1993).

We initially thought that water coordination and H-bonding to it by His<sup>64</sup> played the most crucial role in stabilizing bound hemin. However, H64G metmyoglobin loses heme 40 and 75 times faster than wild type protein at pH 5 and 7, respectively, even though coordinated H<sub>2</sub>O is present. Increasing the size of residue 64 causes significant decreases in k-H at both pH values. The H64F mutant has only a four-fold higher k-H value at pH 5 and shows the same rate of hemin loss at pH 7 as wild type metmyoglobin, even though the hemin group is pentacoordinate. This result suggests that hydrophobic contacts and exclusion of solvent from the distal pocket are as important in retaining heme as direct hydrogen bonding to coordinated water. This conclusion is supported by the results for the H64Q mutation. The O<sub>E</sub> atom of Gln<sup>64</sup> appears to accept a hydrogen bond from coordinated water in the aquomet form of the mutant (Quillin et al. 1993). However, the rate of hemin loss from H64Q metmyoglobin is 4 to 12-fold greater than that for wild-type myoglobin and only 2 to 3-fold lower than that of the H64L mutant, which is pentacoordinate. As is the case at
the 89 and 43 positions, tryptophan substitution at position 64 causes an increase in the rate of hemin loss presumably due to unfavorable steric contacts.

The H64Y mutation also enhances hemin loss despite direct coordination between the phenoxide side chain and the iron atom (Fig. 6.4). The inability of the Tyr64 to accept π electron density from the iron weakens the His93-iron bond through a trans effect (Traylor and Sharma, 1992). The phenoxide side chain of this residue actually destabilizes the heme-globin linkage, and a longer His93-iron bond is found in the structure of Tyr64 metmyoglobin (Hargrove et al. 1994a, Maurus et al., 1994).

Val68(E11): After His64, Val68 is perhaps the most important residue regulating ligand binding in myoglobin. The Val side chain at this position creates the proper distal pocket volume and polarity for ligand binding to deoxymyoglobin and for preventing autooxidation (Quillin et al., 1995). Accordingly, there are several different pathologies associated with the stability and ligand binding properties of Val68(E11) mutants in hemoglobin (Dickerson and Geis 1983).

As shown in Fig. 6.1, the Val68→Ala substitution causes 6 and 10-fold increases in the rate of hemin loss at pH 5.0 and 7.0, respectively. Curiously, the additional contacts with the heme group produced by the V68F mutation do not decrease the rate of hemin loss at either pH, suggesting that some of the new contacts with the benzyl side chain are unfavorable. Similarly, the V68W replacement has no favorable effects on hemin loss. However, the Trp68 and Phe68 side chains do appear to be
Figure 6.4: Effects of H64Y and F46V mutations on heme affinity. 
A) The distal His$^{64}(E7)$ in wild type myoglobin stabilizes coordinated water through a hydrogen bond. B) The Tyr$^{64}$ side chain displaces coordinated water but is a much poorer $\pi$ accepting ligand. The latter effect results in elongation and destabilization of the His$^{93}$-iron bond. C) In F46V metmyoglobin, the distal His$^{64}(E7)$ swings up into the cavity created by the smaller Val side chain. Consequently, the stabilizing hydrogen bond to the coordinated water provided by His$^{64}$ is no longer present, the distal surface of the heme is exposed to solvent, and heme affinity is lowered.
accommodated by the empty space located on the distal side of the
porphyrin ring directly above the A and B pyrroles and do not enhance
hemin loss (Figs. 6.1-6.3).

Human V68G metmyoglobin loses heme at an abnormally high rate of
41 h\(^{-1}\). This large increase in the rate of hemin loss in going from Ala to
Gly might be due to increased solvation of the distal pocket due to the
formation of a direct channel from the distal pocket out into the solvent
phase. This hypothesis is supported by the observation that the rate of
hemin loss from V68N is significantly faster than that of wild type
myoglobin. Asn\(^{68}\) increases the rate of hemin loss almost as much as
Ala\(^{68}\) presumably by increasing the polarity of the distal pocket. This result
suggests that Val\(^{68}\) helps prevent hemin loss by preserving the
hydrophobicity of the heme pocket. A similar effect is seen in hemoglobin
Sydney in which \(\beta\) Val (E11) is changed to Ala. In this case, destabilization
also appears to be due to solvent entering the heme pocket (Dickerson and
Geis, 1983).

Surprisingly, V68S does not increase hemin loss and V68T actually
slows hemin loss dramatically. These results can only be explained by
additional hydrogen bonding to coordinated water (Fig. 6.5). Normally, the
coordinated water is only hydrogen bonded to His\(^{64}\). However, Smerdon et
al. (1991) have shown that Thr\(^{68}\) forms another hydrogen bond to
covalently bound water resulting in even greater stabilization than that seen
in wild type metmyoglobin. Ironically, the polarity introduced by same
mutation results in a much less stable apomyoglobin (Chapter 4, Table
**Figure 6.5:** Effects of Thr$^{68}$ and His$^{68}$ substitutions on heme affinity. A) Coordinated water in wild type metmyoglobin is stabilized by a hydrogen bond from His$^{64}$. B) In V68T metmyoglobin, the Thr$^{68}$ side chain forms a second hydrogen bond with the coordinated water. This bond holds the water in place more tightly than in wild type myoglobin, and heme affinity is increased. C) The His$^{68}$ side chain in H64V/V68H pig myoglobin bonds directly to the sixth coordination position of the heme iron. In this case, the imidazole nitrogen is a better π accepting ligand than water and the His$^{98}$(F8)-iron bond is stabilized.
4.2). However, once heme is bound, the resulting V68T metmyoglobin is much more stable to denaturation due to its increased affinity for hemin.

The same effects on hemin affinity and globin stability are produced by mutants introducing His at position 68, but the effects are more extreme than in the threonine substitution. His$^{68}$ displaces the covalently bound water and coordinates the heme-iron directly (Fig. 6.5). Because His$^{68}$ is a better \( \pi \) accepting ligand and is held in place more rigidly than water, V68H mutants have a higher heme affinity and lower rate of hemin loss than V68T myoglobin. However, the His$^{68}$ substitution causes marked unfolding of the corresponding apoglobin and inhibits \( \text{O}_2 \), CO, and NO binding due to coordination of the His$^{68}$ side chain (Dou et al. 1995; Mauk et al. 1995).

**His$^{97}$ (FG3):** His$^{97}$ appears to be a barrier to solvent entering the proximal portion of the heme pocket and forms a hydrogen bond with the heme-7-propionate. The H97A mutation results in a 40-fold increase in hemin loss and the H97E replacement increases the rate of hemin loss even further, presumably by repulsing the heme-7-propionate.

The rate of hemin loss from H97F is similar to that of wild type myoglobin at pH 5; but at pH 7, H97F myoglobin loses hemin 13 times faster than the wild type protein. This result suggests that the electrostatic interaction between His$^{97}$ and the heme-7-propionate is important only at the higher pH. At pH 5, it appears that only the hydrophobicity of residue 97 and its role in preventing hydration of the proximal heme pocket is important in stabilizing bound heme. It is possible that protonation of propionate carboxyl groups at pH 5 decreases the electrostatic interaction between this group and the His$^{97}$ side chain, accounting for some of the pH
dependence observed for hemin loss from wild type metmyoglobin. Thus at pH 5, Phe$^{97}$ can substitute for His with little effect on hemin affinity.

**Ile$^{99}$ (FG5):** Ile$^{99}$ is another heme-contact residue which fills the space on the proximal side of the heme group with an apolar side chain. The FG5 residue is a valine in human α and β chains, and several unstable hemoglobins result from mutations at this position: α Met (FG5), Koln: α Gly (FG5), Nottingham: α Ala (FG5), Djelfa. As shown in Table 6.1, mutation of this residue to Ser in myoglobin results in large increases in hemin loss whereas more moderate increases are observed for the I99A replacement. This pattern again suggests that the heme pocket needs to be kept anhydrous with apolar amino acid side chains.

**Thr$^{39}$ (C4):** This residue is highly conserved in mammalian and bird hemoglobin and myoglobins but its specific function is not clear. Thr$^{39}$ is in the back corner of the heme pocket and makes only one contact with the porphyrin ring. The polarity of this side chain seems necessary as indicated by the 6-fold increase in $k_{-H}$ caused by the T39V mutation at both pH 5 and 7. The dramatic 50-fold increase in hemin loss caused by the T39Y mutation is almost certainly due to the large size difference between the Thr and Tyr side chains which is likely to disrupt the relatively tight packing in this region of myoglobin structure.

**Tyr$^{103}$ (G4):** The Tyr$^{103}$ side chain is coplanar with the porphyrin ring and is located in the back of the heme pocket with the aromatic hydroxyl pointing into the interior of the protein. The residue at this position is polar in most myoglobins and hemoglobin. When Tyr$^{103}$ is changed to Leu or Ala, the resulting myoglobins lose hemin at rates of 41...
and 21 h\(^{-1}\), respectively, at pH 5.0. However, the Y103F mutation has little effect on hemin dissociation at pH 5 or 7. Interestingly, the rate of hemin loss from Y103A at pH 7.0 is only 3 times greater than that of wild type protein, but \(k_{-H}\) for Y103L myoglobin at pH 7 is 16-fold faster.

**Electrostatic interactions with the heme propionates** - The residue at position 45 (CD3) in most myoglobins is Lys or Arg. The positively charged side chain of this residue interacts with solvent molecules and hydrogen bonds to the heme-6-propionate. Lys and Arg appear to function equally well at retaining hemin since there is little effect produced by the R45K mutation in sperm whale myoglobin. Other mutations at this position in sperm whale and pig myoglobins have relatively modest effects on heme affinity. The R45E (sperm whale) and K45E (pig) mutations cause 5.5 and 3-fold increases in \(k_{-H}\) compared to the corresponding wild type values despite the fact that electrostatic repulsion should occur between the acid side chain and the heme propionate. The R45Y mutation causes the largest effect, a 7 to 8-fold increase in \(k_{-H}\) at both pH values. The R45H and R45S mutations were made to simulate the His(CD3) and Ser(CD3) residues found in \(\alpha\) and \(\beta\) chains of hemoglobin. These mutations cause only 3-fold increases in \(k_{-H}\) relative to wild type myoglobins. Thus, the electrostatic interaction between Arg/Lys 45(CD3) and the heme-6-propionate makes only a modest favorable contribution to heme affinity.

When Ala or Leu is substituted for Ser\(^{92}\)(F7), the rate of hemin loss increases from 1.0 to \(\approx 3.0 \, \text{h}^{-1}\) at pH 5.0, 37°C. Somewhat larger effects are observed at lower temperatures (Smerdon *et al.* 1993). These results
are similar to those observed for Arg$^{45}$/Lys$^{45}$ mutations. The hydrogen bond between Ser$^{92}$(F7) and the side chain of His$^{93}$ does help to stabilize bound heme perhaps by influencing the strength of the His$^{93}$-iron bond, but the effect is relatively small compared to the interactions involving residues 43, 64, 89, 97, and 103.

In most myoglobin crystal structures, there is a lattice of fixed solvent water molecules interconnecting protein residues at the CD corner, E-helix, F-helix, and FG corner. It is possible that deletion of a single electrostatic interaction between the porphyrin and an amino acid side chain will have little effect on hemin affinity because of the multiplicity of other interactions at the solvent interface. This possibility was explored by measuring rates of hemin loss from myoglobin reconstituted with hemin dimethylester. The presence of the methyl esters in place of the acidic propionates should decrease electrostatic interactions between the prosthetic group and nearby polar residues. A time course for hemin loss from dimethyl ester hemin myoglobin (DMEH) is shown in Fig. 6.6B. Surprisingly, myoglobin appears to have equal affinities for hemin and hemin dimethyl ester at both pH 5.0 and 7.0 as judged by the identical values for k$_{-}$H in Table 6.1.

As in the case of the H64F and H97F mutations, the loss of favorable electrostatic interactions appear to be compensated by more favorable apolar partitioning into the protein matrix. Thus, ionized propionates are not required for tight binding. This idea is further supported by the ease with which octaethyl-Fe-porphyrin is incorporated into myoglobin (Neya et al.)
**Figure 6.6:** The kinetics of hemin loss from reconstituted myoglobins. A) The spectra of apomyoglobin (solid line labeled apoMb) before and after (dashed line) addition of hemin are compared to the spectrum of wild type metmyoglobin (solid line). Reconstitution was clearly complete, and no free hemin was present. Similar spectra were obtained for the reconstitution of apomyoglobin with dimethylester hemin. B) Time courses for hemin dissociation from wild type and the hemin dimethylester complex with myoglobin (DMEH) are shown with their fitted curves. The rate constant for hemin dissociation from DMEH is 1.5 h\(^{-1}\) which is very similar to that for wild type metmyoglobin. C) The time courses for hemin loss from wild type and freshly reconstituted wild type (Rcon) myoglobin are shown along with fitted curves. The rate constant for hemin loss from wild type myoglobin is 1 h\(^{-1}\) and that from reconstituted myoglobin is 1.1 h\(^{-1}\).
1991). This complex, which is unable to form any electrostatic interactions between the porphyrin and protein, forms a stable complex because of the strong hydrophobic forces partitioning heme into the apolar heme pocket of the protein.

Non-contact mutations resulting in > 10 fold increases in $k_{H^{-}}$-Leu$^{29}$(B10): The effects of Leu$^{29}$ on ligand binding have been studied in much detail because the isobutyl side chain is located just above the bound ligand on the distal side of the heme pocket. Its physiological function is to slow autooxidation while still allowing high rates of reversible oxygen binding (Carver et al., 1992). Decreasing the size of this residue to valine produces 10-fold increases in $k_{H^{-}}$ at both pH 5 and 7. Increasing the size to Phe has little or no effect, whereas adding a polar side chain (V68N) causes an 8 to 10-fold increase in the rate of hemin loss. Thus, increasing the size of the cavity in the distal pocket increases the rate of hemin loss, presumably by increasing exposure of the heme to solvent. A similar effect can be produced by replacing Leu$^{29}$ with a polar residue, which also tends to hydrate the protein interior. In agreement with this interpretation, several natural hemoglobin mutants at B10 also result in unstable proteins because they introduce polar residues in the heme pocket or open the heme pocket to solvation (St. Louis: $\beta$ Gln(B10), Genova: $\beta$ Pro(B10)).

Phe$^{46}$(CD4): The phenyl side chain of Phe$^{46}$ does not contact the heme group directly, but does limit movement of His$^{64}$ (Lai et al., 1995). The rate of hemin loss increases significantly as the size of residue 46 is decreased, from 2.0 h$^{-1}$ for Leu$^{46}$ to 10-20 h$^{-1}$ for Val$^{46}$ and Ala$^{46}$ at pH
5 (Table 6.1). At pH 7, $k_{H}$ for Ala$^{46}$ myoglobin is 60 times greater than for wild type myoglobin. These results are readily interpreted on the basis of the structures of Leu$^{46}$ and Val$^{46}$ aquometmyoglobins (Lai et al. 1995).

In wild type metmyoglobin the His$^{64}$ side chain is well defined in a "down" position with its $\text{N}_{\varepsilon}$ forming a hydrogen bond to coordinated water. Lai et al. (1995) have shown the His$^{64}$ is highly disordered in the F46V metmyoglobin structure. In this mutant, the imidazole side chain of His$^{64}$ is free to rotate and move between the "down" and "up" conformations. In the latter "up" position, the imidazole ring occupies the space which is normally filled by the Phe$^{46}$ side chain (Fig. 6.4; Romo et al., 1995; Lai et al., 1995). An intermediate result is observed for Ile$^{46}$ metmyoglobin. In this case His$^{64}$ is almost completely in the down conformation but the electron density associated with the imidazole ring is almost spherical, indicating relatively free rotation about the $\text{C}_\beta$-$\text{C}_\gamma$ bond.

When the distal histidine is in the "up" position, a direct channel to the solvent phase is opened and lined with water molecules. This opening is seen clearly in the structure of deoxy F46V myoglobin and is probably a major cause of the 20 to 30-fold higher rate of hemin loss from this mutant. In addition, hydrogen bonding between His$^{64}$ and coordinated water is also disrupted. The larger Ile$^{46}$ and Leu$^{46}$ residues offer more restriction to His$^{64}$ movement and less solvation of the heme pocket.

**Gly$^{65}$ (E8):** This residue is located far from the heme on a section of E helix that points away from the distal pocket. The lack of a side chain at the 65 position allows the B-helix to fit into a notch located roughly in the middle of the E-helix. The net result is a tightly packed heme pocket in
which residues Leu\(^{29}\)(B10) and Leu\(^{32}\)(B13) form part of the surface of the distal cavity that surrounds the ligand binding site. Replacement of Gly\(^{65}\) with Val or Ile will remove the notch, presumably causing the B-helix to move up and away from the heme group. This would expand the size of the distal cavity and, at the least, cause disorder in this region. This disruption of helix packing probably accounts for the dramatic 20 to 70-fold increases in \(k_{H}\) produced by the G65V and G65I mutations.

In contrast, the rate of hemin loss from the G65A mutant is nearly as slow as that of wild type myoglobin, supporting the view that the size of residue 65 is the key factor governing the increase in hemin dissociation. Surprisingly, the G65T mutation has very little effect on hemin loss even though the side chain of Thr is isosteric with that of Val. It is possible that the Thr \(\beta\)-hydroxyl forms a stabilizing electrostatic interaction with the B helix or rotates toward the solvent phase to minimize unfavorable steric interactions with the B helix. Finally, non-conservative mutations at the next position along the E-helix, V66K or V66G, have little effect on hemin loss, suggesting that the effects observed for the Gly\(^{65}\) mutants are highly specific to the E8 position.

**Roles of residues located in the back of the heme pocket**—One of the most surprising results in Table 6.1 is the lack of effect of amino acid substitutions at Ile\(^{107}\)(G8). Neither increasing or decreasing the size of this residue produces much effect on hemin dissociation. Similarly, I111V and I111F mutations produce only small changes in \(k_{H}\). Thus, the interior portion of the heme pocket appears to be more plastic. The simplest interpretation is that these residues are not part of a barrier to solvent
penetration since they are located deep within the protein matrix. However, crystal structures are needed for a complete description.

**Hemin loss from reconstituted myoglobin-** Time courses showing hemin dissociation from wild type and reconstituted wild type sperm whale myoglobin are shown in Fig. 6.6. The rate of hemin loss from both proteins is \( \approx 1 \, \text{h}^{-1} \). The reconstituted protein does show more heterogeneity than the wild type sample due to the presence of a small slow phase. However, there is no evidence for a faster phase of hemin loss from the reconstituted protein. These results are puzzling since La Mar and coworkers have suggested that when apomyoglobin is reconstituted with hemin, two equal populations of orientational conformers are observed depending on the arrangements of the interior heme vinyl and methyl groups. This result indicates equal association rate constants for the binding of these two conformations. However, at equilibrium the distribution of conformers is 95:5 in favor of the orientation found in the crystal structure shown in Figs. 6.1-6.3. This re-orientation implies a 20-fold difference in affinity for the two conformers and suggest that the rate of hemin dissociation from the abnormal orientation should be 20-fold greater than that from the native conformer. Thus, freshly reconstituted myoglobin was expected to show two rates of hemin loss, 20 and 1 \( \text{h}^{-1} \) at pH 5.0. This result is clearly not observed and suggests that further investigations of heme orientational disorder are needed.

**Effects pH on hemin dissociation-** The large pH dependence of hemin loss from myoglobin is partly due to protonation of His\(^{93} \) and disruption of the His\(^{93} \)-Fe bond under acidic conditions. At neutral and
high pH, where the extent of His\textsuperscript{93} protonation is effectively zero, the rate of hemin loss from metmyoglobin is very slow. It could be argued that the effects of mutagenesis on rates of hemin loss should be less at higher pH where heme affinity may be more influenced by the strength of the His\textsuperscript{93} iron than by non-covalent interactions. Although the data are more limited due to the difficulty of measuring such slow reactions, the effects of mutagenesis on hemin loss are roughly the same at pH 7 and the ratio of the rate constant at pH 5 to those at pH 7 is \(~\)100 for most of the mutants listed in Table 6.1.

The exceptions appear to involve those proteins with abnormally fast rates of hemin loss (i.e. F43V, F43I, F46A, L89G, L89A, H93G, and H97A myoglobins). In these cases, the ratio of the rate constants at pH 5 and 7 are 5 to 20 instead of \(\geq 100\). This decrease in pH dependence is most readily understood for the H93G mutant since the proximal base is no longer attached covalently to the protein, and its state of ionization is less important. In the case of F46V, the distal histidine is already in the "up" position, exposing the heme pocket to solvent. In wild type metmyoglobin, the same upward movement of the distal histidine is observed at low pH due to protonation of His\textsuperscript{64} (Yang et al. 1996). This conformational change clearly accounts for part of the pH dependence observed for native myoglobin and is missing in the F46V mutant. The cause of the decrease in pH dependencies of the other mutants is less clear and requires further study.
CONCLUSIONS

The high affinity of myoglobin for hemin results from a combination of factors which include: (1) extrusion of the amphipathic porphyrin from the solvent into the hydrophobic heme pocket, (2) formation of the His$^{93}$-iron bond and changes in its strength due to oxidation and ligand binding, and (3) electrostatic interactions with coordinated water and the heme propionates. These factors are clearly interrelated. For example, the apolar character of the heme pocket has a large effect on the strength of the Fe$^{3+}$-His$^{93}$ bond. Electrostatic interactions between the protein, heme, and bound ligand also affect hemin affinity.

The strength of the His$^{93}$-iron bond is a function of the ligand and oxidation state of the heme. Distal ligands such as CO and CN$^-$ can receive $\pi$-$\pi^*$ back-bonding from the iron which stabilizes the His$^{93}$-iron bond. For example, the heme dissociation rate of cyanometmyoglobin is too small to measure conveniently even at pH 5.0. Distal ligands such as phenoxide, as in the case of the H64Y mutant, cannot accept back-bonding electrons and as a result disrupt the Fe$^{3+}$-His$^{93}$ bond (Traylor and Sharma, 1992; Momenteau and Reed, 1994; Hargrove et al., 1994a). This effect is evident from the structures of wild type and H64Y ferric myoglobins in which the His$^{93}$-Fe bond lengths are 2.2 and 2.6 Å, respectively. This mechanism could also explain why hemin loss from metmyoglobin increases slightly as pH is increased from 6 to 10 (Hargrove et al., 1994a). At higher pH, hydroxide replaces water as the sixth ligand in metmyoglobin. Hydroxide like phenoxide is a poor $\pi$ acceptor and, when bound, is likely to destabilize
the His$^{93}$-iron bond. Reduction to the ferrous state markedly ($\geq 60$-fold) strengthens the iron-His$^{93}$ bond, regardless of the presence or absence of a sixth ligand (Hargrove and Olson, 1996).

Favorable partitioning of the hydrophobic portion of the hemin group into the protein interior is probably the single most important force holding the oxidized prosthetic group in myoglobin. This process accounts for roughly half of the free energy released during hemin binding and is non-specific. The next most important factor is the strength of the Fe$^{3+}$-His$^{93}$ bond. When hemin is not covalently linked to the protein, as in H93G metmyoglobin, the overall association equilibrium constant decreases from $10^{14}$ to $10^9$ M$^{-1}$ (Hargrove et al., 1996). The apolar environment of the heme pocket strengthens the Fe$^{3+}$-His$^{93}$ bond by lowering the pK$_a$ of the imidazole side chain, and other proximal residues hold His$^{93}$ in a fixed orientation for coordination with the iron atom. The most important residues in the proximal pocket which create this environment are Leu$^{89}$ and Ile$^{99}$ which also function to exclude solvent. Replacement of these amino acids with smaller residues results in a myoglobin with a much larger rate constant for hemin dissociation.

The results in Table 1 also provide the background data for designing recombinant hemoglobins and myoglobins with greater resistance to hemin loss. The greatest decreases in k$_{-H}$ are observed when coordination to the heme iron atom is enhanced either by additional hydrogen bonding to bound water (V68T) or replacing the coordinated water with a histidine (V68H). However, this strategy is inappropriate if resistance to autooxidation and moderate O$_2$ affinity are to be retained. Both the V68T and V68H mutants
oxidize rapidly and react poorly with $O_2$. Decreasing the polarity of the heme pocket is another approach; however, the apolar nature of this region of the active site appears to have been maximized by evolutionary processes. The most viable strategy is to enhance favorable electrostatic interactions with the heme propionates, even though the effects may be small (i.e. only 3 to 4-fold). Alterations in the residues around the heme propionates normally have little effect on ligand binding and autooxidation (Carver et al., 1991; Brantley et al., 1993; Smerdon et al., 1993). This strategy has been applied successfully to the $\beta$ subunits of human hemoglobin. When $\beta$ Ser CD3 is replaced by His, a 3 to 4 fold decrease in $k-H$ was observed with little or no change in the ligand binding properties of the R-state form of the mutant hemoglobin (Whitaker, 1995).
Chapter 7
The Stability of Myoglobin is Determined by Heme Affinity

INTRODUCTION

Myoglobin and hemoglobin are perhaps the most thoroughly studied proteins with respect to structure and function and yet the complex interaction between heme and globin prevents a simple understanding of holoprotein stability. The development of extracellular heme proteins for use as O2 delivering pharmaceuticals has generated even more interest in understanding heme binding and globin folding. In vivo and in vitro stabilities of potential blood substitute products are determined by the resistance of the holoprotein to heme loss and subsequent denaturation. On the other hand, expression yields appear to be determined primarily by the rate and extent of apoglobin folding which need to be optimized for commercial production of recombinant hemoglobins (Hargrove et al., 1994b; Chapter 4).

Heme clearly stabilizes intact myoglobins and hemoglobins with respect to their corresponding apoglobins (Crumpton and Polson, 1965; Kawahara et al., 1965). Removal of heme from myoglobin results in a decrease in helicity, swelling of the resulting apoprotein, and a marked decrease in resistance to denaturation (Schechter and Epstein 1968; Hughson and Baldwin 1989). Baldwin, Wright, and coworkers have characterized the acid and urea induced unfolding pathway of apomyoglobin in terms of a three state model (Hughson et al., 1991; Barrick and Baldwin 1993; Jennings and Wright 1993). The native (N) apoglobin state unfolds
first into a molten globule intermediate (I) in which the A, G, and H helices are still intact and appear to form a hydrophobic core. This intermediate then unfolds completely to form the U state at low pH or high concentrations of denaturant. This model has been successful for explaining the effects of mutagenesis on apomyoglobin stability (Hughson and Baldwin 1989; Hargrove et al., 1994b).

In contrast to the work with apoglobins, recent and past studies have not been successful at correlating effects such as helix propensity, helix pairing, and electrostatic interactions with holomyoglobin stability (Flanagan et al., 1983; Garcia-Moreno 1985; Hughson and Baldwin 1989; Pinker et al., 1993; Lin et al., 1993, 1994). Acampora and Hermans (1967) examined far-uv circular dichroism (CD) and Soret absorbance changes during acid and temperature induced unfolding of sperm whale myoglobin. They showed coincidence of both measurements during unfolding and noted that cyanide binding increases the melting temperature. Shen and Hermans (1972 a,b,c) carried out a comprehensive set of experiments analyzing the nature and kinetics of pH-induced sperm whale myoglobin unfolding and refolding. A complex, four state model was developed which proposed that the native state passes through a short lived intermediate on its way to two possible denatured states. Analysis of the denatured states revealed that one was very much like apomyoglobin, readily refolded, and contained substantial helicity but very little Soret absorbance. The second contained little helicity, appeared to be aggregated with heme, and refolded poorly. The low rate of renaturation of the second denatured state was attributed to the slow break up of heme-protein aggregates. Helicity
returned prior to Soret absorbance in all renaturation experiments, suggesting that refolding of apomyoglobin must occur before heme binding.

The stabilities of myoglobin from many different species have been compared in an effort to understand how the subtle differences in amino acid sequences cause changes in the overall structure (Schechter and Epstein 1968; Hapner et al., 1968; Hartzell et al., 1968; Puett 1973; Flanagan et al., 1983; Hargrove et al., 1994b). Sperm whale myoglobin was found to be the most resistant to denaturation. Flanagan et al., (1983) compared calculations of stability based on electrostatic stabilization and buried surface area to the measured denaturation parameters for a variety of Cetacean myoglobins. Only the buried surface area calculations predicted sperm whale myoglobin to be the most stable. Electrostatic calculations were unable to predict the order of stability among the different intact myoglobins. However, it should be noted that the differences in holoprotein stability are very small compared to those observed between the corresponding apoglobins (Hargrove et al., 1994b). Pinker et al. (1993) and Lin et al. (1993) also attempted to alter the stability of sperm whale myoglobin by inserting helix-stabilizing alanine residues into different sites in the protein. Although they were able to affect the observed stability of the holoprotein, the results suggested that helix propensity does not play a dominant role in stabilizing the native conformation.

Most evidence suggests that the unfolding of myoglobin results in heme dissociation. Schechter and Epstein (1968) showed that quenching of tryptophan fluorescence by heme is lost when myoglobin is unfolded. The work of Shen and Hermans (1972b) suggests that heme is no longer
specifically bound to apomyoglobin in the denatured state. More recently, Goto and Fink (1994) described a phase diagram of myoglobin as a function of ionic strength and pH. Under their conditions metmyoglobin, like apomyoglobin, unfolds via an intermediate. The transition from the metmyoglobin native state to the intermediate state requires lower pH values than those for the N→I transition of apomyoglobin, but the second transition occurs at the same pH regardless of whether the starting protein is met- or apomyoglobin. The lack of heme-dependence of the second transition suggests that the prosthetic group is no longer bound in the intermediate.

Despite the large number of previous studies, the contribution of heme to the stability of myoglobin has not been determined quantitatively (Goto and Fink, 1994). In the present work, the stabilities of three recombinant sperm whale myoglobins with vary different heme affinities and apoglobin stabilities were compared to examine the relative contributions of these two properties to the overall stability of the holoprotein. Wild type myoglobin served as a control. The internal Val68(E11) residue was replaced with a polar Thr residue which is known to decrease apoglobin stability but, at the same time, increase resistance to hemin loss by forming a strong hydrogen bond to the water molecule coordinated to the iron atom (Smerdon et al., 1991; Hargrove et al. 1994b). The external His97(FG3) residue was replaced with Asp to disrupt interactions with the heme-7-propionate and allow solvent into the proximal heme pocket. This surface replacement causes a large decrease in heme affinity without significantly affecting apoglobin stability. The aquomet,
cyanomet, deoxy ferrous, and apoglobin forms of each mutant were unfolded by titration with guanidinium chloride. In combination, these experiments help to define the role of heme in stabilizing holo-myoglobin. The results show that denaturation parameters obtained from analyses of holoprotein unfolding reflect primarily heme affinity and do not usually correlate with the stability of the globin portion of the molecule.

METHODS

Preparation of Proteins- Recombinant wild type and mutant sperm whale myoglobins were expressed and purified as described by Springer and Sligar (1987) and Carver et al (1992). The V68T mutation in sperm whale myoglobin was constructed using cassette mutagenesis of the wild type sperm whale myoglobin gene. H97D was constructed using the Kunkel method of oligonucleotide directed mutagenesis. Apomyoglobins were prepared using the methylethyl ketone method described by Ascoli et al (1981) and Hargrove et al (1994). Protein concentrations were determined using the following extinction coefficients: Apomyoglobin: ε\(_{280}\) = 15.2 mM\(^{-1}\) cm\(^{-1}\) (Light 1987); metmyoglobins: ε\(_{409}\) = 157 mM\(^{-1}\) cm\(^{-1}\); cyanomyoglobins: ε\(_{423}\) = 187 mM\(^{-1}\) cm\(^{-1}\); deoxymyoglobins: ε\(_{434}\) = 115 mM\(^{-1}\) cm\(^{-1}\); (Antonini and Brunori 1971).

Reaction Conditions- All experiments were carried out in 50 mM Tris, 50 mM NaCl, pH 8.0 in order to compare the results with heme binding studies under the same conditions. A stock 6.5 M GdmCl solution was prepared in this buffer as described by Pace et al. (1990). In each unfolding experiment the appropriate volume of buffer and stock GdmCl
were mixed to achieve the desired GdmCl concentration (0 to 6.5 M) in a final volume of 1 ml. A 10 μl aliquot of a concentrated (~1 mM) solution of myoglobin was then added to 1 ml of each GdmCl concentration. The resultant solutions were equilibrated for 1 hour prior to measurement. All absorbance spectra were measured with a SLM/Aminco 3000 diode-array spectrophotometer. Steady-state fluorescence emission was measured with a SLM 8100 spectrofluorimeter. Circular Dichroism was measured with an Aviv 62DS spectropolarimeter.

All apomyoglobin and native and H97D metmyoglobins reached equilibrium in a few minutes after which no further absorbance or fluorescence changes occurred (Chapter 4). However, all the cyanomet and deoxy myoglobins and V68T metmyoglobin required at least 30 to 45 minutes to reach equilibrium. Unfolding of CO-myoglobins was attempted. Extremely long incubation times were required to reach equilibrium, and often oxidation of dissociated CO heme occurred. Reversibility of holomyoglobin unfolding reactions was very much dependent on conditions and is compromised by aggregation of free heme and non-specific heme binding to the apoprotein (Acampora and Hermans 1967; Shen and Hermans 1972 a,b,c). True equilibrium was reached in most cases, particularly in the presence of GdmCl; however, caution must be exercised when using these results to obtain thermodynamic parameters (Acampura and Hermans 1967).

Rate constants for hemin loss from wild type and mutant myoglobins were measured as described in Chapter 2. The reaction conditions were 0.15 M potassium phosphate, 0.45 M sucrose, pH 5.0, at 37°C. Hemin loss
experiments at pH 8.0 were carried out in 50 mM Tris, 50 mM NaCl at 37°C. The rate constant for hemin loss, \( k_{H} \), was obtained from fits of each time course to a single exponential expression with an offset.

**Unfolding reactions- Apomyoglobins:** Total tryptophan fluorescence emission was measured with a 320 nm cutoff filter and excitation at 285 nm. These data show very clearly the formation of the I state which results in a peak in plots of fluorescence intensity vs. [GdmCl] (Chapter 4). CD at 222 nm was also monitored to follow changes in helicity during unfolding (Barrick and Baldwin 1993).

**Metmyoglobins:** Soret absorbance spectra were measured at each GdmCl concentration, and the spectra of free hemin in 6.5 M GdmCl was recorded as a control (Fig. 7.1A). Absorbance changes at 409 nm were plotted for analysis. The fluorescence of holomyoglobin is highly quenched when heme is bound in the heme pocket, and measurement of holomyoglobin fluorescence during unfolding monitors primarily heme dissociation (Schechter and Epstein 1968). Excitation and emission were as described for apomyoglobin, and CD was monitored at 222 nm.

**Cyanomyoglobins:** Unfolding of cyanomyoglobins was carried out as described above except that the buffer and GdmCl stock solutions contained 0.5 mM KCN (Pinker et al. 1993). In addition, the myoglobin sample was mixed with KCN prior to dilution into GdmCl to ensure that cyanide was bound before the protein was unfolded. An absorbance spectrum was measured at each GdmCl concentration, and a spectrum of free cyanohemin in 6.5 M GdmCl was recorded as a control (Fig. 7.1 B).
**Deoxymyoglobins:** Unfolding was carried out anaerobically in the presence of a small amount of sodium dithionite to prevent oxygenation and oxidation. The buffer and GdmCl/buffer solutions were equilibrated with 1 atm nitrogen prior to mixing with myoglobin, and the mixtures were sealed in air tight cuvettes which had been flushed with nitrogen. CD and fluorescence measurements of deoxymyoglobin were not possible due to the large absorbance of sodium dithionite at wavelengths less than ~ 315 nm. An absorbance spectrum of each GdmCl/deoxymyoglobin mixture was recorded at, and a spectrum of free deoxy heme in 6.5 M GdmCl was measured as a control (Fig. 7.1 C).

**Thermodynamic Analysis**— Apomyoglobin unfolding curves were fitted to the three state model of Barrick and Baldwin (1993), and the same model modified for fluorescence analysis described in Chapter 4 (Equation 4.1). Each holomyoglobin unfolding curve was initially fit to a two state unfolding transition as described by Pace *et al.* (1990). A rationale for this method is given at the beginning of the discussion section. In these analyses, unfolding transitions are fitted using the following equation:

\[ K_{\text{obs}} = K_0 e^{mx} \quad (7.1) \]

where \( x = [\text{GdmCl}] \), \( K_0 \) is the unfolding equilibrium constant at \([\text{GdmCl}] = 0\), and \( K_{\text{obs}} = [\text{denatured myoglobin}]/[\text{holomyoglobin}] \) at various \([\text{GdmCl}]\). At first, pre and post-transitional slopes were fixed at 0, and \( K_0 \) and \( m \) were allowed to vary. In each case, \( m \) was near 4.1 M\(^{-1}\), the value for the GdmCl induced N to I transition of apomyoglobin determined in Chapter 4.
In subsequent analyses, m was fixed at 4.1 M⁻¹ and K₀ was varied. In the cases of V68T deoxy, wild type cyano, and H97D myoglobins, the pre-transitional slope was allowed to vary. In all other proteins, the pre and post-transitional slopes were fixed at 0. Wild type and H97D metmyoglobin melting curves measured by CD were fitted to the three state model described by Barrick and Baldwin allowing the CD signal of the intermediate to vary. The m value for the second transition was fixed at 2.8 M⁻¹ based on the results for the I to U transition presented in Chapter 4.

RESULTS

Denaturation of holomyoglobin results in heme dissociation- The Soret spectra of free hemin, cyanohemin, and deoxyheme in 6.5 M GdmCl are similar, with the exception of the protein peak at 280 nm, to the final spectra observed when the corresponding complex of myoglobin is titrated with this denaturant (Fig. 7.1). As [GdmCl] is increased, the fluorescence emission intensity of each complex increases (data not shown) concomitant with the decreases in Soret absorbance. The fluorescence increase is due to removal of tryptophan quenching by the heme group as the latter dissociates from the protein. The correspondence between the protein fluorescence changes, the heme absorbance changes, and the initial portion of the CD change indicates that heme dissociation occurs at the same time as or prior to denaturation of the globin.

Thermodynamic analysis of GdmCl induced heme dissociation- Thermodynamic analyses of holomyoglobin denaturation are complex because of heme aggregation and non-specific interactions between heme
Figure 7.1: Absorbance spectra for the titration of met, cyano, and deoxymyoglobin with GdmCl. A) metmyoglobin; B) cyanomyoglobin; C) deoxymyoglobin. In each case the range of [GdmCl] was 0 to 6.5 M. The dotted line in each figure is a spectrum of free heme under the same buffer conditions in 6.5 M GdmCl. It is clear that each of these unfolding reactions results in removal of heme from myoglobin.
and the different folded and unfolded states of the apoprotein (Shen and Hermans 1972 a,b,c). Because heme dissociation accompanies myoglobin denaturation, a model for the denaturation of holomyoglobin must take into account the effects of heme affinity on stability. The simplest expression for this unfolding reaction is given in Equation 7.2. In this case the fraction of holomyoglobin, \( Y_{Mb} \), is described by Equation 7.3:

\[
\begin{align*}
\text{Mb} & \xrightarrow{K_{Mb,U}} U + H \\
Y_{Mb} &= \frac{2 + \frac{K_{Mb,U}}{P_0}}{2 + \left( \frac{K_{Mb,U}}{P_0} \right)^2 - 4 }
\end{align*}
\]  

(7.2)  

(7.3)

where \( K_{Mb,U} \) is the unfolding equilibrium constant, and \( P_0 \) is the total protein or heme concentration. Most unfolding reactions involve only isomerization. However, holoprotein denaturation results in two products and should show dependence on protein concentration. Fig. 7.2A shows denaturation curves simulated using Equation 7.3 with values of 6, 60, and 160 \( \mu \text{M} \) for the total protein concentration, \( P_0 \). It is clear from these curves that a reaction described by Equation 7.3 should exhibit a noticeable dependence on protein or heme concentration. The experimental denaturation curves in Fig. 7.2B do not show a large dependence on protein concentration, suggesting that some mechanism acts to diminish heme induced refolding at high protein concentrations.

The dependence of unfolding on heme concentration can be diminished by taking into account non-specific heme binding to unfolded
Figure 7.2: GdmCl titration of metmyoglobin at different protein concentrations. A) These curves were generated in Matlab™ using Equation 7.3 with $K_{Mb,U} = 1 \times 10^{-11}M$, and $P_0 = 6, 60, \text{ and } 160 \mu M$. The m value for the transition was fixed at $6M^{-1}$. B) Plots of experimental data under the same conditions. The smooth lines were generated using a cubic spline routine in Igor™. C) These curves are simulations of $Y_{Mb}$ and $Y_{UH}$ as a function of GdmCl concentration using Equations 7.5 and 7.6. The sum of these curves approximates the data in Fig. 7.2B.
myoglobin. The simplest two step mechanism is shown in Equation 7.4. The
dependence of the fractions of Mb and the non-specific heme-unfolded
globin complex, UH, on [GdmCl] are given by Equations 7.5 and 7.6,
respectively.

\[
\text{Mb} \xleftrightarrow{K_{\text{Mb,U}}^{-1}} U + H \xleftrightarrow{K_{\text{NS}^{-1}}} UH_{\text{NS}} \quad (7.4)
\]

\[
Y_{\text{Mb}} = \frac{\left(2 + \frac{K_{\text{Mb,U}}}{K_{\text{NS}}} + \frac{K_{\text{Mb,U}}}{P_0}\right) \left(2 + \frac{K_{\text{Mb,U}}}{K_{\text{NS}}} + \frac{K_{\text{Mb,U}}}{P_0}\right)^2 - 4 \left(\frac{K_{\text{Mb,U}}}{K_{\text{NS}}}\right)^2}{2 \left(1 + \frac{K_{\text{Mb,U}}}{K_{\text{NS}}}\right)^2} \quad (7.5)
\]

\[
Y_{\text{UH}} = \left(\frac{K_{\text{Mb,U}}}{K_{\text{NS}}}\right)Y_{\text{Mb}} \quad (7.6)
\]

\[
K_{\text{Mb,U}}(x) = K_{\text{Mb,U}} \exp(m_{\text{Mb,D}}X), \quad K_{\text{NS}}(x) = K_{\text{NS}} \exp(m_{\text{NS,X}}), \quad \text{and} \quad X = [\text{GdmCl}].
\]

Denaturation curves simulated using Equations 7.5 and 7.6 are shown in Fig. 7.2C. This figure shows that non-specific heme binding
during myoglobin unfolding prevents observation of a large dependence on
protein or heme concentration. The mechanism described in Equation 7.4
also accounts for the second absorbance transition seen at high [GdmCl] in
the presence of high concentrations (60 and 160 \(\mu\)M) of protein. This
transition appears to involve GdmCl induced dissociation of non-specifically
bound heme. This phenomenon is seen in the simulations shown in Fig.
7.2C and corresponds with the increase in YUH which is observed at moderate [GdmCl].

Ideally, experimental data at varying protein concentrations could be fitted to the sum of Equations 7.5 and 7.6 to yield values of K_{Mb,U} and K_{NS}. However, it is very difficult to obtain data with enough precision and resolution to assign individual equilibrium constants. Since little dependence on protein concentration is observed and most experiments were carried out at 6 μM heme, we have fitted the observed denaturation curves to a simple two state unfolding reaction, \( \text{Mb} \rightleftharpoons \text{D} \), with a single equilibrium constant, K_{Mb,D}. The fraction of holoprotein is assumed to be:

\[
Y_{Mb} = \frac{1}{1 + K_{Mb,D}} \quad (7.7)
\]

where K_{Mb,D}(x) = K_{Mb,D} \exp(m_{Mb,D}[\text{GdmCl}]) and K_{Mb,D} is the isomerization constant for denaturation of the holoprotein in buffer. Good fits of the observed data to the simple expression in Equation 7.7 were obtained, and the fitted value of K_{Mb,D} is \( \approx \) K_{H}/K_{NS}. This ratio of the specific to non-specific equilibrium dissociation constants for heme binding is expected to be unitless, and should be significantly smaller (10^4 to 10^7-fold) than the independently measured value of K_{H}.

The effects of different heme ligands on myoglobin stability - A comparison of the melting curves for wild type met-, cyanomet-, and deoxymyoglobinins is shown in Fig. 7.3. The fitted values for the single
Figure 7.3: Comparison of Soret absorbance, fluorescence, and CD changes resulting from the unfolding of wild type myoglobin. A) GdmCl-induced aquometmyoglobin unfolding monitored by Soret absorbance, fluorescence, and circular dichroism. B) Apo-, aquomet-, cyanomet-, and deoxymyoglobin unfolding induced by GdmCl. Unfolding of apomyoglobin was monitored with CD only; met and cyanometmyoglobin unfolding was measured by the decrease in Soret absorbance and increase in fluorescence accompanying denaturation and heme dissociation. Unfolding of deoxymyoglobin was monitored by measuring the decrease in the Soret absorbance signal. In each applicable case, the fluorescence and Soret melting curves were coincident. This figure indicates that unfolding of holomyoglobin is a direct measure of heme dissociation and that the ligand oxidation state of the heme greatly influences the observed stability of the protein.
Table 7.1: Equilibrium constants for GdmCl induced unfolding of wild-type, V68T, and H97D myoglobins. In most cases, the unfolding equilibrium constant $K_{Mb,D}$ was determined from fits to a single unfolding event (Equation 1). The circular dichroism data for wild type and H97D metmyoglobins were fitted to a three state transition.

<table>
<thead>
<tr>
<th>Protein</th>
<th>aquometMb $K_{Mb,D}$</th>
<th>cyanometMb $K_{Mb,D}$</th>
<th>deoxyMb $K_{Mb,D}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type holoMb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soret absorbance</td>
<td>$140 \times 10^{-6}$</td>
<td>$6.2 \times 10^{-6}$</td>
<td>$1.9 \times 10^{-6}$</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$180 \times 10^{-6}$</td>
<td>$8.0 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>CD (222 nm)</td>
<td>$140 \times 10^{-6}$</td>
<td>$3.8 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>V68T holoMb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soret absorbance</td>
<td>$13 \times 10^{-6}$</td>
<td>$23 \times 10^{-6}$</td>
<td>$5.2 \times 10^{-6}$</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$14 \times 10^{-6}$</td>
<td>$20 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>CD (222 nm)</td>
<td>$25 \times 10^{-6}$</td>
<td>$14 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>H97D holoMb</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Soret absorbance</td>
<td>$6,000 \times 10^{-6}$</td>
<td>$120 \times 10^{-6}$</td>
<td>$130 \times 10^{-6}$</td>
</tr>
<tr>
<td>Fluorescence</td>
<td>$4,200 \times 10^{-6}$</td>
<td>$140 \times 10^{-6}$</td>
<td></td>
</tr>
<tr>
<td>CD (222 nm)</td>
<td>$4,800 \times 10^{-6}$</td>
<td>$170 \times 10^{-6}$</td>
<td></td>
</tr>
</tbody>
</table>
transition, $K_{Mb,D}$, are listed in Table 7.1. The relative stabilities ($1/K_{Mb,D}$) of the different liganded states of wild type myoglobin are $\text{deoxyMb} \geq \text{cyanometMb} \gg \text{metMb}$. The increased resistance to denaturation incurred by cyanide coordination has been observed previously. Cyanide has greater ability to accept $\pi$ electrons from the heme iron than coordinated water, and this interaction results in trans-stabilization of the $\text{Fe}^{3+}$-$\text{His}^{93}$ bond (Momenteau and Reed 1994). The proximal $\text{Fe}^{2+}$-$\text{His}^{93}$ linkage in deoxymyoglobin is also known to be stronger than the more ionic bond with $\text{Fe}^{3+}$ (Bunn and Jandel, 1968; Allis and Steinhardt, 1970).

The fluorescence, Soret absorbance, and CD signals change simultaneously when cyanometmyoglobin is titrated with GdmCl, and these transitions fit reasonably well to a two state model (Fig. 7.3B). The unfolding of deoxymyoglobin can also be fitted to a simple, two state model. In contrast, the CD changes accompanying metmyoglobin denaturation show the presence of an intermediate much like that seen with apomyoglobin (Fig. 7.3A; Goto and Fink, 1994). These results suggest a three state process for the denaturation of metmyoglobin. The first transition involves hemin loss and formation of the partially folded I state with $\sim 30\%$ of the CD signal of the original holoprotein. Higher [GdmCl] is required to unfold this intermediate to the U state of apomyoglobin. The value of $K_{I,U}$ measured starting from holometmyoglobin is on the same order as that measured by CD for $K_{I,U}$ starting from apoprotein (Fig. 7.3B, Table 7.2). The reason the I to U transition is not seen when denaturing cyanometmyoglobin and deoxymyoglobin is that these holoproteins are much more resistant to heme loss. As a result, the higher [GdmCl] required
to cause heme loss (~ 3 M) is also sufficient to unfold the intermediate, causing the two processes to occur simultaneously.

**Apoglobin stabilities and hemin dissociation equilibrium constants**
The unfolding transitions of wild type, V68T and H97D apomyoglobin are shown in Figs. 7.4A and B. Time courses for hemin dissociation from the corresponding aquometmyoglobin derivatives are shown in Fig. 7.4C. When apomyoglobin unfolding is monitored by fluorescence, an initial increase in total emission intensity is observed for the native apoglobin (N) to intermediate (I) transition followed by a decrease as the protein becomes completely unfolded (Hargrove et al., 1994b). The peak corresponds to the formation of the intermediate state which is thought to contain intact A, G, and H helices and retain 30 to 40% of the CD of the starting apoprotein. The corresponding CD changes also show a two step unfolding process. There is an initial 50 to 60% decrease with a mid point at ~ 1.0 M GdmCl and a second transition centered around 2.4 M GdmCl. The data in Figs. 7.4A and B show that the stabilities of wild type and H97D apomyoglobins are identical, as measured by either fluorescence or CD, whereas V68T apomyoglobin is considerably less stable and unfolds at significantly lower [GdmCl].

Although the fluorescence and CD changes give qualitatively the same results, the fitted values for KN,I and KI,U differ (Table 7.2). In general, the parameters obtained from CD data are 3 to 5 times smaller than those obtained from the fluorescence data. The unfolding data are obtained on a logarithmic x-axis scale since K unfolding is proportional to
**Figure 7.4:** Apomyoglobin stabilities and rates of hemin loss from wild type, V68T, and H97D myoglobins. A) and B) Fluorescence and CD changes during unfolding. The curves indicate that V68T apomyoglobin is less stable than the wild type and H97D apoproteins, which are similar in stability. Fitted equilibrium constants for these transitions are given in Table 7.2. C) Rates of hemin loss from these mutants indicate that V68T metmyoglobin has a much lower rate of hemin loss (>0.1 h⁻¹) than wild type protein (1 h⁻¹), whereas the H97D mutant has a much higher rate (38 h⁻¹). These results show that apomyoglobin stability is not correlated with hemin affinity in these two mutants and wild type metmyoglobin.
Table 7.2: Equilibrium constants for GdmCl induced unfolding of wild-type, V68T, and H97D apomyoglobinins. Unfolding reactions were carried out in 0.2 M phosphate, pH 7.0 at 25°C. Values of $K_{N,I}$ and $K_{I,U}$ were obtained from fits to Equation 4.1.

<table>
<thead>
<tr>
<th>Protein</th>
<th>ApoMb Unfolding</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>$K_{N,I}$</td>
</tr>
<tr>
<td><strong>Wild-type Mb</strong></td>
<td></td>
</tr>
<tr>
<td>Soret absorbance</td>
<td></td>
</tr>
<tr>
<td>Fluorescence</td>
<td>0.011</td>
</tr>
<tr>
<td>CD (222 nm)</td>
<td>0.003</td>
</tr>
<tr>
<td><strong>V68T Mb</strong></td>
<td></td>
</tr>
<tr>
<td>Soret absorbance</td>
<td></td>
</tr>
<tr>
<td>Fluorescence</td>
<td>0.068</td>
</tr>
<tr>
<td>CD (222 nm)</td>
<td>0.025</td>
</tr>
<tr>
<td><strong>H97D Mb</strong></td>
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<td>Soret absorbance</td>
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</tr>
<tr>
<td>Fluorescence</td>
<td>0.009</td>
</tr>
<tr>
<td>CD (222 nm)</td>
<td>0.003</td>
</tr>
</tbody>
</table>
exp(m[GdmCl]), where $m \approx 4.1 \, M^{-1}$. The errors in the $x$ axis position are $= \pm 0.1 \, M$ which lead to errors in the unfolding constants of factors of $\sim 2$. Thus, although still apparent when superimposing the curves, the discrepancies between the CD and fluorescence data are small on a free energy scale. The fluorescence changes are due to tryptophans 7 and 14 and may result from conformational transitions which differ somewhat from those involving large changes in helical content which are monitored by CD (Fink 1995). Resolution of these transitions will require combined CD/fluorescence titrations using a wider variety of myoglobins. However, the main conclusion is clear. The N state of V68T apomyoglobin is much less stable than the corresponding state for either wild type or H97D apomyoglobin (Table 7.2, second and fourth columns).

In contrast to its relatively low apoglobin stability, V68T metmyoglobin shows the greatest resistance to hemin loss (Fig. 7.4C and Table 7.3). The rate of hemin dissociation from this mutant is 25-fold less than that for wild type metmyoglobin at pH 5, and too slow to be measured at pH 8.0. We have shown that the bimolecular association rate constant for monomeric hemin, CO-heme, and cyanohemin binding to apoglobins is $\sim 1 \times 10^8 \, M^{-1} s^{-1}$ at $20^\circ C$ and roughly independent of protein structure, pH, and state of ligation (Hargrove et al. 1996a). Thus, the equilibrium dissociation constants ($K_{-H}$) for hemin binding can be estimated as $k_{-H}/1 \times 10^8 \, M^{-1} s^{-1}$ and are given in Table 7.3 for wild type, V68T, and H97D metmyoglobins.

The hemin dissociation equilibrium constants, $K_{-H}$, differ significantly in the order V68T<wild type<<H97D aquometmyoglobin at both pH values.
This order corresponds with that observed for the holoprotein denaturation constants ($K_{Mb,D}$ in Table 7.1) but not with the apoglobin unfolding constants ($K_{N,I}$ or $K_{I,U}$ in Table 7.2). The high affinity of V68T metmyoglobin for hemin is due to an additional hydrogen bond between the threonine hydroxyl group and the coordinated water molecule (Smerdon et al., 1991; Hargrove et al., 1996b). The low affinity of H97D metmyoglobin for hemin is due to disruption of the proximal hydrogen bonding lattice involving His$^{97}$, Ser$^{92}$, His$^{93}$, and the heme-7-propionate and hydration of this region of the proximal heme pocket (Chapter 6).

**Stability of mutant holoproteins** - Unfolding of wild type, V68T, and H97D metmyoglobins is shown in Fig. 7.5. The fitted values of $K_{Mb,D}$ are listed in Table 7.1. The order of stability of the aquometmyoglobins is V68T > wild type > H97D myoglobin. As was the case for wild type protein, the CD data for H97D metmyoglobin required fitting to a three state process with $K_{Mb,I}$ and $K_{I,U}$ being $\sim 5 \times 10^{-3}$ and $\sim 1 \times 10^{-4}$, respectively. The biphasic character of the H97D metmyoglobin curve is more pronounced than that of wild-type myoglobin due to dissociation of hemin from the mutant at lower [GdmCl]. The I to U transition occurs at the same [GdmCl] for both proteins ($\sim 2.5 - 3$ M). In contrast, the absorbance and CD data for V68T metmyoglobin were readily fitted with a single unfolding constant, $K_{Mb,D} = 2 \times 10^{-5}$.

In the case of the cyanomet derivatives, the observed stabilities are wild type > V68T > H97D myoglobin, and simple two state transitions are observed (Fig. 7.6A; Table 7.1). The change in order of wild type and
Figure 7.5: Comparison of the stabilities of wild type, V68T, and H97D metmyoglobins. The V68T mutant appears to be the most stable of the three myoglobins, and H97D metmyoglobin is clearly the least stable. The I to U transitions of apomyoglobin can still be observed in the unfolding curves for wild type and H97D metmyoglobins as measured by CD, but not by Soret absorbance or fluorescence emission.
Table 7.3: Rate constants for hemin loss from wild-type, V68T, and H97D metmyoglobin. Reactions at pH 5 were carried out in 0.2 M sodium acetate, 0.45 M sucrose, at 37°C. Reactions at pH 8.0 were in 50 mM Tris, 50 mM NaCl, at 37°C. Values of $K_{H}$ were calculated using $1 \times 10^{-8}$ M$^{-1}$s$^{-1}$ for the association rate constant, $k_{H}$.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$k_{H}$ (pH 5) h$^{-1}$</th>
<th>$K_{H}$ (pH 5) M</th>
<th>$k_{H}$ (pH 8) h$^{-1}$</th>
<th>$K_{H}$ (pH 8) M</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Mb</td>
<td>1.0±0.5</td>
<td>$2.8\pm5 \times 10^{-12}$</td>
<td>0.01</td>
<td>$2.8\pm5 \times 10^{-14}$</td>
</tr>
<tr>
<td>V68T Mb</td>
<td>0.04</td>
<td>$0.1 \times 10^{-12}$</td>
<td>&lt;0.005</td>
<td>&lt;0.05 $\times 10^{-14}$</td>
</tr>
<tr>
<td>H97D Mb</td>
<td>38</td>
<td>$110 \times 10^{-12}$</td>
<td>8</td>
<td>$2200 \times 10^{-14}$</td>
</tr>
</tbody>
</table>
Figure 7.6: Comparison of the stabilities of wild type, V68T, and H97D cyanomet- and deoxymyoglobins. Cyanohemin binding clearly stabilizes myoglobin relative to apo- and aquometmyoglobin forms. The H97D mutant is the least stable of the cyanomyoglobins but, under these conditions, the V68T mutant is less stable than wild type protein. The unfolding curves measured by CD for the cyanomyoglobins, including the H97D mutant, fit well to a two state unfolding model. Wild type and V68T deoxymyoglobins show about the same degree of stability whereas the H97D mutant is much less stable. Even in the ferrous form, heme affinity determines the stability of the holoprotein.
V68T myoglobin is due to unfavorable electrostatic interactions between the negative portion of the threonine hydroxyl and bound cyanide (Brancaccio et al., 1994). In the case of the deoxy forms, wild type and V68T myoglobins are equally stable, and the H97D mutant again denatures at much lower [GdmCl] (Fig. 7.6B). We attempted to unfold the ferrous CO forms of myoglobins. Unfortunately, the extremely long periods of time required for the CO samples to reach equilibrium resulted in competition between unfolding, CO-heme loss, and oxidation. However, CO binding does clearly result in even greater stability than that observed for five-coordinated deoxymyoglobin. GdmCl concentrations on the order of 5 M were required to see significant (~ 50%) absorbance changes due to CO-heme loss.

**DISCUSSION**

The denaturation of holomyoglobin can be interpreted in terms of the following three step scheme:

\[
\text{Mb} \overset{K_{-H}}{\rightleftharpoons} \text{N + H} \overset{K_{N,I}}{\rightleftharpoons} \text{I} \overset{K_{I,U}}{\rightleftharpoons} \text{U} \quad (7.8)
\]

Each equilibrium constant shows an exponential dependence on [GdmCl]. Most apoproteins show a two step denaturation curve both in fluorescence and CD experiments (Fig. 7.4). However, the number of discrete transitions seen for denaturation of holoprotein is variable and depends on the oxidation state, iron coordination, and protein structure, all of which regulate hemin affinity. In the case of wild type apomyoglobin, the
concentration of GdmCl required for $K_{N,I}(x)$ and $K_{I,U}(x)$ to be 1.0 are ~ 1 and 2.5 M, respectively, whereas the concentration of GdmCl required for 50% dissociation of hemin, cyanoheamin, and deoxyheme from the holoprotein are ~ 2, ~ 2.9, and ~ 3.2 M, respectively. Two transitions are observed for the denaturation of wild type metmyoglobin: \(\text{Mb} \rightarrow \text{H}+\text{I}\) and \(\text{I} \rightarrow \text{U}\), since the \([\text{GdmCl}]\) causing hemin loss (2 to 2.5 M) is insufficient to unfold completely the apoprotein intermediate, I. In contrast, only one transition is observed for cyanomet- and deoxymyoglobin unfolding since the \([\text{GdmCl}]\) required for heme dissociation (in these cases 3 to 3.5 M) is greater than that required for complete unfolding of apomyoglobin (2.5 to 3 M). A summary of $K_{\text{Mb,D}}$, $K_{N,I}$, and $K_{-H}$ values at pH 8 for wild type, V68T, and H97D sperm whale metmyoglobins is given in Table 7.4.

**Heme affinity is the primary determinant of holomyoglobin stability**- The results in Tables 7.1-7.4 indicate that the factors governing the stability of holomyoglobin are the same as those which determine heme affinity. The strength of the His$^{93}$ (F8)-iron bond decreases in the order ferrous pentacoordinate $\geq$ cyanomet $>$ aquomet which is the order of the stabilities of the corresponding myoglobin derivatives based on our GdmCl induced unfolding studies (Table 7.1). As shown in Table 7.4, there is also a direct correlation between the first equilibrium unfolding constant of aquometmyoglobin, $K_{\text{Mb,D}}$, and the equilibrium dissociation constant for hemin binding $K_{-H}$. The V68T mutant shows lower values for both constants due to an "extra" hydrogen bond between the Thr$^{68}$ hydroxyl and coordinated water. The H97D mutant shows higher values for both constants due to disruption of hydrogen bonding lattice between His$^{97}$,
Table 7.4: Comparison of the stability constants of met-and apomyoglobin with their corresponding hemin affinities. $K_{Mb,U}$ and $K_{N,I}$ were determined as described in Tables 7.2 and 7.1, respectively. $K_{-H}$ was determined as described in Table 7.3.

<table>
<thead>
<tr>
<th>Protein</th>
<th>$K_{Mb,U}$</th>
<th>$K_{N,I}$</th>
<th>$K_{-H}$ (M$^{-1}$)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Wild-type Mb</td>
<td>$150 \times 10^{-6}$</td>
<td>0.011</td>
<td>$2.8 \times 10^{-4}$</td>
</tr>
<tr>
<td>V68T Mb</td>
<td>$14 \times 10^{-6}$</td>
<td>0.068</td>
<td>&lt; $0.05 \times 10^{-4}$</td>
</tr>
<tr>
<td>H97D Mb</td>
<td>$5000 \times 10^{-6}$</td>
<td>0.009</td>
<td>$22002 \times 10^{-4}$</td>
</tr>
</tbody>
</table>
Ser$^9_2$, and the heme-7-propionate and hydration of this region of the heme pocket. In contrast, there is no correlation between the unfolding first constants of the holoproteins and those for the corresponding apoglobins (i.e. $K_{Mb,D}$ vs. $K_{N,I}$). V68T apomyoglobin is the least stable apoprotein, and wild type and H97D apoglobins show almost identical stabilities (Table 7.4; Fig. 7.4).

**Discrepancies between Soret absorbance and far-uv CD changes** - When the unfolding of holomyoglobins is monitored by fluorescence or Soret absorbance, the observed changes reflect the release of heme from the protein. In contrast, CD changes at 222 nm measure primarily alterations in globin secondary structure. In the case of cyanomet and ferrous myoglobin derivatives, heme dissociation occurs at GdmCl concentrations high enough to fully denature the corresponding apoglobins. As a result the $CD_{222nm}$ changes associated with loss of myoglobin secondary structure follow closely the absorbance and fluorescence changes associated with heme dissociation (Fig. 7.6). However, if heme dissociation occurs at GdmCl concentrations which are insufficient to fully denature the apoprotein, then residual $CD_{222nm}$ changes will occur at higher [GdmCl], even though the fluorescence and absorbance changes associated with hemin loss are complete. Thus, the discrepancy between CD, absorbance, and fluorescence changes is most pronounced for the aquomet form of the H97D mutant which has the poorest affinity for hemin of the proteins studied (Fig. 7.4)

**Interpretations of heme protein stability studies** - Heme binding clearly stabilizes the globin tertiary structure with respect to both the
intermediate and unfolded states (e.g. $K_{Mb,D}$ vs. $K_{N,I}$ in Table 7.4). The physiologically relevant parameter is the stability constant for the holoprotein which is determined primarily by hemin affinity. Pinker et al. (1993) and Lin et al. (1993) have also shown that it is not possible to decipher the effects of mutations on protein-backbone stability by measuring the unfolding of cyanomyoglobins. For protein folding studies, the stability of apomyoglobin must be measured directly. It is certainly possible that apomyoglobin stability could affect heme affinity and *vice versa*, but a direct relationship is not required.

As shown in Chapter 4, constitutive expression of myoglobin from *E. coli* is governed by both apomyoglobin stability and heme affinity. Apomyoglobin must be able to fold properly to prevent aggregation and precipitation from competing with heme binding. As a result, mutants with moderately poor heme affinities but very stable apoglobin structures can express as well as or better than those with high heme affinities (e.g. V68T vs. H97F in Table 7.1; Chapter 4). However, the holoproteins of mutants with high rates of hemin dissociation are much less stable to long term storage at room temperature, repeated freezing and thawing, and other conditions that promote denaturation.
Chapter 8: Conclusions

Traditionally, stability of myoglobin had been measured as though it were a simple small globular protein. This thesis shows that folding and stability of myoglobin is much more complex, involving heme affinity and protein backbone stability. Studied in this way, myoglobin can serve as a model system for studies of the stability of proteins containing prosthetic groups.

When I started my graduate work, there was very little known about specific interactions between heme and globin and much of the information on ferric hemoglobin and myoglobin stability was qualitative and based on relative resistance to heat or chemical denaturation. Clear distinctions between loss of the prosthetic group and globin unfolding had not been made. This situation was due to the lack of a convenient assay for measuring rates of hemin dissociation. Chapter 1 gives some background information helpful for understanding studies of myoglobin stability. The following chapters provide a detailed study of the factors governing the stability of myoglobin and hemoglobin.

Chapter 2 describes the development of an assay for hemin dissociation from myoglobin and hemoglobin. His$^{64}$(E7) was replaced by Tyr in sperm whale myoglobin to produce a holoprotein with a distinct green color due to an intense absorption band at 600 nm. Val$^{68}$(E11) was replaced by Phe in the same protein to increase its stability. When excess Tyr$^{64}$Val$^{68}$ apoglobin is mixed with either metmyoglobin or methemoglobin, the solution turns from brown to green, and the absorbance
changes can be used to measure complete time courses for hemin dissociation from either holoprotein. This assay was used to measure rates of hemin dissociation from native metmyoglobin, four myoglobin mutants (Ala^{64}(E7), Ala^{68}(E11), Phe^{68}(E11), and Glu^{45}(CD3)), and native methemoglobin.

In Chapter 3, this assay was used to study the heme affinity of human hemoglobin in more detail. Two kinetic phases were observed for hemin dissociation from native human hemoglobin at pH 7.0 and 37°C. Valence and mutant hybrid hemoglobins were used to assign the faster phase \( (k = 7.8 \pm 2.0 \ hr^{-1}) \) to hemin dissociation from ferric \( \beta \) subunits and the slower \( (k=0.6\pm0.15 \ hr^{-1}) \) to dissociation from \( \alpha \) subunits. The corresponding rate for wild-type metmyoglobin is \( 0.007 \pm 0.001 \ hr^{-1} \). Rates of hemin loss from the \( \alpha \) and \( \beta \) subunits of native and recombinant human hemoglobin were measured as a function of protein concentration at pH 7.0, 37°C to determine the effects of quaternary structure on hemin affinity. Rates of hemin loss were also measured for isolated chains, genetically stabilized tetrameric hemoglobin, and hemoglobin in the presence of inositol hexaphosphate. The value of \( k-\mathcal{H} \) for \( \beta \) subunits in native hemoglobin shows a large dependence on protein concentration, increasing from \( \sim 1.6 \ h^{-1} \) for tetramers at mM protein levels to \( \sim 15 \ h^{-1} \) for dimers at \( \mu M \) levels. In contrast, \( k-\mathcal{H} \) for \( \alpha \) subunits in native hemoglobin is significantly smaller and roughly independent of protein concentration. Stabilization of hemoglobin tetramers by genetically linking the \( \alpha \) subunits removes the concentration dependence of hemin loss and results in a molecule with \( \alpha \) and \( \beta \) hemin loss rates identical to those observed for native hemoglobin at
high concentrations. Rates of hemin loss from monomeric α and β chains were estimated to be 12 and 40 h⁻¹, respectively at pH 7, 37°C. Thus, the equilibrium constant for hemin binding depends strongly on quaternary state. The order of affinity constants for α subunits is: $3 \times 10^{10} \text{M}^{-1}$ (monomers) $< 70 \times 10^{10} \text{M}^{-1}$ (dimers) $= 100 \times 10^{10} \text{M}^{-1}$ (tetramers), whereas the order for β subunits: $1 \times 10^{10} \text{M}^{-1}$ (monomers) $= 1.5 \times 10^{10} \text{M}^{-1}$ (dimers) $< 20$ to $30 \times 10^{10} \text{M}^{-1}$ (tetramers). The ability of α subunits to retain hemin is enhanced greatly by monomer aggregation to form the $\alpha_1\beta_1$ interface but is little affected by creation of the $\alpha_1\beta_2$ interface during dimer aggregation. In contrast, the $\alpha_1\beta_1$ interface has little benefit on hemin retention in α subunits, whereas the $\alpha_1\beta_2$ interface increases 25-fold the resistance of β subunits to hemin loss.

After gaining a general understanding of the heme affinity of myoglobin and hemoglobin, I became interested in how heme affinity relates to protein stability. Our experience with trying to express many mutant myoglobins suggested that some factor other than heme affinity must also affect the overall stability of holoprotein. It was clear that the stability and expression of myoglobins is more complex than a simple measure of hemin affinity. The other factor affecting protein expression is the folding and stability of apomyoglobin.

In Chapter 4, the factors governing stability of sperm whale, pig, and human metmyoglobin were examined by: (1) measuring guanidinium chloride induced unfolding of apoglobins containing 22 replacements at positions 29(B10), 43(CD1), 64(E7), 68(E11), and 107(G8); (2) determining the rates of hemin loss from the recombinant holoproteins, and
(3) estimating constitutive expression levels of the corresponding genes in *Escherichia coli* TB-1 cells. The denaturant titrations were analyzed in terms of a two step unfolding reaction, \(N(\text{native apoprotein}) \rightarrow I(\text{intermediate}) \rightarrow U(\text{unfolded})\), in which the intermediate is visualized by an increase in tryptophan fluorescence emission intensity. Two key conclusions were reached. First, high rates of hemin loss are not necessarily correlated with unstable globin structures and *vice versa*. In general, both rates of hemin loss and the equilibrium constants for apoprotein unfolding must be determined in order to understand the overall stability of heme proteins and to predict the efficiency of their expression. Second, polar residues in the distal pocket cause marked decreases in the overall stability of apomyoglobin. Removal of hemin from V68N and L29N sperm whale myoglobins produces the molten globular I state at pH 7, 25\(^\circ\) without addition of denaturant. In contrast, the H64L and H64F mutations produce apoproteins which are 10-30 times more stable than wild-type apoglobin. The latter results show that protein stability is sacrificed in order to have the distal histidine (H64) present to increase O\(_2\) affinity and inhibit autooxidation by direct hydrogen bonding interactions with the bound ligand.

The work in Chapter 4 provides some qualitative insight into the stability of myoglobin, and some quantitative data for the stability of apomyoglobin. But the relative contributions of heme affinity and globin folding to holoprotein stability were still not clear and the structural factors governing heme affinity had not been determined. The next three chapters
(5-7) explore in detail the reaction of heme with apomyoglobin and the effects of heme binding on protein stability.

In Chapter 5, rate constants for heme binding to 35 different recombinant apomyoglobins and several other apoproteins were measured in an effort to understand the factors governing heme affinity and the velocity of the association reaction. Surprisingly, the rate constant for the binding of monomeric heme is \( \approx 1 \times 10^8 \text{ M}^{-1}\text{s}^{-1} \) regardless of the structure or overall affinity of the apoprotein for iron-porphyrin. Major differences between the proteins are reflected primarily in the rates of dissociation of the prosthetic group. Slow phases observed in the reaction of CO heme with excess apomyoglobin result from formation of non-specific heme-protein complexes which must dissociate before heme can bind specifically in the heme pocket. Once the specific heme-globin complex is formed, the heme pocket rapidly collapses around the porphyrin simultaneously forming the bond between the proximal His\(^{93}\) and the heme iron atom. The overall affinity of apomyoglobin for hemin is \( \sim 1 \times 10^{14} \text{ M}^{-1} \). Non-specific hydrophobic interactions between the porphyrin and the apolar heme cavity account for a factor of \( 10^5 \) to \( 10^7 \). Covalent bond formation between Fe\(^{3+}\) and His\(^{93}\)(F8) provides an additional factor of \( 10^3 \) to \( 10^4 \). Specific interactions with conserved amino acids in the heme pocket contribute the final factor of \( 10^3 \) to \( 10^4 \). Reduction of the iron atom strengthens the Fe-histidine bond \( \sim 60 \) fold, and CO coordination makes heme binding effectively irreversible at room temperature and neutral pH (K\(_{\text{CO}}\)-heme \( \approx 10^{19} \text{ M}^{-1} \)).
Chapter 6 describes measurement of rates of hemin dissociation from ~100 different met-myoglobin mutants in an effort to determine which amino acid residues are important for retaining the prosthetic group. Most of the amino acids examined are within 4 Å of the porphyrin ring, but replacements of a number of non-contact residues were also made. Mutations of His\textsuperscript{93}(F8) and Leu\textsuperscript{89}(F4) can result in >100 fold increases in the rate of hemin loss at pH 5 and 7. Some replacements of the contact residues His\textsuperscript{64}(E7), Val\textsuperscript{68}(E11), His\textsuperscript{97}(FG3), Ile\textsuperscript{99}(FG5), Thr\textsuperscript{39}(C4), and Tyr\textsuperscript{103}(G4) cause > 10 fold changes in the rate of hemin dissociation. Substitutions of the non-contact residues Leu\textsuperscript{29}(B10), Phe\textsuperscript{46}(CD4), and Gly\textsuperscript{65}(E8) can also increase the rate of hemin loss > 10-fold. The principal causes of hemin retention are: (1) hydrophobic interactions between apolar residues in the heme pocket and the porphyrin ring, (2) the covalent bond between His\textsuperscript{93}(F8) and the Fe\textsuperscript{3+} atom, and (3) hydrogen bonding between distal residues and coordinated water. Although significant, electrostatic interactions between the heme propionates and amino acids at the surface of the protein are less important. Rates of hemin dissociation from newly reconstituted myoglobin and myoglobin containing dimethyl-ester hemin are identical to those of native and wild type myoglobin, raising the question of the structural cause of heme orientational disorder.

In Chapter 7, the properties of wild type, V68T, and H97D sperm whale myoglobins were compared to determine the relative importance of heme affinity and globin stability on the resistance of the holoprotein to denaturation. The V68T mutation decreases apoglobin stability by placing a polar side chain in the interior heme pocket. However, this substitution
increases hemin affinity by formation of a strong hydrogen bond between coordinated water and the Thr$^{68}$E11 side chain. The H97D substitution disrupts favorable electrostatic interactions with the heme-7-propionate, allows solvent into the proximal heme pocket, and causes a large increase in the rate of hemin dissociation. However, the Asp replacement has little affect on apoglobin stability because His$^{97}$(FG3) is a surface residue. The aquomet, cyanomet, deoxyferrous, and apoglobin forms of each mutant and wild type myoglobin were unfolded by titration with guanidinium chloride. Even though holomyoglobin denaturation involves the dissociation of heme and should be dependent on protein concentration, non-specific heme binding to unfolded states makes the overall process appear to be a simple, unimolecular unfolding transition. The equilibrium constants for the denaturation of holomyoglobin are governed almost exclusively by heme affinity and do not correlate with the stability of the globin portion of the molecule. Thus, the design of more stable recombinant hemoglobin and myoglobins requires the use of mutations that inhibit hemin loss. However, the expression yields of these proteins do depend strongly on the ability of the apoprotein to produce a stable native-like tertiary structure which is capable of binding heme rapidly.

Chapters 5, 6, and 7 provide, for the first time, a complete analysis of heme binding to apomyoglobin and its effect on protein stability. Chapter 5 shows that the major driving force for heme association with apoproteins is a non-specific hydrophobic partitioning effect. As a result, the association rate constant for heme binding is very large, $\sim 1 \times 10^8 \text{M}^{-1}\text{s}^{-1}$ and roughly the same for all proteins and mutants. Chapter 6 describes the roles of
individual amino acids in retaining heme and possible strategies for designing more stable recombinant myoglobins and hemoglobins. Chapter 7 shows unequivocally that the resistance of holomyoglobin to denaturation is determined by its affinity for heme and not by the stability of its apoglobin structure. The latter observation will require a re-evaluation of previous folding studies carried out with intact hemoglobins and myoglobins.

All of this work with myoglobin and apomyoglobin provides a relatively detailed understanding of the complex stability of this protein. However, another way to approach the stability of myoglobin is to first put the question in context. Simply asking "what determines the stability of myoglobin" can be answered by showing that heme affinity is the major determinant. But this is only true of the folded, heme-bound, holoprotein. And this type of "stability" has little to do with the ability of a myoglobin to be expressed and fold in *E. coli* or *in vivo*. For proper expression and folding, the stability of apomyoglobin is the most important factor. So the answer to the question "what determines the stability of myoglobin" should be answered with "which type of stability do you mean?"
References


Barrick, D. (1994) *Biochemistry* 33, 6546-6554


Gibson, Q. H., and Antonini, E. (1960) *Biochem. J.* 77, 328-341


Hargrove, M.S., Barrick, D., and Olson, J.S. (1996a) *Biochemistry*

Hargrove, M.S., Wilkinson, A.J., and Olson, J.S. (1996b) *Biochemistry*

Hargrove, M.S., and Olson, J.S. (1996) *Biochemistry*


Hughson, F. M., Barrick, D., & Baldwin, R. L. (1991) Biochemistry 30, 4113-4118


Rohlfs, R. J., Mathews, A. J., Carver, T. E., Olson, J. S., Springer, B. A.,


Sage, J.T., Morikis, D., and Champion, P.M. (1991) *Biochemistry* 30, 1227-
1237


Chapter 5, pp 184-188, Cambridge University Press


Smerdon, J. S., Dodson, G. G., Wilkinson, A. J., Gibson, Q. H., Blackmore, R.

Smerdon, J.S., Kryzwda, S., Wilkinson, A.J., Brantley, R.E., Jr., Carver, T.E.,

*Proc. Natl. Acad. Sci. USA* 88, 882-886

8965

Rev.* 94, 699-714
Stryer, L. (1965) *J. Mol. Biol.* 13, 482-495


