Trans Effects on Cysteine Ligation in the Proximal His93Cys Variant of Horse Heart Myoglobin†

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ABSTRACT: Three variants of horse heart myoglobin (Mb) in which the proximal His93 residue has been replaced with a Cys residue have been constructed and studied by NMR, EPR, and MCD spectroscopy to evaluate the contributions of proximal and distal residues to the coordination environment of the heme iron in these proteins. Although no experimental conditions were identified that allowed quantitative ligation of the cysteine residue to the heme iron in the His93Cys variant, all of the spectroscopic evidence collected for the His93Cys/His64Ile and His93Cys/His64Val double variants supports the assignment of thiolate as the ligand to iron in the oxidized forms of these variants. The double metMb variants exhibit Soret maxima that are considerably blue-shifted, ^{1}H NMR spectra with decreased mean methyl resonances, and EPR spectra with highly rhombic *g* values. These spectroscopic data for the Fe(III) variants resemble the corresponding properties reported for ferricytochrome P-450. The decrease in the reduction potential of the double variants by 280 mV relative to wild-type protein is also consistent with the low midpoint potential of cytochrome P-450. MCD spectroscopy of these variants confirms that the proximal cysteine residue is not bound in the reduced forms of these proteins and, in the case of the His93Cys variant, that the distal histidine is coordinated to the iron. Similar coordination environments were created in the ferrimyoglobin variants by cyanogen bromide modification, which resulted in cyanation of the sulfur atom and prevented the ligation of Cys93 to the heme iron. From these results it is apparent that simple mutagenenic modifications of the active site of horse heart myoglobin can reproduce the characteristics of ferricytochrome P-450, but that reproduction of the spectroscopic properties of ferrocytochrome P-450 will require more subtle modifications.

Previous studies of myoglobin variants in which the proximal (Egeberg et al., 1990; Adachi et al., 1991, 1993; Hildebrand et al., 1995) and distal (Springer et al., 1989; Maurus et al., 1994) His residues have been replaced with alternative coordinating ligands have been undertaken in an attempt to understand the contributions of these residues to the properties of heme enzymes with similar replacements, in the absence of contributions from other structural elements. In related studies, a myoglobin variant in which the proximal His residue is replaced by a glycyl residue has been used for the facile preparation of derivatives in which various exogenously added species can be recruited as proximal ligands (Barrick, 1994; DePillis et al., 1994). Through systematic execution of these and related studies, insight may be gained into the structural origin of the functional diversity exhibited by heme-containing proteins (Dawson, 1988). One variant of particular interest in this regard has involved replacement of the proximal His residue of human myoglobin with a Cys residue (Adachi et al., 1991, 1993). This ligand substitution is of interest insofar as it may provide insight into the contributions of proximal Cys ligation to the functional and catalytic properties of cytochrome P450 (Poulos et al., 1986, 1987; Poulos & Raag, 1992) and chloroperoxidase (Dawson, 1988).

In previous studies of variants in which the distal His64 residue or the proximal His93 residue of horse heart myoglobin was replaced by a tyrosyl residue, we found (Tang et al., 1994; Maurus et al., 1994; Hildebrand et al., 1995) subtle differences in the coordination properties of horse heart myoglobin relative to the reported properties of the corresponding variants of sperm whale (Egeberg et al., 1990) and human proteins (Adachi et al., 1991, 1993). These differences in behavior presumably result from the contributions of other differences between the sequences of these species of myoglobin that have been identified only infrequently (Hildebrand et al., 1995). For this reason, it was of interest to evaluate the possible consequences of these or other unidentified structural differences on the behavior of the His93Cys variant of horse heart myoglobin. In addition, the absence of a distal His residue in the structure of cytochromes P450 raises the possibility that residues in the distal heme pocket might be critical in determining the structural, spectroscopic, and functional properties of proximal ligand variants of myoglobin. To evaluate these potential contributions to the coordination of a proximal Cys residue, we have also studied double variants of horse heart myoglobin that possess both a proximal Cys ligand and replacement of the distal His residue with non-coordinating residues. These studies have been further complemented by spectroscopic characterization of derivatives of the single and double

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variants following cyanation, as previously reported for the wild-type protein (Shiro & Morishima, 1984; Bracete et al., 1991; Adachi & Morishima, 1992).

EXPERIMENTAL PROCEDURES

Site-Directed Mutagenesis, Protein Expression, and Protein Purification. The construction and purification of a synthetic gene coding for wild-type horse heart myoglobin in *Escherichia coli* has been described previously (Guillemette et al., 1991). Oligonucleotide-directed mutagenesis techniques (Zoller & Smith, 1983, 1984) were used to construct the variants used in this work. Potential mutants were screened by single-stranded DNA sequencing (Maniatis et al., 1989). Restriction enzymes were obtained from Pharmacia, and all other enzymes were obtained from Sigma. Cultures of *E. coli* expressing recombinant myoglobins were grown as described previously (Lloyd & Mauk, 1994).

With the following exceptions, the purification procedure used for isolation of the myoglobin variants used in this work is the same as that described previously (Hildebrand et al., 1995). To eliminate possible complications arising from the production of sulfmyoglobin during myoglobin expression in *E. coli* (Lloyd & Mauk, 1994), all of the variants isolated in the present work were converted to the respective apoproteins and reconstituted with protoheme IX (Porphyrin Products, Logan, Utah). Apoprotein was prepared following elution of the recombinant proteins from a chelating Sepharose column (Lloyd & Mauk, 1994). The His64Ile and His64Val variants were kept in the buffer used to elute the recombinant protein from this column [50 mM Tris-HCl buffer containing 50 mM imidazole and 0.5 M NaCl (pH 8.0)], but the His93Cys, His93Cys/His64Ile, and His93Cys/His64Val variants were exchanged into 50 mM Tris-HCl buffer containing 0.5 mM dithiothreitol (DTT) before the heme was removed. These Cys-containing variants were kept in 0.5 mM DTT throughout the heme extraction procedure. An equivalent of hemin dissolved in 0.1 M NaOH was added to these apoproteins, and the DTT was then removed by dialysis against 50 mM Tris-HCl (pH 8.0) prior to gel filtration. Extinction coefficients were determined by the pyridine hemochromogen method (De Duve, 1948).

Spectroscopy. Electronic absorption spectra were recorded at 25 °C with a Cary Model 219 spectrophotometer interfaced to a microcomputer (On-Line-Instrument-Systems, Bogart, GA) and fitted with a water-jacketed cell holder and a circulating water bath. MCD spectra were recorded with a Jasco J-720 spectropolarimeter and an Oxford Instruments SM-4 superconducting solenoid. The spectropolarimeter was calibrated with a solution of camphorsulfonic acid. Spectra were collected at 300 K and 2.0 T using a 1 mm cuvette.

NMR spectra were recorded at 20 °C with a Bruker MSL-200 spectrometer operating in the quadrature mode at 200 MHz. Typical spectra consisted of 30−60K transients of 8192 data points each that were collected over a 62.5 kHz bandwidth using a superWEFT pulse sequence (Inubushi & Becker, 1983). Before Fourier transformation, the free induction decay was apodized by an exponential function that introduced 30 Hz line broadening. Chemical shifts are referenced to DSS (2,2-dimethyl-2-silapentane-5-sulfonate) through the residual water reference.

EPR spectra were obtained at 4 K with a Bruker ESP 300E spectrometer (modulation frequency 100 kHz, modulation amplitude 8 G, microwave frequency 9.46 GHz, power 0.5−1 mW) equipped with a Hewlett-Packard Model 5352B microwave frequency counter and an Oxford Instruments ESR900 continuous flow cryostat.

Electrochemical Measurements. The midpoint reduction potentials of the His93Cys/His64Ile and His93Cys/His64Val variants were determined with a photochemical method that employs riboflavin and EDTA, as previously described (Hildebrand et al., 1995). The protein solutions (20−26 μM) used in these measurements contained 46 mM sodium phosphate buffer (pH 8.0), 10 mM EDTA, and 25−35 μM riboflavin. The potentials of the wild-type protein and of the His64Ile and His64Val variants were determined by spectroelectrochemistry through the use of an optically transparent thin-layer electrode (Reid et al., 1982). The midpoint potentials of wild-type myoglobin and of the single variants (His64Ile and His64Val) were measured in 46 mM sodium phosphate at pH 6.0 and 7.0, respectively. Triply recrystallized $Ru(NH_3)_6Cl_3$ (E° = 51 mV vs SHE; Alfa) (Pladzewicz et al., 1973) was used as a mediator in all spectroelectrochemical experiments. For the single variants, $Ru(NH_3)_5(Im)Cl_3$ (E° = 110 mV vs SHE) (Sundberg et al., 1974) was added as a second mediator. Each mediator was used at a concentration that was one-tenth that of the protein.

Cyanogen Bromide Titrations. Cyanogen bromide (CNBr) titrations were performed with wild-type, His93Cys, His93Cys/His64Val, and His93Cys/His64Ile myoglobins. The wild-type protein was exchanged into sodium phosphate buffer (46 mM, pH 6.0), whereas the cysteine-containing variants were exchanged into Tris-HCl buffer (50 mM, pH 8.9). A stock solution (28 mM) of CNBr (Kodak) was prepared by dissolving the solid in distilled water. Wild-type (8−10 μM) and variant (20 μM) myoglobins were titrated with ~10 equiv of CNBr, and electronic spectra were recorded (300−500 nm, 25 °C) until no further changes were observed.

Kinetics of Reconstitution of the His93Cys/His64Ile Variant. The apoprotein of the variant was prepared as previously described (Teale, 1959) and then dialyzed against Tris-HCl buffer (50 mM, pH 8.0) containing DTT (0.5 mM). Hemin (0.75 equiv) dissolved in a minimum volume of NaOH (0.1 M) was added to a solution of the apoprotein (21 μM), and spectra were recorded at regular intervals until no further change was observed. Alternatively, the same experiment was conducted by monitoring the change in absorbance at 402 nm every 15 s for 4 h. The rate of heme iron coordination to the Cys93 residue monitored in this way (1−4 h) was determined by fitting the data to a single-exponential process.

RESULTS

Electronic Absorption Spectroscopy. The electronic absorption spectra, absorption maxima, and extinction coefficients for the Fe(III), Fe(II), and Fe(II)−CO derivatives of the wild-type and variant proteins are shown in Figure 1 and Table 1. The absorption maxima of the His64Val variant (Figure 1B) are essentially identical to those of the His64Ile variant. The Fe(III) forms of both proteins exhibit Soret maxima that are blue-shifted and less intense than that of the wild-type protein. Such changes are typical of a five-coordinate Fe(III) center and resemble the spectrum of cyanogen bromide-modified wild-type myoglobin (*vide infra*). The crystallographically determined three-dimensional

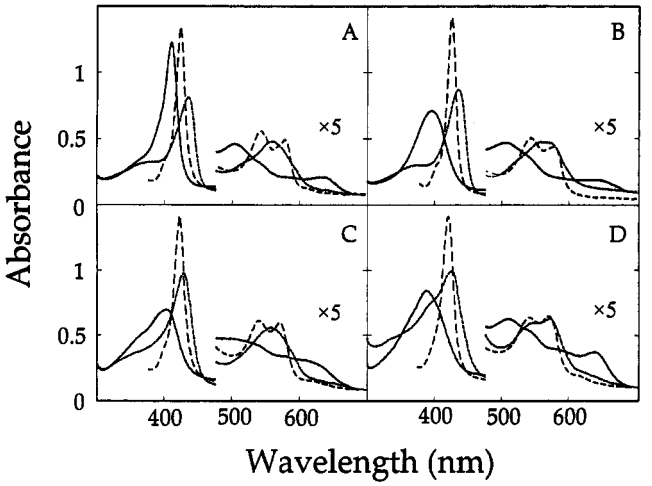

FIGURE 1: Electronic absorption spectra (298 K) of Fe(III) (solid line), Fe(II) (dashed line), and Fe(II)−CO (dotted line) horse heart myoglobin in 46 mM sodium phosphate: (A) wild type, pH 6.5; (B) His64Val, pH 6.5; (C) His93Cys, pH 7.5; (D) H93Cys/His64Val, pH 7.5.

Table 1: Electronic Absorption Spectra of Wild-Type and Variant Forms of Horse Heart Myoglobin

protein	derivative	Soret	visible
wild type[a]	Fe(III)	408 (188)[d]	402 (10.2), 630 (3.9)
	Fe(II)	435 (121)	560 (13.8)
	Fe(II)−CO	424 (207)	540 (15.4), 579 (13.9)
Val64[b]	Fe(III)	395 (98)	506 (11.6), 640 (2.5)
	Fe(II)	434 (121)	560 (12.6)
	Fe(II)−CO	425 (200)	542 (13.4), 576 (11.6)
Cys93[c]	Fe(III)	402 (78)	498 (10.8), 610 (6.7)
	Fe(II)	429 (115)	558 (12.8)
	Fe(II)−CO	422 (171)	541 (14.2), 571 (13.8)
Val64Cys93[c]	Fe(III)	389 (87)	509 (13.3), 634 (7.5)
	Fe(II)	427 (109)	545 (11.8), 570 (13.1)
	Fe(II)−CO	421 (153)	539 (13.4), 569 (13.7)

[a] Antonini & Brunori, 1971. [b] 46 mM sodium phosphate (pH 6.5). [c] 46 mM sodium phosphate (pH 7.5). [d] mM^{-1} cm^{-1}.

structure of the His64Val variant of sperm whale myoglobin indicates that the distal water molecule normally bound as the sixth axial ligand of wild-type metmyoglobin is not present in the variant (Quillin et al., 1993). Substitution of the distal histidine with small, nonpolar residues eliminates the hydrogen bond normally formed between His64 and the coordinated water molecule and thereby destablizes the binding of water as the sixth ligand in the Fe(III) derivative of the variant. Moreover, the electronic spectra of the His64Ile and His64Val variants exhibit an unusual pH dependence that is unlike that seen in the wild-type protein and that has been discussed elsewhere (R. Bogumil et al., in press).

On the other hand, the spectrum of the Fe(III) derivative of the His93Cys variant (Figure 1C) is distinctly different from that of the corresponding human myoglobin variant (Adachi et al., 1991). For example, at pH 6.5 the Soret maximum of the horse heart variant occurs at 406 nm, while the Soret band of the human variant occurs at 391 nm. Raising the pH of the horse heart protein to 9.4 leads to a reversible shift in the Soret maximum to 399 nm (data not shown). Although raising of the pH slightly favors coordination of the proximal Cys residue to the heme iron, these data and the lower extinction coefficient suggest that the Fe(III) derivative of the horse heart variant predominantly

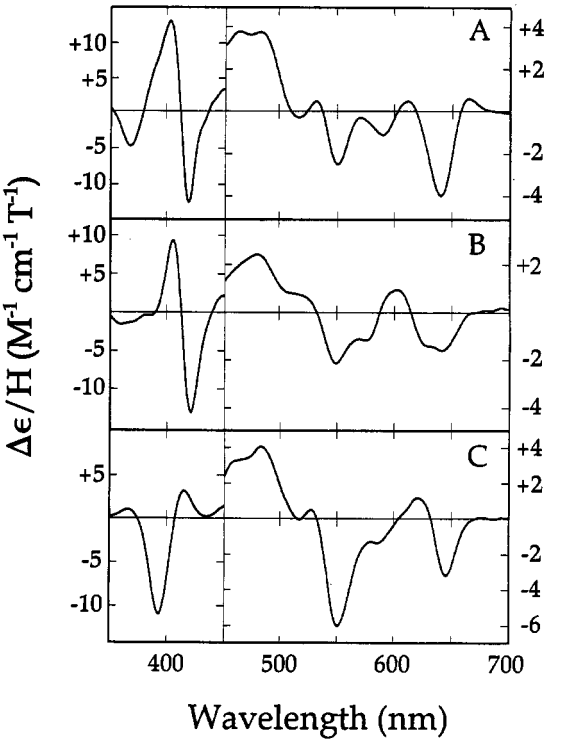

FIGURE 2: MCD spectra of oxidized horse heart myoglobin: (A) wild type, pH 7.0; (B) His93Cys, pH 8.0; (C) His93Cys/His64Val, pH 8.0. Myoglobin samples were prepared in 46 mM sodium phosphate to concentrations of 5×10^{-5} and 5×10^{-4} M in the Soret and visible regions, respectively.

occurs with the Cys93 ligand not bound to the iron. Interestingly, the electronic spectra of the Fe(II) and Fe(II)−CO derivatives of the horse heart variant are nearly identical to those exhibited by the corresponding human variant, suggesting that these derivatives of both species of the variant exhibit similar axial ligation. Reduction of the heme iron in the human variant has been proposed to eliminate coordination by the proximal cysteine ligand in this protein (Adachi et al., 1991, 1993).

As the best characterized heme enzyme possessing a proximal cysteine ligand to the heme iron, cytochrome P-450, does not possess a distal His residue, the effect of eliminating the distal His residue of myoglobin (His64) on the coordination of Cys93 has been evaluated. The spectra of the Fe(III) derivatives of the His93Cys/His64Ile and His93Cys/His64Val variants are similar to the spectrum of the Fe(III) derivative of the His93Cys variant of human myoglobin (λ_{max} = 390 nm, pH 6.5−10.0) (Figure 1D). Thus, it appears that substitution of the distal histidine allows quantitative ligation of the proximal cysteine of the horse heart protein when the heme iron is in the Fe(III) state. The reduced forms of the double variants have Soret maxima that are blue-shifted and decreased in intensity compared to the reduced His93Cys variant. The spectra of the Fe(II)−CO forms of the cysteine-containing variants are not typical of thiolate ligation (see the following).

Magnetic Circular Dichroism. To address the nature of the ligation state(s) in these variants, further, MCD spectra were obtained for the oxidized (Figure 2) and reduced (Figure 3) forms at pH 7 for the wild-type protein and at pH 8 for the variants. The spectroscopic features of wild-type ferrimyoglobin observed in this study are typical of six-coordinate, predominately high-spin, heme proteins, and the

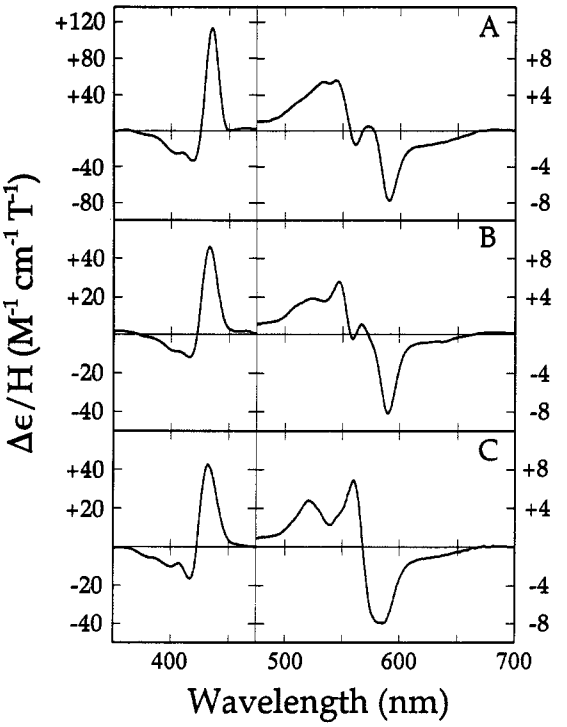

FIGURE 3: MCD spectra of reduced horse heart myoglobin: (A) wild type, pH 7.0; (B) His93Cys, pH 8.0; (C) His93Cys/His64Val, pH 8.0. Myoglobin samples were prepared in 46 mM sodium phosphate to concentrations of 3×10^{-5} and 5×10^{-4} M in the Soret and visible regions, respectively.

strongly positive ellipticity in the Soret region is typical of oxygen- or nitrogen-containing ligands (Vickery et al., 1976). The spectrum of the His93Cys/His64Val variant is similar to those of oxidized cytochrome P-450, chloroperoxidase, and thiolate-containing protoporphyrin models (Dawson et al., 1976a). The strong negative intensity in the Soret region is diagnostic of five-coordinate thiolate-bound protoporphyrin IX. The His93Cys variant shares some spectroscopic similarity with the double variants in the visible region, but the shape and intensity of the Soret band are not consistent with cysteinate axial ligation, and it is more likely that a water molecule serves as the proximal ligand in this variant.

Upon reduction of wild-type myoglobin to ferromyoglobin (Figure 3), the coordination environment of the heme iron undergoes loss of the distally bound water molecule to produce a five-coordinate environment that can be identified by a characteristic MCD spectrum (Vickery et al., 1976). The spectrum of the His93Cys/His64Val variant is clearly distinct from that of wild-type metmyoglobin and that of cytochrome P-450 (Dawson et al., 1976b). This observation confirms that cysteinate is not a ligand to the heme iron in the reduced double variant and leads us to propose that water is the proximal ligand in this case. With the His93Cys variant, however, spectroscopic similarities with wild-type myoglobin suggest the presence of a five-coordinate heme iron in which the distal histidyl residue is bound. These observations are consistent with those of the corresponding human variant (Adachi et al., 1991, 1993). The differences in the Soret and visible regions between the His93Cys variant and wild-type horse heart protein probably arise from incomplete ligation by the distal histidine and from the presence of a mixture in which some of the variant possesses a coordinated His64 and the remainder possesses a coordinated water molecule.

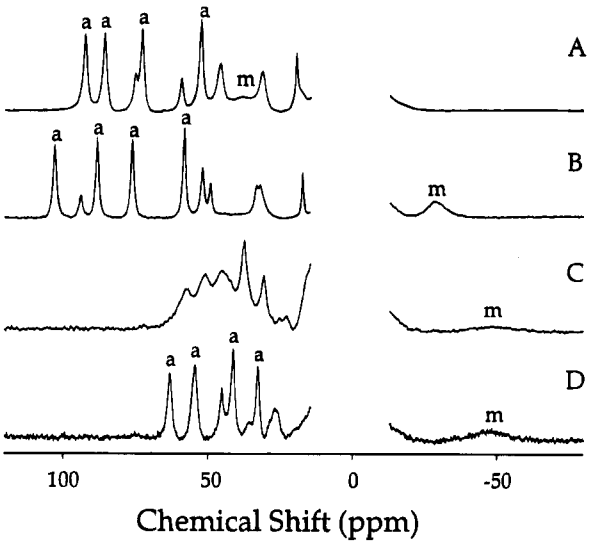

FIGURE 4: Hyperfine-shifted region of the 200 MHz ^{1}H NMR spectra of wild-type and variant forms of horse heart metmyoglobin: (A) wild type; (B) His64Val; (C) His93Cys; (D)His93Cys/His64Val. Only the heme methyl (a) and *meso* protons (m) are labeled. Myoglobin samples (~2 mM) are prepared in 46 mM deuterated sodium phosphate (pD 7.0).

The MCD spectra of the oxidized and reduced His93Cys/His64Ile double variant are essentially identical to those of the His93Cys/His64Val form. The MCD spectra of oxidized His64Val and His64Leu variants of human myoglobin have been reported previously (Ikeda-Saito et al., 1992) and are similar to those of the His64Val and His64Ile variants of horse heart myoglobin (data not shown). These spectra are characterized by very weak Soret bands and are similar to the MCD spectra of naturally occurring, five−coordinate heme proteins (Ikeda-Saito et al., 1992).

Nuclear Magnetic Resonance (NMR) Spectroscopy. The hyperfine-shifted regions of the ^{1}H NMR spectra of wild-type and the His64Val variant of horse metmyoglobin (Figure 4A,B) are similar to those of sperm whale and human metmyoglobins (Rajarathnam et al., 1991; Ikeda-Saito et al., 1992). The upfield resonance observed for the *meso* protons is a characteristic feature of five-coordinate heme systems (Arasasingham et al., 1990). The *meso* protons that appear downfield at ~40 ppm in the spectrum of wild-type, hexacoordinate metmyoglobin are shifted to −29 ppm in the spectra of His64 variants. In addition, the mean heme methyl resonance shifts approximately 5 ppm downfield upon the loss of the axially bound water molecule. The heme methyl resonances of the wild-type protein appear at 92.4, 85.6, 72.7, and 52.5 ppm, whereas they occur at 103.0, 88.3, 76.2, and 58.3 ppm in the spectrum of the His64Val variant.

The ^{1}H NMR spectrum of the His93Cys Fe(III) derivative has fewer resonances relative to the corresponding spectra of the His93Cys/His64Ile and His93Cys/His64Val variants (Figure 4C). The spectrum of the His93Cys variant, however, exhibits the broad upfield *meso* proton resonance that is indicative of a five-coordinate species. The spectra of the double variants (Figure 4D) more closely resemble the spectrum of the human myoglobin His93Cys variant (Adachi et al., 1991, 1993). The downfield regions of these spectra exhibit methyl resonances at 62.9, 54.5, 41.4, and 32.7 ppm. In addition, a broad upfield resonance (ca. −48

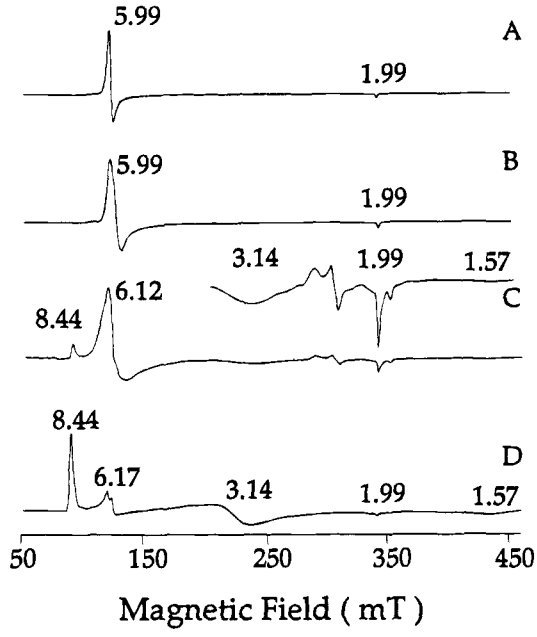

FIGURE 5: EPR spectra of horse metmyoglobin: (A) wild type; (B) His64Val; (C) His93Cys (low-spin and $g = 2$ region expanded); (D) His93Cys/His64Val. Myoglobin samples (~1 mM) were prepared in 46 mM sodium phosphate (pH 7.0).

ppm) is observed that was not reported for the human variant and that is presumably attributable to the *meso* protons.

Electron Paramagnetic Resonance (EPR) Spectroscopy. All of the proteins in this study exhibit high-spin EPR spectra at pH 7. At this pH, the EPR spectrum of wild-type metmyoglobin is that of a high-spin, axially symmetric system having g values of 5.99 and 1.99 (Figure 5A). The EPR spectra of the His64Val and His64Ile variants are similar to that of wild-type myoglobin (Figure 5B) (Ikedo-Saito et al., 1992; Bogumil et al., in press). However, the broadening of the low-field region in the spectra of these proteins suggests that all of the His64 variants are slightly more rhombic than the wild-type protein.

The EPR spectrum of the His93Cys Fe(III) variant (Figure 5C) exhibits a mixture of high-spin components. The major species has g values of 6.12 and 1.99 and is attributable to a form of the protein in which the Cys93 ligand is not bound to the heme iron. A minor, highly rhombic species having g values of 8.44, 3.14, and 1.57 is attributable to a pentacoordinate form of the protein in which the Cys93 residue is coordinated to the heme iron. There is also evidence for a low-spin species (expanded region) having g values of 2.37, 2.24, and 1.94 that presumably arises from a hexacoordinate derivative in which the Cys93 residue and a distal water molecule are bound to the heme iron. The high- and low-spin cysteine-ligated forms exhibit EPR signals similar to those reported for the His93Cys variant of human metmyoglobin, which is six-coordinate (Adachi et al., 1991, 1993). It has been suggested that the low-spin form of the human metmyoglobin variant results from freezing the sample (Adachi et al., 1991, 1993).

The EPR spectra of the His93Cys/His64Ile and His93Cys/His64Val double variants are identical to each other (Figure 5D) ($g = 8.44$, 3.14, and 1.57) and are similar to those of the high-spin species reported for the His93Cys variant of human metmyoglobin. The horse heart variants also exhibit a minor component having g values of 6.17 and 1.99 that results from a fraction of the protein in which the Cys93 residue is not coordinated to the iron. This species only appears after heme extraction and reconstitution and apparently results from modification of the cysteine residue in the apoprotein. This conclusion is based on the observation that the contribution of this form of the protein to the spectrum is greater if no dithiothreitol is used during apoprotein preparation. The EPR spectra of the double variants exhibit no evidence of the low-spin form present in the spectra of the His93Cys horse heart and human variants, presumably due to destabilization of the bound water ligand in variants lacking the distal histidine.

Table 2: Midpoint Reduction Potentials of Wild-Type and Variant Forms of Horse Heart Myoglobin

protein	E_m (mV vs SHE)	method
wild type	63	spectroelectrochemistry
His64Val	87	spectroelectrochemistry
His64Ile	95	spectroelectrochemistry
His93Cys/His64Val	−217	photoreduction
His93Cys/His64Ile	−219	photoreduction

Electrochemistry. Substitution of the proximal histidine with anionic ligands such as phenolate and thiolate has been shown to cause a large decrease in the reduction potential of the protein (Adachi et al., 1991; Hildebrand et al., 1995). For the His93Cys/His64Val and His93Cys/His64Ile variants of horse heart myoglobin, the introduction of a proximal cysteine residue lowers the reduction potential by 280 mV relative to that of the wild-type protein, and it lowers the potential by 305 and 313 mV relative to the His64Val and His64Ile variants, respectively (Table 2). This decrease in the reduction potential is further evidence of thiolate ligation and is similar to the potential reported for the human His93Cys variant (Adachi et al., 1991). Removal of the distal histidine increases the reduction potential of myoglobin by eliminating the distally bound water molecule and decreasing the electron density around the iron.

Cyanogen Bromide (CNBr) Titration. Addition of CNBr to wild-type horse heart metmyoglobin causes a blue shift in the Soret band (408 to 398 nm) and decreases its intensity (Figure 6A) (Shiro & Morishima, 1984; Bracete et al., 1991; Adachi & Morishima, 1992). This modification is believed to involve cyanation of the distal histidine to produce a pentacoordinate heme iron as the result of disruption of the hydrogen bond between His64 and the water molecule normally coordinated to the heme iron.

This experiment was repeated with the His93Cys variant in an attempt to explain the mechanism of ligation of the double variants (Figure 6B). At pH 8.9, titration of this variant with CNBr shifts the Soret maximum from 399 nm in the unmodified protein to 405 nm to produce a spectrum similar to that observed for the His93Cys variant at low pH (Figure 1 and Table 1). Cyanogen bromide, therefore, disrupts the bond between the iron and the proximal ligand. The same effect is seen for the double variants (Figure 6C), in which case the Soret maximum shifts from 389 to 404 nm. It appears that, in the His93Cys variants, CNBr causes cyanation of the Cys93 sulfur atom, which is known to be more reactive toward this reagent than histidine (Shiro & Morishima, 1984), and that this reaction, therefore, competes with the ligation of Cys93 to iron.

Although the electronic spectrum of the Fe(II)−CO derivative of the His93Cys/His64Val variant indicates that

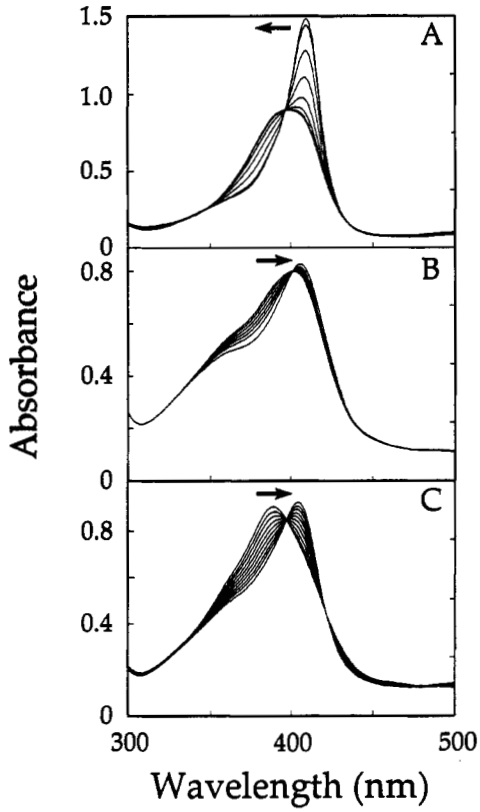

FIGURE 6: Titration of horse metmyoglobins with cyanogen bromide: (A) wild type; (B) His93Cys; (C) His93Cys/His64Val. The arrows indicate the direction of the shift in the Soret maximum.

the Fe−Cys bond is broken in this form of the protein, it is known from studies with model compounds that a Soret maximum at 422 nm can result from the axial coordination of sulfur-containing ligands (Collman & Sorrell, 1975). A Soret maximum at 450 nm occurs only if the cysteine ligand is deprotonated. As further proof to exclude the possibility of a protonated cysteine giving rise to the electronic spectra observed here, we prepared the reduced and CO forms of the His93Cys/His64Val variant after treatment with sufficient cyanogen bromide to modify the Fe(III) derivative. The resulting CNBr-modified double variant exhibits electronic spectra that are essentially identical to those of the reduced and CO-bound derivatives of the unmodified proteins (data not shown) and indicates that the proximal ligand is no longer coordinated to the heme iron after conversion of the protein to ferromyoglobin.

Reconstitution of the His93Cys/His64Ile Variant with Heme. During reconstitution of the double variants with heme, time-dependent changes in the electronic spectra are observed (Figure 7). Reaction of hemin with the apo-His93Cys/His64Ile variant leads to a spectrum with a Soret band initially at 402 nm that promptly shifts to higher energy. The initial spectrum is similar to that of the His93Cys variant at pH 6.5 (Figure 1 and Table 1) and to those of CNBr-modified forms of the His93Cys, His93Cys/His64Ile, and His93Cys/His64Val variants (Figure 6). This spectrum is attributable to a heme group that resides in the active site of the protein, but that is not coordinated to the proximal protein ligand. Within an hour following reconstitution, the Soret maximum shifts to 390 nm. Thus, it appears that reconstitution of these variants occurs in two discrete steps: heme uptake, which is rapid, followed by heme ligation to Cys93, which is slow.

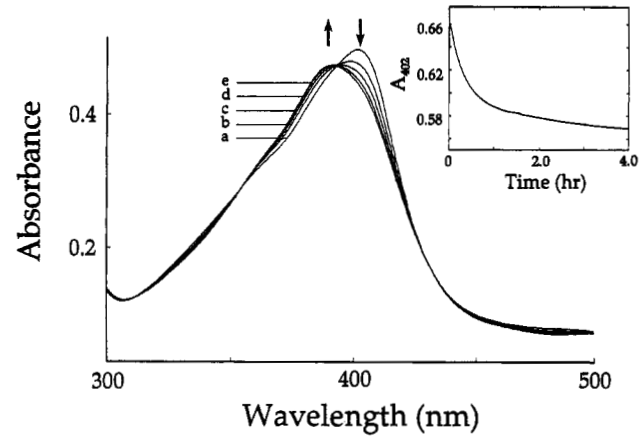

FIGURE 7: Kinetics of heme binding to the His93Cys/His64Ile variant of horse heart apomyoglobin. Sequential spectra recorded after the addition of hemin at time = t_0. For illustrative purposes, only the first five spectra are shown. The time after the addition of hemin at which each scan was begun is as follows: a, 1.5; b, 7; c, 12; d, 18; and e, 24 min. The direction of the shift in the Soret maximum is indicated by the arrows. Inset: Change in absorbance at 402 nm after the addition of hemin.

To examine heme uptake and ligation more thoroughly, the absorbance change at 402 nm (Figure 7, inset) was monitored. Although only small changes are seen in the Soret region of the spectrum after 1 h, the absorbance at 402 nm continues to decrease for at least 4 h. For an accurate estimate of the absorbance of the fully ligated derivative, the spectrum was determined after 24 h. The change in absorbance observed between ~1 and 4 h following reconstitution is consistent with a single-exponential process and has a rate constant of 1.3×10^{-4} s^{-1} that presumably represents ligation of Cys93 to the heme iron.

DISCUSSION

Comparison of Horse Heart and Human Myoglobin. The amino acid sequences of horse heart and human myoglobins exhibit 89% identity. Nevertheless, we have previously noted subtle differences between the proximal His93Tyr variants of horse heart and human myoglobins (Hildebrand et al., 1995) that can be attributed to minor structural differences on the proximal side of the heme-binding pocket. The results reported here demonstrate the existence of similar subtle differences in the properties of the proximal His93Cys variants of these two species of metmyoglobin. We have found no experimental conditions that permit quantitative ligation of the proximal cysteine residue to the heme iron of this variant, as observed for the corresponding variant of human myoglobin (Adachi et al., 1991, 1993). Evidently, the distance between the iron and the proximal Cys residue in the horse heart protein is too great to permit facile coordination of this residue (*vide infra*). From the spectroscopic results described earlier, and by analogy to previous results reported for the human variant (Adachi et al., 1991, 1993), we propose that the principal spectroscopic component of this metMb variant possesses a five-coordinate ferriheme iron center with a distally bound water molecule. Furthermore, we conclude that the coordination of this water molecule is stabilized through a hydrogen-bonding interaction with the distal histidine residue.

From previous crystallographic studies of sperm whale myoglobin variants, it is known that substitution of His64

with apolar residues such as valine or leucine destabilizes distal coordination by an aquo ligand through elimination of the hydrogen bond normally formed with this residue. As a result, the His64Val variant of sperm whale myoglobin undergoes displacement of the iron atom from the pyrrole nitrogen plane by 0.18 Å toward the proximal side relative to the wild-type protein (Quillin et al., 1993). We have now found that when His64 is replaced with Val (or Ile) in the His93Cys variant of horse metmyoglobin, the anticipated proximal displacement of the iron, although small, is sufficient to allow quantitative coordination of Fe(III) by Cys93, so that the double variant possesses a five-coordinate heme center in which the Cys93 residue is coordinated and the distal ligand-binding site is vacant.

The ligation state of the reduced form of the His93Cys variant involves a novel rearrangement of the active site of the protein. Adachi and co-workers (1993) suggest that the iron atom in the reduced form of the human His93Cys myoglobin variant is coordinated by the distal His64 residue and that the proximal ligand-binding site is unoccupied. Furthermore, binding of CO to this variant was proposed to occur at the proximal side of the heme. The electronic spectra of both the reduced human and horse heart His93Cys variants indicate that the behavior of both proteins is similar under these circumstances. The MCD spectrum of reduced His93Cys reported here provides further evidence that it is possible for the distal histidine to coordinate to the heme iron under some conditions. Interestingly, the electronic spectrum of the reduced His93Cys/His64Val variant resembles that of deoxymyoglobin at pH 2.6, in that it possesses a blue-shifted Soret maximum and two discernible maxima in the visible region (Han et al., 1990). At low pH, the proximal histidyl residue of wild-type deoxymyoglobin is thought to be replaced by a weak-field ligand such as water (Han et al., 1990; Palaniappan & Bocian, 1994). Although the similarity of the electronic spectra of five- and six-coordinate model ferroheme complexes (Han et al., 1990; Brault & Rougee, 1974) precludes unambiguous assignemt of the coordination state of the ligand-free Fe(II) derivatives of the His93Cys/His64Val and His93Cys/His64Ile double variants, the carbonyl derivatives of these double variants can be identified as six-coordinate on the basis of the observation that the electronic spectra of five-coordinate carbonyl−ferroheme complexes exhibit Soret maxima at ~390 nm (Traylor et al., 1985).

Trans Effects on Proximal Coordination by Cys93. The *sina qua non* of the cytochrome P-450 family is the red-shifted Soret maximum of the reduced CO derivative (Klingenberg, 1958; Garfinkel, 1958; Omura & Sato, 1962). This spectroscopic feature is generally regarded to be diagnostic of proximal thiolate coordination and has been assigned to a cysteine−heme charge transfer transition (Hanson et al., 1976). The reduced carbonyl forms of the His93Cys and His93Cys/His64Val horse heart myoglobin variants do not exhibit this characteristic Soret maximum. Instead, the Fe(II)−CO derivatives of these variants exhibit electronic spectra similar to those observed for wild-type myoglobin and non-native P-450 (P-420), respectively, both of which possess proximal histidyl ligands (Wells et al., 1992). On the basis of this observation, and by analogy to resonance Raman studies of the corresponding variant of human myoglobin (Adachi & Morishima, 1992), it seems likely the carbonyl derivative of the horse heart His93Cys variant studied here also involves distal coordination by His64 and proximal binding of carbon monoxide. On the other hand, distal coordination by His64 cannot occur in the Fe(II)−CO derivative of the His93Cys/His64Val double variant. In this case, it seems likely that the distal coordination position is occupied by a water molecule. Unfortunately, MCD spectroscopy does not permit identification of the sixth ligand of CO−Fe(II)−heme derivatives unless the sixth ligand is a thiolate group (Collman et al., 1976). Interestingly, a mutant of semisynthetic cytochrome *c* in which one of the natural ligands is replaced with a cysteine residue (Met80Cys) produces a protein with thiolate bound in the oxidized form, but not in the reduced form (Smulevich et al., 1994). These authors suggest that in the Fe(II)−CO form the cysteine ligand may be either protonated or displaced.

Nature of the Cys93−Heme Iron Bond. Molecular graphics replacement of a Cys residue for His93 in the structure of wild-type myoglobin suggests that an S−Fe distance of 3.9 Å would result in the absence of compensatory conformational adjustments by the protein. This distance is clearly greater than the 2.4−2.5 Å anticipated for an S−Fe bond. On the other hand, substitution of His64 by a non-coordinating and non-hydrogen-bonding residue would be expected to displace the iron atom by <0.5 Å toward the proximal side of the heme. On the basis of these simple considerations, it is reasonable to propose that additional structural rearrangement of the active site must occur to permit coordination of Cys93 in the double variants (e.g., movement of the F-helix; *vide infra*). Modification of Cys93 in the double variants by reaction with cyanogen bromide suggests that the interaction of this residue with the iron is weak. Weak proximal coordination in this variant may be consistent either with the requirement for a significant conformational change to permit Cys93 coordination to the heme iron or with a relatively long Fe−S bond. The slow rate of proximal ligand binding to the iron observed here may result from a significant structural rearrangement that is required for ligation by the proximal Cys93.

In a previous report concerning the His93Tyr variant of horse heart myoglobin, we provided structural evidence that a relatively large proximal ligand to the heme iron of horse heart myoglobin can be accommodated by substantial structural rearrangement in which the helical domain in which His93 resides moves away from the heme prosthetic group (Hildebrand et al., 1995). In the current study, we have replaced His93 with a significantly smaller residue and have evaluated the ability of this residue to serve as the proximal iron ligand. Unfortunately, attempts to produce crystals of the variants reported here for crystallographic analysis have not succeeded at this time. Nevertheless, from the spectroscopic results reported here, it is clear that the relatively small Cys93 residue can function as an adequate heme ligand in ferrimyoglobin, particularly in the absence of the distal His residue. The range of ligand size that can be accommodated at position 93 suggests a remarkable degree of conformational flexibility in the active site of myoglobin, although this flexibility is not sufficient to retain coordination by Cys93 upon reduction of the heme iron.

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NOTE ADDED IN PROOF

A different type of *trans* effect in the regulation of ligand binding to myoglobin variants has been studied recently by Boxer and co-workers (S. M. Decatur, S. Franzen, G. D. DePillis, R. B. Dyer, W. H. Woodruff, and S. G. Boxer, *Biochemistry*, in press). In this work, the effect of distal NO binding to the His93Gly variant on proximal ligation of exogenous ligands has been characterized, in part as a model of the active site of guanyl cyclase.

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