Conversion of Myoglobin into a Reversible Electron Transfer Protein That Maintains Bishistidine Axial Ligation

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Abstract: The identity of the axial ligands provided by the protein to complete the coordination environment of the heme iron is one of the major structural determinants of heme protein functional properties. In the current work, the distal valine 68 residue in horse heart myoglobin has been replaced with a histidyl residue (Val68His), and the variant protein has been characterized by electronic absorption, MCD and EPR spectroscopies, and spectroelectrochemistry. The electronic absorption spectrum of the oxidized form of the variant at ambient (25.0 °C) temperatures exhibits absorption maxima similar to those of cytochrome b_5. An additional high-spin component that is not present at 77 K is also apparent. The low-temperature (77 K) spectrum of the reduced form of the variant exhibits the characteristic α and β bands that characterize the spectrum of ferrocytochrome b_5. Near-IR MCD spectroscopy (300 K) of the oxidized derivative reveals an intense transition at 1607 nm that is similar to those observed for cytochrome b_5 and imidazole-Mb. The visible MCD spectrum (300 K) of reduced Val68His Mb is essentially identical to that of reduced cytochrome b_5, but it possesses an additional component at 592 nm that is absent at 77 K. The reduction potential of the variant (pH 7.0, $\mu = 0.10$ M, 25.0 °C) is -110 ± 0.1 mV *vs* SHE, compared to a value of 60.9 ± 0.1 mV for wild-type Mb and 4 mV for cytochrome b_5. The overall spectroscopic properties of the variant are remarkably similar to those of cytochrome b_5, the classical bishitidine ligated heme protein, and direct coordination of His68 to the heme iron of the variant is proposed for both oxidation states in the absence of exogenous ligands (e.g., dioxygen).

The relationships that exist between various classes of heme proteins and the mechanisms by which protein structures control heme chemical reactivity are of considerable current interest.[1] Although many structural variables are influential, one of the major structural determinants of heme protein functional properties is the identity of the axial ligands provided by the protein to complete the coordination environment of the heme iron. Alteration of axial ligands by semisynthesis or site-directed mutagenesis has been reported previously for a number of heme proteins (e.g., myoglobin and cytochrome *c*) in studies designed to rationalize the functional diversity that occurs between various classes of heme proteins.[2] In the current work, the distal valine 68 residue in horse heart myoglobin has been replaced with a histidyl residue, and the variant protein has been characterized by electronic absorption, MCD and EPR spectroscopies, and electrochemistry. Remarkable similarities between the spectroscopic and electrochemical properties of this variant and cytochrome b_5, the prototypical bishistidine ligated heme protein, are observed, and direct coordination of His68 to the heme iron is proposed to occur in both oxidation states of the variant.

Experimental Procedures

Site-Directed Mutagenesis and Protein Expression. Expression of a synthetic gene coding for horse heart myoglobin has been described.[3] Oligonucleotides were synthesized with a modified Applied Biosystems 380A DNA synthesizer and purified by passage through a Millipore C18 Sep Pak column at the UBC Nucleic Acid and Protein Synthesis laboratory. The sequence of the mutagenic oligonucleotide was 5′-CCTAGGGCAGTTAAGTGAACCGGTACCATG-3′.

Protein Purification. The variant myoglobin was purified according to procedures described for preparation of the wild-type protein.[3,4] The resulting protein was reconstituted with fresh protoheme IX (Porphyrin Products, Logan, UT) to remove any sulfmyoglobin formed during fermentation.[4] The resulting purified, reconstituted protein exhibited A_{412}/A_{280} ratios ≥ 4.8.

Electronic Absorption Spectroscopy. Electronic spectra were collected near ambient temperature with a Cary Model 219 spectrophotometer that was equipped with a water-jacketed cell holder connected to a thermostated water bath and that was interfaced to a microcomputer (OLIS, Bogart, GA). Electronic spectra were recorded at 77 K with an SLM-Aminco Model DW-2C dual wavelength spectrophotometer that was fitted with a sample holder having a 2 mm path length; spectra were collected with buffer in the reference beam at the same temperature as the sample. Molar absorption coefficients were determined with the pyridine-hemochromagen method.[5] For the Val68His variant, $\epsilon_{412} = 1.34 \times 10^5$ M^{-1} cm^{-1} (pH 7.0, 25.0 °C).

EPR Spectroscopy. EPR spectra were obtained with a Bruker Model ESP 300E spectrometer that was equipped with an HP5352B

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microwave frequency counter and with an Oxford Instruments ESR900 continuous flow cryostat. The modulation frequency was 100 kHz, the modulation amplitude was 5 G, the microwave frequency was 9.46 GHz, and the microwave power was 0.50−1.0 mW.

MCD Spectroscopy. Near-infrared and uv−visible MCD spectra were collected with Jasco Model J-730 and Model J-720 spectropolarimeters, respectively, and an Oxford Instruments Model SM4 spectromag. Protein samples were placed in a quartz cuvette with a 1 mm path length, and sample temperature was maintained with an Oxford Instruments Model ITC-4 temperature controller.

^{1}H-NMR Spectroscopy. ^{1}H-NMR spectra were recorded at 20 °C with a Bruker MSL-200 spectrometer operating in the quadrature mode at 200 MHz ([Mb] ~ 2 mM in 50 mM sodium phosphate buffer, 20 °C, pD 7.0). Spectra were collected as 10 000 transients over a 38.5 kHz band width with 4096 data points and a recycle delay of 120 ms. The spectra were zero filled to 8192 points and apodized to introduce 0.5−5 Hz line broadening. Chemical shifts were referenced to 2,2-dimethyl-2-silapentane-5-sulfonate (DSS) through the residual water resonance. A Radiometer Model 84 pH meter and an Aldrich (#Z-11,343-3) microcombination electrode were used to measure pH. All pH readings in ^2H_2O were uncorrected for the deuterium isotope effect and are reported as p^{2}H.

Spectroelectrochemistry. Reduction potentials (25.0 °C, μ = 0.10 M) were obtained with an optically-transparent thin-layer electrode ([Mb] = 500 μM; optical path length ~ 0.02 cm).[6] A mixture of 2-hydroxy-1,4-naphthaquinone (50 μM) (Sigma #H-0508) and recrystallized[7] $[Ru(NH_3)_6]Cl_3$ (50 μM) (Strem) ($E°$ = 51 mV vs NHE[7]) was used to mediate electron transfer between the variant and the gold mesh working electrode. The reference electrode was standardized against the quinhydrone couple, and the resulting potentials were converted to the hydrogen scale as described by Dutton.[8] Trace amounts of *Rhus vernicifera* laccase and catalase (Sigma #C100) were added to maintain anaerobic conditions and to avoid possible complications resulting from peroxide formation. The resulting data were fitted to the Nernst equation.

Results

Electronic Absorption Spectra. The electronic absorption spectra of the oxidized and reduced forms of wild-type and Val68His myoglobins are shown in Figure 1. For the oxidized wild-type protein, the absorption maxima at 408, 502, and 630 nm[9] are consistent with a high-spin ferric iron with water as the sixth ligand. The oxidized Val68His derivative has a spectrum that differs substantially (λ_{max} (nm): 412, 527, ~560, ~630) from that of the wild-type protein. Both the red-shifted Soret band and the visible maxima are characteristic of low-spin heme iron centers and are essentially the same as those observed for bovine liver microsomal cytochrome b_5 (λ_{max} (nm): 412.5, 532, 560[10]) which has two axial histidine ligands.[11] However, the variant exhibits an additional feature at ~630 nm that is not seen in cytochrome b_5 and that is indicative of high-spin Fe(III). Similar transitions have been identified in imidazole bound metMb (λ_{max} (nm): 535, 568, and 647[12]) which is known to exist as an equilibrium mixture of high- and low-spin forms. However, from these data it is not possible to distinguish between high-spin metaquo Mb and high-spin bishistidine Mb. The Soret maximum (428 nm) in the reduced form of the variant lies between that expected for ferrous wild-type Mb (435 nm[9]) and ferrous cytochrome b_5 (423 nm[10]), but the broad features in the visible region preclude qualitative interpretation at this temperature.

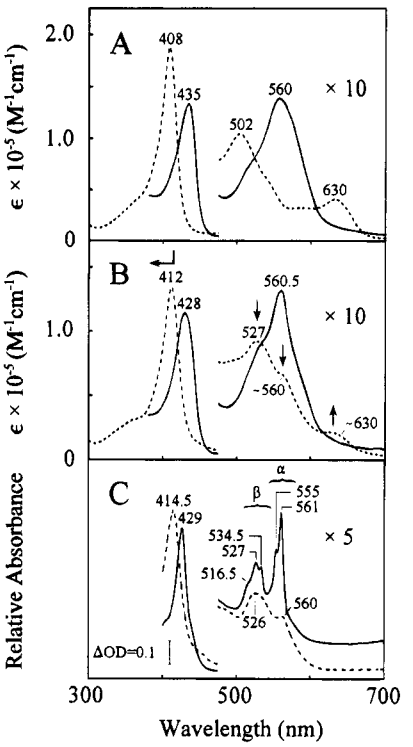

Figure 1. Electronic absorption spectra of wild-type and Val68His myoglobins. (A) oxidized (- - -) and reduced (—) wild-type Mb (sodium phosphate, pH 6.0, μ = 0.10 M) at 25.0 °C. (B) Oxidized (- - -) and reduced (—) Val68His Mb (sodium phosphate, pH 7.0, μ = 0.10 M) at 25.0 °C. The arrows indicate the absorbance changes that occur in the oxidized derivative as the temperature is increased (see text). (C) Low-temperature (77 K) spectra of oxidized (- - -) and reduced (—) Val68His Mb (sodium phosphate, pH 7.0, μ = 0.10 M). For clarity, the spectrum of the reduced derivative in the visible region (475−700 nm) is offset by +0.1 OD units against the oxidized derivative. Molar absorption coefficients were determined using the pyridine hemochromagen method.[5] Absorption maxima (nm) are indicated.

To determine the origin of the high-spin component in the oxidized derivative, the electronic spectra of the oxidized and reduced variant were studied as a function of temperature (77-323 K). Small but systematic changes in absorption maxima were identified. As the temperature was raised, the maxima at 527 and ~560 nm decreased in intensity while the band at ~630 nm simultaneously increased (Figure 1B). A shift (λ_{max} (nm): 412 (10 °C), 411.5 (50 °C)) and a decrease in intensity of the Soret maximum was also observed (Figure 1B). As the temperature is lowered further, the spectra exhibit additional changes (Figure 1C). At 77 K, the oxidized derivative exhibits maxima (λ_{max} (nm): 414.5, 526, 560) that compare with those for cytochrome b_5 at 77 K (λ_{max} (nm): 413, 531, 559[13]). The disappearance of the broad maximum at ~630 nm is particularly noteworthy. These observations are consistent with the existence of a thermal equilibrium between high- and low-spin iron, with the $S = {}^1/_2$ form dominant at 77 K and the $S = {}^5/_2$ form increasingly favored as the temperature is raised. In the reduced derivative at 77 K (Figure 1C) splitting of the broad feature at 560.5 nm is apparent (λ_{max} (nm): 429, 516.5 (sh), 527, 534.5,

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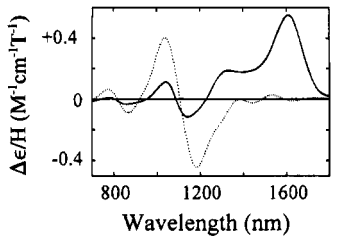

Figure 2. Near-IR MCD spectra of wild-type (- - -) and Val68His (—) metMb (25 mM sodium phosphate, pD 7.0 + 50% glycerol v/v, 300 K, 4.8 T).

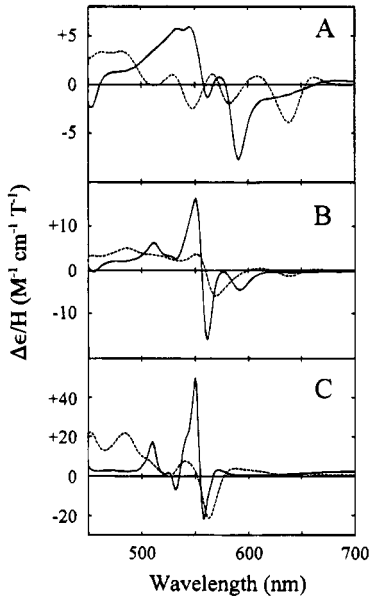

Figure 3. Visible MCD spectra of reduced (—) and oxidized (- - -) wild-type and Val68His Mb (pH 7.0, sodium phosphate buffer (25 mM)): (A) Wild-type Mb, 300 K; (b) Val68His Mb + 50% glycerol v/v, 300 K; (C) Val68His Mb + 50% glycerol v/v, 77 K.

555, 561), producing a spectrum that is similar to the corresponding low-temperature spectrum of reduced cytochrome b_5 (λ_{max} (nm): 422, 514 (sh), 524, 530, 550, 555[13]). Although the maxima differ slightly, the distinct α and β components that characterize ferrous (low-spin) cytochrome b_5 can be identified clearly in the variant.

Titration of the Val68His variant between pH 5.0 and 10.0 resulted in no significant spectroscopic change. Beyond these limits, a decrease in the Soret maximum was observed that can be attributed to protein instability. Addition of azide or fluoride to the oxidized variant produces no change in the spectrum, but a substantial change is observed on addition of cyanide that indicates efficient production of the metMbCN complex (data not shown). Exposure of the reduced protein to carbon monoxide results in formation of a species with a spectrum characteristic of carbonyl Mb (λ_{max} (nm): 422, 541, 536[9]).

MCD Spectroscopy. Additional evidence for bishistidine coordination in the oxidized variant is provided by MCD spectroscopy (Figures 2 and 3). In the near-IR region (300 K) (Figure 2), wild-type metaquoMb exhibits a positive peak at 1038 nm and a negative trough at 1183 nm that are characteristic of high-spin ferric heme iron.[14] The oxidized Val68His variant retains this high-spin transition (1041 nm and 1143 nm) as a minor feature but has an additional, dominant transition at 1607 nm that is similar to those observed for imidazole-Mb (1600 nm)[15] and cytochrome b_5 (1630 nm).[15] This observation, therefore, provides strong evidence for bishistidine ligation at ambient temperature. Although from the MCD data alone it is not possible to identify the nature of the high-spin component (i.e., metaquo vs high-spin bishistidine), there was no indication of any formation of hydroxide-bound Mb in the near-IR MCD spectrum as it was titrated to p^2H 8.6. This result suggests that the high-spin transition is not attributable to metaquoMb.

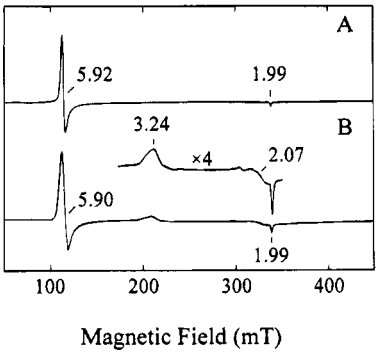

Figure 4. EPR spectra of (A) wild-type and (B) Val68His metmyoglobins (wild-type: 50 mM sodium phosphate, pH 7.0, 4.2K, [Mb] ~ 2mM; Val68His: 25 mM sodium phosphate, pD 7.0 + 50% glycerol v/v, 10K, [Mb] ~ 2mM). The expanded region shows the spectrum of the minor, low-spin component.

The MCD spectrum in the visible region of reduced Val68His Mb at 300 K (Figure 3) exhibits little resemblance to the spectrum of the reduced wild-type protein.[16] With the exception of the 592-nm band and the overall intensity, this spectrum (λ_{max} (nm): 511, 550, 561, 592) is essentially identical to that of reduced cytochrome b_5.[13,17] At 77 K the spectrum sharpens (λ_{max} (nm): 510, 549, 558) and is also very similar to that of cytochrome b_5 at 77 K.[13] Although the origin of the 592-nm band is uncertain, the fact that it is absent at 77 K is indicative of a spin-equilibrium as identified for the oxidized form. Together with the low-temperature electronic absorption data (Figure 1C), this finding provides strong evidence for low-spin, bishistidine ligation at 77 K.

EPR Spectroscopy. The EPR spectrum (10 K) of oxidized Val68His Mb suggests that at this temperature the variant is a mixture of two species (Figure 4). The major component is high spin with g values (g = 5.90, 1.99) similar to those of wild-type Mb (g = 5.92, 1.99). A minor low-spin component (g = 3.24, 2.07) is also apparent although its intensity is quite small at 10 K, and it almost completely disappears on cooling to 4.2 K (data not shown). Interestingly, the EPR spectrum of this low-spin component resembles that reported by Walker and co-workers (g_{max} ~ 3.4) for model compounds in which the planes of the coordinated imidazole groups are orthogonal to each other,[18] in contrast to the EPR spectrum exhibited by cytochrome b_5 (g_z ~ 3.06)[15,19] in which the planes of the coordinate imidazole groups are essentially parallel. As the electronic spectra at 77 K clearly indicate the presence of bishistidine ligation, the variant evidently undergoes a temper-

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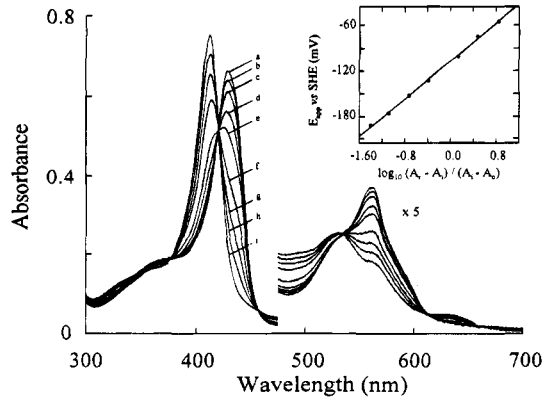

Figure 5. Spectroelectrochemical thin-layer spectra of the Val68His variant obtained as described in the text. The insert represents a Nernst plot derived from the dependence of the absorbance at 412 nm on the solution potential. The potentials at which the spectra were recorded are as follows (mV vs SHE): (a) −366; (b) −193; (c) −176; (d) −153; (e) −133; (f) −101; (g) −75; (h) −55; (i) 561.

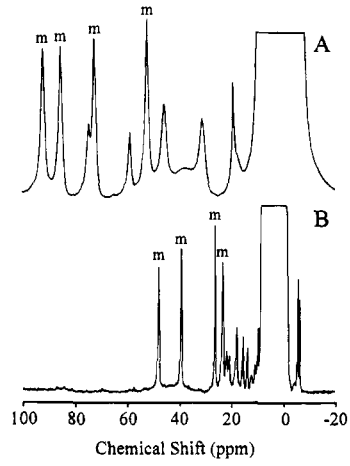

Figure 6. 200-MHz ^{1}H-NMR spectra of wild-type and Val68His metMb recorded as described in the text. The methyl resonances are labeled *m*.

ature-dependent ligation change with the histidine ligand largely displaced at 10 K.[20]

Spectroelectrochemistry. A family of spectra collected during a spectroelectrochemical titration of the variant is shown in Figure 5 along with a Nernst plot of the data (Figure 5, inset). The reduction potential of the variant was determined to be -110 ± 0.1 mV *vs* SHE (pH 7.0, 25.0 °C, $\mu = 0.10$ M) (Nernst slope = 61.5 mV), which compares to the corresponding value determined for the wild-type protein of 60.9 ± 0.1 mV (pH 7.0, 25.0 °C, $\mu = 0.10$ M).[21] The reduction potential for cytochrome b_5 under identical conditions is 4 mV.[6]

NMR Spectroscopy. The ^{1}H-NMR spectrum of the oxidized variant (Figure 6) is essentially the same as that previously reported for the His64Val/Val68His double variant of sperm whale Mb (which also exists as a spin equilibrium),[2i] with the mean methyl chemical shift, the line width, and the temperature dependence of the heme resonances also consistent with the existence of a thermal spin equilibrium. From the mean methyl shift of the variant (34 ppm) and values of ~15 ± 2 and ~80 ± 3 ppm for purely low-spin and purely high-spin Fe(III) heme, respectively,[2i] we estimate[2i] that 29% of the variant exists in the high-spin form at 20 °C. Significantly, we see no resonances in the high-field region that correspond to those resonances observed in wild-type aquometMb.

Discussion

The results presented above for the oxidized and reduced Val68His variant are fully consistent with the formation of a bishistidine ligated Mb in both oxidation states of the variant in the absence of exogenous ligands. Wild-type metMb is known to exhibit six-coordinate ligation of this type only at high pressure[22] or after reaction with cyanogen bromide and azide.[23] While variant myoglobins have been reported in which substitution of the distal His residue (His64) results in six-coordinate metMb variants in which both proximal and distal ligands are provided by amino acid residues, the new distal ligand is not retained upon reduction of the heme iron in these cases. The double variant His64Val/Val68His of sperm whale metMb also appears to possess bisimidazole axial ligation,[2i] but information concerning the reduced form of this protein has not been reported. Nevertheless, based on the information that is available for the oxidized form of this double variant, it seems likely that the sixth ligand of the single variant of the horse heart protein studied here is provided by His68 rather than His64. The difference between cytochrome b_5, which is entirely low spin at ambient temperatures, and the variant, which is only partially low spin, can be rationalized by assuming a weak iron–histidine bond in our myoglobin variant. This assumption is consistent with the observation that strong ligands (e.g., cyanide, CO) can displace distal binding by the His residue. A unique characteristic of the variant is the ability of the sixth histidine ligand to coordinate to the iron in both the ferric and ferrous derivatives. In general, axial ligands bound at the distal site of metMb dissociate when the iron is reduced, whereas the axial coordination of cytochrome b_5 is stable to changes in iron oxidation state.[8] Hence, wild-type metaquoMb,[9] imidazole-Mb, *N*-tetrazole-substituted imidazole-Mb,[23] and the His64Tyr variants of sperm whale Mb[2h] and horse heart metMb,[2b] in which the distal tyrosine residue is coordinated to the heme iron, all undergo an oxidation-state-dependent ligation change upon reduction (from six to five coordinate) and exist as five-coordinate high-spin derivatives in the ferrous state.

Comparison of the visible absorption, NMR, and NIR-MCD spectrum of the oxidized variant obtained at 293–300 K with the electronic spectrum obtained at 77 K and the EPR spectrum obtained at 10 K indicates a variety of thermal effects on the active site of this form of the protein. These spectra are consistent with the presence of bishistidine coordination with a spin-equilibrium at 298–300 K that becomes predominantly low spin at 77 K. At 10 K, the EPR spectrum indicates the presence

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of a large high-spin component and a smaller amount of a low-spin species. Interestingly, the spectrum of the low-spin component suggests that the orientation of the axial ligands is such that the planes of the bound imidazole groups are orthogonal to each other.[24] Consequently, it seems possible that upon cooling the sample to 10 K, the structure of the active site changes such that the distal imidazole ligand moves away from the iron atom to generate a significant amount of high-spin component.

As noted above, exposure of the reduced Val68His variant to carbon monoxide results in generation of a species with the electronic spectrum of carbonyl Mb, indicating displacement of the distally-bound His ligand by CO (data not shown). On the other hand, exposure of the reduced variant to air in the absence of reducing agent leads to the transient formation of a species with the spectrum of MbO_2 followed by rapid autoxidation (data not shown). The ability of CO to replace the distal His ligand in the variant differs from the behavior of reduced cytochrome b_5, which exhibits no response to such treatment.

The finding that the variant exhibits a lower reduction potential than the wild-type protein is strongly indicative of bishistidine coordination, although the large magnitude of the decrease (161 mV) is more difficult to understand. In considering the factors most likely to contribute to this relatively low reduction potential, attention is first directed at the proximal and distal histidyl residues. In particular, we note that there is no evidence for the existence of an imidazolate ligand,[25] which would lower the potential relative to cytochrome b_5. From studies with model heme compounds, the relative orientations of the axially coordinated imidazole groups have been argued to influence the midpoint potential of the heme iron atom such that the less stable perpendicular orientation is expected to increase the midpoint potential as much as 50 mV over that observed for the parallel orientation.[24a] To the extent that the EPR spectrum obtained at 10 K reflects the coordination geometry of the active site at 25 °C, the perpendicular orientation of the axial ligands observed in the low-spin component at low temperature does not help explain the relatively low potential of the variant. Hydrogen bonding interactions involving either of the coordinated imidazole groups could, of course, reduce the potential, though it seems likely that this property of the proximal His residue remains unchanged in the variant.[26] Nevertheless, the hydrogen bonding interactions of the distally coordinated His residue cannot be assessed clearly without detailed structural characterization and may be a significant contributory factor. The contribution of the spin equilibrium observed in the oxidized form of the variant to the midpoint reduction potential is difficult to evaluate at present.

In the absence of a three-dimensional structure for the variant, it is also difficult to speculate regarding the contributions of the second, unbound histidyl residue in the distal heme pocket and the consequences of its interactions with the adjacent, coordinated His residue. This interaction combined with a distorted coordination geometry resulting from the proximity of these two residues also presumably contributes to the spin equilibrium and temperature-dependent behavior of the variant that also relate to the observed midpoint reduction potential. Further analysis of the electronic properties of this variant should include consideration of the temperature dependence of the reduction potential and correlation of these results with corresponding analysis of the temperature dependence of the electronic, MCD, and NMR spectra. Ultimately, it will be necessary to determine the structures of the variant in both the reduced and oxidized states to permit assessment of structural changes introduced at the active site and the consequences of juxtaposition of two histidyl residues in the distal heme pocket.

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(25) Ligation by imidazole can be clearly identified by near-IR MCD spectroscopy.[15] Our spectra provide no evidence for deprotonation of either ligand.

(26) The influence of hydrogen bonding interactions of axially bound imidazole on the reduction potentials of heme proteins and model heme compounds has been discussed previously: (a) Valentine, J. S.; Sheridan, R. P.; Allen, L. C.; Kahn, P. C. *Proc. Natl. Acad. Sci. U.S.A.* **1979**, *76*, 1009−1013. (b) Doeff, M. M.; Sweigart, D. A.; O'Brien, P. *Inorg. Chem.* **1983**, *22* 851−852. (c) O'Brien, P.; Sweigart, D. A. *Inorg. Chem.* **1985**, *24*, 1405−1409.