The H93G Myoglobin Cavity Mutant as a Versatile Template for Modeling Heme Proteins: Ferrous, Ferric, and Ferryl Mixed-Ligand Complexes with Imidazole in the Cavity

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One of the difficulties in preparing accurate ambient-temperature model complexes for heme proteins, particularly in the ferric state, has been the generation of mixed-ligand adducts: complexes with different ligands on either side of the heme. The difference in the accessibility of the two sides of the heme in the H93G cavity mutant of myoglobin (Mb) provides a potential general solution to this problem. To demonstrate the versatility of H93G Mb for the preparation of heme protein models, numerous mixed-ligand adducts of ferrous, ferric, and ferryl imidazole-ligated H93G (H93G(Im) Mb) have been prepared. The complexes have been characterized by electronic absorption and magnetic circular dichroism (MCD) spectroscopy in comparison to analogous derivatives of wild type Mb. The starting ferric H93G(Im) Mb state spectroscopically resembles wild-type ferric Mb as expected for a complex containing a single imidazole in the proximal cavity and water bound on the distal side. Addition of a sixth ligand to ferric H93G(Im) Mb, whether charge neutral (imidazole) or anionic (cyanide and azide), results in formation of six-coordinate low-spin complexes with MCD characteristics similar to those of parallel derivatives of wild-type ferric Mb. Reduction of ferric H93G(Im) Mb and subsequent exposure to either CO, NO, or O_2 produces ferrous complexes (deoxy, CO, NO, and O_2) that consistently exhibit MCD spectra similar to the analogous ferrous species of wild-type ferrous Mb. Most interestingly, reaction of ferric H93G(Im) Mb with H_2O_2 results in the formation of a stable high-valent oxoferryl complex with MCD characteristics that are essentially identical to those of oxoferryl wild-type Mb. The generation of such a wide array of mixed-ligand heme complexes demonstrates the efficacy of the H93G Mb cavity mutant as a template for the preparation of heme protein model complexes.

Introduction

The desire to produce structural and mechanistic models for heme-containing proteins has led researchers to investigate both synthetic models as well as natural protein systems. Synthetic models allow researchers to focus exclusively on the heme group and its direct interaction with axial ligands. While these models offer an excellent starting point in the characterization of unknown heme systems, they are necessarily incomplete. The protein environment surrounding the heme prosthetic group is known to be as important as the oxidation and coordination state of the iron in the determination of enzymatic activity. For example, in peroxidase systems such as horseradish peroxidase (HRP) and cytochrome *c* peroxidase (CCP), the distal His/Arg dyad assists in the heterolytic cleavage of the bound peroxide O−O bond to form the high-valent intermediate known as compound I.[1,2] Additionally, steric constraints in the distal pocket restrict solvent access to the active intermediate directing protein function toward substrate oxidation rather than oxygenation.[3−5] Clearly the protein environment strongly influences the detailed spectral as well as catalytic properties of the heme iron prosthetic group. Consequently, more sophisticated models are needed to better mimic the heme center within the protein system.

To this end, the technique of site-directed mutagenesis has been invaluable. As catalytically and structurally important residues in a protein are identified, researchers can reengineer the protein by mutating key residues. Conversion of the CCP active site into a cytochrome P450-like active site has recently been accomplished by Sigman and co-workers by mutating two proximal CCP residues (His175 and Asp235) into Cys and Leu, respectively.[6] The resulting H175C/D235L CCP double mutant spectroscopically resembles substrate-bound P450−CAM in the ferric state, indicating the potential of that system as a P450 model. This redesign strategy has also produced models for wild-type CCP in F221W HRP[7] and for catalase in H93Y myoglobin (Mb).[8]

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Cavity mutants are a new class of reengineered proteins in which amino acid residues having large side chains have been replaced with residues having small side chains such as glycine and alanine. This creates a cavity that can accommodate exogenous ligands. In cases where the deleted amino acid side chain has a specialized function, exogenous ligands are sometimes able to occupy the cavity and reconstitute the function deleted by the mutation. This rescue of activity has been observed for the cavity mutants of azurin,[9] carbonic anhydrase,[10] hexose-1-phosphate uridyltransferase,[11] and several heme proteins.[12−16] In the cavity mutants of heme proteins, the proximal His heme ligand is replaced with smaller, noncoordinating residues. First used for H93G Mb,[12,13] this approach has also been successful for CCP,[14] heme oxygenase,[15] and HRP.[16] The structures of the ferric heme cavity mutants have been studied spectroscopically and/or crystallographically in the cases of H175G CCP,[14,17] H25A heme oxygenase,[15] and H93G Mb[18] but have not yet been addressed in detail for H170A HRP.

The magnetic circular dichroism (MCD) spectra of iron porphyrins are very sensitive to the nature of the axial ligands. Thus, the technique is especially well suited for investigations of axial ligation in structurally undefined heme iron proteins.[19] Ligation assignments are made through comparisons of the spectra of the structurally uncharacterized heme protein with those of structurally defined heme iron centers. In this manner, MCD spectroscopy has been used to characterize the coordination structure of numerous heme proteins including *A. ornata* dehaloperoxidase,[20] *N. lobatus* chloroperoxidase,[20] and nitric oxide synthase.[21] The technique has recently been used in the characterization of the ligand-free states of the H93G Mb and H25A heme oxygenase[18] cavity mutants as well as thiolate adducts of H93G Mb.[22]

In this study, we have used electronic absorption and MCD spectroscopy to probe the coordination properties of ferrous, ferric, and ferryl H93G Mb with imidazole in the cavity, H93G(Im) Mb, and with various ligands trans to imidazole. The MCD spectra of the various H93G(Im) Mb species have been compared to the spectra of the parallel derivatives of wild-type Mb to illustrate the expediency of the H93G(Im) Mb cavity mutant as a template for the preparation of mixed-ligand heme iron complexes. The present results also lay the foundation for the use of the H93G Mb cavity mutant to study other combinations of proximal and distal ligands.

Experimental Section

Materials. Sperm whale H93G Mb was expressed and purified in the presence of 10 mM imidazole as previously described.[12] As isolated, the protein contains imidazole as its proximal ligand and exists in a mixture of ferric and oxyferrous states. Complete oxidation of the heme iron is accomplished by addition of a few crystals of potassium ferricyanide (Fluka). Imidazole was removed from the proximal pocket as previously reported.[18,22] Horse heart Mb and sodium dithionite were obtained from Sigma; CO, NO, and O_2 gases were purchased from Matheson; and KCN, NaN_3, imidazole, and H_2O_2 were procured from Aldrich.

Sample Preparation. Derivatives of wild-type Mb were prepared as described previously.[23] H93G Mb concentrations were determined by the pyridine hemochromogen method.[24] Samples were examined at 4 °C in 100 mM potassium phosphate pH 7.0 buffer unless otherwise stated. The ferric imidazole-bound form of the cavity mutant, H93G(Im) Mb, was prepared by microliter additions from a 10 mM imidazole stock solution until the electronic absorption spectrum resembled that of ferric wild type Mb. The ferric and ferrous H93G(Im) Mb ligand adducts were prepared as previously described.[22,23] The oxoferryl states of H93G(Im) Mb and wild-type Mb were produced by the addition of 1.05 and 1.0 equiv of H_2O_2, respectively.

Spectroscopic Techniques. Electronic absorption spectra were recorded with a Cary 210 spectrophotometer interfaced to an IBM PC. Magnetic circular dichroism (MCD) spectra were measured using a 0.2 or 1.0 cm cuvette at 1.41 T with a JASCO J500-A spectropolarimeter. This instrument was equipped with a JASCO MCD-1B electromagnet and interfaced with a Gateway 2000 4DX2-66V PC through a JASCO IF-500-2 interface unit. All spectral measurements were performed at ~4 °C. Data acquisition and manipulation has been described elsewhere.[25] Electronic absorption spectra were recorded before and after the MCD measurements to verify sample integrity. The spectra of H93G(Im) Mb presented here are overplotted with spectra of parallel derivatives of Mb that have been recorded in this study.

Results and Discussion

In earlier studies, we examined H93G myoglobin (Mb) in the absence of exogenously added ligands with magnetic circular dichroism (MCD) spectroscopy.[18] Here, we investigate ferrous and ferric H93G Mb with imidazole bound in the cavity, H93G(Im) Mb, in the presence and absence of numerous exogenous sixth ligands such as imidazole, cyanide, azide, CO, NO, and O_2 as well as its high-valent intermediate, compound II, using electronic absorption and MCD spectroscopy. The MCD data for most of these H93G(Im) Mb derivatives are shown along with the spectra of the parallel derivatives of wild-type Mb. Figure 1 illustrates the active site environment of H93G Mb in the presence of exogenous imidazole as determined by Barrick.[12] Figures 2 and 3 compare the MCD and, for a select case, the electronic absorption spectra of various ferric derivatives of the two proteins while Figures 4−6 compare the MCD of the variousferrous and high-valent complexes of the two proteins. Tables 1 and 2 in the Supporting Information summarize the electronic absorption and MCD spectral data of the ferrous, ferric, and ferryl derivatives of H93G(Im) Mb examined.

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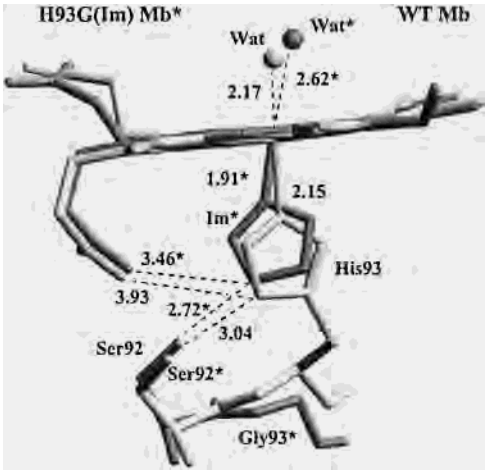

Figure 1. Schematic representation of the active sites of wild-type myoglobin (light) and the myoglobin cavity mutant H93G with imidazole in the cavity (dark) as determined from X-ray coordinates[12,47] using PDB files 1IRC for H93G Mb and 1VXH for wild-type Mb. Dashed lines represent hydrogen-bonding interactions. The asterisk denotes H93G(Im) Mb interactions.

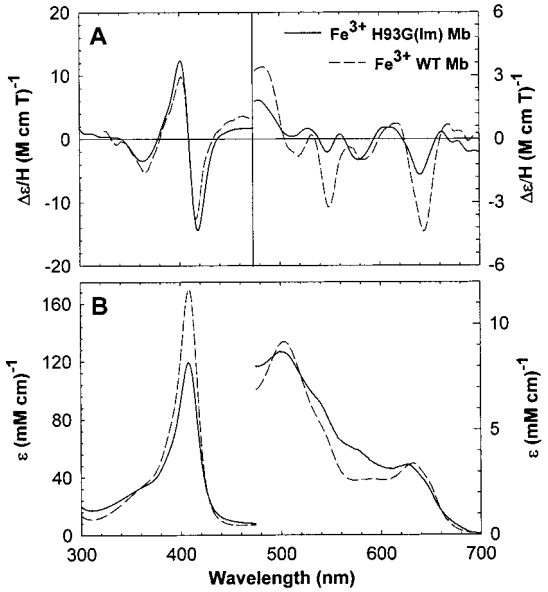

Figure 2. MCD (A) and electronic absorption (B) spectra of ferric H93G(Im) Mb (1 mM Im) (solid line) and of wild-type ferric Mb (dashed line). The samples were examined in 100 mM potassium phosphate pH 7.0 buffer at 4 °C. The spectra of wild type ferric myoglobin are similar to those previously reported.[23,48]

Several lines of evidence indicate that the proximal pocket of H93G Mb is the preferred binding site of exogenously added imidazole. Boxer and co-workers have shown with NMR spectroscopy that various substituted imidazoles bind in the proximal pocket of H93G in the formation of mono(imidazole) complexes.[26] In addition, NMR studies of the CO complex of H93G Mb in the presence of imidazole ([Im] = ~1 mM) shows that the resonance of Val 68 CγH$_3$ is the same in H93G(Im)-CO and WT Mb.[27] As adjustments in the conformation of Val 68 have been shown to result in sizable changes in its chemical shift,[28] this similarity between the mutant and WT Mb indicates that the imidazole binds in the proximal and not the distal cavity.

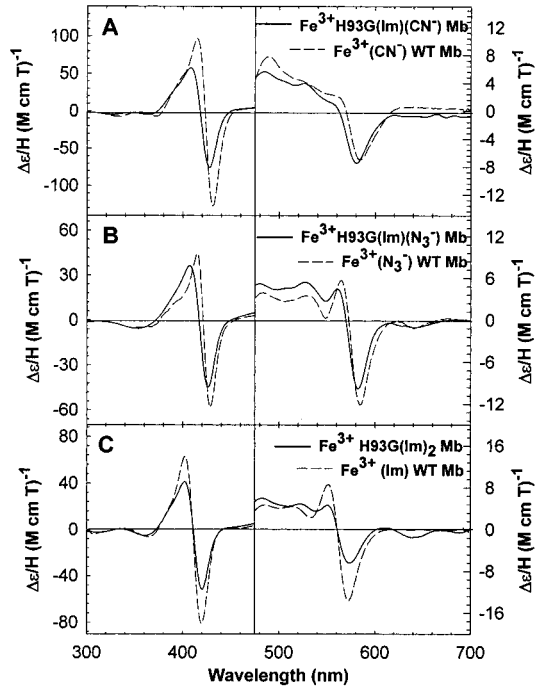

Figure 3. MCD spectra of the ferric cyanide (A), ferric azide (B), and ferric bis(imidazole) (C) derivatives of H93G(Im) Mb (solid) and wild-type Mb (dashed). For the H93G(Im) Mb complexes, [CN^-] = 8.9 mM, [N_3^-] = 3.62 mM, and [Im] = 1.0, 1.0, and 5.4 mM, respectively. The samples were examined in 100 mM potassium phosphate pH 7.0 buffer at 4 °C. The spectra of the wild-type ferric myoglobin derivatives are similar to those previously reported.[23,48]

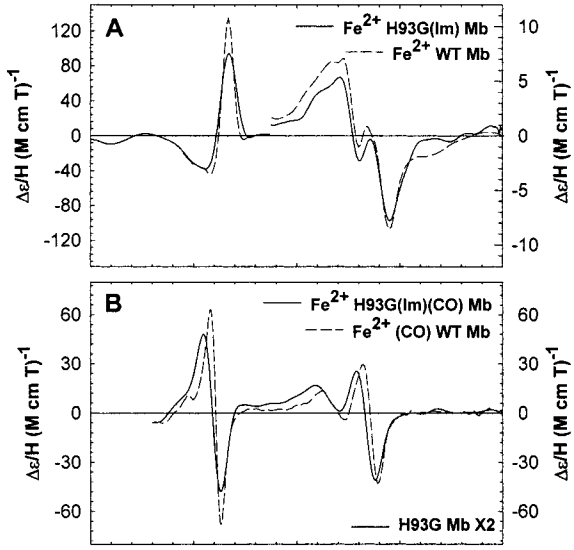

Figure 4. MCD spectra of the deoxyferrous (A) and ferrous-CO (B) derivatives of H93G(Im) Mb (solid) and wild-type Mb (dashed). The samples were examined in 100 mM potassium phosphate pH 7.0 buffer at 4 °C and [Im] = 1.0 mM. The spectra of the wild type ferric myoglobin derivatives are similar to those previously reported.[23,49]

Formation of a bis(imidazole) adduct requires substantially higher concentrations of imidazole.[29] This clearly demonstrates that the two sides of the heme differ in their interaction with exogenous ligands. Furthermore, when the distal histidine is replaced with aspartate in the H64D/H93G double mutant, imidazole binds simultaneously to both axial coordination positions due to the reduction of steric hindrance on the distal

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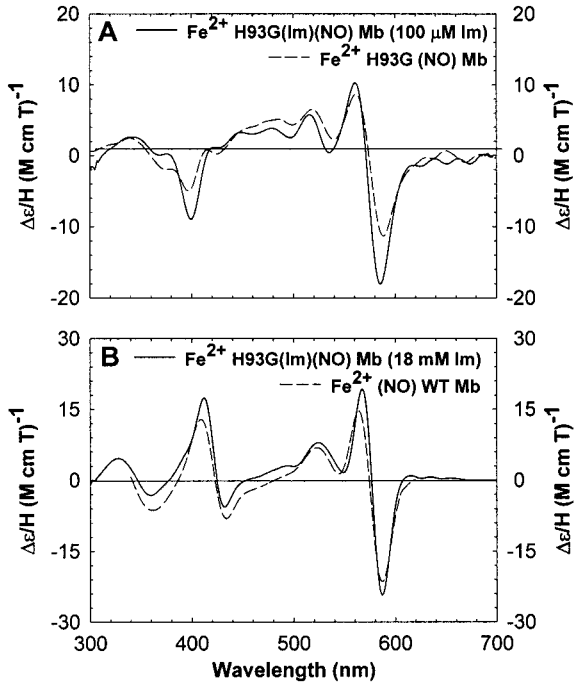

Figure 5. MCD spectra of (A) the five-coordinate ferrous-NO derivative of H93G (Im) Mb (solid) and H93G (ligand free) Mb (dashed) and of (B) the six-coordinate ferrous-NO derivatives of H93G-(Im) Mb ([Im] = 18.0 mM) (solid) and wild-type Mb (dashed). The samples were examined in 100 mM potassium phosphate pH 7.0 buffer at 4 °C. The MCD spectrum of ferrous-NO Mb is very similar to that previously reported.[49]

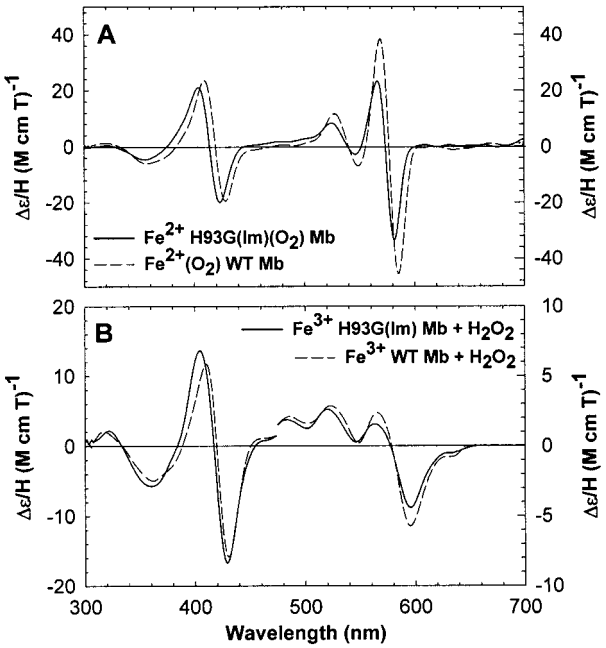

Figure 6. MCD spectra of the oxyferrous (A) and compound II (B) derivatives of H93G(Im) Mb (solid) and wild-type Mb (dashed). The samples were examined in 100 mM potassium phosphate pH 7.0 buffer at 4 °C and [Im] = 1.0 mM. The spectra of the wild type ferric myoglobin derivatives are similar to those previously reported.[49,50]

side of the heme.[29] Taken together, these observations indicate that the proximal binding site is the preferred location for initial ligand binding in H93G Mb.

Ferric Derivatives. Upon the addition of 10 equiv of imidazole to the ligand-free ferric form of H93G Mb, the Soret absorption band in the electronic absorption spectrum red-shifts and increases in intensity (Figure 2). These changes indicate the formation of a six-coordinate high-spin complex with imidazole serving as one of the axial ligands. This assignment is substantiated by previous resonance Raman investigations of H93G Mb.[18] These earlier studies indicate that the addition of imidazole to the ligand-free form of the mutant results in a complex with ν_3 and ν_2 modes at 1482 and 1507 cm^{-1} and 1561 and 1580 cm^{-1}, respectively, denoting conversion to a six-coordinate species with both high- and low-spin components. The observation of both high- and low-spin character seen for H93G(Im) Mb is also observed for wild-type ferric Mb.[30] The overall band pattern of the MCD spectrum of ferric H93G(Im) Mb is quite similar to that of wild type Mb. The only distinguishing characteristics of the spectrum of ferric H93G-(Im) Mb are an overall weaker intensity of all features in the visible region (between 450 and 700 nm) and a higher energy charge transfer band at 638 nm. These dissimilarities are probably due to the slight variation in imidazole orientation relative to the His93 orientation in wild-type Mb.

By examination of superimposed representations of the heme and selected residues in the active sites of H93G(Im) Mb and wild-type Mb, it is obvious that the rotational freedom of the imidazole results in structural changes (Figure 1). The plane of the exogenous proximal imidazole of H93G(Im) Mb is rotated about the Fe−N_i bond by ∼40° relative to the imidazole ligand of His93 in wild-type Mb.[12,26] This orientation aligns the plane of the imidazole ring of H93G(Im) Mb with the heme *meso* carbons rather than eclipsing the pyrrole nitrogens of the heme as in wild-type Mb. Attraction of the imidazole by the ferric heme iron results in an imidazole−iron bond length 0.24 Å shorter than that observed for wild-type Mb. Concomitantly, the Fe−O bond to the distal water is lengthened by 0.45 Å relative to the same bond in ferric wild type Mb. In addition, the interactions of the imidazole N-1 with a heme propionate oxygen atom and with the side chain hydroxyl group of Ser92 results in shorter distances and an increased tilt of the imidazole ring relative to His93 in wild type Mb. These variations would be expected to result in an alteration of the energy of the imidazole−iron covalent bond, and these variations are probably the origin of the small spectral variations observed in the MCD and electronic absorption spectra of ferric H93G(Im) Mb relative to the analogous spectra of wild-type Mb.

The consistent similarity between the MCD spectra of parallel ligand adducts of H93G(Im) Mb and wild type Mb seen in Figure 3A−C demonstrates that the electronic properties of the two proteins are comparable when a sixth ligand is present. Again, in this series of spectra, a common observation is that the spectra of H93G(Im) Mb mixed-ligand adducts are typically blue-shifted relative to the corresponding adducts of wild type Mb. The altered imidazole ligation (relative to His93 of wild type Mb) is probably responsible. The ability of the cavity mutant system to retain imidazole ligation in the presence of both neutral and anionic sixth ligands indicates the stability of the proximal ligation in this system.

Ferrous Derivatives. Proximal ligation by imidazole is preserved in the reduced form of H93G(Im) Mb. Reduction of ferric H93G(Im) Mb with sodium dithionite results in a five-coordinate high-spin complex with electronic absorption and MCD characteristics nearly identical to those of deoxyferrous wild-type Mb (Figure 4A). Similar to the situation seen for the ferric ligand adducts, the reduction of the heme iron appears to counteract any influence the replacement of the His93 with

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glycine and exogenous imidazole may have on the electronic nature of the deoxyferrous state of the cavity mutant. The striking similarity between the MCD spectra of the deoxyferrous complexes of H93G(Im) Mb and wild-type Mb confirms the similar coordination structures for the two proteins.

The addition of CO to the deoxyferrous complex of H93G-(Im) Mb results in a heme derivative with a Soret absorption band at 422 nm, indicative of six-coordinate low-spin complex with Im−Fe−CO ligation (Table 1, Supporting Information). This form of the cavity mutant has also been structurally characterized by ^{1}H NMR.[27] Overall, the NMR features of the ferrous-CO H93G(Im) Mb complex are very similar to those of the ferrous-CO complex of wild type Mb with the exception of subtle differences for the heme meso proton resonances. These differences are attributed to variations in the conformation of the proximal imidazole ring between H93G(Im) Mb and wild type Mb. These variations do not, however, perturb the MCD of the ferrous-CO complex of the cavity mutant on the basis of its similarity to the MCD spectrum of the analogous form of the wild-type protein except possibly in the ∼3 nm blue-shift of its MCD features (Figure 4B).

A second strongly binding ligand in the ferrous state of heme proteins is NO. Unlike CO, NO complexes have a large repulsive trans effect such that when NO binds to a five-coordinate ferrous heme the bond to the axial ligand trans to the NO is weakened. This trans effect results in the weakening or breakage of the proximal axial bond in several heme protein systems, most notably in the guanylate cyclase system.[31−33] Conversely, the addition of NO to the deoxyferrous complex of wild-type Mb results in the formation of a stable six-coordinate NO complex.[34] Only at low pH, when the proximal His is protonated, can a five-coordinate NO complex be formed.[34] The formation of the NO complex of H93G Mb has been extensively studied by Boxer and co-workers.[35] The coordination of NO is dependent on the concentration of exogenous imidazole present in the sample. At low imidazole concentrations (∼1 mM), the binding of NO causes a rupturing of the Fe−Im bond, yielding a five-coordinate NO complex. However, addition of an excess of imidazole (∼18 mM) results in a six-coordinate H93G(Im) NO complex.

These findings are supported here by the MCD data for the NO complexes of H93G Mb (Figure 5). We compare the MCD spectrum of the H93G(Im) Mb NO ([Im] = 1 mM) complex with the spectrum of the ferrous-NO complex of the ligand free form of H93G Mb (i.e. no exogenous imidazole added). In the MCD spectra of these complexes (Figure 5A), a trough centered just below 400 nm can be seen in the Soret region and a derivative-shaped feature centered at 570 nm dominates the visible region. These spectra closely match the spectrum previously reported for a five-coordinate ferrous-NO model complex.[36] The similarity between the two NO complexes confirms that the addition of NO to the deoxyferrous cavity mutant causes the imidazole−iron bond to weaken and break. Addition of excess imidazole to this five-coordinate NO complex ([Im] = 18 mM) is the only manner by which the six-coordinate NO complex can be formed. The six-coordinate state of this species is confirmed by MCD similarity between it and the NO adduct of ferrous wild type Mb at neutral pH (Figure 5B). These spectra also closely match the spectrum previously reported for a six-coordinate imidazole-ligated ferrous-NO model complex.[36]

In the context of the location of the ligands, the NO case is subtle. For the six-coordinate H93G(Im) NO complex ([Im] = 18 mM), the similarity of the MCD spectra of the mutant and WT Mb indicates that the two enzymes bind their respective ligands in a similar manner (i.e., the imidazole is in the proximal pocket and the NO is in the distal site). The five-coordinate H93G(NO) complex is more difficult to characterize as the EPR[35] and the MCD data do not distinguish NO binding on the proximal or distal side. However, ^{19}F MNR studies are consistent with NO binding on the distal side of the heme in the five-coordinate NO complex of H93G Mb.[37]

As the biological function of Mb is the transport of O_2 in respiring muscle, the stability of the oxyferrous complex is vital to its physiological role. The protein environment surrounding the heme group is believed to be instrumental for the stabilization of the oxyferrous complex. In particular, the removal of the distal His (His64) by site-directed mutagenesis increases the rate of autoxidation 40−350-fold.[38] These results suggest that the distal His discriminates between CO and O_2 binding by both sterically hindering bound CO and stabilizing bound O_2 through hydrogen bonding. Addition of O_2 to deoxyferrous H93G(Im) Mb yields a very stable oxyferrous complex as determined by its MCD spectral similarity to oxyferrous wild type Mb (Figure 6A). Unlike the relatively unstable oxyferrous states of HRP and P450−CAM, oxyferrous H93G(Im) Mb system is stable at 4 °C for at least 48 h. This stability implies that the removal of the protein tether does not disrupt the interaction between the distal His and the bound oxygen, verifying that the oxyferrous species of H93G(Im) Mb is an excellent model of the physiologically important complex of wild-type Mb. On the other hand, in the absence of imidazole (i.e. exogenous ligand free H93G) the ferrous species rapidly autooxidizes when exposed to O_2 (data not shown).

Ferryl Derivative. Incubation of wild-type Mb with hydrogen peroxide causes a slow conversion ($\sim 10^2$ M^{-1} s^{-1}) of the ferric species to an oxoferryl complex (Fe^{VI}═O) spectrally comparable to compound II of the peroxidases.[39−42] Conversely, peroxidases react readily with hydrogen peroxide ($\sim 10^7$ M^{-1} s^{-1}) to yield compound I, an oxoferryl heme coupled to a porphyrin cation radical (Fe^{IV}═O $Por^{\cdot+}$).[42,43] A compound I state has not been observed for wild-type Mb despite the heterolytic cleavage of the peroxide bond.[44] The absence of an observable compound I species is likely due to its rapid decay to compound II. The instability of compound I is believed to be due to the malfunction of the distal histidine (His64) as a general acid−base catalyst. Instead, this residue may provide a conduit for reduction of compound I yielding a surface tyrosine radical and an

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oxoferryl heme.[42,45,46] Here, we have investigated the reaction of the imidazole-bound H93G Mb with hydrogen peroxide to determine if the subtle differences in the imidazole orientation and hydrogen-bonding interactions seen in the cavity mutant affect its ability to form a high-valent intermediate. Comparison of the MCD spectrum of the product of this reaction with compound II of wild-type Mb (Figure 6B) indicates that the cavity mutant is competent to form the oxoferryl species lacking the porphyrin π-cation radical. The result argues that the constriction of the imidazole ring imposed by the protein tether is not important in the formation of an oxoferryl heme species.

Conclusions

The ability of the H93G Mb mutant to spectrally mimic wild-type Mb when imidazole is bound in the proximal cavity is of great importance in the effort to produce more sophisticated model systems for heme-containing proteins. The H93G(Im) Mb system, in both ferric and ferrous oxidation states, is able to successfully form mixed-ligand complexes with both neutral and anionic ligands that exhibit electronic absorption and MCD spectra similar to those of parallel derivatives of wild-type Mb. Additionally, the ability of the H93G(Im) Mb system to form a stable oxoferryl compound II complex observable by MCD indicates the excellent stability of the model system. The H93G Mb cavity mutant serves as an excellent structural model for the His-ligated wild type protein and can potentially be developed into a model for other heme-containing protein systems by titration of exogenous ligands other than imidazole into the proximal cavity.

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Supporting Information Available: Tables 1 and 2, listing the electronic absorption and magnetic circular dichroism parameters for the derivatives of H93G myoglobin examined in this study. This material is available free of charge via the Internet at http://pubs.acs.org.

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