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## Heme-Fe Proteins

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# CHEMICAL REACTIVITY OF THE ACTIVE SITE OF MYOGLOBIN

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## I. Introduction

The discovery of myoglobin in skeletal muscle occurred during the last half of the 19th century though exact attribution is complicated by uncertainty at the time regarding the possible contamination of preparations by hemoglobin and by the difficulty in spectroscopic differentiation of hemoglobin from myoglobin (*1*). The first preparation of myoglobin that was spectroscopically distinguished from hemoglobin




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was obtained from dog muscle by Mörner in 1897 (*2*). Although Mörner referred to this material as myochrome, Günther subsequently proposed the term myoglobin to emphasize the similarity of the protein to hemoglobin (*1, 3*).

Myoglobin is now known to be a protein of about 150 amino acid residues that binds a single protoheme IX prosthetic group through coordination of the heme iron by a histidyl residue and a variety of noncovalent interactions between the protein and the heme. The principal functional role of myoglobin is based on its ability to bind dioxygen reversibly at the heme iron, a property of the heme that results from the environment provided for it by the protein. This fundamental property of myoglobin combined with comparative studies of a wide range of animal species led logically to the view that myoglobin serves a role in oxygen storage (*4, 5*) that is particularly important in diving mammals. Subsequent work led to the suggestion that another role of myoglobin that may be more important in other species is to facilitate the diffusion of dioxygen through muscle tissue to mitochondria (*6–9*). Theoretical (*10, 11*) and experimental (*12*) studies have challenged this view. Surprisingly, a mutant mouse devoid of myoglobin has subsequently been shown to be viable (*13*) owing to the effectiveness of a variety of compensatory physiological processes (*14*). Ironically, therefore, the true biological role of one of the most thoroughly studied proteins remains a matter of continuing investigation.

Because the apparent functional role of myoglobin is reversible binding of dioxygen, a considerable literature has developed that concerns the binding of gaseous ligands to ferromyoglobin and the binding of anionic ligands to ferrimyoglobin (metMb). With the advent of site-directed mutagenesis as a means of manipulating the structure of proteins, increasing attention has been directed toward understanding the chemical reactivity of myoglobin with a particular emphasis on understanding the chemistry of the active site of the protein. In part, this growing activity has arisen from renewed interest in understanding the way in which the protein environment of the heme dictates the chemical reactivity of this prosthetic group.

Myoglobin in many respects is the prototypical example of the larger family of heme containing proteins and enzymes that vary in function from the relatively simple process of reversible binding of an electron to the activation of dioxygen for substrate hydroxylation. The relationship between members of this family of proteins is not based simply on structural similarities but on similarities in chemical reactivity as well. As the structure of myoglobin is relatively simple compared to other heme proteins and as it was the first for which the three-dimensional

structure was determined, myoglobin serves as a model for understanding the manner in which the protein environment determines reactivity of the heme.

Interest in this relationship has led to demonstration that myoglobin can participate in a number of reactions that are related to those catalyzed with far greater efficiency by heme proteins that are true enzymes. In the context of the current volume, this chemical reactivity of the active site of myoglobin is our focus of attention. Studies of the ligand binding properties of myoglobin have been reviewed elsewhere (e.g., *15–17*) and are not considered here. In view of the essential role of molecular genetics in the development of understanding of the chemical reactivity of myoglobin and the anticipation that this role will expand in the future, we begin this survey by considering advances in the engineering of myoglobin structure before considering the chemical reactivity of heme at the active site of myoglobin. In subsequent discussion of the chemistry of the Mb active site, emphasis is given in many cases to consideration of studies involving variants of Mb and references are provided to reviews of related literature that does not involve variants.

## II. Cloning and Expression of Recombinant Myoglobin

Prior to the development of recombinant DNA technology, most functional studies of myoglobin concerned protein isolated from sperm whale and horse heart muscle because these sources were abundant and they were available from commercial sources. With the cloning of cDNAs for human (*18–21*), porcine (*22*), mouse (*23*), grey seal (*24, 25*), bovine (*26*), icefish (*Chionodraco rastrospinosus, 27*), and mollusk (*Aplysia limacina* (*28*) and *Biomphalaria glabrata* (*29*)) myoglobins,[1] expression systems have been developed for production of the human, porcine, and *A. limacina* proteins in *Escherichia coli* and for bovine myoglobin in yeast (*Saccharomyces cerevisiae*) (*19, 26, 28, 35, 36*). In cases for which a cDNA has not been isolated, synthetic genes have been prepared and overexpressed. This approach has been used to prepare recombinant sperm whale (*37, 38*) and horse (*39*) Mbs.[2] Amino acid sequences for the proteins that have been studied most extensively in recombinant form and for which the greatest number of variants has

[1] cDNAs for a new class of Mb with close similarities to the heme enzyme indoleamine dioxygenase have also recently appeared (*30–34*).

[2] For sperm whale Mb, an error (corresponding to a D122N mutation) was incorporated into the synthetic gene (*37*) and, in this case, the recombinant wild-type sequence (*37*) is not identical to that of the authentic wild type.

```
                    10        20        30        40        50        60
                    |         |         |         |         |         |
Human               GLSDGEWQLVLNVWGKVEADIPGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASED
Porcine             GLSDGEWQLVLNVWGKVEADVAGHGQEVLIRLFKGHPETLEKFDKFKHLKSEDEMKASED
Horse heart         GLSDGEWQQVLNVWGKVEADIAGHGQEVLIRLFTGHPETLEKFDKFKHLKTEAEMKASED
Sperm whale         VLSEGEWQLVLHVWAKVEADVAGHGQDILIRLFKSHPETLEKFDRFKHLKTEAEMKASED

                    70        80        90        100       110       120
                    |         |         |         |         |         |
Human               LKKHGATVLTALGGILKKKGHHEAEIKPLAQSHATKHKIPVKYLEFISECIIQVLQSKHP
Porcine             LKKHGNTVLTALGGILKKKGHHEAELTPLAQSHATKHKIPVKYLEFISEAIIQVLQSKHP
Horse heart         LKKHGTVVLTALGGILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISDAIIHVLHSKHP
Sperm whale         LKKHGVTVLTALGAILKKKGHHEAELKPLAQSHATKHKIPIKYLEFISEAIIHVLHSRHP

                    130       140       150
                    |         |         |
Human               GDFGADAQGAMNKALELFRKDMASNYKELGFQG
Porcine             GDFGADAQGAMSKALELFRNDMAAKYKELGFQG
Horse heart         GDFGADAQGAMTKALELFRNDIAAKYKELGFQG
Sperm whale         GDFGADAQGAMNKALELFRKDIAAKYKELGYQG
```

FIG. 1. Alignment of amino acid sequences for human (*18, 19, 21*), porcine (*22, 36*), horse heart (*39*), and sperm whale (*37, 38*) myoglobins.

been produced (human, porcine, horse, and sperm whale) are aligned in Fig. 1.

Compared with recombinant expression of most proteins, expression of heme proteins presents the additional challenge that arises from the need to coordinate synthesis of the prosthetic group with synthesis of the protein and from the chemical reactivity of heme. For example, significant quantities of sulfmyoglobin (*vide infra*) are generated during expression of horse heart and sperm whale Mbs in *E. coli* (*42, 43*). Other anomalous spectroscopic features have also been noted for recombinant human hemoglobin (*44*) and for recombinant cytochrome *c* peroxidase (*45*). These observations illustrate the necessity of rigorous characterization of recombinant heme proteins to ensure that subsequent functional studies are not influenced by the presence of unanticipated protein derivatives resulting from recombinant expression or from mutations of the protein.

## III. Active Site Variants of Myoglobin

With the development of bacterial expression systems for Mb described earlier and with the known diversity of metal coordination

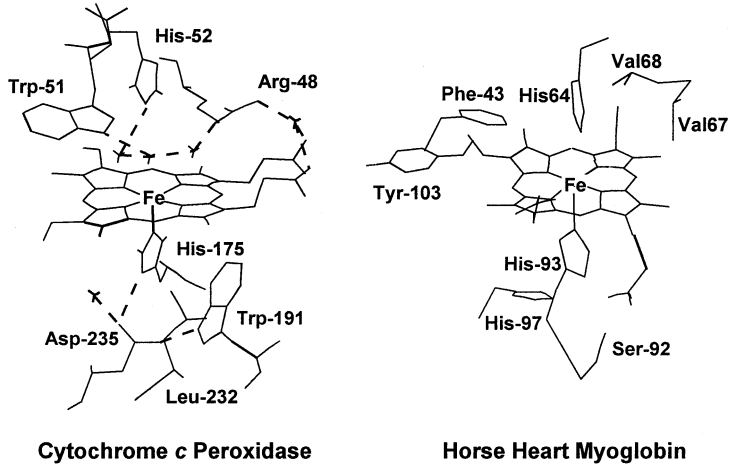


FIG. 2. Comparison of the heme environments at the active sites of horse heart myoglobin (*40*) and yeast cytochrome *c* peroxidase (*41*).

environments among heme proteins (*46–48*), considerable attention has been focused on examining the role of the axial ligands to the heme iron in determining the spectroscopic and functional properties of the protein. Although it is clear that the active site coordination environments of cytochromes P450, peroxidases, catalases, globins, and other heme proteins differ significantly from each other, the extent to which this difference contributes to the functional diversity of this family of proteins relative to the contribution provided by other structural attributes of the active sites of these proteins is not fully understood (*47–49*). For the purpose of comparison, critical residues in the heme binding pocket of horse heart Mb and yeast cytochrome *c* peroxidase are illustrated in Fig. 2. As a result, considerable interest has been directed toward changing the coordination environment of Mb in an attempt to mimic the diverse range of spectroscopic and chemical reactivities exhibited by other heme proteins. Although it is undeniably naïve to expect that identity of axial ligands alone is sufficient to reproduce the spectroscopic and functional properties of one heme protein through mutagenesis of another, such work inevitably produces new, interesting, and informative variants that provide a logical starting point for more subtle and sophisticated protein engineering efforts. For these reasons and others, a significant fraction of the myoglobin variants that have been reported at present involve modification of the heme binding pocket. As the chemical reactivity of the myoglobin active site can respond significantly to amino acid substitutions in this region of the protein, it is useful to begin by surveying the active site variants for the species

of Mb that have received the greatest attention (sperm whale, horse heart, and human Mb) and by considering some of the consequences of these structural modifications.

### A. Mutagenesis of the Distal Heme Binding Pocket

The distal H64 residue of Mb has been investigated extensively by site-directed mutagenesis. The emphasis of most of this effort has concerned the participation of this residue in ligand binding reactions. These studies have been reviewed in detail elsewhere (*16*) and are beyond the scope of the current discussion. The simplest mechanism by which replacement of H64 influences axial ligation results when residues incapable of H-bonding interactions are introduced at this position. Evidently, the hydrogen bond normally formed by H64 and the water molecule coordinated as the sixth ligand is sufficiently important to the stability of water coordination that such variants typically exhibit five-coordinate, high-spin metMb derivatives (*50*) with altered ligand binding properties (*16*). In a few cases, on the other hand, substitution of H64 with appropriate residues may result in coordination of the new residue to the heme iron. Variants of this type are generally less reactive in the reactions considered in this review, but they provide useful spectroscopic species for comparative studies with other types of heme proteins.

Distal pocket substitutions of various species of Mb in which a new distal ligand is provided by the protein include V68H, H64V/V68H (*51–56*), H64Y (*57–62*), V68E (*57, 63–65*), and possibly the H64R variant of sperm whale Mb (*60*). For the ferric H64Y variants of sperm whale and horse heart Mbs, electronic (*57, 61*), EPR (*57, 61, 62*), and resonance Raman (*57, 60*) spectroscopy, XANES (*61*), and X-ray crystallographic results (*62*) are all consistent with a six-coordinate species in which direct coordination of the distal tyrosyl residue occurs; however, this ligand is not coordinated following reduction of the heme iron (*57*). In this regard, the H64Y variants reproduce the behavior of HbM Saskatoon (*66, 67*) in which the distal histidyl residue of human hemoglobin is replaced with a tyrosyl residue. Demonstration of carboxylate coordination to the heme iron was provided independently by electronic (*57, 63, 64*), EPR (*57, 65*), and NMR (*64, 65*) spectroscopy of the V68E variants of sperm whale (*57*) and human (*63, 64*) Mbs. These results correspond to behavior of HbM Milwaukee, in which a valyl residue in the distal pocket is replaced with a glutamyl residue. MetHbM Milwaukee is known (*68*) to possess a high-spin, six-coordinate heme iron with a distal glutamyl residue providing the sixth ligand. As is

the case for HbM Saskatoon, this variant ligand dissociates from the iron upon reduction. Coordination of an argininyl residue has been suggested from the low-spin characteristics in the resonance Raman spectrum of the H64R variant of sperm whale metMb (*60*). However, as there is no unambiguous precedent for a heme protein with a coordinated argininyl residue, the spectroscopic criteria for establishing this coordination environment are insufficient to provide a compelling argument.

### B. Mutagenesis of the Proximal Heme Binding Pocket

Preparation of Mb variants in which the proximal H93 ligand is substituted is intrinsically more difficult than preparation of distal ligand variants. In all cases of which we are aware, the H93 variants are expressed as the apo-protein and require reconstitution with exogenous heme during purification. The first successful preparation of a proximal ligand variant of Mb was reported in 1990 (*57*) for the H93Y variant of sperm whale Mb. Subsequently, H93Y variants of both human (*69–71*) and horse (*72*) Mbs were reported. The spectroscopic properties of the ferric derivatives of these variants are uniformly consistent. The electronic spectra (sperm whale (*57*), human (*70*), horse (*72*)), EPR spectra (sperm whale (*57*), human (*70*), horse (*72*)), and resonance Raman spectra (sperm whale (*57*) and human (*70*)) are in agreement and are consistent with the presence of a high-spin, five-coordinate iron with tyrosine providing the fifth axial ligand. The tyrosine coordination and absence of a distally coordinate water molecule were confirmed by X-ray diffraction analysis of the H93Y variant of horse heart Mb (*72*). Interestingly, NMR spectra for this variant were consistent with rotation of the phenolate ligand on the time scale of the NMR experiment, so the crystal structure, therefore, probably represents one of a number of possible conformational orientations. NMR spectra of the corresponding variant of human Mb, however, are not consistent with phenolate rotation. This species-specific difference in behavior has been attributed (*72*) to a difference in the residue present at position 142 and emphasizes the subtle distinctions that can exist between closely related proteins.

Introduction of thiolate ligation at position 93 has also been reported (*69, 70, 73*). Again subtle species-specific differences in sequence on the proximal side of the heme binding pocket result in significant differences in behavior between closely related proteins. In this case, the spectroscopic properties of the human H93C variant (*69, 70*) were consistent with the presence of five-coordinate, cysteine-coordinated high-spin Fe(III). On the other hand, similar studies of the corresponding

horse heart metMb variant (*73*) failed to indicate thiolate coordination under any conditions. In this case, the principal spectroscopic component probably comprises a five-coordinate, heme iron with a coordinated water molecule. Proximal cysteinyl coordination in the horse heart protein could be achieved, however, through concurrent replacement of the distal H64 residue with apolar valyl and isoleucyl residues (*73*) that are known (*50*) to disfavor distal coordination of a water molecule. In these double variants, the heme iron presumably lacks a distal ligand and is displaced toward the proximal C93 residue. Unfortunately, none of the H93C variants of either human or horse Mb retained the proximal ligand upon reduction of the heme iron, so the complex formed with carbon monoxide fails to exhibit a Soret maximum at 450 nm that is characteristic of cytochromes P450. In fact, spectroscopic evidence suggests that upon reduction of the H93C (and H93Y) variants of the human protein, not only does the proximal cysteinyl ligand dissociate from the heme iron, but the distal histidyl residue becomes the fifth ligand (*69, 70*).

An alternative strategy for introduction of alternative proximal ligands involves replacement of H93 with the glycyl residue to produce a so-called proximal "cavity" variant (H93G) (*74–80*) in which the proximal ligand may consist of a hydroxyl group (*78, 79*). The advantage of this variant is that it can be functionalized conveniently by the introduction of other small molecules (**X**) that can coordinate to the iron (*74*). In addition to imine ligands (**X** = imidazole, pyridine), this exchange strategy has been used to introduce both phenolate (**X** = phenol) and thiolate (**X** = ethanethiol) groups that closely resemble the spectroscopic features of the ferric catalases and cytochromes P450, respectively.

## IV. Electron Transfer Reactions of Myoglobin

### A. Electrochemistry

Perhaps the most fundamental functional property of a heme prosthetic group at the active site of a heme protein is the relative stability of the reduced and oxidized states of the heme iron. A number of structural characteristics of the heme binding environment provided by the apo-protein have been identified as contributing to the regulation of this equilibrium and have been reviewed elsewhere (*82–84*). Although a comprehensive discussion of these factors is not possible in the space available here, they can be summarized briefly. The two most significant influences of the reduction potential of the heme iron appear to be the dielectric constant of the heme environment (*81, 83*) and the chemical

identity of the axial ligands of the heme iron (*81*). These characteristics are further modified by the orientation and hydrogen bonding interactions of the axial ligands (*85–89*) and by the orientation of the heme group in the heme binding pocket (*90*). In addition, the orientation of the heme vinyl groups and the hydrogen bonding interactions of the heme propionate groups have also been implicated as modulating influences (*91–93*). Although semiquantitative correlation of these structural features with reduction potentials is possible in some cases, comprehensive, quantitative understanding of all contributions has been elusive. Most notably, unambiguous methods for quantification of the dielectric of the heme pocket are not established. The primary experimental problem, however, has been the difficulty in varying any one of these characteristics individually without simultaneously changing another. For this reason, it is imperative that any attempt to address this issue entail a rigorous characterization of the variant or chemically modified protein to ensure that some unanticipated structural consequence of the modification will not be overlooked.

*1. Methods*

The first electrochemical studies of Mb were reported for the horse heart protein in 1942 (*94*) and subsequently for sperm whale Mb (e.g., *95*) through use of potentiometric titrations employing a mediator to achieve efficient equilibriation of the protein with the electrode (*96*). More recently, spectroelectrochemical measurements have also been employed (*97, 98*). The alternative methods of direct electrochemistry (*99–102*) that are used widely for other heme proteins (e.g., cytochrome *c,* cytochrome $b_5$) have not been as readily applied to the study of myoglobin because coupling the oxidation–reduction equilibrium of this protein to a modified working electrode surface has been more difficult to achieve. As a result, most published electrochemical studies of wild-type and variant myoglobins have involved measurements at equilibrium rather than dynamic techniques.

Recent work has resolved some of the issues that complicate direct electrochemistry of myoglobin, and, in fact, it has been demonstrated that Mb can interact effectively with a suitable electrode surface (*103–113*). This achievement has permitted the investigation of more complex aspects of Mb oxidation–reduction behavior (e.g., *106*). In general, it appears that the primary difficulty in performing direct electrochemistry of myoglobin results from the change in coordination number that accompanies conversion of metMb (six-coordinate) to reduced (deoxy) Mb (five-coordinate) and the concomitant dissociation of the water molecule (or hydroxide at alkaline pH) that provides the distal ligand to the heme iron of metMb.

A summary of reduction potentials reported for the Fe(III)/Fe(II) couple for wild-type and variant forms of horse, human and sperm whale Mbs is given in Table I. As indicated earlier, the structural characteristics of a heme protein that exert the greatest influence on the reduction potential are the axial ligands provided to the metal ion and the dielectric constant of the heme environment. For this reason, we first consider the results presented in Table I for variants involving changes in the distal and proximal ligand and then consider results for variants in which the electrostatic properties of the heme pocket have been changed without replacement of the axial ligands. Emphasis on axial ligation in this survey reflects the interest in comparison of such variants with the behavior of heme proteins that possess axial ligands different from those of wild-type myoglobin and reflects the dominance of electrochemical information for such variants in the literature.

*2. Electrochemistry of Variants with an Altered Distal Ligand*

The H64 distal ligand of wild-type myoglobin does not coordinate to the heme iron in either the reduced or the oxidized form of the native protein but stabilizes the coordination of a distally bound water molecule of metMb. Replacement of H64 with other amino acid residues can, therefore, change the coordination environment of the heme iron in two ways. Such variants either may possess a distal residue that is able to coordinate to the heme iron or may possess a distal residue that is incapable of either coordinating to the iron or of forming a hydrogen bond with a coordinated water molecule.

The H64Y variant of Mb is an example of the former situation in that the tyrosyl side chain coordinates to the heme iron of the oxidized variant. As expected for a variant with an anionic phenolate ligand, the reduction potential of this variant is ~40 mV lower than that of the wild-type protein (Table I). Although this change is consistent with stabilization of the oxidized form of the protein, the fact that the tyrosyl ligand is not coordinated in the reduced protein complicates quantitative interpretation of this shift in potential.

On the other hand, the H64L, H64V, and H64F variants constitute a group of proteins in which H64 is replaced by residues with side chains that are incapable of coordinating to the heme iron atom and that do not stabilize a distally coordinated water molecule through hydrogen bonding interactions. As a result, both the metMb and deoxyMb derivatives of these variants are five-coordinate. Interestingly, the reduction potentials of these variants are all somewhat greater than that for the wild-type protein and are within ~30 mV of each other ($E^\circ = 76$–$109$ mV) in both oxidation states. Interestingly, the H64G variant exhibits a

coordination chemistry and midpoint potential that are virtually identical to those of the wild-type protein. A common characteristic of all the H64 variants is that their electron transfer activity as estimated from direct electrochemical measurements is enhanced relative to wild-type Mb. As observed by Van Dyke *et al.* (*106*), Mb electron transfer at the electrode surface appears to be controlled not only by oxidation-state-dependent changes in coordination number (from six- to five-coordinate) but also by the ease with which these changes are communicated to the surface of the molecule through the hydrogen bond network that links His64 to the bulk solvent (*106*). For wild-type protein, the change in oxidation state is linked through the hydrogen-bond network to the reorganization of bulk solvent; for the variants, this is not the case, and electron transfer is more facile.

In some cases, axial ligation may be modified by substitution of residues other than those that provide the axial ligands in the wild-type protein. For example, replacement of V68 with a histidyl residue results in coordination of H68 to the heme iron in both oxidation states of the protein, as is the case for cytochrome $b_5$. This behavior has been established both for the single variant (V68H) of the horse heart protein (*51*) and for the double variant H64V/V68H of the human and porcine proteins (*52*). Although the reduction potentials for the human H64V/V68H and horse V68H variants are similar to each other ($-128$ and $-110$ mV, respectively; Table I), they are 170 to 190 mV lower than the values of corresponding wild-type proteins. Notably, these potentials are much lower than that of microsomal cytochrome $b_5$ ($+5$ mV (*122*)) even though the heme group of the cytochrome is more exposed to bulk solvent at the surface of the protein. Furthermore, EPR data (*51*) indicate that the planes of the axial imidazole ligands in the variant Mb are presumably oriented perpendicular to each other (*123–132*), which should increase the potential as much as 50 mV relative to the parallel orientation present in cytochrome $b_5$.

### *3. Electrochemistry of Variants with an Altered Proximal Ligand*

Evaluation of the contribution made by the proximal ligand to the oxidation-reduction equilibrium of Mb (H93 in the wild-type protein (Fig. 2)) has been more difficult because substitution of the proximal residue results in expression of apo-Mb without heme incorporation. All of the available data for these variants (Table I), therefore, derive from proteins prepared by reconstitution of purified recombinant apoprotein with exogenous heme (*69, 70, 72*). In those cases where trace quantities of native Mb are produced (*73*), heme extraction followed by reconstitution was undertaken to eliminate complications from sulfMb

TABLE I

REDUCTION POTENTIALS FOR SITE-DIRECTED VARIANTS OF HORSE, HUMAN, AND SPERM WHALE MBS

| Derivative | Source | Variant | E°[a] | Axial ligation[b] | | Methods[c] | Conditions | Ref. |
|---|---|---|---|---|---|---|---|---|
| | | | | *Oxidized* | *Reduced* | | | |
| MetMb[d] | Horse | T39I/K45D/F46L/I107F | 24 | His/$H_2O$ | His/– | S | Sodium phosphate, $I$ = 0.1 M, pH 6.0, 25.0°C | *116* |
| | | H64Y | 20 | His/Tyr[e] | His/– | S | Sodium phosphate, $I$ = 0.1 M, pH 7.0, 25.0°C | *117* |
| | | H64V | 87 | His/– | n.d. | S | Sodium phosphate (46 mM), pH 7.0, 25.0°C | *73* |
| | | H64I | 95 | His/– | n.d. | S | Sodium phosphate (46 mM), pH 6.0, 25.0°C | *73* |
| | | H64V/H93C | –217 | Cys/– | n.d. | P | Sodium phosphate (46 mM), EDTA (10 mM), pH 8.0, 25.0°C | *73* |
| | | H64I/H93C | –219 | Cys/– | n.d. | P | Sodium phosphate (46 mM), EDTA (10 mM), pH 8.0, 25.0°C | *73* |
| | | V67R | 106 | His/$H_2O$ | His/– | S | Sodium phosphate, $I$ = 0.1 M, pH 6.0, 25.0°C | *118* |
| | | V67A/V68S | –23 | His/$H_2O$[f] | His/– | S | Sodium phosphate, $I$ = 0.1 M, pH 7.0, 25.0°C | *119* |
| | | V68H | –110 | His/His | His/His | S | Sodium phosphate, $I$ = 0.1 M, pH 7.0, 25.0°C | *51* |
| | | S92D | 72 | His/$H_2O$[f] | His/– | S | Sodium phosphate, $I$ = 0.1 M, pH 6.0, 25.0°C | *120* |
| | | H93Y | –208 | Tyr/–[f] | n.d | P | Sodium phosphate (35 mM), EDTA (10 mM), pH 8.0, 25.0°C | *72* |
| MetMb[g] | Human | V68E | –137 | His/Glu | His/– | S | Sodium phosphate, $I$ = 0.1 M, pH 7.0, 25.0°C | *63, 64* |
| | | V68D | –132 | His/$H_2O$ | His/– | S | Sodium phosphate, $I$ = 0.1 M, pH 7.0, 25.0°C | *63, 64* |
| | | V68N | –24 | His/$H_2O$ | His/– | S | Sodium phosphate, $I$ = 0.1 M, pH 7.0, 25.0°C | *63, 64* |
| | | H64V/V68H | –128 | His/His[h] | His/His | R | Phosphate (0.1 M), pH 7, 20°C | *52* |
| | | H93Y | –190 | Tyr/– | –/His[i] | T | Not reported | *69, 70* |
| | | H93C | –230 | Cys/– | –/His[i] | T | Not reported | *69, 70* |
| MetMb[j] | Sperm whale | H64L | 83 | His/–[k] | His/–[k] | P | Phosphate (50 mM), pH 7.0, 25°C | *121* |
| | | H64L | 84 | His/–[k] | His/–[k] | CV | Hepes (0.1 M), pH 7.0, 22°C | *106* |
| | | H64L/F43H | 88 | His/$H_2O$[f] | His/– | P | Phosphate (50 mM), pH 7.0, 25°C | *121* |
| | | H64L/I107L | 60 | *** | n.d. | P | Phosphate (50 mM), pH 7.0, 25°C | *121* |

| | | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | | H64L/L29H | −22 | His/$H_2O$[f] | His/– | P | Phosphate (50 mM), pH 7.0, 25°C | *121* |
| | | H64G | 65 | His/$H_2O$[k] | His/– | CV | Hepes (0.1 M), pH 7.0, 22°C | *106* |
| | | H64L | 84 | His/– | His/– | CV | Hepes (0.1 M), pH 7.0, 22°C | *106* |
| | | H64V | 76 | His/–[k] | His/– | CV | Hepes (0.1 M), pH 7.0, 22°C | *106* |
| | | H64M | 98 | His/– | His/– | CV | Hepes (0.1 M), pH 7.0, 22°C | *106* |
| | | H64F | 109 | His/– | His/– | CV | Hepes (0.1 M), pH 7.0, 22°C | *106* |
| CyanometMb[l] | Horse | V67R | −392 | His/$CN^-$ | His/–[n] | CV | Tris/cacodylate, pH 7.0, $I = 0.1$ M, 20°C | *118* |
| | | V68H | –257 | His/$CN^-$ | His/–[n] | CV | Tris/cacodylate, pH 7.0, $I = 0.1$ M, 20°C | *118* |
| | | S92D | −412 | His/$CN^-$ | His/–[m] | CV | Tris/cacodylate, pH 7.0, $I = 0.1$ M, 20°C | *118* |

[a] mV vs SHE.

[b] From analysis of various spectroscopic data unless otherwise stated. Given as proximal/distal.

[c] S, spectroelectrochemistry; P, photochemical reduction; T, reductive titration; CV, cyclic voltammetry; R, redox potentiometry.

[d] Values of $E^\circ$ for wild-type horse Mb have been reported as 61 mV (pH 7.0, $I = 0.1$ M, 25.0°C), 64 mV (pH 6.0, $I = 0.1$ M, 25.0°C), and 45 mV (pH 8.0, $I = 0.1$ M, 25.0°C) (*114*); 46 mV (pH 6.95, phosphate, $I = 0.2$ M, 30°C) (*94*); 46 mV (pH 7.0, 0.1 M phosphate, 0.1 M NaCl) (*115*); 19 mV (pH 7.1, 0.05 M MOPS, 0.1 mM EDTA, 20°C) (*98*).

[e] Determined by X-ray crystallography (*62*).

[f] Determined by X-ray crystallography (*119*).

[g] Values of $E^\circ$ for the wild-type protein have been reported as 59 mV (pH 7.0, $I = 0.1$ M, 25.0°C) (*63*); 50 mV (conditions not reported) (*69*).

[h] Determined from analysis of spectroscopic data. X-ray crystallographic information (*52*) for the corresponding H64V/V68H variant in porcine Mb confirmed bis-histidine axial ligation in the ferric derivative.

[i] Distal ligand proposed as arising from ligation of His64.

[j] Values for wild-type sperm whale Mb have been reported as 59 mV (pH 7.0, $I = 0.1$ M, 25.0°C) (*63*); 59 mV (0.1 M Hepes, pH 7.0, 22°C) (*106*); 52 mV (0.05 mM phosphate, pH 7.0, 25°C) (*121*); 47 mV (pH 7, 30°C) (*95*); 14 mV (pH 7.1, 0.05 M MOPS, 0.1 mM EDTA, 20°C) (*98*).

[k] Determined by X-ray crystallography (*50*).

[l] Values of E° for the wild type protein have been reported as −385 mV (pH 7.0, $I = 0.1$ M, Tris/cacodylate, 20°C) (*113*).

[m] Thermodynamic product. The initial product of the reaction is Fe(II)–$CN^-$ (*113*).

[n] The nature of reduced form in this variant (His/His or His/– ) is not known with certainty. However, CO is known to displace the distal histidine ligand in ferrous V68H, and it is, therefore, likely that the reduced form in these experiments is deoxy Mb (His/–).

(Section IX) formation to ensure homogeneity of the protein sample. This approach has been required, for example, in characterization of H93C and H93Y variants of horse heart and human Mb (*69, 72, 133*).

Replacement of histidine 93 with a tyrosyl residue (H93Y variant) in horse and human Mb leads to a dramatic decrease in reduction potential (−208 and −190 mV respectively; Table I). This finding can be understood in terms of stabilization of the oxidized protein by the anionic (electron-donating) properties of the phenolate ligand. The corresponding cysteine variant (H93C) of human Mb has a reduction potential (−230 mV) that is, again, consistent with increased electron density on the metal. This value is intermediate between that of the high-spin, five-coordinate derivative of cytochrome $P450_{cam}$ (−170 mV (*134*)) and the low-spin, six-coordinate derivative of the enzyme (−270 mV (*134*)). Similarly, the H64V/H93C and H64I/H93C variants of horse heart Mb, in which coordination of cysteine to the iron has been established (*73*), exhibit reduction potentials of −217 and −219 mV, respectively.

### *4. Electrochemistry of Variants with an Electrostatically Altered Heme Binding Pocket*

Electrostatic interactions on the surface and the interior of the protein can modulate the electrochemical properties of the heme center, although the magnitude of the effect of surface electrostatic changes is more variable (*83*). Nevertheless, several attempts to rationalize these effects in terms of Coulombic interactions in other proteins have been reported (e.g., cytochrome $b_5$ (*135*) and high potential iron protein (*136, 137*)). However, the effects of surface electrostatic charges are not explained so simply for other proteins (e.g., cytochrome *c* (*83*) and ruberythrin (*138*)). The first report to consider electrostatic modulation of the oxidation–reduction equilibrium of myoglobin involved investigation of a human Mb variant in which the hydrophobic V68 residue (Fig. 2) was replaced with anionic (V68E and V68D variants) and neutral (V68N variant) residues (*63, 64*). In this work, the V68D and V68E variants exhibited reduction potentials 200 mV lower than that of the wild-type protein, and the V68N variant exhibited a potential ~80 mV lower than wild-type Mb (Table I). Although the magnitude of these changes is difficult to explain quantitatively, the direction of the change for the variants with an acidic residue at this position can be understood qualitatively in terms of destabilization of the reduced protein. The behavior of the V68E variant is, however, complicated by coordination of the glutamate side chain to the heme iron in the ferric form of the protein. This is not the case, however, for the V68D variant, suggesting that the observed decrease in potential is purely a reflection of the electrostatic nature of this residue.

Seemingly complementary experiments involving horse heart metMb, on the other hand, are not readily explained (*120*). In this case, replacement of the proximal S92 residue with an aspartyl residue (S92D) increases the potential by 8 mV relative to the potential of the wild-type protein. This is a surprising result, particularly insofar as the S92 side chain is in contact with the proximal H93 heme iron ligand in the wild-type protein (Fig. 2). An extensive spectroscopic analysis of the possible basis for the increase in potential revealed that a number of small but significant and unanticipated secondary alterations are induced by this substitution. These changes include alteration in solvent accessibility of the heme, the p$K_a$ of H97, the orientation of the axial ligands, and the hydrogen bonding properties of the proximal ligand. Evidently, these changes in structure exert mutually compensating influences on the oxidation–reduction equilibrium of the protein to result in a relatively small increase in potential. This example emphasizes the importance of assessing all the functional and spectroscopic properties of a new variant and not simply those of immediate interest.

The effect of electrostatic modifications and the role of charge compensation on the electrochemical properties of cyanmetMb (Table I) have been addressed through analysis of a series of horse heart Mb variants in which both proximal (S92D variant) and distal (V68H and V67R variants) amino acids were replaced with titratable residues (*118*). The midpoint potential of the S92D variant is 8 mV higher than that of the wild-type protein, while the potential of the V67R variant is 42 mV higher. On the other hand, the cyanide complexes of the S92D and V67R variants exhibit potentials that are 27 and 7 mV lower than that of wild-type cyanometMb, respectively. These results have been interpreted in terms of a thermodynamic driving force for electroneutrality that helps to compensate for the additional charge within the active site introduced by mutagenesis. Unfortunately, analysis of this type was not possible for the V68H variant owing to the more profound alteration in coordination environment of this variant in the oxidized form.

### *5. Electrochemistry of Higher Oxidation States of Myoglobin*

Although electrochemical studies of the Fe(III)/Fe(II) couple are of considerable interest in understanding many electron transfer reactions of heme proteins, the catalytic activities of these proteins involve higher oxidation states of these proteins. Rigorous understanding of the thermodynamics of these reactions requires knowledge of the potentials for compound II/Fe(III) (i.e., Fe(IV)═O/Fe(II)) and compound I/compound II (i.e., Fe(IV)═O$^{\cdot}$/Fe(IV)═O) equilibria. The reduction potentials for interconversion of these forms of heme proteins are experimentally challenging because they are generally quite high (0.8–1.0 V)

and because they are usually quite similar in value to each other. At present, these values have been reported only for wild-type sperm whale Mb (0.887 V (20°C) (*139, 140*); 0.896 V (pH 7.0, 15°C) (*141*)). Similar studies of selected variants would contribute significantly to our understanding of the catalytic activities exhibited by these variants and are discussed further in the discussion that follows.

### B. Electron Transfer Kinetics

The kinetics of myoglobin oxidation and reduction have been studied by a variety of experimental techniques that include stopped-flow kinetics, pulse radiolysis, and flash photolysis. In considering this work, attention is directed first at studies of the wild-type protein and then at experiments involving variants of Mb.

#### *1. Stopped-Flow Kinetics*

Stopped-flow kinetics studies of metMb reduction have investigated reduction of the protein by a number of inorganic and organic reducing agents. For example, Fleischer and co-workers studied the anaerobic reduction of metMb by $[Cr(H_2O)_6]^{2-}$ (*142*) while others used dithionite (*143–145*), $[Fe(CDTA)]^-$ (*146*) and $[Fe(EDTA)]^{2-}$ (*147, 148*), $[Fe(bpy)]^{2+}$ and $[Fe(NTA)]^{2+}$ (*149*), and ascorbate (*150*). Each of these studies revealed different aspects of the reaction. For example, dithionite reduction of various ligand-bound complexes of metMb at alkaline pH was found to require dissociation of the bound ligand prior to reduction of the iron by $SO^{2-}$ radical, except for the imidazole and cyanide complexes, which were reduced prior to ligand dissociation (*144*). On the other hand, at acidic pH, only the cyanide complex was found to undergo reduction prior to ligand dissociation (*143*). The similarity in reduction potentials of Mb and $Fe(CDTA)^-$ permitted use of this reagent to study the reduction of the cyanide and nitric oxide complexes of metMb and the oxidation kinetics of $MbO_2$ (*146*). The kinetics by which $Fe(EDTA)^{2-}$ reduces metMb were analyzed by Marcus theory to demonstrate the relative inefficiency of Mb in electron transfer relative to cytochromes (*147*) and to study the thermodynamics and pH dependence of reaction (*148*).

#### *2. Pulse Radiolysis*

Pulse radiolysis has also been used to study the reduction of various Mb derivatives by hydrated electrons (*151–154*). With this technique, it was possible to study reduction of ligand-bound forms of metMb at cryogenic temperature and thereby identify reduced, ligand-bound forms of the protein (*152*) and to reduce oxyMb to produce ferryl (Fe(IV)═O)