Structural Factors Governing Hemin Dissociation from Metmyoglobin†

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ABSTRACT: Rates of hemin dissociation from ∼100 different metmyoglobin mutants were measured 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 noncontact residues were also made. Mutations of His^{93}(F8) and Leu^{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^{64}(E7), Val^{68}(E11), His^{97}(FG3), Ile^{99}(FG5), Thr^{39}(C4), and Tyr^{103}(G4) cause >10-fold changes in the rate of hemin dissociation. Substitutions of the noncontact residues Leu^{29}(B10), Phe^{46}(CD4), and Gly^{65}(E8) can also increase the rate of hemin loss >10-fold. The key structural factors stabilizing bound hemin in myoglobin are (1) hydrophobic interactions between apolar residues in the heme pocket and the porphyrin ring, (2) the covalent bond between His^{93}(F8) and the Fe^{3+} atom, and (3) hydrogen bonding between distal residues and coordinated water. Specific electrostatic interactions between the heme propionates and amino acids at the surface of the protein appear to be less important. Loss of these polar interactions can be compensated by increasing the apolar character of either the heme group by esterification of the propionates or replacement of charged surface residues with large apolar side chains [*e.g.*, replacing His^{97}(FG3) with Phe].

The association equilibrium constant for hemin binding to apomyoglobin is approximately 10^{14} M^{-1} at neutral pH and is determined by a complex set of hydrophobic, ionic, and covalent interactions [Dickerson & Geis, 1983; Hargrove *et al*. (1996) and references therein]. The tetrapyrrole ring makes multiple contacts with residues in the heme pocket, and the propionate carboxyl groups are involved in electrostatic interactions 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 Nϵ of 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 & Brunori, 1971; Dickerson & 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 (Figure 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 an ∼20% increase in helicity (Griko *et al*., 1988). Three edges of the porphyrin are buried in the protein interior and are protected from solvent. The fourth side contains the heme-6- and heme-7-propionates, which interact with solvent and several polar surface residues.

At room temperature, 40−50% of the free energy released during the formation of aquometmyoglobin comes from nonspecific, hydrophobic partitioning of the hemin group into the apoprotein (Hargrove *et al*., 1996). The remaining free energy changes are due to specific interactions between the porphyrin ring and amino acid side chains lining the heme pocket and to formation of the Fe^{3+}−His^{93} bond. The strength of the latter interaction can be estimated from the association equilibrium constant for hemin binding to H93G myoglobin, which lacks the proximal histidine residue. The affinity of H93G apoglobin for hemin is $\approx 10^9$ M^{-1}, suggesting that the Fe^{3+}−His^{93} bond accounts for a factor of 10^4 in the overall association equilibrium constant for 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 Fe^{3+}−His^{93} bond as well as through specific interactions with the tetrapyrrole ring (Momenteau & Reed, 1994). In addition, reduction to Fe^{2+} and coordination by π electron acceptors such as CN^- and CO increase the strength of the iron−His^{93} bond and, therefore, the affinity of myoglobin for its prosthetic group (Hargrove & Olson, 1996).

The overall association rate constant for the binding of monomeric heme to apomyoglobin is $\sim 1 \times 10^8$ M^{-1} s^{-1} regardless of the oxidation state and nature of the globin molecule (Hargrove *et al*., 1996). As a result, hemin affinity is governed almost exclusively by the rate constant for dissociation. Until recently, direct measurement of hemin loss from metmyoglobin was difficult due to its unusually high affinity for the prosthetic group and the small spectral changes associated with transfer from myoglobin to other

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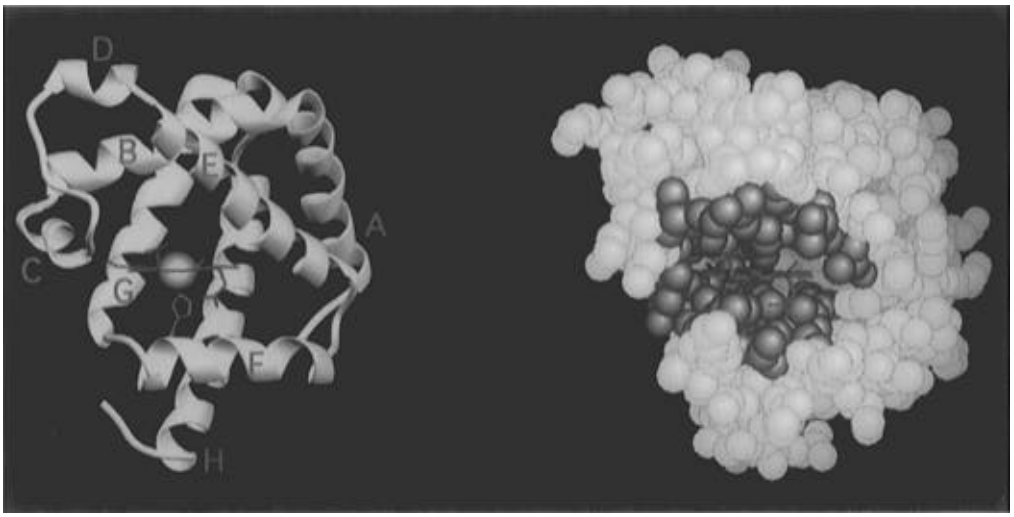

FIGURE 1: Structure of sperm whale metmyoglobin. (A, left) Ribbon drawing of the eight helices (labeled A through H). The heme group is shown in red, and the proximal His^{93}(F8), to which the heme iron is covalently bound, is shown in pink. (B, right) Space-filling representation of myoglobin. Residues making contacts with the heme group are in blue, and other protein residues are in yellow. The heme group is shown in red. Three edges of the heme are buried in the protein, and the fourth, containing the heme propionates, is exposed to solvent.

hemin binding proteins. Human hemoglobin has a significantly lower affinity for hemin, and methods for measuring hemin dissociation from this protein have been developed. The results for hemoglobin have led to some understanding of the interaction of iron-porphyrin with globin (see Banerjee, 1962a−c; Bunn & Jandel, 1968; Benesch & Kwong, 1990, 1995).

Two years ago Hargrove *et al*. (1994a) developed a relatively fast, convenient assay to measure hemin dissociation from almost any heme protein, including myoglobin. In combination with site-directed mutagenesis, this assay was used to examine some of the factors that had been presumed to regulate the affinity of myoglobin for hemin. At pH 5, 37 °C, they observed rates of hemin loss ranging from < 0.1 h^{-1} to 54 h^{-1} for a series of single point mutations at five different amino acid positions. However, the study was not systematic and, to date, there has been no comprehensive description of the factors regulating the affinity of myoglobin for hemin nor of the roles of specific amino acid residues in the active site. In this work, we have systematically mutated almost all 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 96 different myoglobins have been determined, often at both pH 5 and 7. The results have been used to evaluate the chemical mechanisms governing hemin affinity and to suggest possible mutations for enhancing its retention in recombinant myoglobins and hemoglobins.

MATERIALS AND METHODS

Preparation of Proteins. Native sperm whale myoglobin was obtained by special permit from Sigma. The initial single mutants at positions 68 and 64 in sperm whale myoglobin were constructed using the cassette mutagenesis system developed by Barry A. Springer and Karen D. Egeberg at the University of Illinois (H64L, H64F, H64Q, V68A, and V68F; Springer & Sligar, 1987; 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, also by cassette mutagenesis. Sperm whale myoglobin mutants at positions 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 Leu^{29} 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 methyl ethyl 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 1 atm of 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 determining concentrations (ϵ_{407nm} = 147 mM^{-1} cm^{-1}; Light, 1987). A slight excess of apomyoglobin (≈0.5 mM) was mixed with hemin (≈0.45 mM) 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 hemin peak at ≈390 nm (Figure 6A). Reconstitution of myoglobin with hemin dimethyl ester was performed as described by La Mar *et al*. (1986). Hemin dimethyl ester 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 Hargrove *et al*. (1994a). 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 and 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 2101UVPC spectrophotometer. Hemin loss from

Table 1: Survey of the Rates of Hemin Loss from Myoglobin Mutants[a]

protein	k_{-H} at pH 5 (h^{-1})	k_{-H} at pH 7 (h^{-1})	protein	k_{-H} at pH 5 (h^{-1})	k_{-H} at pH 7 (h^{-1})	protein	k_{-H} at pH 5 (h^{-1})	k_{-H} at pH 7 (h^{-1})
			(A) Sperm Whale Myoglobin					
native	1.0 ± 0.5	0.01 ± 0.01	F46I	5.8		V68S	1.0	0.10
old wild type	1.0	0.01	F46W	2.6		V68T	<0.1	<0.01
new wild type	1.0	0.01	**H64G**	**38**	**0.75**	A71F	2.2	
real wild type	1.0		**H64A**	**17**	**0.40**	**L89G**	**≈700**	**140**
L29V	**11**	**0.10**	H64V	7.3		**L89A**	**56**	**5.7**
L29I	5.6		**H64L**	**11**	**0.20**	**L89S**	**≈500**	**15**
L29F	2.5	0.01	H64I	8		L89F	2.5	0.1
L29N	8.3	0.10	H64F	4.8	0.01	**L89W**	**17**	**0.40**
L29W	**11**	**0.1**	**H64W**	**24**		**H93G**	**660**	**140**
F43V	**54**	**2**	H64Q	3.5	0.12	**H97A**	**39**	**2.0**
F43I	**38**	**2**	**H64Y**	**16**	**0.04**	**H97V**	**23**	**0.84**
F43L	**14**	**0.2**	G65A	1.7	0.03	H97F	1.5	**0.14**
F43W	8.9		**G65V**	**39**	**0.70**	**H97D**	**38**	**5**
R45K	1.7	0.039	G65T	3.1	0.01	**H97E**	**56**	**3**
R45H	3.4	0.029	**G65I**	**21**	**11/0.47**	I99A	8	0.2
R45A	3.7	0.053	V66K	1.5		**I99S**	**41**	**0.9**
R45T	3.1	0.040	V66G	1.2		I107A	1.1	
R45S	3.1	0.035	V68A	5.8	0.10	I107V	1.3	
R45Y	8.0	0.066	V68L	2.3		I107T	1.4	
R45D	3.7	0.033	V68I	1.1		I107L	1.3	
R45E	5.5	0.08	V68F	2.0	0.01	I107F	1.1	
F46A	**13**	**0.60**	V68W	2.0		I111V	1.7	
F46V	**23**	**0.30**	V68Q	2.4	0.03	I111F	2.8	
F46L	1.9		V68N	5.2	0.01			
			(B) Pig Myoglobin					
wild type	1.0	0.01	T39V	6.0	0.06	S92L	2.8	
K45E	3		V68T	0.5	<0.01	**Y103A**	**24**	**0.03**
K45S	2.6		V68S	1.6	<0.01	**Y103L**	**41**	**0.16**
K45H	2.6		V68N	8.7	0.07	Y103F	2.6	0.05
T39Y	**51**	**3.8**	S92A	3.3				
			(C) Human Myoglobin					
wild type	3	0.01	H64G/V68H	0.38		H64I/V68H	<0.1	
V68G	**41**		H64V/V68H	<0.1	≪0.01	H64F/V68H	1.0	
V68A	3.2							
			(D) Other Myoglobins					
Horse Mb	2.5		Cow Mb	1.5		sperm whale Mb		
						reconstituted	1.1	
						DME-hemin	1.5	0.02

[a] The hemin loss properties of all the proteins were surveyed in 0.15 M acetate and 0.45 M sucrose, pH 5, 37 °C. Selected mutants were examined in 0.15 M phosphate and 0.45 M sucrose, pH 7.0, 37 °C. Mb mutants that cause >10-fold changes in k_{-H} are shown in boldface type. Old wild type contains Asn122, new wild type (corrected) contains Asp122, and real wild-type is new wild type without the *N*-formylmethionine. 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 the dimethyl ester of hemin.

H93G, L89A, L89G, and L89S myoglobins was measured using a Gibson-Dionex stopped-flow apparatus.

Reconstituted wild-type sperm whale myoglobin was prepared in 20 mM potassium phosphate, pH 7.0, stored on ice, and used within 30 min of preparation. La Mar *et al*. (1984) used NMR techniques to show that the half-time for hemin reorientation at pH 7 is ∼12 h at room temperature, and the reaction is even slower at 4 °C. Our laboratory confirmed their results by CD measurements [Light *et al*., 1987; see also Aojula *et al*. (1986)]. Thus, under our experimental conditions, no hemin reorientation occurs prior to dilution into the pH 5 buffer containing excess H64Y/V68F apoprotein and subsequent measurement of hemin loss. Myoglobin reconstituted with dimethyl ester hemin was allowed time for reorientation at pH 5 (∼4 h; Light *et al*., 1987) prior to the measurement of hemin loss.

In general, hemin dissociation data were collected for 200 min at pH 5, and for over 1000 min at pH 7. It was not always possible to collect end points for experiments carried out 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 apoprotein denaturation at pH 5.0. These rates were obtained from fits to a two-exponential expression; the larger fast phase was attributed to hemin loss. Two rate constants for hemin loss from G65I metmyoglobin at pH 7 were reported because the time courses for this mutant are clearly biphasic even though no precipitation is observed.

RESULTS AND DISCUSSION

Hemin Loss from Myoglobin. Table 1 lists rates of hemin loss from native sperm whale, horse, and cow myoglobins, recombinant wild-type sperm whale, pig, and human myoglobins, 67 sperm whale myoglobin mutants at 15 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 ≥ 10-fold increase in the rate of hemin loss are shown in boldface type, and those which clearly decrease hemin loss are underlined. The hemin loss

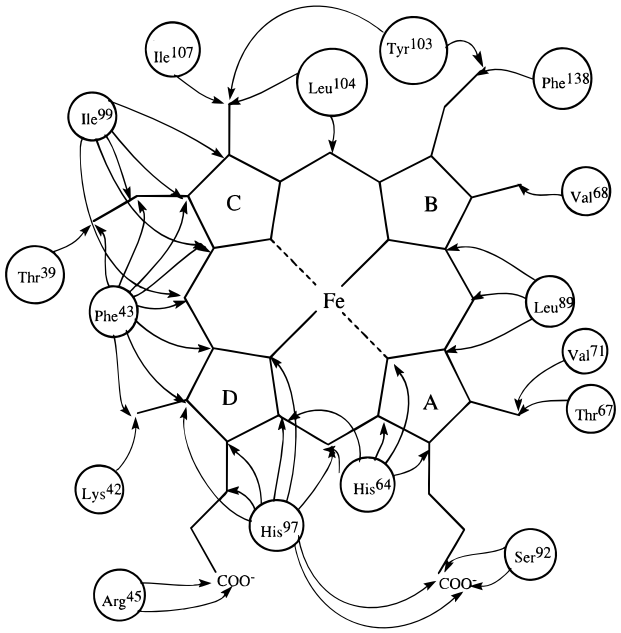

FIGURE 2: Heme−protein contacts. Each residue within 4 Å of the heme group in sperm whale metmyoglobin is shown as a circle. Each contact is shown as an arrow to the appropriate porphyrin atom.

reaction is fairly rapid at pH 5.0 and is readily measured for all the proteins examined. At pH 7.0, the dissociation reaction is usually slower and technically more difficult to measure. As a result, a more limited set of mutants was surveyed at this pH.

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

A schematic drawing of interactions in the heme pocket of sperm whale metmyoglobin is shown in Figure 2. Residues within 4 Å of the heme are connected by arrows to the heme atom(s) they contact. The view is looking down onto the distal face of the porphyrin ring with His^{93} below the page and not shown. Figure 1B shows these residues (in blue) in relation to the entire protein.

Figure 3 presents expanded views of the heme pocket looking in from the solvent phase. The side chains of each heme pocket residue in Table 1 are shown as stick figures. The heme contact residues which were not mutated in this study are shown as light green space-filling atoms. Figure 3B presents the same view in which the contact side chains have been labeled and all other protein residues have been deleted. At least one mutation of the residues shown in blue results in a ≥10-fold change in the rate constant for hemin loss, k_{-H}, and at least one mutation of the residues shown in red alters this parameter by ≥100-fold. Mutations of the residues shown in white and gray have smaller effects on the kinetics of hemin loss.

Mutations Causing >100-Fold Increases in k_{-H}: His^{93}(F8). It is no surprise that removal of His^{93} results in a myoglobin with a very 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 spectrum very much like that of the native and wild-type proteins. However, the lack of a covalent connection to the globin increases the rate of hemin loss nearly 1000-fold with respect to that of wild-type metmyoglobin, regardless of pH (Hargrove *et al.*, 1996).

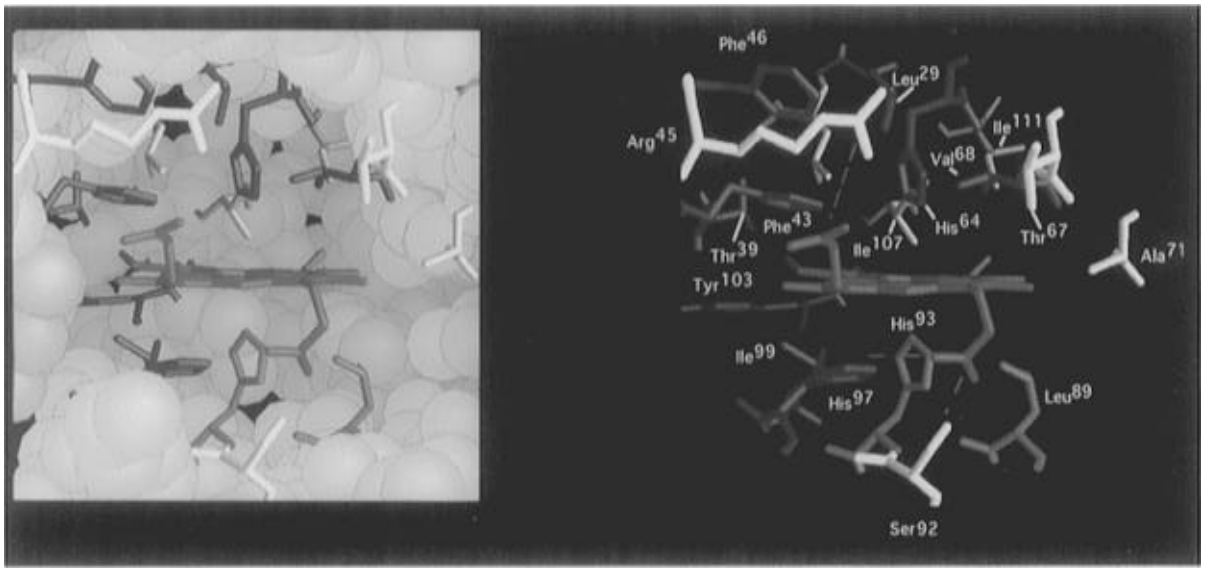

FIGURE 3: Key residues in the heme pocket of myoglobin. (A, left) This view of the heme pocket shows the outline of each heme pocket residue mutated in this study. At least one mutation of the pink residues (His^{93} and Leu^{89}) results in a >100-fold increase in the rate of hemin loss at pH 5.0, 37 °C. Mutation of the residues shown in blue can result in a >10-fold increase in hemin loss, and mutation of those residues shown in white and gray have smaller effects on hemin affinity. The residues shaded in green are within 4 Å of the heme group but were not mutated. (B, right) 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.

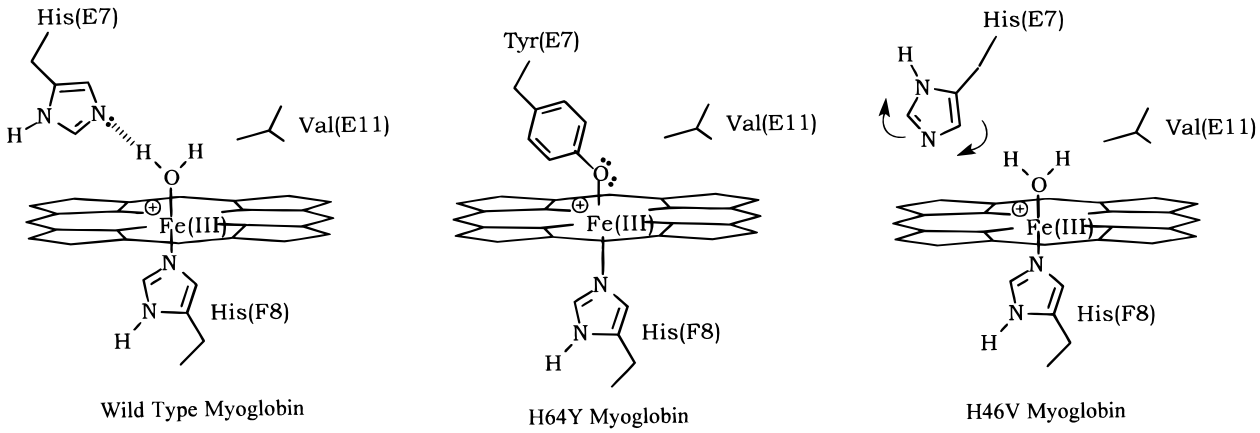

FIGURE 4: Effects of H64Y and F46V mutations on heme affinity. (A) The distal His[64](E7) in wild-type myoglobin stabilizes coordinated water through a hydrogen bond. (B) The Tyr[64] side chain displaces coordinated water but is a much poorer π-electron-accepting ligand. The latter effect results in elongation and destabilization of the His[93]−iron bond (Hargrove *et al.*, 1994a). (C) In F46V metmyoglobin, the distal His[64](E7) swings up into the cavity created by the smaller Val[46] 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 hemin affinity is lowered.

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 the disruption of the heme−imidazole bond and then the loss of both free hemin and imidazole. The rate of dissociation of coordinated free imidazole from the ligand binding site of native metmyoglobin is $\approx 1 \times 10^4$ h^{-1}, which is only 15-fold greater than the rate of hemin loss from H93G myoglobin (Antonini & Brunori, 1971; Table 1). The rate of hemin loss from H93G myoglobin reported in Table 1 was measured by diluting the protein into imidazole-free buffer containing the H64Y/V68F apoglobin reagent. Thus, the free imidazole concentration was effectively zero. The value of k_{-H} does become smaller at high concentrations of exogenous imidazole (10 mM) at both pH 5.0 and 7.0. The latter observation suggests that the imidazole−Fe^{3+} bond may break prior to hemin dissociation, since higher free base concentrations would lower the fraction of noncoordinated hemin present in the mutant protein.

Leu[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[89](F4) is located at the base of the proximal heme pocket next to an internal cavity, referred to as the xenon binding site, and contributes to the apolar environment around His[93] (Tilton *et al.*, 1984). Hemoglobin Boras, in which β Leu-F4 is changed to Arg, results in a pathology associated with hemin loss and formation of semihemoglobins. This effect has been attributed to solvation of the proximal pocket (Antonini & Brunori, 1971; Dickerson & Geis, 1983; Bunn & Forget, 1986). It is likely that the L89G and L89S mutations have the same effect on the proximal pocket in myoglobin. The increase in polarity and space around His[93] destabilizes bonding to 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 increase 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 benzyl side chain are similar to those of the naturally occurring isobutyl group. The large indole side chain of the L89W mutant appears to hinder the porphyrin sterically, resulting in moderate increases in the rate of hemin loss at both pH 5 and 7.

Mutations Resulting in >10-Fold Increases in k_{-H}: Phe[43](CD1). Several hemoglobin pathologies result from mutation of the conserved Phe(CD1) residue. Hemoglobins Torino (α Val-CD1), Louisville (β Leu-CD1), Hammersmith (β Ser-CD1), and Warsaw (β Val-CD1) are all associated with heme loss and unstable proteins. The most severe, Hb Hammersmith, results in the presence of extra waters in the heme pocket and causes disruption of other regions of the protein (Dickerson & Geis, 1983; Bunn & Forget, 1986). Mutation of the corresponding residue in myoglobin produces similar effects. F43V and F43I metmyoglobins lose hemin with rates of 54 h^{-1} and 38 h^{-1}, respectively, at pH 5.0, and the relative effects of these substitutions are even greater at pH 7.0 (Table 1). The decrease in hemin affinity caused by the F43V mutation is enhanced by greater apoglobin unfolding, and the net result is a markedly unstable holoprotein (Hargrove *et al.*, 1994b). Although the F43V mutation does not result in additional water in the heme pocket, it does disrupt the structure of the CD corner (Whitaker, 1995).

Replacing Phe[43](CD1) with leucine does not increase hemin loss as much as the isoleucine replacement. This is probably because the two δ methyl groups of Leu are better able to interact hydrophobically with the heme group than the single δ carbon of Ile. The γ methyl group of isoleucine in the F43I mutant 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[64](E7). The distal histidine regulates ligand binding in myoglobin and hemoglobin by stabilizing bound oxygen through a hydrogen bond which enhances O_2 affinity and prevents autooxidation of the heme iron (Springer *et al.*, 1994; Brantley *et al.*, 1993). In metmyoglobin, His[64] also forms a hydrogen bond to the coordinated water and provides important steric contacts on the distal side of the heme (Figure 4; Quillin *et al.*, 1993).

We initially thought that water coordination and H-bonding to it by His[64] played the most crucial role in stabilizing bound hemin. However, H64G metmyoglobin loses heme 40 and

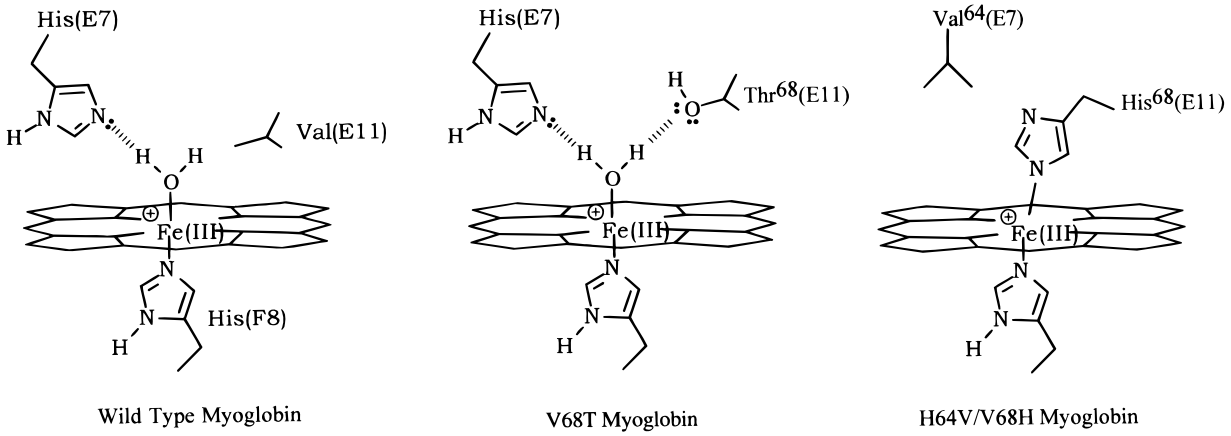

FIGURE 5: Effects of V68T and V68H substitutions on hemin affinity. (A) Coordinated water in wild-type metmyoglobin is stabilized by a hydrogen bond from His^{64}. (B) In V68T metmyoglobin, the Thr^{68} side chain forms a second hydrogen bond with the coordinated water. This bond holds the water in place 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 hemin iron. In this case, the imidazole nitrogen is a better π-electron-accepting ligand than water and the His^{98}(F8)−iron bond is stabilized.

75 times faster than wild-type protein at pH 5 and 7, respectively, even though coordinated H_2O is present. Increasing the size of residue 64 causes significant decreases in k_{-H} at both pH values. The H64F mutant has only a 4-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_ϵ atom of Gln^{64} 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 4−12-fold greater than that for wild-type myoglobin and only 2−3-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, again 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 (Figure 4). The inability of Tyr^{64} to accept π electron density from the iron weakens the His^{93}−iron bond through a trans effect (Traylor & Sharma, 1992). The phenoxide side chain of this residue actually destabilizes the heme−globin linkage, and a longer His^{93}−iron bond is found in the structure of Tyr^{64} metmyoglobin (Hargrove *et al*., 1994a; Maurus *et al*., 1994).

Val^{68}(E11). After the distal histidine, Val^{68} is perhaps the most important residue regulating ligand binding in myoglobin. The isopropyl side chain at this position creates the proper distal pocket volume and apolar environment 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 Val(E11) mutants in hemoglobin (Dickerson & Geis 1983; Bunn & Forget, 1986).

As shown in Table 1, the V68A substitution causes 6- and 10-fold increases in the rate of hemin loss at pH 5.0 and 7.0, respectively. A similar effect is seen in hemoglobin Sydney, in which β Val(E11) is changed to Ala. Destabilization of this mutant Hb appears to be due to solvent entering the heme pocket (Tucker *et al*., 1978). Curiously, the additional contacts with the heme group produced by the V68F mutation do not affect the rate of hemin loss at either pH, and the V68W replacement also has little effect. The Trp^{68} and Phe^{68} side chains appear to be accommodated readily by the empty space located on the distal side of the porphyrin ring, directly above the B pyrrole (Figures 1−3).

Human V68G metmyoglobin loses heme at a rate of 41 h^{-1}. This large increase in the rate of hemin loss is probably a result of increased solvation of the distal pocket due to the formation of a direct channel to solvent. This hypothesis is supported by the observation that the rate of hemin loss from V68N is significantly faster than that of wild-type myoglobin. The Asn^{68} replacement increases the rate of hemin loss almost as much as the Ala^{68} mutation, presumably by increasing the polarity of the distal pocket. Taken together, all of these results suggest that Val^{68} helps prevent hemin loss by preserving the hydrophobicity of the heme pocket.

Surprisingly, the V68S mutation does not increase hemin loss, and the V68T replacement actually slows hemin loss dramatically. These results can only be explained by additional hydrogen bonding to coordinated water (Figure 5). In native myoglobin, the coordinated water is hydrogen-bonded to only His^{64}. However, Smerdon *et al*. (1991) have shown that Thr^{68} forms another strong hydrogen bond to covalently bound water, resulting in even greater stabilization than that seen in wild-type metmyoglobin. The polarity introduced by the V68T mutation results in a less stable apomyoglobin; however, once hemin is bound, the resulting holoprotein is much more resistant to denaturation due to its increased affinity for hemin (Hargrove *et al*., 1994b; Hargrove & Olson, 1996).

Even larger increases in hemin affinity and decreases in globin stability are observed for the V68H mutation. His^{68} displaces the covalently bound water and coordinates directly to the heme−iron (Figure 5). Because His^{68} is a better π 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 O_2, CO, and NO binding due to direct coordination of the His^{68} side chain to the heme iron atom

(Hargrove *et al*., 1994b; Dou *et al*., 1995).

His^{97}(FG3). His^{97} appears to be a barrier to solvent entering into 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 repelling 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 favorable electrostatic interactions between His^{97} and the heme-7-propionate occur only at the higher pH. Below pH 5, the heme propionates become protonated. Under these acid conditions, the hydrophobicity of residue 97 and its role in preventing hydration of the proximal heme pocket is the more important factor in stabilizing bound hemin. As a result, Phe^{97} can substitute for His with little effect on hemin affinity at low pH.

Ile^{99}(FG5). Ile^{99} is a heme contact residue which fills interior 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. As shown in Table 1, mutation of this residue to Ser in myoglobin results in large (40−400-fold) increases in hemin loss, whereas more moderate (8−20-fold) increases are observed for the I99A replacement. This pattern again suggests that the internal regions of the heme pocket need to be kept anhydrous with apolar amino acid side chains.

Thr^{39}(C4). This residue is highly conserved in mammalian and bird hemoglobins 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 in pig myoglobin 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 the 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 hemoglobins. When Tyr^{103} is changed to Leu or Ala in pig myoglobin, the resulting mutants lose hemin at rates of 41 h^{-1} and 21 h^{-1}, respectively, at pH 5.0. However, the Y103F mutation has a much smaller effect on hemin dissociation at both pH 5 and 7. Curiously, the rate of hemin loss from Y103A at pH 7.0 is only 3 times greater than that of the wild-type pig protein, but k_{-H} for Y103L myoglobin at pH 7 is 16-fold faster.

Electrostatic Interactions with the Heme Propionates. In most myoglobins, the residue at position 45 (CD3) is Lys or Arg. The positively charged side chain of this residue hydrogen-bonds to solvent molecules and the heme-6-propionate in the crystal structures of all mammalian myoglobins that have been studied. However, mutations at this position in sperm whale and pig myoglobins have only modest effects on the rate of hemin loss. The R45E (sperm whale) and K45E (pig) mutations cause only 3−8-fold increases in k_{-H} compared to the corresponding wild-type values, despite the fact that strong electrostatic repulsion should occur between the acid side chain and the heme propionate. The R45Y mutation also causes a significant effect, a 7−8-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 α and β chains of hemoglobin, respectively. 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 modestly favorable contribution to hemin affinity, at least compared to those made by the residues shown in blue in Figure 3.

Ser^{92}(F7) forms a hydrogen bond with the side chain of His^{93} which is thought to increase the strength of the Fe^{3+}−His^{93} bond and stabilize bound hemin (Smerdon *et al*., 1993). The results in Table 1 suggest that this effect is relatively small, at least compared to the interactions involving residues 43, 64, 89, 97, and 103. When Ala or Leu is substituted for Ser^{92}(F7) in pig myoglobin, the rate of hemin loss increases from 1.0 to only 3.0 h^{-1} at pH 5.0, 37 °C. However, somewhat larger effects are observed at lower temperatures (Smerdon *et al*., 1993).

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 appears that deletion of a single electrostatic interaction between the porphyrin and an amino acid side chain has only a small effect on hemin affinity because of the multiplicity of other interactions at the solvent interface. This conclusion was tested by measuring rates of hemin loss from myoglobin reconstituted with hemin dimethyl ester. The presence of methyl esters in place of the acidic propionates should decrease the strength of the electrostatic interaction between the prosthetic group and nearby polar residues.

A time course for hemin loss from dimethyl ester hemin myoglobin is shown in Figure 6B. Surprisingly, wild-type myoglobin shows the same rates of hemin and hemin dimethyl ester dissociation at both pH 5.0 and 7.0. As in the case of the H64F and H97F mutations, the loss of electrostatic interactions appears to be compensated by favorable partitioning of a more hydrophobic heme group into the protein matrix. Thus, ionized propionates are not required for tight binding. This idea is supported by the ease with which octaethyl Fe−porphyrin is incorporated into myoglobin (Neya *et al*., 1991). This porphyrin, which is unable to interact electrostatically with surrounding amino acids, forms a stable complex because of the strong hydrophobic effect driving the completely apolar heme group into the protein.

Noncontact Mutations Resulting in >10-Fold Increases in k_{-H}: Leu^{29}(B10). The effects of Leu^{29} on ligand binding have been studied in 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 (V29N) causes an 8−10-fold increase in the rate of hemin loss. These results suggest that increasing the size of the cavity in the distal pocket enhances exposure of the porphyrin to solvent which, in turn, facilitates hemin loss. A similar effect can be produced by replacing Leu^{29} with a polar residue, which

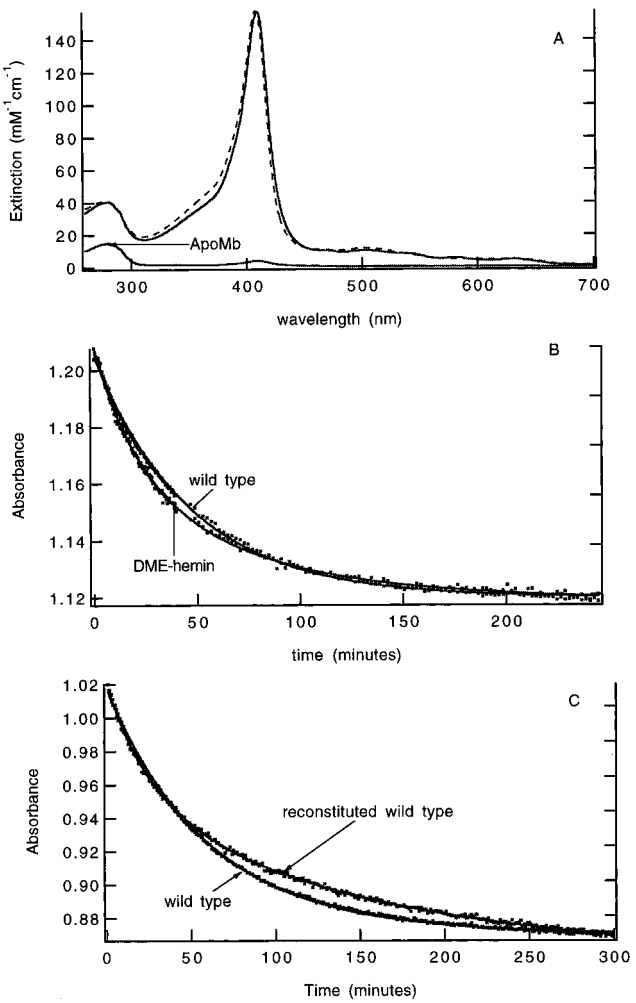

FIGURE 6: Kinetics of hemin loss from reconstituted myoglobins. (A) The spectra of apomyoglobin before (solid line labeled apoMb) and after (dashed line) addition of hemin are compared to the spectrum of wild-type metmyoglobin (solid line). It is evident that reconstitution was complete, and no free hemin was present. Similar spectra were obtained for the reconstitution of apomyoglobin with the dimethyl ester of hemin. (B) Time courses for dissociation of hemin and the dimethyl ester of hemin (DME-hemin) from myoglobin are shown with fitted curves. The rate constant for DME-hemin dissociation from wild-type metmyoglobin is 1.5 h^{-1}, which is almost identical to that of wild-type metmyoglobin. (C) The time courses for hemin loss from wild-type and freshly reconstituted wild-type myoglobin are shown along with fitted curves. The rate constant for hemin loss from wild-type myoglobin is 1 h^{-1}. The time course for hemin loss from reconstituted myoglobin was fitted to a two-exponential expression. The two fitted rate constants were 1.1 h^{-1}, presumably representing k_{-H} for hemin dissociation, and 0.05 h^{-1}, which is presumably associated with precipitation of excess apomyoglobin.

also leads to hydration of the protein interior. In agreement with this interpretation, naturally occurring hemoglobin mutants which introduce polar residues into the heme pocket at the B10 position also result in very unstable proteins, *e.g.*, St. Louis (β Gln-B10) and Genova (β Pro-B10) (Dickerson & Geis, 1983).

Phe^{46}(CD4). The phenyl side chain of Phe^{46} does not contact the heme group directly but does restrict movement of His^{64}. 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 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 crystal structures of Leu^{46} and Val^{46} aquomet-myoglobins (Lai *et al.*, 1995).

In wild-type metmyoglobin the His^{64} side chain is well-defined in a "down" position with Nϵ accepting a hydrogen bond from coordinated water. Lai *et al.* (1995) have shown that the His^{64} side chain 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 (Figure 4). In the up position, the imidazole ring occupies the space which is normally filled by the Phe^{46} side chain (Lai *et al.*, 1995). An intermediate result is observed for Leu^{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 C_β−C_γ 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 structures of F46V CO- and deoxymyoglobin and is probably the major cause of the 20−30-fold higher rate of hemin loss from this mutant. 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 along the E helix at a position facing 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. Disruption of helix packing probably accounts for the dramatic 20−70-fold increases in k_{-H} produced by the G65V and G65I mutations. However, 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 β-hydroxyl forms a stabilizing electrostatic interaction with the B helix. Nonconservative 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 1 is the lack of effect of amino acid substitutions at Ile^{107}(G8). Neither increasing or decreasing the size of the 107 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 more detailed interpretation.

Hemin Loss from Reconstituted Myoglobin. Time courses showing hemin dissociation from wild type and sperm whale myoglobin reconstituted at pH 7 are shown in Figure 6. The rate of hemin loss from both proteins is ≈1 h^{-1} when assayed at pH 5. 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 co-workers have suggested that when apomyoglobin is reconstituted with hemin, two equal populations of orientational conformers are formed (La Mar *et al.*, 1978, 1983, 1984, 1986; Aojula *et al.*, 1986; Light *et al.*, 1987). These conformers are

defined by the arrangements of the interior heme vinyl and methyl groups. Nuclear magnetic resonance results indicate equal association rate constants for the formation 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 Figures 1−3. This reorientation implies a 20-fold difference in affinity for the two conformers and suggests that the rate of hemin dissociation from the abnormal orientation should be ∼20-fold greater than that from the native conformer.

In our experiment, apomyoglobin was reconstituted with 1 equiv of hemin at pH 7, kept at 0 °C, and assayed within 30 min of preparation. La Mar *et al*. (1984) and Light *et al.* (1987) have shown that no significant hemin reorientation takes place under these conditions. Thus, the freshly reconstituted myoglobin sample was expected to show two rates of hemin loss: ∼20 and 1 h^{-1} at pH 5.0. This result is clearly not observed and suggests that further investigations of heme orientational disorder are needed.

Effects of pH on Hemin Dissociation. The large pH dependence of hemin loss from myoglobin is partly due to protonation of His^{93} and concomitant disruption of the proximal imidazole−Fe bond under acidic conditions. At neutral and high pH, where the extent of His^{93} protonation is effectively zero, the rate of hemin loss from metmyoglobin is very slow and difficult to measure. In most cases the effects of mutagenesis on hemin loss are roughly the same at pH 7 and 5. The ratios of the rate constants at pH 5 to those at pH 7 are ∼100 for most of the proteins listed in Table 1.

The exceptions appear to involve proteins with abnormally large rates of hemin loss (*i.e.*, F43V, F43I, F46A, L89G, L89A, H93G, and H97A myoglobins). In these cases, the ratios of the rate constants at pH 5 and 7 are 5−20 instead of ≥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 coordination with the heme iron atom is less important. Presumably hemin can dissociate from H93G myoglobin either with or without the free base attached. In the case of F46V, the distal histidine is already in the up position, exposing the heme pocket to solvent (Figure 4). In wild-type metmyoglobin, the same upward movement of the distal histidine is observed only at low pH due to protonation of His^{64} (Yang & Phillips, 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.

CONCLUSIONS

The high affinity of myoglobin for hemin results from a combination of factors which include in order of importance: (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.

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 $\pi-\pi^*$ backbonding 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 backbonding electrons and as a result disrupt the $Fe^{3+}-His^{93}$ bond (Traylor & Sharma, 1992; Momenteau & 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 π acceptor and, when bound, is likely to destabilize the His^{93}−iron bond. Reduction to the ferrous state markedly (≥60-fold) strengthens the iron−His^{93} bond, regardless of the presence or absence of a sixth ligand (Hargrove & 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 nonspecific. 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 by 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.*, ≤3−4-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). In the case of sperm whale myoglobin, favorable interaction with the heme-6-propionate has been maximized with an Arg residue at the CD3 position. Replacement with any other residue including Lys causes small increases in k_{-H} (Table 1). However, in the case of human hemoglobin β subunits, the CD3 residue is a serine, and the methoxy side chain is too short to interact with the heme-6-propionate. When β

Ser(CD3) is replaced by His, a 3−4-fold decrease in the rate constant for hemin dissociation is observed with little or no change in the ligand binding properties of the mutant human hemoglobin (Whitaker, 1995). Thus, Table 1 has already provided useful background data for designing more stable recombinant heme proteins.

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