The Role of the Distal and Proximal Protein Environments in Controlling the Ferric Spin State and in Stabilizing Thiolate Ligation in Heme Systems: Thiolate Adducts of the Myoglobin H93G Cavity Mutant

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Abstract: Recently, heme protein cavity mutants have been engineered in which the proximal coordinating amino acid has been replaced by a smaller, noncoordinating residue leaving a cavity that can be filled by exogenous axial ligands. This approach was pioneered by Barrick (*Biochemistry* **1994**, *33*, 6546−6554) with H93G sperm whale myoglobin where the coordinating histidine is replaced by glycine and the proximal cavity is filled with imidazole. In the present study, models for cysteine thiolate-ligated ferric cytochrome P450 have been prepared using H93G myoglobin containing thiolate ligands in the cavity. Despite the availability of water to serve as a distal ligand as occurs in both ferric wild type myoglobin and ferric H93G myoglobin with imidazole in the cavity, the ferric H93G thiolate complexes spectroscopically resemble five-coordinate high-spin substrate-bound ferric P450, which contains a thiolate proximal ligand and *lacks* a water distal ligand. Thus, the distal protein environment plays a crucial role in controlling whether a five-coordinate thiolate-ligated ferric heme binds water or not. Two advantages pertain to the present thiolate-ligated heme protein model relative to purely synthetic thiolate-ligated ferric porphyrins: (a) aliphatic thiols can form complexes without reduction of the ferric iron and (b) mixed ligand complexes that are stable at ambient temperatures can be prepared with a neutral ligand such as imidazole trans to thiolate. However, when anionic ligands are added to the ferric thiolate adduct in an attempt to prepare mixed ligand complexes with two anionic ligands, the thiolate ligand is displaced (or lost) without formation of a stable mixed ligand derivative. Further, reduction of the ferric-thiolate complex leads to loss of the thiolate ligand even in the presence of CO. The data presented for the thiolate adducts of ferric H93G myoglobin are analyzed in the context of the spectrally related H93C myoglobin mutants. The inability of the thiolate adducts of H93G myoglobin to accommodate a second anionic ligand in the ferric state or to remain thiolate-ligated in the ferrous state is likely due to the lack of (a) correctly positioned hydrogen bond donating groups and (b) a properly oriented helix dipole to stabilize the thiolate ligand as occurs in the proximal protein environment of P450. The present results illustrate the important role of the distal and proximal heme environments in controlling the ferric spin state and in stabilizing thiolate ligation in heme systems, respectively.

Introduction

Cytochrome P450 enzymes utilize a cysteine thiolate-ligated heme iron to activate dioxygen for ambient temperature oxygen atom insertion into C−H bonds likely via a thiolate "push" mechanism involving a highly reactive oxo-ferryl intermediate.[1−3] Thiolate-ligated iron porphyrin complexes as models for the ferric and ferrous states of P450 have been extensively characterized by spectroscopic methods and by X-ray crystallography.[1,4,5] Construction of thiolate-ligated mutant heme proteins by replacement of the endogenous proximal histidine of myoglobin with cysteine to model the P450 active site has been achieved in human myoglobins H93C and H93C/H64V[6−8] and horse heart myoglobin H93C/H64V.[9] Further, the human mutant myoglobins exhibit enhanced reactivity toward heterolytic cleavage of bound peroxides and increased monooxygenase

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activity[7,8] in accordance with the proposed thiolate "push" mechanism for dioxygen activation by P450.[1−3]

A recently developed protein-based model system for investigating ligand binding to heme iron has been the engineering of cavity mutant heme proteins where the proximal ligating residue is replaced with a smaller, noncoordinating residue. The resulting proximal cavity can then be filled with exogenous ligands. This approach was pioneered by Barrick with H93G sperm whale myoglobin where the coordinating histidine was replaced by glycine and the cavity was filled with imidazole.[10−12] Cavity mutant heme proteins have subsequently been prepared with cytochrome *c* peroxidase,[13] heme oxygenase,[14] and horseradish peroxidase.[15]

The high degree of spectral detail seen in the magnetic circular dichroism (MCD) of iron porphyrins makes this technique especially well suited for investigations of axial ligation in structurally undefined iron porphyrins and heme proteins.[16] Ligation assignments can often be made through comparisons of the spectra of a structurally uncharacterized heme complex with those of structurally defined heme iron centers. The method has been applied in this manner to study numerous heme proteins including P450,[17] *Caldariomyces fumago* chloroperoxidase,[17] nitric oxide synthase,[18] guanylate cyclase,[19] and heme oxygenase.[20] In the present study, we have investigated the H93G cavity mutant of sperm whale myoglobin in the presence of an extensive series of thiols to generate ferric-thiolate adducts as mimics for the heme iron coordination structure of ferric P450. A significant advantage to this protein-based P450 model system is the ability to form mixed ligand adducts in the ferric state with neutral ligands trans to thiolate. The accuracy of the model system has been demonstrated using MCD spectroscopy. Because the thiolate ligand to the heme iron in this system is not covalently linked to the peptide backbone, it is free to dissociate when the conditions do not stabilize the Fe−S(thiolate) bond. Consequently, the results described herein probe the role of the proximal heme environment in stabilizing thiolate ligation. A recent paper by Green focused on the role of the thiolate proximal ligand in P450 in determining the low-spin state of the water-ligated six-coordinate ferric resting state of the protein.[21] The present study sheds light on the factors that influence the binding of water trans to thiolate in ferric heme complexes in proteins.

Experimental Section

Protein Preparation and Purification. The sperm whale myoglobin H93G mutant was expressed and purified as previously described.[10] 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 can be removed from the proximal pocket by two different methods. Gel filtration on a P6DG (BioRad) size exclusion column typically removes approximately 90% of iron-bound imidazole as judged by resonance Raman spectroscopy.[22] Extraction of the heme from H93G, followed by reconstitution with hemin, yields ferric H93G which is 100% free of imidazole.[22] Preparation of *apo*-H93G was done by the method of Teale.[23] *apo*-H93G was reconstituted with hemin by addition of one equivalent of hemin in 0.1 N NaOH. After standing at 4 °C for 1 h, the sample was concentrated and exchanged into 10 mM potassium phosphate buffer at pH 7.0 on a P6DG gel filtration column.

Preparation of Ligand Adducts. Imidazole, potassium cyanide, sodium dithionite and the thiols used in this study were obtained from Aldrich and used without further purification. All protein samples with the exception of those used for determination of dissociation constants were handled at 4 °C at concentrations of approximately 60 mM in 100 mM potassium phosphate buffer at pH 7.0. The ferric thiolate adducts were prepared from exogenous ligand-free ferric H93G through addition of minimal volumes of 2 mM thiol stock solutions. Because the thiol pK_a values are approximately 8 or lower, they are referred to in the text as thiols before addition to H93G and as thiolates after complexation since ligation of a thiol to Fe invariably results in deprotonation. The ferric H93G ethanethiolate/imidazole adduct was prepared by addition of minimal volumes of a 6.8 mM imidazole stock solution to the ferric H93G ethanethiolate sample. The cyanoferric adduct was prepared from both the ferric H93G−ethanethiolate adduct and ferric exogenous ligand-freeH93G through addition of minimal volumes of a 1 M KCN stock solution. The deoxyferrous samples were prepared from the ferric H93G−ethanethiolate adduct and exogenous ligand-free ferric H93G by exchanging the atmosphere of the cuvette with nitrogen followed by addition of solid sodium dithionite.

Determination of Dissociation Constants for the Aromatic Thiolate Adducts. Deoxygenated ferric H93G samples free of exogenous ligands were handled at 25 °C in 100 mM potassium phosphate buffer at pH 7.0 in concentrations near 2 mM which were determined spectrophotometrically using the molar absorptivity of 107 mM^{-1} cm^{-1} for the Soret absorption maximum at 405 nm for exogenous ligand-free H93G at pH 7.0.[22,24] The aromatic thiol ligand solutions were prepared in concentrations near 200 mM by addition of 1 M ligand solutions in ethanol to cold deoxygenated 100 mM potassium phosphate buffer. Accurate concentrations of the aqueous ligand solutions were determined by iodometric titration. Dissociation constants for the binding of aromatic thiols to H93G were then determined in triplicate by Hill analysis of ligand titration data obtained by addition of microliter aliquots of ligand solution to the protein.[25]

Spectroscopic Techniques. Electronic absorption spectra were obtained on a Cary 210 spectrophotometer interfaced to an IBM PC. MCD spectra were recorded at a magnetic field strength of 1.41 T with a JASCO J500A spectropolarimeter equipped with a JASCO MCD 1B electromagnet and interfaced to a Gateway 2000 4DX2-66V PC through a JASCO IF-500-2 interface unit. Spectroscopic data handling has been described elsewhere.[26]

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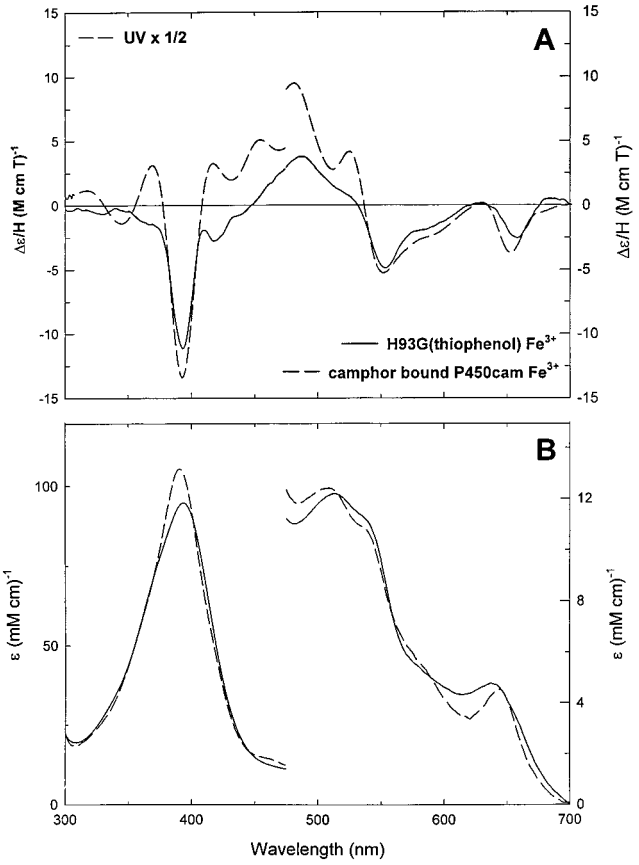

Figure 1. Magnetic circular dichroism (A) and electronic absorption (B) spectra of ferric thiophenolate-bound H93G myoglobin (51 μM) at pH 7.0 + 110 mM thiophenol (solid line) and of ferric camphor-bound cytochrome P450-CAM at pH 7.0 (dot−dashed line) (re-plotted using data from ref 27). Samples were examined in 100 mM potassium phosphate buffer at 4 °C.

Results

The magnetic circular dichroism (MCD) and electronic absorption spectra of the thiophenolate adduct of ferric H93G are compared to those of the five-coordinate, camphor-bound, high-spin derivative of ferric cytochrome P450-CAM[27,28] in Figure 1. Ferric−thiolate complexes of ferric H93G have been prepared with several aliphatic and aromatic thiols and the resulting electronic absorption maxima have been collected in Table 1. The adducts formed with aliphatic thiols have Soret absorption peaks at 391 ± 1 nm, identical in wavelength with that of high-spin ferric P450-CAM. In contrast, when ferric−thiolate adducts were formed with a series of para-substituted aromatic thiols, the Soret peak positions vary from 390 to 400 nm and the peak position correlates with the Hammett σ parameter (Figure 2), which measures the electron donating capability of the para substituent. The Soret absorption peak is found at 390 nm for *p*-nitrothiophenolate (electron-withdrawing substituent) and at 400 nm for *p*-methoxythiophenolate (electron-donating substituent). One of the most characteristic electronic absorption spectral features of high-spin ferric P450-CAM is the charge transfer band at 644 nm. This band is much more well resolved and is found at nearly the same wavelength (637−643 nm) in the spectra of the ferric−thiolate adducts of H93G formed with aromatic thiols whereas the complexes with aliphatic thiols exhibit poorly resolved charge-transfer bands at about 620 nm (Table 1). Dissociation constants have been determined for four of the five aromatic thiolate−H93G complexes examined in this study and range from 0.4 to 1.1 μM. These data are presented as an inset to the Hammett plot in Figure 2. Clearly, the aromatic thiols bind very strongly to ferric H93G. The K_d values follow an unexpected trend where the more weakly donating thiolate ligands (those with electron-withdrawing substituents) bind more tightly than the more strongly donating ones. Apparently the binding constants are less influenced by the electronic character of the ligand than by steric and/or thermodynamic factors. In any event, the dissociation constants are found in a very narrow range indicating that the substituents have only a minor influence on their binding affinity.

Upon addition of imidazole to ferric H93G−ethanethiolate, a species is formed whose MCD and electronic absorption spectra are presented in Figure 3 in comparison to the spectra of the ferric P450-CAM imidazole complex.[30] Although the intensities of the Soret absorption peak and MCD trough of the H93G−ethanethiolate/imidazole complex are weaker than those observed for the P450 imidazole adduct, the band shapes and MCD crossover points are almost identical. The MCD crossover point in the Soret region is at 424.5 nm for ferric H93G−ethanthiolate/imidazole and at 425.5 nm for imidazole-bound ferric P450.

Figure 4 indicates that addition of potassium cyanide to the ferric H93G−ethanethiolate causes the ethanethiolate ligand to be displaced from the heme iron. If both ethanethiolate and cyanide remained bound, the MCD and electronic absorption spectra would be expected to resemble those of the cyanoferric adduct of P450.[31] Instead these spectra resemble those of cyanoferric wild-type myoglobin.[32] Relative to cyanoferric wild-type myoglobin, the Soret crossover and the electronic absorption maximum of cyanoferric P450 is red-shifted by approximately 25 nm. Because of this similarity to cyanoferric myoglobin, it is likely that the distal histidine 64 of H93G is the ligand trans to cyanide in this case.

Upon anaerobic reduction of the ferric ethanethiolate adduct of H93G with sodium dithionite, the resulting H93G MCD and electronic absorption spectra are quite distinct from the corresponding spectra of deoxyferrous P450 (Figure 5). The prominent Soret feature in the MCD spectrum of deoxyferrous P450 is a broad trough centered at 419 nm. The MCD spectrum observed following reduction of the ferric ethanethiolate adduct of H93G, on the other hand, has a sharp positive band with a maximum at 427 nm. The spectrum resembles that of wild-type deoxyferrous myoglobin in overall band pattern but is much weaker in intensity. The Soret absorption peak of the sample is also blue-shifted relative to that of wild-type deoxyferrous myoglobin. Resonance Raman investigations of this species[29] have indicated that it lacks the low-frequency iron−histidine stretch in the 218−221 cm^{-1} range.[33,34]

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Table 1. Wavelength Absorption Maxima for Wild-type and Thiolate-Ligated Ferric Mutant Myoglobins (molar absorptivities in mM^{-1} cm^{-1})

protein	source	Soret	CT1	CT2	ref
Cyt P450cam + camphor	*Ps. Putida*	391 (102)	509	644	26
H93C Mb	human	391 (115)	509	629	7
H93C Mb	horse heart	406 (78)	498	610	9
H93C/H64V Mb	horse heart	391 (95)	508	637	8
H93C/H64V Mb	human	391 (100)	508	637	8
H93G Mb + ethanethiol	sperm whale	391(74)	508	620	this work
H93G Mb + benzylthiol	sperm whale	391 (79)	508	623	this work
H93G Mb + cyclopentanethiol	sperm whale	392 (77)	510	622	this work
H93G Mb + cyclohexanethiol	sperm whale	392 (80)	508	623	this work
H93G Mb + *p*-nitrothiophenol	sperm whale	390 (77)	515	643	this work
H93G Mb + *p*-chlorothiophenol	sperm whale	396 (93)	515	639	this work
H93G Mb + thiophenol	sperm whale	395 (97)	514	637	this work
H93G Mb + *p*-methylthiophenol	sperm whale	397 (97)	519	638	this work
H93G Mb + *p*-methoxythiophenol	sperm whale	400 (98)	524	638	this work

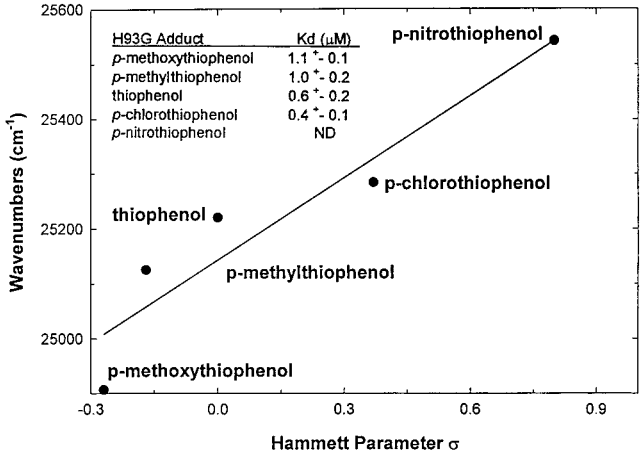

Figure 2. Hammett correlation plot of Soret absorption peak position vs Hammett parameter σ. H93G concentrations ranged from 49 to 66 mM in 100 mM potassium phosphate buffer pH 7.0 at 4 °C. Ligand concentrations were typically 2-fold in excess of the protein concentration. The K_d values for the aromatic thiolates are shown in the inset.

A similar result occurs upon reduction of the ferric H93G−ethanethiolate adduct in the presence of CO. The resulting ferrous−CO complex has a Soret absorption peak near 420 nm rather than at 450 nm as would be expected for a thiolate-ligated ferrous−CO complex. The MCD spectrum of the sample (data not shown) is again similar in shape and band pattern to that of wild-type ferrous−CO myoglobin, but is much weaker in intensity and slightly blue-shifted in wavelength. Identification of the fifth ligand in the deoxyferrous and ferrous−CO cases is under investigation.

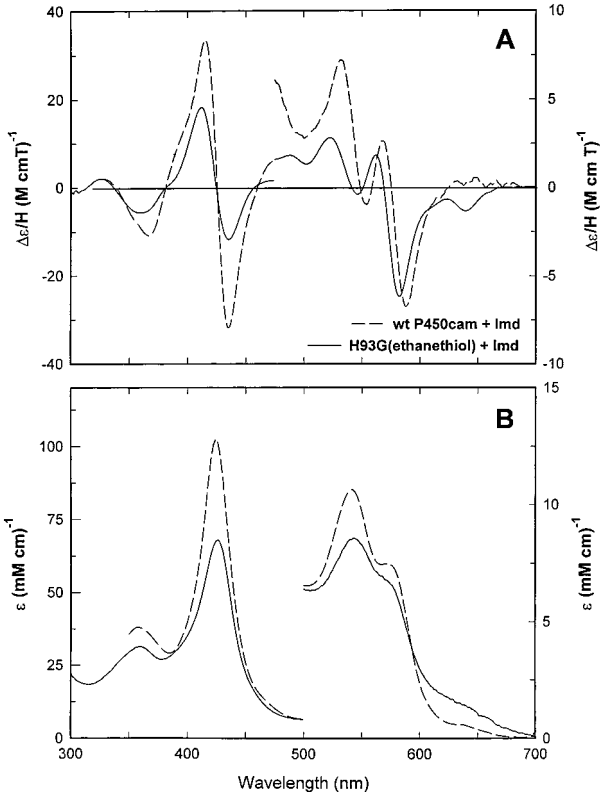

Figure 3. Magnetic circular dichroism (A) and electronic absorption (B) spectra of ethanethiolate (EtSH) and imidazole (Im)-bound ferric H93G myoglobin (H93G(EtSH)(Im)) pH 7.0, 30 mM EtSH, 7 mM Im (solid line) and of ferric Im-bound cytochrome P450-CAM, pH 7.0 (dashed line) (re-plotted using data from ref 30). Samples were examined in 100 mM potassium phosphate buffer at 4 °C.

Discussion

Thiolate Ligation to the Ferric H93G Myoglobin Cavity Mutant. Addition of a wide variety of thiolates to the ferric H93G myoglobin cavity mutant consistently leads to complexes that have Soret absorption peaks between 390 and 400 nm (Table 1) and display MCD spectra that closely resemble that of ferric high-spin cytochrome P450-CAM (Figure 1). The MCD spectrum of high-spin ferric P450 is quite distinctive;[1,3] clearly the ferric H93G thiolate adducts described herein are five-coordinate thiolate-ligated ferric heme adducts. Although we have no direct evidence that the proximal pocket is the primary binding site for exogenous ligands added to ferric H93G, there are several indirect lines of evidence to support that supposition. (a) Boxer and co-workers have used NMR spectroscopy to prove that various substituted imidazoles bind in the proximal pocket of H93G in the formation of mono-imidazole adducts.[12] (b) To form a bis-imidazole adduct, it is necessary to use substantially higher imidazole concentrations.[35] This clearly demonstrates that the two sides of the heme differ in their accessibility to exogenous ligands. (c) 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 in steric hindrance on the distal side of the heme.[35] All of these observations taken together indicate that the proximal binding site is the preferred location for initial ligand binding in H93G.

The formation of stable thiolate-ligated ferric heme complexes with aliphatic thiols (Table 1) is somewhat unexpected. In preparing five-coordinate thiolate-ligated ferric porphyrin mod-

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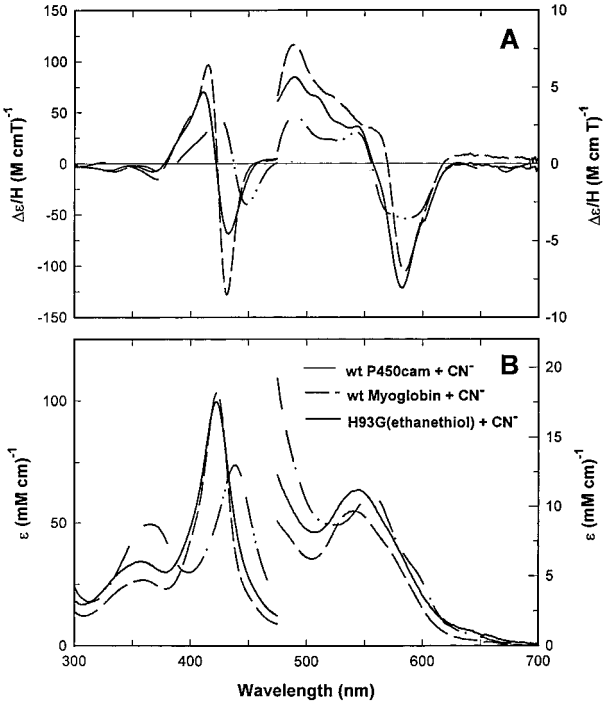

Figure 4. Magnetic circular dichroism (A) and electronic absorption (B) spectra of the reaction product of ferric H93G(EtSH) + potassium cyanide, pH 7.0, 30 mM EtSH, 30 mM potassium cyanide (solid line); of cyanoferric cytochrome P450-CAM, pH 7.0 (dash−dot line) (re-plotted using data from ref 31); and of cyanoferric myoglobin, pH 7.0 (dashed line) (re-plotted using data from ref 32). Samples were examined in 100 mM potassium phosphate buffer at 4 °C.

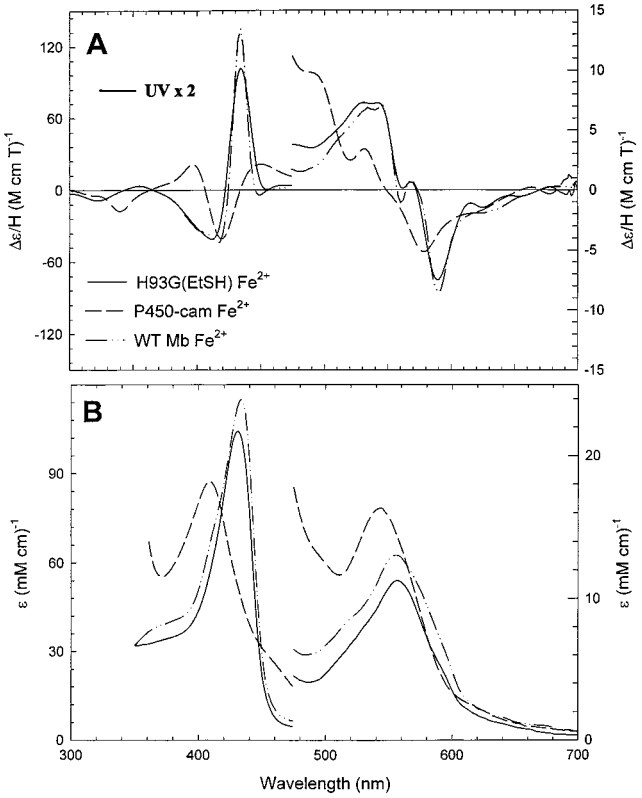

Figure 5. Magnetic circular dichroism (A) and electronic absorption (B) spectra of H93G(EtSH) following the addition of solid sodium dithionite, pH 7.0, 30 mM EtSH, (solid line); of deoxyferrous cytochrome P450-CAM, pH 7.0 (dashed line) (re-plotted using data from ref 27); and of deoxyferrous myoglobin, pH 7.0 (dashed−dot line) (re-plotted using data from ref 27). Samples were examined in 100 mM potassium phosphate buffer at 4 °C.

els, Holm and co-workers used *p*-nitrobenzenethiolate and *p*-chlorobenzenethiolate to avoid reducing the iron and forming disulfides as occurred with aliphatic thiolates.[4] The present study also reveals that for aromatic thiolates, the energy of the Soret absorption transition correlates with the electron-donating properties of the para substituent (Figure 2). This opens up a new avenue to probe the unusual spectroscopic properties of thiolate-ligated ferric hemes. Dawson and co-workers have extensively studied the effect of changing the donor properties of the distal ligand trans to cysteinate in ferric P450 and *C. fumago* chloroperoxidase;[31] now it will be possible to keep the distal ligand constant and vary the donor properties of the thiolate in studies of the spectroscopic properties of such systems.

Preparation of Six-Coordinate Ferric Complexes With Two Different Axial Ligands. Addition of a neutral ligand such as imidazole to ferric ethanethiolate-bound H93G myoglobin leads to formation of a derivative that spectroscopically resembles imidazole-bound ferric P450-CAM (Figure 3). This spectral similarity is consistent with formation of a six-coordinate mixed ligand thiolate/imidazole adduct. The spectra displayed in Figure 3 are also distinct from those that would be expected for a bis-imidazole complex.[36] Preparation of ferric heme derivatives with two different axial ligands is difficult to achieve in synthetic model heme systems. In most cases, the affinity of one of the two ligands for the heme iron is sufficiently great to lead to formation of a bis-ligated adduct. Clearly, the ability of the cavity mutant system to bind two different ligands to generate mixed ligand adducts is potentially useful and will be further investigated.

Effect of the Distal Environment. Holm and co-workers have reported the crystal structure of a five-coordinate ferric porphyrin complex with *p*-nitrobenzenethiolate as the fifth ligand.[4] Addition of any ligand trans to thiolate, even a weak donor such as tetrahydrofuran as a model for water ligation, converts the complex to low-spin.[4,5,17] Similar effects were reported by Dawson and co-workers working with the enzyme itself.[17,37] The crystal structure of low-spin resting ferric P450-CAM contains a six-coordinate thiolate-ligated heme center with water as the distal ligand.[38] Addition of camphor displaces the water distal ligand and converts the structure to five-coordinate high-spin.[39]

Wild-type human, horse heart, and sperm whale myoglobins in the ferric state are known to exist as six-coordinate high-spin complexes with histidine and water as proximal and distal ligands, respectively.[40] Replacement of the proximal histidine with a thiolate as in the ferric thiolate-ligated H93G cavity mutant might therefore have been expected to produce a six-coordinate low-spin ferric complex with water as the distal ligand, as is the case for resting ferric P450.[38] In fact, the ferric H93C human myoglobin[7] and H93C/H64V horse heart myoglobin[9] mutants have previously been reported to exist as five-coordinate high-spin heme-thiolate complexes. However, the

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replacement of the proximal histidine with cysteine exchanges a larger amino acid with a smaller one. Thus, the fact that the H93C myoglobin mutants were found to be five-coordinate could have logically been the result of difficulty forming a low-spin six-coordinate derivative due to the inability of the cysteine-ligated iron to move into the plane of the porphyrin. Such an effect has been invoked to explain why H175C cytochrome *c* peroxidase does not form a stable thiolate-ligated complex but instead oxidizes the cysteine to cysteic acid.[41] The thiolate ligand in the H93G myoglobin cavity mutant is unconstrained and yet still forms a five-coordinate high-spin complex.

The P450-CAM distal pocket is generally nonpolar and consequently when water is in the pocket it can be stabilized by binding to the heme iron. The myoglobin distal pocket is more polar with a distal histidine in close proximity to the heme iron. Apparently, in thiolate-ligated H93G (as well as in H93C) myoglobin, the water in the distal pocket is stabilized through interactions with the more polar amino acids of the pocket and does not bind to the heme iron. Green has recently theoretically shown that the thiolate ligand in the six-coordinate water-ligated ferric P450 resting state is responsible for its low-spin state.[21] That result is entirely consistent with the extensive model chemistry done by Holm and Collman and their co-workers.[4,5,17] The present results show that the binding of water to generate the low-spin resting state of ferric P450 also depends on the nature of the distal environment of the heme to promote water ligation.

Effect of the Proximal Environment. Attempts to form mixed ligand adducts of ferric H93G with anionic ligands trans to thiolate or to reduce the iron to the ferrous state while retaining the thiolate ligand have so far been unsuccessful. This is likely because the heme iron complex of H93G cannot tolerate an overall −1 charge. A ferric (porphyrin dianion) with a thiolate ligand is a neutral complex. Addition of a second anionic ligand in the ferric state or reduction of the iron to the ferrous state would give the complex a charge of −1. This behavior clearly contrasts to that of P450 and *C. fumago* chloroperoxidase which retain cysteinate ligation in the ferric states with distal anionic ligands bound and upon reduction to the ferrous state.

Poulos has suggested that the anionic thiolate in P450 and chloroperoxidase is stabilized through peptide backbone amide N−H hydrogen bond donation to the anionic sulfur and by the favorable location of the Fe−S unit near the positive end of the proximal helix dipole.[42] In contrast, the environment on this side of the heme in myoglobin features hydrogen bond acceptors to interact with the histidine ligand. In addition, the bound histidine in myoglobin is near the negative end of the proximal helix dipole. These differences between the proximal heme environment of myoglobin and those of P450 and chloroperoxidase are most likely responsible for the loss of thiolate ligation in H93G myoglobin when the net charge of the heme iron complex is −1. These same factors may also contribute to the destabilization of distal water binding in ferric thiolate-ligated H93G myoglobin. With no hydrogen bond donors to draw electron density away from the thiolate (as occurs in P450), the ferric iron in H93G myoglobin is more electron rich. This would decrease the tendency of the iron to bind water trans to thiolate. Thus, the proximal environment also appears to play an important role in controlling the ferric spin and coordination state.

The results described herein for thiolate-ligated H93G myoglobin are entirely consistent with those previously reported for the two H93C[7−9] myoglobin mutants which both failed to retain thiolate ligation following reduction to the ferrous state. As mentioned above, however, the H93C myoglobin mutants involved replacement of the larger histidine proximal ligand with a smaller cysteine that might not have been able to bind in an optimum manner. Thus the present system with an unconstrained thiolate ligand provides a better measure of the role of the proximal heme environment on the stabilization of the thiolate ligand.

Conclusions

A model for high-spin ferric cytochrome P450 has been prepared using the H93G cavity mutant of myoglobin with thiolate ligands in the cavity. In contrast to resting low-spin ferric P450, which has water bound trans to thiolate, the resulting thiolate H93G adduct is five-coordinate despite the availability of water to serve as a distal ligand as in ferric wild-type myoglobin. The distal and proximal protein environments appear to play critical roles in determining whether a five-coordinate thiolate-ligated ferric heme binds water or not. The H93G cavity mutant system has significant advantages relative to purely synthetic thiolate-ligated ferric porphyrins for the study of ligand binding in that aliphatic thiolates bind without reduction of the ferric iron and stable mixed ligand complexes can be prepared with a neutral ligand such as imidazole trans to thiolate. Unfortunately, however, thiolate ligation is not retained following addition of a second anionic ligand to the ferric−thiolate adduct or upon reduction of the heme iron to the ferrous state. Loss of thiolate ligation in these latter cases is attributed to lack of correctly positioned hydrogen bond donors or properly oriented helix dipole. The results described herein demonstrate the crucial role of the distal and proximal heme environments in controlling the ferric spin state and in stabilizing thiolate ligation in heme systems, respectively.

Note Added in Proof: Since the submission of this article, Lu, Dawson, and co-workers have reported the successful preparation of a cysteine-ligated mutant of cytochrome *c* peroxidase.[43]

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