Biophysical and Structural Analysis of a Novel Heme b Iron Ligation in the Flavocytochrome Cellobiose Dehydrogenase*

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Running Title:  Engineering Novel Heme Ligation in Cellobiose Dehydrogenase

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SUMMARY

The fungal extracellular flavocytochrome, cellobiose dehydrogenase (CDH), participates in lignocellulose degradation. The enzyme has a tripartite organization with a 190-residue cytochrome domain connected to a flavin-binding domain by a flexible peptide linker. The CDH cytochrome domain contains a 6-coordinate low-spin \( b \)-type heme with unusual iron ligands and coordination geometry. Wild-type CDH is only the second example of a \( b \)-type heme with the Met-His ligation, and it is the first example of a Met-His ligation of heme \( b \) where the ligands are arranged in a near-perpendicular orientation. To investigate the ligation further, Met65 was replaced with a histidine to create a \( bis \)-histidyl ligated iron typical of \( b \)-type cytochromes. The variant is expressed as a stable 90 kDa protein which retains the flavin domain catalytic reactivity. However, the ability of the mutant to reduce external one-electron acceptors such as cytochrome \( c \) is impaired. Furthermore, electrochemical measurements demonstrate a decrease in the redox midpoint potential of the heme by 210 mV. In contrast to the wild-type enzyme, the ferric state of the protoheme iron displays a mixed low-spin/high-spin state at room temperature and low-spin character at 90 K, as determined by resonance Raman spectroscopy. The wild-type cytochrome does not bind CO, but the ferrous state of the variant forms a CO complex, although the association rate is very low. The crystal structure of the M65H cytochrome domain has been determined and refined at 1.9 Å resolution. The variant structure confirms a \( bis \)-histidyl ligation of heme \( b \), but reveals unusual features. As for the wild-type enzyme, the ligands have a nearly perpendicular arrangement. Furthermore, the iron is bound by imidazole \( \text{N}^{\delta 1} \) and \( \text{N}^{\varepsilon 2} \) nitrogen atoms, rather than the typical \( \text{N}^{\varepsilon 2}/\text{N}^{\varepsilon 2} \) coordination encountered in \( bis \)-histidyl ligated heme proteins. To our knowledge, this is the first example of a \( bis \)-histidyl \( \text{N}^{\delta 1}/\text{N}^{\varepsilon 2} \) coordinated protoporphyrin IX iron.
INTRODUCTION

Cellobiose dehydrogenases (CDHs)\(^1\) are extracellular fungal flavocytochromes with a role in the biodegradation of lignocellulose (1). The CDH gene from the white-rot fungus *Phanerochaete chrysosporium* has been cloned and sequenced (2, 3), revealing a full-length protein of 755 amino acids, partitioned into a cytochrome domain (residues 1–190) and a flavodehydrogenase domain (residues 216–755), connected by a 25-residue peptide linker. A flavin adenine dinucleotide cofactor is bound to the flavoprotein domain, while the cytochrome domain contains a 6-coordinated low-spin (6cLS) Fe-protoporphyrin IX (4, 5). In the reductive half reaction, the flavodehydrogenase domain catalyzes the oxidation of cellobiose to yield cellobiono-1,5-lactone (6), with the concomitant reduction of flavin adenine dinucleotide. During the ensuing oxidative half reaction, the flavin is re-oxidized by an electron acceptor, either directly for two-electron acceptors such as 2,6-dichlorophenol-indophenol (DCPIP), or via the cytochrome domain for one-electron acceptors, such as cytochrome *c* (cyt *c*).

The 1.9 Å resolution crystal structure of the wild-type *P. chrysosporium* CDH cytochrome domain has been reported elsewhere (7). The heme-binding module features an unusual fold among cytochromes: an immunoglobulin-like β-sandwich consisting of a five-stranded and a six-stranded β-sheet. The protoheme group is bound in a hydrophobic pocket at one face of the β-core with one heme edge exposed to solvent. Three loops protrude from the β-sheet and wedge the *b*-type heme. The packing of the heme pocket formed by various non-polar residues is tight, leaving little space for exogenous molecules. The crystal structure (7) confirmed earlier spectroscopic predictions (4), that the heme iron is ligated by a methionine and histidine with an unusual, near-perpendicular arrangement (~100º) of the two planes defined by the methionine thioether group and the His163 imidazole ring. The distances of the Fe–N and Fe–S bonds, 2.0 Å and 2.3 Å, respectively, are typical of those observed in *c*-type cytochromes with Met–His iron ligation.

Results from site-directed mutagenesis of the two protoheme-iron ligands confirmed their importance (8). Substitution of either residue with an alanine demonstrated that the Met–His

\(^{1}\) CDHs: Cellobiose dehydrogenases.
coordination is essential for heme reactivity, i.e., the electron transfer (ET) to one-electron acceptors. In addition, the loss of an axial protein ligand rendered the cytochrome domain highly susceptible to degradation. Indeed, similar mutant studies in other b-type cytochromes reveal a weaker binding (9) or non-incorporation of the heme (10–12). Loss of the protoheme in the alanine variants of CDH may lead to unfolding of the cytochrome domain, rendering it more susceptible to proteolytic cleavage. In contrast, in c-type cytochromes, replacing the axially ligated methionine with a histidine produced a stable protein with some properties similar to the wild type (13–16). Speculation about the coordination geometry to the protoheme in these variants has been advanced (14, 16), but no structural studies have been reported. Herein, we report the results from site-directed mutagenesis, kinetic, electrochemical, spectroscopic, and crystallographic studies on the M65H variant of P. chrysosporium CDH.

EXPERIMENTAL PROCEDURES

Organism — Growth and maintenance of the auxotrophic strain OGC316-7 (Ura 11) and prototrophic transformants were as described previously (17, 18). Escherichia coli DH5α was used for subcloning plasmids.

Construction of the Mutant Plasmid pM65H — The M65H site-directed mutation was introduced into pUGC1 using the Transform™ site-directed mutagenesis kit (Clontech Laboratories, Palo Alto, CA) (8). The mutant primer converted the ATG codon (Met) to the CAC codon (His). The mutant plasmid pM65H was isolated and the mutation was confirmed by sequencing.

Transformation of P. chrysosporium with pM65H — Protoplasts of P. chrysosporium OGC316-7 (Ura11), a uracil auxotroph, were prepared as described previously (19, 20), transformed with EcoRI-linearized pM65H (2 µg), and potential transformants were screened for uracil prototrophy (19, 20). Conidia from prototrophs were then cultured in high-carbon high-nitrogen (HCHN) stationary liquid cultures with glucose as the sole carbon source (8, 19) and assayed for extracellular CDH activity using both the cyt c and DCPIP assays (8, 19). The
transformant exhibiting the highest activity was purified by isolating single basidiospores as described elsewhere (21, 22), and progeny were re-screened for CDH activity in liquid cultures.

Production and Purification of the M65H Variant — The M65H strain was grown for 7 days at 37 °C from conidial inocula in HCHN stationary liquid cultures with glucose as the sole carbon source. The extracellular fluid from 7-day-old cultures was concentrated and dialyzed against 20 mM potassium phosphate, pH 6. Subsequently, the variant protein was purified by cellulose-affinity chromatography, gel filtration (Sephacryl S200 HR), and fast protein liquid chromatography using a MonoQ HR5/5 anion exchanger, as described previously (8).

Preparation of Cytochrome Domains — The cytochrome domains of recombinant wild-type CDH (rCDH) and M65H (CYT<sub>M65H</sub>) were obtained by limited proteolysis with papain (5, 23), and purified by fast protein liquid chromatography using a MonoQ anion exchanger with a 0–1 M NaCl gradient in 10 mM Tris-HCl, pH 8.

SDS-PAGE and Western Blot Analysis — Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) was performed using a 12% Tris-glycine system (24) in a Miniprotean II apparatus (Bio-Rad, Hercules, CA), and gels were stained with Coomassie blue. Western blot analysis was performed as described previously (8).

Estimation of Protein and Heme Content — Protein concentration was determined by the bicinchoninic acid method (25). Heme content was estimated by the pyridine hemochromogen procedure (26).

Spectroscopic Procedures — Electronic absorption spectra of rCDH and the M65H variant were recorded at room temperature with a Cary 100 spectrophotometer (Varian, Australia). Spectra were obtained in 20 mM Na-succinate, pH 4.5. The enzymes were reduced under aerobic or anaerobic conditions by addition of cellobiose (200 µM) or excess dithionite. The CO adduct of the reduced form of the M65H variant was obtained by briefly bubbling CO gas through a cellobiose- or dithionite-reduced enzyme solution under anaerobic conditions. To measure the association rate of CO, native M65H variant (~1.5 µM) was added to an anaerobic
solution of 20 mM Na-succinate, pH 4.5, containing 60–240 µM CO and >100 µM dithionite. Ligand association was followed by the change in the absorbance at 431 nm.

**Resonance Raman Spectroscopy** — Resonance Raman spectra were measured on 15 µL of each sample sealed in a glass melting-point capillary tube, using a custom McPherson 2061/207 spectrograph equipped with a Princeton Instruments LN1100PB liquid N2-cooled CCD detector and Kaiser Optical Systems holographic notch filter. Excitation light was provided by an Innova 302 krypton laser (413 nm). The laser power at the sample was ~40 mW. Plasma emission lines were removed by an Applied Photophysics prism monochromator. Data at room temperature and 90 K were collected in a back-scattering geometry with the sample capillary placed in a copper cold finger. For the 90 K experiments, the capillary was cooled by liquid nitrogen. Spectral data were processed using GRAMS/386 (Galactic Industries) and Origin (Microcal) data analysis programs. Spectra were calibrated against indene as an external standard. Frequencies are estimated to be accurate to ±1 cm⁻¹.

**Enzyme Assays and Kinetic Procedure** — CDH activity was measured using either the cyt c or the DCPIP assay (8, 19). The steady-state kinetic parameters for cellobiose oxidation were determined by monitoring ferrocytochrome c formation (ε₅₅₀ = 28 mM⁻¹ cm⁻¹) or DCPIP reduction (ε₅₁₅ = 6.8 mM⁻¹ cm⁻¹). The assays contained a fixed level of ferricytochrome c (12.5 µM) or DCPIP (35 µM) and varying levels of cellobiose (5–200 µM) in 20 mM Na-succinate, pH 4.5. The steady-state kinetics for cyt c and DCPIP reduction were determined with a fixed cellobiose concentration (200 µM) and variable cyt c and DCPIP concentrations (0.2–40 µM).

**Potentiometric Titration** — Potentiometric titrations were carried out at room temperature in a borosilicate glass cell, similar to that described previously (27). The potential was measured with a platinum electrode versus a REF401 calomel electrode (Radiometer). All values are expressed with respect to the normal hydrogen electrode (NHE). The electrodes were calibrated against a pH 7 standard solution of quinhydrone (Eₘ = +293 mV vs. NHE) with a Metrohm 632 pH meter (Metrohm, Herisau, Switzerland). The redox midpoint potential was determined in 50 mM Na-succinate, pH 4.5. Redox equilibration between the protein and the
electrode was achieved by the use of a mixture of dyes: phenazine methosulfate, phenazine ethosulfate, 2-hydroxy-1,4-naphthoquinone, anthraquinone-1,5-disulfonate, anthraquinone-2,6-disulfonate, anthraquinone-2-sulfonate, and/or Fe$^{3+}$-EDTA. The redox titration was carried out with stirring of the buffered solution (5.5 ml), containing 5 µM enzyme, the mediator dyes (20 µM each), and 50 µM Fe$^{3+}$-EDTA. Prior to the reductive titration, the solution of enzyme and mediators was flushed with argon. The solution was then allowed to reach equilibrium, and the first UV-vis spectrum was recorded with an HP 8353 Diode Array spectrophotometer (Hewlett Packard, Palo Alto, CA). The redox potential of the system was adjusted by the addition of a small volume of 10 or 100 mM dithionite via a Hamilton syringe. After equilibration (constant reading of absorbance and potential), a spectrum was recorded, the potential noted, and an additional small volume of dithionite was added. This process was repeated until the enzyme was completely reduced. The oxidative titration was carried out by the addition of small amounts of air to the cell, followed by flushing with argon. The system was allowed to equilibrate, a spectrum was recorded, and the potential noted. This procedure was repeated until the enzyme was completely oxidized. The redox state of CDH was determined from the size of $\alpha$ band of the heme $b$: 562 nm for rCDH and 560 nm for M65H. The absorbance at this wavelength, corrected for the absorbance at 800 nm, was plotted against the potential of the system. The graph was fitted against the Nernst equation to obtain the redox potential $E_m$. The Nernst plot for both oxidative and reductive titration exhibited no hysteresis, confirming that the system was at equilibrium.

**Protein Preparation for Crystallization** — The M65H variant was cleaved proteolytically with papain to yield distinct cytochrome and flavin fragments as described previously (5, 23). The fragments were fractionated on a MonoQ HR 5/5 anion exchanger in 20 mM Tris-HCl, pH 8.0, using a linear NaCl gradient (0–1 M), followed by re-fractionation of the samples containing CYT$_{M65H}$ at pH 4.2, using a linear Na-acetate gradient (50 mM–1 M). Crystals of CYT$_{M65H}$ were grown at room temperature using the hanging-drop vapor-diffusion method (28). Hanging drops were prepared by mixing equal volumes of protein solution (3 mg/ml) and reservoir. The
reservoir contained 30% (w/v) polyethylene glycol 4000, 5% (v/v) 2-methyl 2,4-pentanediol, 100 mM HEPES, pH 7.5, and 10 mM CaCl₂. Crystals appeared as red hexagonal rods of space group P6₅ with cell constants \(a = b = 139.0\ \text{Å}\) and \(c = 52.67\ \text{Å}\), and with two molecules in the asymmetric unit.

**X-ray Crystallographic Data Collection and Refinement** — Data were collected at 100 K using synchrotron radiation (source ID14-EH4, ESRF, Grenoble, France, \(\lambda = 0.9763\ \text{Å}\)). Data reduction and scaling were carried out using MOSFLM (29) and SCALA (30), respectively. The previously reported structure of the *P. chrysosporium* CDH cytochrome domain at 1.9 Å resolution (PDB ID code 1D7C) (7) was used as a starting model for crystallographic refinement against CYT\(_{M65H}\) amplitudes. Initial refinement and manual model building were performed with the programs CNS (31) and O (32), respectively. Final refinement was done with REFMAC5 (33) at 1.9 Å resolution, using anisotropic scaling, hydrogens in their riding positions, and atomic displacement parameter refinement, using the “translation, libration, screw-rotation” (TLS) model. The two non-crystallographically related molecules were defined as rigid bodies during TLS refinement. All least-squares planes and angles between normals and least-squares planes were calculated, using the program MOLEMAN2 (34).

**RESULTS**

*Expression and Purification of the M65H Variant* — The M65H mutation was verified by DNA sequencing. Transformation of the Ura⁻ strain (Ura11) with linearized pM65H resulted in the isolation of several prototrophic transformants. Each was grown in liquid HCHN cultures in the presence of glucose, conditions under which endogenous wild-type CDH is not expressed (2, 19). The extracellular medium was monitored for CDH activity, using the cyt c and DCPIP reduction assays. Several transformants exhibited significant DCPIP reduction activity, but none efficiently reduced cyt c. The transformant exhibiting the highest DCPIP activity was purified by fruiting and isolating single basidiospore-derived colonies (21, 22). The purified transformant was incubated at 37 °C in HCHN medium for 7 days. The amount of CDH secreted
was ~50% of rCDH cultures, based on the DCPIP reduction assay. Western blot analysis of the extracellular medium over the 7-day-old culture period indicated the presence of a 90 kDa CDH-like protein. The M65H protein was purified to homogeneity by cellulose affinity chromatography, gel-filtration, and anionic exchange chromatography. The \( R_z \) value (\( A_{411}/A_{280} \)) was 0.77, and the extinction coefficient of the Soret maximum at 411 nm was 133 mM\(^{-1}\) cm\(^{-1}\).

**Steady-state Kinetics** — Measuring CDH activity in the extracellular medium of M65H transformants suggested that the cytochrome variant efficiently reduced DCPIP, but its ability to reduce cyt \( c \) was significantly impaired. Under steady-state conditions, linear double-reciprocal plots were obtained in 20 mM Na-succinate, pH 4.5, for the purified variant and for the rCDH protein. The apparent \( K_m \) values for cellobiose and DCPIP and \( k_{cat} \) values for cellobiose oxidation and DCPIP reduction were similar for both CDH proteins (Table I). However, the specific activity for cyt \( c \) reduction by the M65H variant was approximately 100-fold lower than that for rCDH (Table I).

**UV-Vis Spectroscopy of rCDH and the M65H Variant** — The electronic absorption spectra for both rCDH and the M65H variant were dominated by the heme \( b \) spectrum, with a weak absorbance near 450 nm attributed to the flavin. The ferric heme spectrum of rCDH was typical for a low-spin (LS) heme iron, with a Soret maximum at 421 nm and visible bands at 530 and 570 nm (Fig. 1A, Table II). The M65H substitution altered the optical properties of the ferric heme, giving rise to a spectrum that contained a mixture of LS and high-spin (HS) protoheme iron signals (Table II). Moreover, the Soret band was blue-shifted to 411 nm in the variant, and the band at 730 nm, characteristic of a Met–Fe ligation (35), disappeared. A new weak band indicative of a HS species in the ferric state is present at 630 nm. Analysis of possible heme absorbances near 500 nm was compromised by the flavin absorbance. Therefore, the truncated cytochrome domain was obtained by limited proteolysis, and the resulting electronic absorption spectrum showed a maximum at 495 and shoulders at 530 and 560 nm (Fig. 1A). As was observed with rCDH, the ferric heme in M65H is unreactive with both cyanide and imidazole (50 mM).
The optical properties of the dithionite-reduced recombinant wild-type and variant CDHs were similar and were typical of a LS ferrous heme. The main differences were (i) the intensity of the absorptions, and (ii) the slightly blue-shifted $\alpha$ and $\beta$ bands in the variant (Fig. 1B, Table II). Cellobiose rapidly reduced both the flavin and heme in the wild-type enzyme (35a). Using it was demonstrated stopped-flow spectrophotometry, that both redox centers in the wild-type enzyme are reduced within less than one second if no terminal substrate is present (35a), and we confirmed this with the recombinant wild-type enzyme. Addition of a large excess of cellobiose to the M65H variant appeared to reduce the flavin completely, whereas the heme iron was only partially reduced (Fig. 1B). The extent of heme reduction of the variant was approximately 20% under aerobic conditions (Fig. 1B). Under anaerobic conditions, only 50% reduction of the heme occurred in one minute, and only 90% reduction within 45 min. The ferrous wild-type $b$-heme did not bind CO, whereas the variant formed a ferrous–CO complex, exhibiting a Soret maximum at 425 nm, and $\alpha$ and $\beta$ bands at 540 and 572 nm, respectively (Fig. 2A). Binding of carbon monoxide was a slow process (Fig. 2B), and the formation of the Fe$^{2+}$–CO complex could be monitored on a conventional spectrophotometer. The observed time courses were dominated by a single exponential process (Fig. 2B) and were linearly dependent on the CO concentration from 60 to 240 µM (Fig. 2B insert). The association rate constant was calculated to be $8.6 \times 10^{-5}$ µM$^{-1}$ s$^{-1}$.

**Resonance Raman Spectroscopy** — To further investigate the coordination and spin states in wild-type CDH and the M65H variant, resonance Raman (RR) high-frequency spectra were obtained, using Soret excitation (Figs. 3 and 4, Table III). The spectral data for rCDH were similar to those reported for the wild-type CDH (5). The oxidation marker $\nu_4$ was observed at 1371 cm$^{-1}$ in both enzymes (Fig. 3), indicating a ferric heme. In the case of rCDH, the core-size marker bands $\nu_2$ and $\nu_3$ at 1575 and 1505 cm$^{-1}$, respectively, identified the ferric heme as a 6cLS heme species. Essentially identical RR data were obtained with the truncated heme domain with only minor differences, attributed to a contribution from the flavin cofactor (5). In the M65H variant, $\nu_3$ was observed at 1480 cm$^{-1}$, indicating 6-coordinated high-spin heme species (36).
Weak shoulders at 1638 ($\nu_{10}$) and at 1505 cm$^{-1}$ ($\nu_3$) reflected the presence of a minor population of 6cLS heme. The LS $\nu_3$ band was obscured by a band at 1515 cm$^{-1}$ assigned as $\nu_{38}$ (36). When the temperature was lowered to 90 K, both enzymes exhibited similar RR spectra, with $\nu_2$, $\nu_3$, and $\nu_{10}$ at 1577, 1507, and 1642 cm$^{-1}$, respectively, characteristic of a 6cLS heme. The electronic absorption spectra of the reduced CDH proteins (Fig. 2B) were both indicative of a 6cLS system, and the RR spectra confirm this conclusion, with $\nu_2$ and $\nu_3$ at 1580 and 1494 cm$^{-1}$, respectively, for both CDH proteins (Table III).

**Optical Potentiometric Titration** — The redox midpoint potential of the heme prosthetic group was obtained by optical potentiometric titration. The extent of reduction of the heme could readily be determined from the $\alpha$ band, a wavelength where the absorbances of the flavin cofactor and the redox mediators were negligible. The heme in the holo-wild-type enzyme and its truncated heme domain exhibited a similar redox midpoint potential at pH 4.5 (+164 mV vs. NHE) (Fig. 4, Table II). This value was in close agreement with previous electrochemical measurements of native CDH (37) and its truncated heme domain (38) and similar to the value of the heme group in cytochrome $b_{562}$ (39), a second example of a $b$-type heme with a Met–His coordination. Substitution of Met65 by histidine resulted in a 210 mV drop to –53 mV (Fig. 4, Table II). This value was in the range for bis-histidyl cytochromes, such as cytochrome $b_5$ (40).

**Overall Crystal Structure of CYT$_{M65H}$** — Data collection and model refinement statistics to 1.9 Å resolution are summarized in Table IV. The final model contained two protein molecules (residues 1–186), 336 water molecules, six cadmium ions, two protoheme groups, one polyethylene glycol molecule (modeled as C$_{11}$O$_6$), and two $N$-linked carbohydrate chains at Asn111, each with two $N$-acetyl glucosamine residues. This model had $R$ and $R_{free}$ values of 0.17 and 0.20, respectively. The CYT$_{M65H}$ structure was similar to that of the wild type with root-mean-square deviation values of 0.20 Å for 186 $C^\alpha$ atoms and 0.28 Å for all atoms in the residue zone 1–186. The electron density for the CYT$_{M65H}$ molecule was of good quality (Fig. 5A), and the only region with less well-ordered electron density was found in a loop composed of residues 36–39 in one of the non-crystallographically related molecules (molecule A).
Compared with the wild-type CDH cytochrome, differences in the protein occurred, as expected, exclusively in close proximity to the substitution site (Fig. 5B). Local protein backbone displacements of 0.5–0.6 Å occurred at residues 63–65, and of 0.5–0.7 Å at residues 87–90. In the wild-type cytochrome, Tyr90 was positioned close to the heme-ligating residue Met65, and the Tyr90 hydroxyl group formed a hydrogen bond to the D-propionate side chain of the protoporphyrin. To accommodate the bulkier histidyl imidazole ring at position 65, the backbone of Tyr90 was displaced by 0.6 Å away from the protoporphyrin ring (Fig. 5B). At position 87, the backbone was displaced by 0.5 Å due to steric hindrance between the Cβ atom of Ala87 and the imidazole ring of His65. However, the His65 backbone moved closer to the protoporphyrin ring by 0.7–0.8 Å.

**Structural Details of the Heme-Binding Site** — Wild-type CDH featured an unusual type of protoporphyrin ligation: Met65–His163 with the plane defined by the methionine CH3–S–CH2 group almost perpendicular (100°) to the plane of the histidyl imidazole ring. Introducing a histidine residue at position 65 in CYT<sub>M65H</sub> resulted in a histidine side chain conformation similar to that of the original methionine (Fig. 6B). In this conformation, the Nδ1 atom of His65 was suitably positioned to ligate the heme iron. Given the backbone conformation at residue 65, ligation through the histidyl Nε2 was highly unlikely. The Fe–His65 Nδ1 bond (2.1 Å) was shorter than the wild-type Fe–Met65 Sγ bond (2.3 Å), whereas the length of the Fe–His163 Nε2 bond remained unchanged (2.1 Å). The His65 χ1 torsion angle assumed favorable values of 180.0°/181.5° (mol A/B; trans); whereas those of His163 deviated more than 3σ from ideal values: χ1 of 34.5°/32.6° (mol A/B; gauche−). The χ2 values were 103°/98° (mol A/B) for His65 and 72°/74° (mol A/B) for His163.

The angle between the normals to the planes of the two histidyl imidazole rings was slightly larger, 114°/118° (mol A/B), than the angle between Met65 CH3–S–CH2 and the His163 imidazole ring (~100°) in the wild-type, thus deviating further from a perpendicular arrangement in the mutant. The orientation of the His65 imidazole ring was further stabilized by a hydrogen bond formed between His65 Nε2 and the main-chain carbonyl oxygen of Val91. The average
temperature factor for the His163 imidazole ring (mol A, 21.9 Å²; mol B, 22.0 Å²) was higher than that for the mutant His65 side chain (mol A, 18.9 Å²; mol B, 19.7 Å²), indicating that local discrete disorder was introduced at the unsubstituted rather than at the substituted ligand. This was also manifested as a strained conformation of the His163 side chain as judged by the deviation from ideal torsion-angle values. The discrepancy in temperature factors for the two ligands was not observed in the wild type. The electron density was of excellent quality throughout the heme-binding pocket, and the crystal packing at the exposed heme site was well defined, thus the discrete disorder at His163 was not due to large perturbations in the region. In both wild type and mutant, the protoporphyrin ring adopted a nearly planar conformation (~170°; corresponding to the angle between the normals to the two planes defined by pyrrole atoms C2A–C3D–C4A–C1D and C1B–C4C–C3D–C2C, respectively).

DISCUSSION

Axial Coordination of the M65H Variant — The CYT<sub>M65H</sub> structure was determined at 100 K, and the protoheme Fe<sup>2+</sup> is stably 6-coordinated by the two His ligands, His65 and His163, and the four pyrrole nitrogens (Fig. 5). The Fe–N(His) distances and the angles defined by N(His)–Fe–N(pyrrole) are typical for bis-histidyl ligated cytochromes, 2.1 Å and ~90°, respectively. A unique feature of the axial coordination is the Fe–His65 N<sup>δ1</sup> bond. Although histidyl N<sup>δ1</sup> ligation occurs in non-heme iron and copper complexes, it is rarely encountered in heme proteins. Indeed, the only previous example of heme-Fe ligation through a histidyl N<sup>δ1</sup> is that of the c-type, LS Heme-1 in the tetra-heme cytochrome c<sub>554</sub> (cyt c<sub>554</sub>) from the bacterium <i>Nitrosomonas europaea</i> (41). The structure of cyt c<sub>554</sub> has been determined in the reduced form at 1.6 Å resolution and in the oxidized form at 1.8 Å resolution, PDB ID codes 1FT5 and 1FT6, respectively (42), and the structure of the Heme-1 site is essentially identical in the two oxidation states. The cyt c<sub>554</sub> Heme-1 is coordinated by His102 N<sup>δ1</sup> and His15 N<sup>ϵ2</sup>. In addition, on the His N<sup>ϵ2</sup> side of the porphyrin ring, a common C-x-y-C-H motif covalently attaches the heme to the protein through Cys11 and Cys14. The heme-binding sites in CYT<sub>M65H</sub> and cyt c<sub>554</sub>
(Heme-1) are very similar: the histidine residues have favorable side-chain torsion angles, the length of the Fe–His Nδ1 bonds are identical (2.1 Å), and the angle to the normals of the planes is nearly perpendicular. The orientation of the His102 imidazole ring in cyt c554 is stabilized by a hydrogen bond between His102 Nε2 and Gln126 Oε1, equivalent to the hydrogen bond between His65 Nε2 and the main-chain carbonyl oxygen of Val91 in CYT<sub>M65H</sub>.

Rearrangements in CDH Met65His occur, not unexpectedly, at the His65 site of the heme-binding pocket. This site is formed by two loops: residues 61–69 (loop A) and 87–93 (loop C). To accommodate the bulkier histidine side chain at position 65 and to properly orient its Nδ1 atom for ligation (∠Nε2-Fe-Nδ1 ~180°), minor backbone displacements of 0.5–0.7 Å are required in loop A and at Tyr90 in the adjacent C-loop (Fig. 5A), indicating that these loops have a degree of conformational freedom. The dense packing around His65 and the hydrogen bond between His65 Nε2 and Val91 O may stabilize this alternative coordination. In addition, an extended hydrogen-bonding network is present within the backbone of loop A, and between the Tyr90 hydroxyl group and the D-propionate carboxylic acid group. The displacement of backbone atoms may introduce main-chain strain, causing the observed thermally induced spin-state transition and the binding of carbon monoxide.

**Spectroscopic Studies** — The electronic absorption and RR spectra of the ferrous M65H variant (Fig. 1, Table III) confirm a bis-histidyl coordination at room temperature, deduced from the cryogenic tertiary structure of ferrous CYT<sub>M65H</sub>. The cryogenic RR spectrum of the variant in the ferric state is also indicative of 6eLS heme species (Fig. 3); thus, it is likely that both histidines are coordinated to the heme iron as shown in the ferrous CYT<sub>M65H</sub> structure. At ambient temperature, however, the ferric heme undergoes a spin-state conversion to a predominantly 6-coordinated high-spin species. This suggests coordination of a water molecule, implying replacement of the histidine ligand upon oxidation or conversion to a HS histidine residue. Although the room temperature data cannot rule out a His/aquo coordination, considering the bis-histidyl coordination in the ferrous state as well as the slow formation of a Fe<sup>2+</sup>-CO adduct (see below), we favor a model where both histidines, His65 and His163,
coordinate to the heme iron in both ferric and ferrous states. In support, redox state-dependent,
thermally induced, spin-state transitions have been previously observed in a variant of
myoglobin (Mb), Mb-H64V/V68H (43, 44), which contained an engineered bis-histidyl heme.
The 30% population of the ferric HS state at ambient temperature is attributed to a weaker ligand
field, the result of a tilted His68 (43, 44) and a longer Fe-imidazole N$^{\varepsilon 2}$ bond (43). The
weakened bis-histidyl ligation is not obvious in the tertiary structure of CYT$_{M65H}$; however, it
may arise from a strained backbone, the alteration in proper axial coordination, heme-ring
distortion, and/or discrete disorder at His163.

**Heme Reactivity** — The M65H mutation results in a marked decrease in catalytic
reactivity, i.e., a significantly reduced rate for inter-domain ET (Fig. 2A) as well as negligible
cyt c reductase activity (Table I). The drop in the redox potential by 210 mV for the heme in the
M65H variant (Fig. 4, Table III) is a likely explanation, lowering the thermodynamic driving
force for ET between the flavin and cytochrome domain in the M65H variant. On the basis of
earlier work (45), it was estimated that histidine versus methionine ligation should account for a
redox midpoint potential difference of 160–168 mV (35), similar to that observed. Previous
mutant studies in horse heart cyt c (16), *Rhodobacter capsulatus* cytochrome c$_1$ heme (14),
*Pseudomonas* cytochrome c$_{551}$ (15), and cytochrome c$_{555}$ from *Aquifex aeolicus* (13) demonstrate
that substitution of an axially ligated methionine by a histidine lowers the midpoint potential by
200–400 mV. Other factors, such as the dielectric constant, the hydrogen bonding network, and
electrostatic interactions can also modulate the redox properties of the heme group (46). Thus,
the range indicates that other modifications can occur in the heme-binding pocket upon a change
in ligands.

A second factor, possibly responsible for the low rate of electron transfer to cyt c, is the
difference in spin states for the ferric and ferrous M65H heme iron. To lower the
reorganizational energy, thus facilitating ET, cytochromes invariably contain a strongly ligated
heme iron that is LS in both redox states. The spin-state conversions of the heme iron during the
catalytic cycle of the M65H variant will likely impair electron transfer between the two redox
moieties. The residual cyt c activity is similar to the activity of the truncated flavin domain (47), possibly suggesting a weak direct flavin-to-cyt c electron-transfer pathway rather than via the heme domain.

With the drop in redox potential, the reactivity of the heme iron atom with carbon monoxide is another feature of cytochrome variants with substitution of a ligated methionine by a histidine. For CO to bind to either side of the iron, significant rotation about the $\chi^1$ of the axial ligands is needed in addition to backbone shifts. Indeed, the M65H variant exhibits an unusually low CO association rate of $8.6 \times 10^{-5} \text{µM}^{-1} \text{s}^{-1}$ (Fig. 2B), a value ~10,000-fold lower than that for Mb (43) and 20-fold lower than that for the heme-based oxygen sensor Dos (48). The sensor protein requires the displacement of the endogenous methionine ligand upon binding of CO and other gaseous molecules (49). The CO association rate for the M65H variant reflects the displacement of a histidine ligand and possibly the rearrangement of the backbone. The tertiary structure of CYT$_{M65H}$ at 100 K (Fig. 5) displays discrete disorder at His163, and this likely increases with temperature. In addition, His163 appears to be slightly more exposed than the M65H site. However, His163 is partially shielded by the side chains of Glu162 and Phe166, and there are no significant structural changes introduced in this region of the heme pocket (loop B, residues 147–164) in the M65H variant. These structural observations, together with the inability of the wild-type Met65 protein to bind CO, argue against His163 as the site of CO entry and favor His65 as the CO binding site.

Conclusions — The M65H variant of the flavocytochrome CDH displays a novel bis-histidyl coordination to heme $b$ iron, involving the $\text{N}^{\delta 1}$ nitrogen atom of His65 and $\text{N}^{\varepsilon 2}$ nitrogen atom of His163. As expected, flavin reactivity is retained, but flavin-to-heme ET is essentially abolished, most likely owing to a decrease in the redox potential of the protoheme cofactor. The spin state of the heme iron is dependent on temperature as well as redox state, but both histidines remain coordinated in the absence of exogenous ligands. In contrast to the wild-type protein, the heme iron in the M65H variant binds CO, which apparently replaces His65 as a ligand. Finally, the tertiary structure of the M65H cytochrome indicates that an iron $\text{N}^{\varepsilon 2}/\text{N}^{\delta 1}$ coordination is
neither sterically nor energetically unfavorable (35, 50). However, restraints on the heme-ligand orientation and backbone conformation may aggravate fine-tuning of the microenvironment around the heme, constituting a possible bottleneck for heme-iron–N$^{δ1}$ ligation. Thus, this may have resulted in a strong preference for ligation through His–N$^{ε2}$ as is observed in almost all heme proteins examined to date.
REFERENCES


FOOTNOTES

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§ Joint first authorship.

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The abbreviations used are: 6cLS, 6-coordinated low-spin; CDH, cellobiose dehydrogenase; cyt c, cytochrome c; CYT M65H, M65H cytochrome domain; DCPIP, 2,6-dichlorophenol-indophenol; EDTA, ethylenediaminetetraacetic acid; Em, redox midpoint potential; ET, electron transfer; HCHN, high carbon-high nitrogen; HS, high spin; LS, low spin; Mb, myoglobin; NHE, normal hydrogen electrode; rCDH, recombinant wild-type CDH; RR, resonance Raman; SDS-PAGE, sodium dodecyl sulfate-polyacrylamide gel electrophoresis; TLS, translation, libration, screw-rotation.

The atomic coordinates and structure factors (code 1PL3) have been deposited with the Protein Data Bank, Research Collaboratory for Structural Bioinformatics, Rutgers University, New Brunswick, NJ (http://www.rcsb.org/).
FIGURE LEGENDS

Fig. 1. **Electronic absorption spectra of rCDH (---) and the M65H (—) variant in 20 mM Na-succinate, pH 4.5.** A, oxidized, and B, reduced with dithionite. Insert Fig. 1A: Oxidized M65H variant, flavocytochrome (—), cytochrome domain (---). Insert Fig. 1B: M65H variant, oxidized (—), reduced with 200 µM cellobiose under aerobic conditions (---).

Fig. 2. **Electronic absorption spectra of M65H variant (~1.5 µM) in 20 mM Na-succinate, pH 4.5.** A, reduced with cellobiose (—), Fe²⁺–CO complex (---) under anaerobic conditions. B, kinetic trace and fit for conversion of ferrous M65H variant to ferrous CO complex in the presence of 60 µM CO. Insert Fig. 2B, observed rate of association of CO to the ferrous M65 variant in 20 mM Na-succinate, pH 4.5, at room temperature.

Fig. 3. **High frequency RR spectra of the oxidized rCDH and the M65H variant, obtained at room temperature (A) and 90 K (B), with Soret excitation (413 nm) in 20 mM Na-succinate pH 4.5.** HS, high spin; LS, low spin; 6c, 6-coordinated.

Fig. 4. **Oxidative redox titration of rCDH (□) and the M65H CDH variant (■).** Optical potentiometric titrations were performed at pH 4.5, as described in Experimental Procedures.

Fig. 5. A, the σA-weighted F₀–Fₐ electron density around the Fe-protoporphyrin-IX ring in the M65H cytochrome domain. The 6-coordinated heme iron is ligated by His65 Nδ¹ and His163 Nε². B, superposition of the M65H variant (green) and wild-type (violet) heme-binding pocket (PDB ID code 1D7C) (7). The drawings were made with the program pymol (http://pymol.sourceforge.net).
Table I

*Steady-state parameters for rCDH and the M65H variant at pH 4.5*\(^a\)

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cellobiose oxidation (DCPIP)</th>
<th>DCPIP reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_\text{cat} (\text{s}^{-1}))</td>
<td>(K_m (\mu\text{M}))</td>
</tr>
<tr>
<td>rCDH</td>
<td>25.7</td>
<td>40</td>
</tr>
<tr>
<td>M65H</td>
<td>26.0</td>
<td>35</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Cellobiose oxidation (cyt c)</th>
<th>Cyt c reduction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>(k_\text{cat} (\text{s}^{-1}))</td>
<td>(K_m (\mu\text{M}))</td>
</tr>
<tr>
<td>rCDH</td>
<td>10.5</td>
<td>18</td>
</tr>
<tr>
<td>M65H</td>
<td>0.1</td>
<td>NA(^b)</td>
</tr>
</tbody>
</table>

\(^a\) Reactions were performed in 20 mM Na-succinate, pH 4.5. \(K_m\) and \(k_\text{cat}\) for cellobiose were determined using 40 \(\mu\text{M}\) DCPIP or 10 \(\mu\text{M}\) cyt c. \(K_m\) and \(k_\text{cat}\) for DCPIP and cyt c were determined using 200 \(\mu\text{M}\) cellobiose.

\(^b\) NA = not applicable.
Table II

*Spectral features and heme redox midpoint potential for rCDH, the M65H variant, and selected heme proteins with histidine ligation*

<table>
<thead>
<tr>
<th>Protein</th>
<th>Oxidized</th>
<th>Reduced</th>
<th>$E_m$ (mV vs NHE)</th>
</tr>
</thead>
<tbody>
<tr>
<td>rCDH</td>
<td>421 530 570</td>
<td>429 532 562$^a$</td>
<td>+164</td>
</tr>
<tr>
<td>rCDH (cyt domain)</td>
<td>421 530 570</td>
<td>429 532 562$^a$</td>
<td>+161</td>
</tr>
<tr>
<td>M65H</td>
<td>411 530 560 630</td>
<td>428 530 560$^a$</td>
<td>−53</td>
</tr>
<tr>
<td>M65H (cyt domain)</td>
<td>411 495 530 560</td>
<td>428 530 560$^a$</td>
<td>ND$^b$</td>
</tr>
<tr>
<td>cytochrome $b_5^c$</td>
<td>413 532 560</td>
<td>423 526 556</td>
<td>+4</td>
</tr>
<tr>
<td>cytochrome $b_{562}^d$</td>
<td>418 529 558</td>
<td>427 531 562</td>
<td>+167</td>
</tr>
<tr>
<td>horseradish peroxidase$^e$</td>
<td>403 500 641</td>
<td>437 556</td>
<td></td>
</tr>
<tr>
<td>MetMb$^f$</td>
<td>410 505 635</td>
<td>434 556</td>
<td>+61</td>
</tr>
</tbody>
</table>

$^a$ Addition of 400 µM cellobiose (rCDH) or grain of dithionite (rCDH, M65H, cytochrome domains).

$^b$ ND = not determined.

$^c$ UV-vis from ref. 51; redox potential from ref. 40.

$^d$ UV-vis from ref. 52; redox potential from ref. 39.

$^e$ UV-vis from ref. 53.

$^f$ UV-vis from ref. 54; redox potential from ref. 55.
### Table III

*High frequency resonance Raman vibration modes*

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>T(K)</th>
<th>v&lt;sub&gt;4&lt;/sub&gt;</th>
<th>v&lt;sub&gt;3&lt;/sub&gt;</th>
<th>v&lt;sub&gt;2&lt;/sub&gt;</th>
<th>v&lt;sub&gt;10&lt;/sub&gt;</th>
</tr>
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<tbody>
<tr>
<td>Ferric rCDH</td>
<td>295</td>
<td>1371</td>
<td>1505</td>
<td>1575</td>
<td>1638</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>1371</td>
<td>1507</td>
<td>1577</td>
<td>1642</td>
</tr>
<tr>
<td>Ferric M65H</td>
<td>295</td>
<td>1371</td>
<td>1480</td>
<td>1555/1578</td>
<td>1621</td>
</tr>
<tr>
<td></td>
<td>90</td>
<td>1375</td>
<td>1507</td>
<td>1577</td>
<td>1642</td>
</tr>
<tr>
<td>Ferric cytochrome b&lt;sub&gt;5&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>1373</td>
<td>1502</td>
<td>1579</td>
<td>1640</td>
</tr>
<tr>
<td>Aquomet myoglobin&lt;sup&gt;b&lt;/sup&gt;</td>
<td></td>
<td>1373</td>
<td>1483</td>
<td>1563</td>
<td>1621</td>
</tr>
<tr>
<td>Ferrous rCDH</td>
<td>295</td>
<td>1362</td>
<td>1494</td>
<td>1580</td>
<td>1615</td>
</tr>
<tr>
<td>Ferrous M65H</td>
<td>90</td>
<td>1362</td>
<td>1494</td>
<td>1580</td>
<td>1615</td>
</tr>
<tr>
<td>Ferrous cytochrome b&lt;sub&gt;5&lt;/sub&gt;&lt;sup&gt;a&lt;/sup&gt;</td>
<td></td>
<td>1359</td>
<td>1493</td>
<td>1584</td>
<td>1615</td>
</tr>
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</table>

<sup>a</sup> Ref. 56.

<sup>b</sup> Ref. 57.
Table IV

*Statistics for data collection and refinement*

<table>
<thead>
<tr>
<th>Data collection&lt;sup&gt;a&lt;/sup&gt;</th>
<th></th>
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<tbody>
<tr>
<td>Resolution (Å) full range / outer shell</td>
<td>48–1.90 / 2.00–1.90</td>
</tr>
<tr>
<td>Observations (measured/unique)</td>
<td>256,083 / 45,850</td>
</tr>
<tr>
<td>Multiplicity</td>
<td>5.6 (4.9)</td>
</tr>
<tr>
<td>Completeness (%)</td>
<td>99.4 (97.5)</td>
</tr>
<tr>
<td>&lt;I / σ(I)&gt;</td>
<td>5.0 (1.1)</td>
</tr>
<tr>
<td>(R_{\text{merge}}) (%)</td>
<td>11.3 (57.5)</td>
</tr>
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<table>
<thead>
<tr>
<th>Refinement</th>
<th></th>
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<tr>
<td>Resolution range (Å)</td>
<td>28–1.90</td>
</tr>
<tr>
<td>Completeness for range (%)</td>
<td>99.2</td>
</tr>
<tr>
<td>(R_{\text{factor}}) / number of reflections (work)</td>
<td>0.173 / 43,870</td>
</tr>
<tr>
<td>(R_{\text{free}}) / number of reflections (free)</td>
<td>0.198 / 1,852</td>
</tr>
<tr>
<td>Number of non-hydrogen atoms</td>
<td>3,341</td>
</tr>
<tr>
<td>Mean (B) values (Å&lt;sup&gt;2&lt;/sup&gt;) protein all atoms (A/B)</td>
<td>26.1 / 30.4</td>
</tr>
<tr>
<td>Rmsd bond lengths (Å) / angles (°)</td>
<td>0.019 / 1.73</td>
</tr>
<tr>
<td>Ramachandran plot outliers&lt;sup&gt;d&lt;/sup&gt; (%)</td>
<td>2.1</td>
</tr>
</tbody>
</table>

<sup>a</sup> The outer shell statistics using 5% of the reflections are in soft brackets.

<sup>b</sup> \(R_{\text{merge}} = \left[ \Sigma_{hkl} \Sigma_i |I−<I>| / \Sigma_{hkl} \Sigma_i |I| \right] \times 100\%\).

<sup>c</sup> \(R_{\text{factor}} = \Sigma_{hkl} | |F_o|−|F_c|| / \Sigma_{hkl} |F_o|\)

<sup>d</sup> Percentage of residues that fall outside core regions of the Ramachandran plot (58) as defined by Kleywegt and Jones (59).
Figure 4

- M65H: -53 mV
- rCDH: +164 mV

Fraction reduced vs E (mV vs NHE)