The Redox Behavior of the Heme in Cystathionine β-synthase Is Sensitive to pH[†]

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ABSTRACT: Human cystathionine β-synthase (CBS) is a unique pyridoxal-5′-phosphate-dependent enzyme in which heme is also present as a cofactor. Because the function of heme in this enzyme has yet to be elucidated, the study presented herein investigated possible relationships between the chemistry of the heme and the strong pH dependence of CBS activity. This study revealed, via study of a truncation variant, that the catalytic core of the enzyme governs the pH dependence of the activity. The heme moiety was found to play no discernible role in regulating CBS enzyme activity by sensing changes in pH, because the coordination sphere of the heme is not altered by changes in pH over a range of pH 6−9. Instead, pH was found to control the equilibrium amount of ferric and ferrous heme present after reaction of CBS with one-electron reducing agents. A variety of spectroscopic techniques, including resonance Raman, magnetic circular dichroism, and electron paramagnetic resonance, demonstrated that at pH 9 Fe(II) CBS is dominant while at pH 6 Fe(III) CBS is favored. At low pH, Fe(II) CBS forms transiently but reoxidizes by an apparent proton-gated electron-transfer mechanism. Regulation of CBS activity by the iron redox state has been proposed as the role of the heme moiety in this enzyme. Given that the redox behavior of the CBS heme appears to be controlled by pH, interplay of pH and oxidation state effects must occur if CBS activity is redox regulated.

Cystathionine β-synthase (CBS)[1] catalyzes the condensation of serine and homocysteine to yield cystathionine, an intermediary yet critical step in the biological conservation of sulfur. CBS, positioned at a branch point in the methionine metabolic cycle that converts methionine to cysteine and ultimately to glutathione, is one of two enzymes responsible for removing toxic homocysteine from the bloodstream (*1*). Deficiency of CBS activity in humans is the most common cause of homocystinuria, an inherited metabolic disease characterized by elevated levels of homocysteine in the blood. The most extreme symptoms of homocystinuria are dislocated eye lenses, skeletal problems, vascular disease, and mental retardation. More than 100 pathological variants of CBS that result in a deficiency of enzyme activity have been described in homocystinuric patients (*2*). Recent reports have also associated elevated homocysteine levels with neural tube defects and Alzheimer's disease (*3*, *4*).

Human CBS is the only known enzyme that requires both heme and pyridoxal-5′-phosphate (PLP) for maximal activity (*5*). CBS binds two substrates, homocysteine and serine, and turnover is regulated by the allosteric affector *S*-adenosylmethionine (SAM) (*6*). The human enzyme is an α_4-tetramer composed of 63 kDa subunits that each contain 551 amino acids (*7*, *8*). Each subunit has a defined organization in which the N-terminal, middle, and C-terminal regions of the protein serve as the binding sites for heme, PLP, and SAM, respectively (*9*). The heme moiety is axially coordinated by the thiolate of Cys^{52} and the $N_{\epsilon 2}$ atom of His^{65}, both located on a loop within the N-terminal domain (*10*−*13*). Naturally occurring Cys(thiolate)/His-coordinated hemes are rare; another interesting example is provided by the multiheme protein SoxAX from *Rhodovulvum sulfidophilum* (*14*, *15*).

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[1] Abbreviations: βLys[87], a lysine located at position 87 in the β-subunit of tryptophan synthase; Δ1−40 CBS-45, an N-terminal truncation variant of CBS-45 the amino acid sequence of which spans residues 41−413; C420, an inactive form of chloroperoxidase; CBS, cystathionine β-synthase; CBS-45, a truncation variant of cystathionine β-synthase in which the C-terminal autoinhibitory domain (residues 413−551) has been deleted from the enzyme; CBS-63, full-length cystathionine β-synthase; CD, circular dichroism; CHES, 2-(*N*-cyclohexylamino)ethanesulfonic acid; CooA, a CO-sensing heme protein that regulates the transcription of the *coo* operon encoding a CO-oxidation system in *R. rubrum*; cyt *c*, cytochrome *c*; cyt *c* M80C, cytochrome *c* where the methionine ligand is replaced by cysteine; DMF, dimethylformamide; DTT, dithiothreitol; EPPS, *N*-(2-hydroxyethyl)piperazine-*N*′-(3-propanesulfonic acid); EPR, electron paramagnetic resonance; FixL, an O_2-sensing heme protein that regulates *nif* and *fix* gene expression in plant-associated N_2-fixing rhizobia; H450, hemoprotein 450, a heme protein isolated in 1976 that was later identified as cystathionine β-synthase by sequence alignment; MCD, magnetic circular dichroism; MES, 2-(*N*-morpholino)ethanesulfonic acid; MOPS, 3-(*N*-morpholino)propanesulfonic acid; NMR, nuclear magnetic resonance; PLP, pyridoxal-5′-phosphate; rR, resonance Raman; SAM, *S*-adenosylmethionine; SoxAX, multiheme enzyme encoded by the *soxA* gene and an essential component of the thiosulfate-oxidizing multienzyme system (TOMES) of *Rhodovulum sulfidophilum*; Tris, tris(hydroxymethyl)aminomethane; VT-MCD, variable-temperature magnetic circular dichroism.

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Two forms of CBS, termed CBS-63 and CBS-45, are available for study. Homogeneity of the full-length human enzyme (referred to subsequently as CBS-63) is difficult to maintain because of its propensity to aggregate (*16*). Limited proteolysis of CBS-63 results in the separation of the N-terminal "catalytic core", which is composed of the heme- and PLP-binding domains, from the inhibitory C-terminal SAM-binding domain (*8*). Thus, the catalytic core (referred to subsequently as CBS-45) is approximately twice as active as CBS-63 but is insensitive to SAM. Truncation of CBS-63 also results in a change in the quaternary structure of the enzyme from a homotetramer to a homodimer, reducing the propensity of the enzyme to aggregate. A system for the overexpression of CBS-45 in *Escherichia coli* has recently been developed, and the X-ray crystal structure of CBS-45 has been solved (*11*, *13*, *17*).

CBS was first characterized spectroscopically as "hemoprotein H450," a peculiar heme protein of unknown function that displayed spectroscopic similarities to the P450 cytochromes (*18*−*21*). The most striking of these similarities was an intense, red-shifted Soret absorbance (λ_{max} = 450 nm) that H450 displayed upon reduction, which was suggestive of cysteinate coordination. H450, however, also displayed spectral features uncharacteristic of the P450 cytochromes such as pH-dependent EPR and ferrous heme absorption spectra. H450 was identified as CBS when the cDNA sequences of rat H450 and rat CBS became available and were compared (*5*). The early spectroscopic work performed on H450 serves as a solid basis for current studies of CBS, such as the one presented here.

The role of the heme in cystathionine β-synthase remains unknown. The catalytic condensation reaction performed by CBS can be explained solely by the participation of PLP in the reaction mechanism (*22*). The heme-free enzyme is catalytically competent, though the specific activity is lower than that of the holo-enzyme (*9*, *23*, *24*). Intriguingly, the dependence on heme appears to be an evolutionarily new acquisition for this enzyme. Deduced amino acid sequences of CBS from lower eukaryotes, for example, *Saccharomyces cerevisiae* (*25*−*27*), *Trypanosoma cruzi* (*28*), and *Leishmania tarentolae* (*29*), contain the conserved lysine residue responsible for PLP binding but do not contain the critical heme-binding residues characteristic of CBS from higher organisms. Furthermore, *S. cerevisiae* CBS has been shown spectroscopically to contain PLP but not heme (*25*). Nevertheless, heme is essential for maximal activity in the human enzyme, because activity decreases substantially upon deletion of the heme-binding domain (*9*). The studies presented herein were originally an attempt to connect the pronounced pH-dependence of CBS activity with changes in the axial coordination of the heme center. Our initial hypothesis was that pH-dependent changes to the heme ligand environment affected activity, implicating the CBS heme as a sensor of intracellular proton concentrations. Our studies, however, demonstrate that this is not the case. Instead, we have found a more profound connection between the CBS heme and pH in that the redox state of the heme in this enzyme appears to be pH-sensitive. This finding has implications for the existing hypothesis that the heme of CBS regulates activity through changes in redox state, because the heme prefers to be in the ferric state at physiological pH.

EXPERIMENTAL PROCEDURES

Materials. Titanium(III) chloride was purchased from Acros, L-[U-^{14}C]serine was obtained from Perkin-Elmer Life Sciences, and all other chemicals were purchased from Sigma. All chemicals were used as received. Titanium(III) citrate was prepared from titanium chloride and sodium citrate in buffers of the appropriate pH as described previously (*30*).

Recombinant Human CBS. The cloning of human CBS-63 and CBS-45 cDNAs, the expression of the proteins in *E. coli*, and the purification of the enzymes to homogeneity were performed as described previously (*17*, *31*). Another form of CBS-45, Δ1−40 CBS-45, the amino acid sequence of which spans residues 41−413, was also utilized in these studies and was prepared by digesting CBS-63 with trypsin (*8*). CBS-45 and Δ1−40 CBS-45 display identical spectral behavior. Protein concentrations were determined by the Lowry method (*32*) using bovine serum albumin as a standard. Heme saturation was determined using the pyridine hemochromagen assay (*33*).

CBS Activity Assay. The enzyme activity at various pH values was determined as described previously (*34*). Briefly, this method involves incubating the enzyme with substrates homocysteine and L-[^{14}C]serine in a Bis-Tris propane buffer of the appropriate pH, separating the product cystathionine by descending paper chromatography, and counting the activity of cystathionine by scintillation. Each assay was performed in a total volume of 200 μL and contained 50 ng of CBS-63 or 25 ng of CBS-45, 5 mM homocysteine, and 5 mM serine. One unit of activity is defined as the amount of CBS that catalyzes the formation of 1 μmol of cystathionine in 1 h at 37 °C. The pH dependence of specific activity (SA) was fitted to the bell-shaped curve described by eq 1:

$$SA = \frac{(SA)_{max}}{1 + 10^{(pK_{A1} - pH)} + 10^{(pH - pK_{A2})}} \quad (1)$$

Electronic Absorption Spectroscopy. Electronic absorption spectra were recorded on a double-beam Cary 4 Bio spectrophotometer equipped with a temperature controller and set to a spectral bandwidth of 0.5 nm. Spectra were obtained at 25 °C with CBS-45 or CBS-63 in 25 mM buffer (MES for pH 6.0−6.5, MOPS or sodium phosphate for pH 6.50−7.75, EPPS for pH 7.75−9.0, and CHES for pH 9.0−9.5) with an additional 100 mM NaCl, unless stated otherwise. Reactions of the ferric enzyme with reducing agents (titanium citrate, sodium dithionite, or dithiothreitol) were performed anaerobically under an atmosphere of argon and were initiated by the addition of a degassed stock solution of reductant to a final concentration of 2 mM. Spectral behavior as a function of pH for samples treated with reducing agent was monitored by two methods: (1) diluting the protein stock into a buffered solution of the appropriate pH and subsequently adding reductant or (2) adjusting the pH of a protein sample containing reductant by adding a small amount of a 0.5 M buffer. Both methods produced identical results. A Corning ion analyzer 250 equipped with an Acumet combination microelectrode was used to verify the pH of every sample at 25 °C. Rate constants for the spontaneous reoxidation of Fe(II) CBS

under reducing conditions were obtained by fitting electronic absorbance data to eq 2, which describes a first-order approach to equilibrium.

$$\mathrm{Abs}_t = \mathrm{Abs}_\infty + (\mathrm{Abs}_0 - \mathrm{Abs}_\infty)\, e^{-k_1 t} \quad (2)$$

The pH dependence of the rate constants obtained from eq 2 was fitted to eq 3 to obtain an apparent kinetic pK_A. Equation 3 describes a model with two active species, a limiting rate constant at lower pH, k_{acid}, one pK_A, and a limiting rate constant at high pH, k_{basic}:

$$k_1 = \frac{k_{acid}(10^{(pK_A - pH)}) + k_{basic}}{1 + 10^{(pK_A - pH)}} \quad (3)$$

Magnetic Circular Dichroism Spectroscopy. Magnetic circular dichroism (MCD) spectra were recorded on a Jasco J715 CD spectropolarimeter with the sample compartment modified to accommodate an SM-4000-8 magnetocryostat (Oxford Instruments). Buffers used for MCD samples were the same as those detailed above. Spectra were obtained with Δ1−40 CBS-45 in a solvent of 55% v/v glycerol with 45 mM buffer. Addition of glycerol had no effect on the absorption spectrum of any sample at room temperature, nor was any change observed at temperatures at which MCD data were collected. Sodium dithionite was introduced in excess as a solid into anaerobic samples of the ferric enzyme. Each sample was transferred to an argon-filled cell via gastight syringe and frozen in liquid nitrogen. All MCD spectra were at 7 T at temperatures ranging from 4.5 to 85 K. Zero-field CD spectra were subtracted from the MCD spectra for all samples.

Resonance Raman Spectroscopy. Resonance Raman (rR) spectra were obtained using 135° backscattering geometry and f_1 collection as described previously (*35*). Either the 413.1 nm emission from a Kr^+ laser or the 441.6 nm emission from a HeCd laser was used for Raman excitation. The laser powers ranged from 12 to 20 mW at the sample, and a cylindrical lens was used to focus the beam on the sample. The sample tube was spun at 20 Hz to minimize laser-induced damage to the protein. Electronic absorption spectra were obtained before and after rR experiments to ensure that the samples had not been irreversibly altered in the laser beam. Spectra were calibrated against toluene, DMF, and CH_2Br_2. CBS-45 and CBS-63 samples for rR experiments were prepared in 5 mm NMR tubes. Buffers for rR samples were the same as described above. The ferric protein solutions were equilibrated with N_2 and reacted with excess dithionite ion by anaerobic transfer of a buffered, N_2-sparged stock solution.

Electron Paramagnetic Resonance Spectroscopy. X-band EPR spectra were collected on a Bruker ESP 300E equipped with an Oxford ESR 900 continuous flow cryostat and an Oxford ITC4 temperature controller. The field was calibrated using a Varian ER 035M gaussmeter. The microwave frequency was monitored using an EIP model 625A CW microwave frequency counter. Spectra of CBS-45 or CBS-63 were recorded in 100 mM buffer with an additional 100 mM NaCl. Buffers used for EPR were the same as detailed above, and the pH of each sample was measured at 25 °C. For samples containing reducing agent, sodium dithionite was added as a degassed stock solution to a final concentration of 4.4 mM. Sodium dithionite-treated samples were monitored by electronic absorption spectroscopy until no further changes to the spectrum occurred. Each sample was transferred to an argon-filled tube via gastight syringe and frozen in liquid nitrogen. For all samples, scans of 0−10 000 G revealed no signals other than those reported. EPR spectral simulations were performed using the Win-EPR SimFonia software package from Bruker, software version 1.2, 1995.

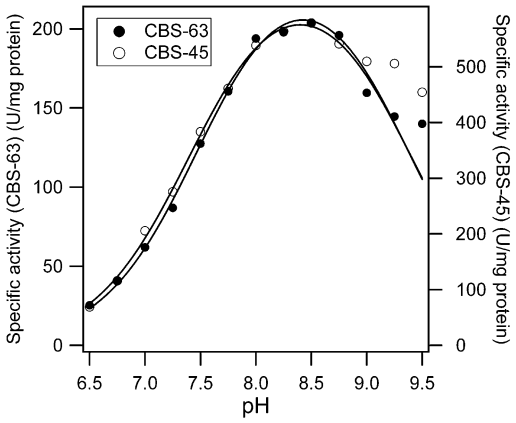

FIGURE 1: pH dependence of enzyme activity for the CBS-45- and CBS-63-catalyzed condensation of L-[U-^{14}C]serine and L-homocysteine to form ^{14}C-L-cystathionine. Reactions were carried out in total volumes of 200 μL at 37 °C in Bis-Tris propane buffer at various pH conditions. Lines represent the nonlinear regression fits of specific activity vs pH to eq 1. Because of base-catalyzed racemization of L-serine at pH > 9, assays carried out at pH ≥ 9 were considered unreliable and were not included in the nonlinear regression fit of the data (*36*).

RESULTS

The Enzyme Activity of CBS-63 and CBS-45 Show Similar pH-Dependent Behavior. The truncation variant, CBS-45, displays pH-dependent behavior very comparable to that of the full-length recombinant enzyme (Figure 1). Both full-length Fe(III) CBS-63 and truncated Fe(III) CBS-45 exhibit a bell-shaped pH dependence with maximal activity at pH 8.5. The enzyme activity increases approximately 10-fold upon increasing the pH from 6.0 to 8.0, and the specific activity of CBS-63 is approximately 2.7-fold lower than that of CBS-45 at the pH of optimal activity, consistent with deletion of the inhibitory C-terminal domain (*8*). A nonlinear regression fit of the activity data to a bell-shaped curve (eq 1) yields two pK_A values for each form of CBS: 7.50 ± 0.03 and 9.35 ± 0.09 for CBS-63 and 7.42 ± 0.02 and 9.38 ± 0.07 for CBS-45. The pK_A values of substrates serine (pK_A = 9) and homocysteine (pK_A = 10) match closely with the higher pK_A value, suggesting that the pH-dependence of CBS is determined in part by the intrinsic acid−base properties of the substrates (*37*, *38*). The lower pK_A value, therefore, can be attributed to the properties of the enzyme, its cofactors, or both. The fact that the pH profile of CBS-45, which lacks the C-terminal domain, is nearly identical to that of CBS-63 suggests that ionizable groups within the active core of the enzyme are responsible for the lower pK_A and thus the dramatic pH dependence observed for CBS.

The Heme Coordination Environment of Fe(III) CBS is Minimally Affected by Changes in pH. Using several spectroscopic techniques, we have determined that pH does not substantially affect the axial ligand field of the heme iron in

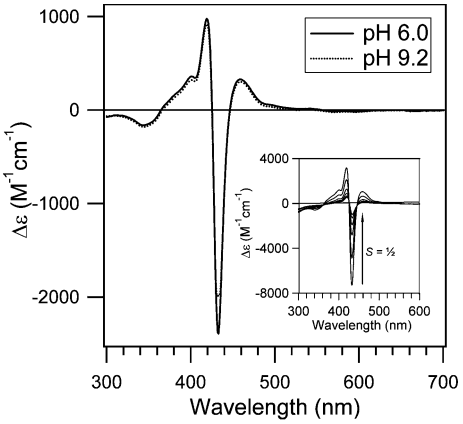

FIGURE 2: Variable temperature MCD spectra of Fe(III) Δ1−40 CBS-45, 21 μM in heme, at low and high pH. Samples were in 45 mM buffer (MES for pH 6.0 and CHES for pH 9.2) and 45 mM NaCl with 55% (v/v) glycerol. The spectra were recorded at 4.0 K in a magnetic field of 7.0 T. The slight variation in signal amplitude is presumably due to inconsistencies in sample preparation. The inset shows the VT-MCD spectra of Fe(III) Δ1−40 CBS-45, 21 μM in heme. This sample was in 45 mM MES buffer, pH 6.0, and 45 mM NaCl with 55% (v/v) glycerol. The spectra were recorded at temperatures of 4.0, 8.0, 15.0, 25.0, 50.0, and 85.0 K in a magnetic field of 7.0 T. Intensities of all MCD bands increase with decreasing temperature.

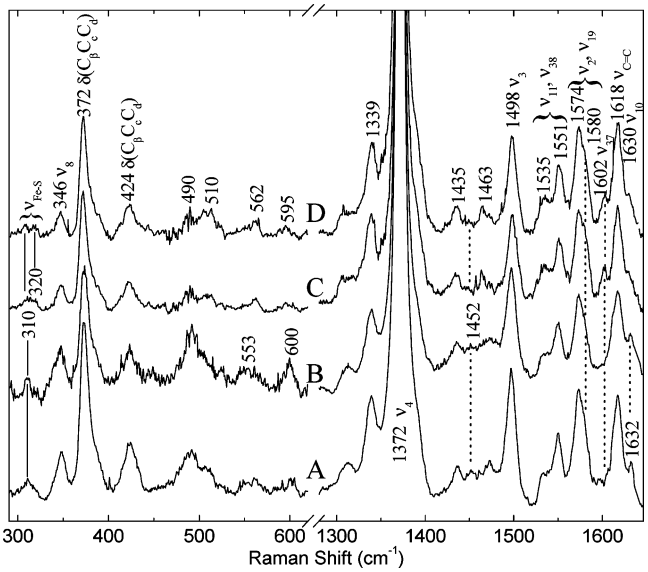

FIGURE 3: Resonance Raman spectra of ferric CBS-63 and CBS-45 obtained with 413.1 nm excitation at low and high pH: (A) 37 μM CBS-45 in 50 mM MES, pH 6.0; (B) 7 μM CBS-63 in 50 mM MES, pH 6.0; (C) 20 μM CBS-45 in 50 mM CHES, pH 9.2; (D) 25 μM CBS-63 in 50 mM CHES, pH 9.2. Spectra for the pH 6 and 9.2 samples were obtained with 12 and 16 mW of laser power at the samples, respectively. Assignments are based on those for ferricytochrome *c* (*45*).

either Fe(III) CBS-45 or Fe(III) CBS-63. Electronic absorption spectra of both enzyme forms display a δ band at 365 nm, a Soret band at 428 nm, and a broad, asymmetric absorption band at 550 nm. Weak thiolate-to-iron charge-transfer (CT) bands are also observed at 645 and 705 nm (Figure S1, Supporting Information). These spectral features are consistent with a thiolate-ligated heme (*20*, *39*, *40*, Table S1 and references therein) and are not altered by changes in pH. The insensitivity of these spectral features to changes in pH is compelling evidence that the heme of Fe(III) CBS-45 remains six-coordinate and retains histidine/cysteine-(thiolate) coordination over the aforementioned pH range (*41*, *42*).

Magnetic circular dichroism spectroscopy also supports the observation that the heme of Fe(III) CBS is unaffected by changes in pH. The MCD spectrum of Fe(III) CBS-45 (Figure 2) is dominated by *C*-terms in the Soret region of the spectrum. The temperature dependence of MCD intensity attributed to the *C*-term (Figure 2, inset) requires ground-state degeneracy and derives from the temperature dependence of the Boltzmann population distribution of the Zeeman-split ground state (*43*). Thus, the MCD spectrum of ferric heme will be dominated by *C*-terms in all cases, because both low-spin ($S = {}^1/_2$) and high-spin ($S = {}^5/_2$) ferric heme are paramagnetic and have degenerate ground states. A magnetization plot of our data reveals an $S = {}^1/_2$ system, consistent with the assignment of a low-spin state (data not shown). The closely spaced, oppositely signed nature of the Soret *C*-terms is also indicative of low-spin, ferric heme (*44*). The features of the Fe(III) CBS-45 MCD spectrum remain unchanged as the pH of the sample is varied from 6.0 to 9.2; the heme remains low-spin at both low and high pH.

Subtle differences are observed between the resonance Raman spectra of Fe(III) CBS at low and high pH (Figure 3). Bands sensitive to oxidation state (ν_4) and spin state (ν_3, ν_2) are observed at 1372, 1498, and 1574 cm^{-1}, respectively, for both CBS-63 and CBS-45. These frequencies are consistent with previously reported resonance Raman data for CBS (*46*) and are indicative of a low-spin, ferric heme. While ν_4, ν_3, and ν_2 of CBS-45 and CBS-63 are relatively unaffected over the pH range of 6.0 to 9.2, there are subtle changes in the high-frequency spectra. The intensity of the shoulder at 1580 cm^{-1} is diminished and the band at 1603 cm^{-1} is lost as the pH is lowered from 9.2 to 6.0. The low-frequency spectra of Fe(III) CBS-45 at pH 6.0 and 9.2 exhibit a band at 310 cm^{-1}. This band has previously been assigned to the ν_{Fe-S} stretching mode at pH 8.6 (*46*), and its presence at low pH indicates that the heme coordination of CBS-45 is not altered. At pH 9.2, a second band occurs at 320 cm^{-1}, whereas at pH 6.0, only the lower frequency ν_{Fe-S} (310 cm^{-1}) band is predominant. The higher-frequency band may be attributable to ν_{51} or it could be due to inhomogeneity in the heme pocket of CBS.

The electron paramagnetic resonance spectrum of Fe(III) CBS-45 contains two overlapping sets of rhombic signals, the ratio of which varies slightly with pH (Figure S2). Both signals are consistent with the type II low-spin ferric heme centers described by Walker (*47*). No high-spin EPR signals were observed in any Fe(III) CBS sample. Both low-spin components were observed in independently isolated CBS-45 samples, in CBS-63 (vide infra), and in different buffers. Simulations of the experimental EPR spectra indicate that the major component ($g_z = 2.49$, $g_y = 2.31$, and $g_x = 1.87$) accounts for approximately 60% and the minor component ($g_z = 2.43$, $g_y = 2.31$, and $g_x = 1.90$) accounts for approximately 40% of the total signal intensity at pH 7.0. The presence of multiple low-spin signals has been observed in the EPR spectra of a number of other thiolate-ligated heme proteins (*20*, *39*, *41*, *48*). EPR simulations suggested that the major and minor signal intensities vary slightly with pH, though shifts in the respective *g*-values were not observed. This observation is in accordance with a previous EPR study of H450 (*20*), although in our study, signal intensities do

Scheme 1

$$\text{Fe(III)CBS} + e^- \xrightarrow{k_0} [449\text{ nm}] \underset{k_{-1}}{\overset{k_1}{\rightleftharpoons}} [428\text{ nm}]$$

not vary with pH in a consistent fashion. The presence of two EPR signals in our preparations is also consistent with early work on H450, but not with recent studies of CBS-45, which report only one rhombic signal for the ferric enzyme (*10*). The presence of major and minor signals among several forms of CBS, that is, H450, CBS-45, and CBS-63, however, suggests that heme inhomogeneity is a characteristic native to CBS.

Reaction of Fe(III) CBS with Reducing Agents Yields Two Distinct Products. Electronic absorption spectroscopy reveals that different species are formed when Fe(III) CBS is treated with one-electron reducing agents at high and low pH. Upon addition of either sodium dithionite or titanium citrate to Fe(III) CBS-45 at pH 9.0, the Soret maximum shifts from 428 to 449 nm. The red shift of the Soret band is accompanied by a sharpening of the $\alpha-\beta$ region into two distinct peaks at 540 and 573 nm. The strongly red-shifted Soret at 449 nm indicates that the thiolate remains bound to the heme (*49*). This spectral change is identical to that noted previously in studies of H450, CBS-63, and CBS-45 (*18*, *50*, *51*) and is indicative of conversion to a six-coordinate, low-spin, ferrous heme complex. When a reducing agent is added to Fe(III) CBS-45 at pH 6.0, a different final product is observed (Figure 4). This new species is characterized by a Soret peak at 428 nm and a broad $\alpha-\beta$ feature at approximately 550 nm. A mixture of the 449 and 428 nm species (alkaline and acid forms, respectively) is observed at pH values between 6.0 and 9.0. The equilibrium of the two different species is shifted upon altering the pH of the reductant-treated sample (Figure 4, inset), allowing the 449 and 428 nm species to be reversibly interconverted. Identical pH-dependent behavior was also observed for the full-length enzyme, CBS-63.

Further examination reveals that reactions of CBS-45 and CBS-63 with dithionite ion at low pH proceed via a transient species, according to Scheme 1. This transient species exhibits a Soret maximum at 449 nm and appears to be the same as that observed in the reaction of the ferric protein with reductants at high pH. Initial formation of the 449 nm species is followed by conversion to the 428 nm species. This change was also observed at high pH, albeit at a much slower rate. Experiments were conducted with two different reductants (titanium citrate and sodium dithionite) that were present in excess at all times, in two different buffer systems (noncomplexing tertiary amine and phosphate), and in the presence of DTT; indistinguishable behavior was observed in all cases. The conversion of the 449 nm species to the 428 nm species was monitored over time (Figure 5, inset) and a value for k_1 was obtained by fitting absorbance data to eq 2 (vide supra). The rate of conversion to the 428 nm species was found to increase dramatically at lower pH (Figure 5). A nonlinear regression fit of the pH-dependence of k_1 to the pH-dependent model described by eq 3 (vide supra) yields an apparent pK_A value of 6.50 $\pm$ 0.09. These data implicate a two-step process for the reaction of Fe(III) CBS with reductants (Scheme 1). Initially, addition of reducing agents to Fe(III) CBS rapidly converts the heme to the ferrous state, as evidenced by the appearance of the species characterized by a 449-nm Soret maximum at both high and low pH. The ferrous heme then undergoes a significant change, the rate of which is inversely dependent on the pH of the medium.

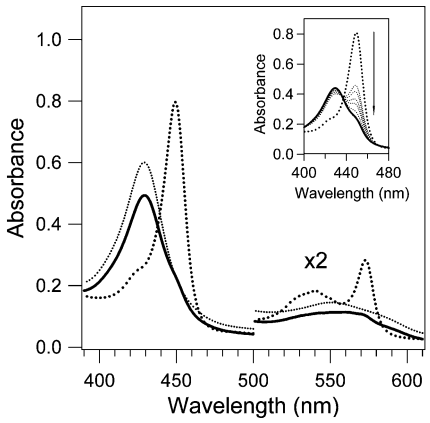

FIGURE 4: Electronic absorption spectra of Fe(III) CBS-45 treated with reductant at pH 9.0 (•••) and 6.0 (—) at 25 °C. Samples (6 μM in heme, 2 mM sodium dithionite) were in 25 mM buffer with an additional 100 mM NaCl. CHES was used as the buffer at pH 9.0, and MES was used at pH 6.0. The pH-independent spectrum of Fe(III) CBS-45 (6 μM in heme) in 25 mM CHES, pH 9.0, with an additional 100 mM NaCl (···) is included for comparison. The inset shows conversion of the 449 nm species to the 428 nm species, initiated by the addition of concentrated, low-pH buffer. MES buffer, 0.5 M, pH 6.0, was added to a pH 9.0 sample (6 μM in heme, 2 mM sodium dithionite, 100 mM NaCl, 25 mM CHES) to a final concentration of 33 mM and a final pH of 6.34. The conversion was followed for 100 min.

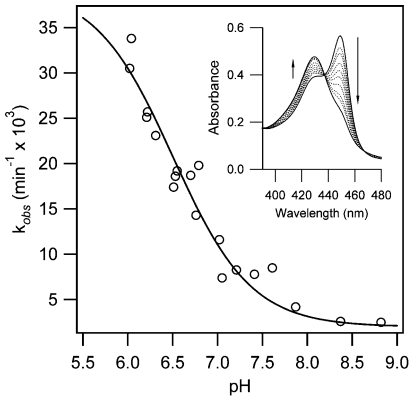

FIGURE 5: Rate of formation of the 428 nm species of CBS-63 as a function of pH at 25 °C. Samples (1.5 μM in heme, 2 mM sodium dithionite) were in 100 mM buffer with an additional 100 mM Na_2SO_4. The buffer used varied with the pH, as noted in the Experimental Procedures. Reactions were initiated by the addition of dithionite stock solution. The absorbance at 449 nm was followed over time and was observed to approach equilibrium by first-order kinetics in all cases. A nonlinear regression fit of the absorbance data to eq 2 yielded rate constants for this process. The line represents a nonlinear regression fit of k_1 vs pH to eq 3. The inset shows representative conversion from the 449 nm species to the 428 nm species of CBS-45 at pH 6.65. Arrows illustrate the growing-in of the 428 nm species and the growing-out of the 449 nm species over time.

Magnetic Circular Dichroism Spectroscopy Indicates That Fe(III) CBS Is Favored at Low pH. MCD spectroscopy reveals that the two products observed when Fe(III) CBS-45 is treated with reducing agents at high and low pH exist in different spin states (Figure 6). At high pH, the MCD spectrum contains signals in both the Soret and $\alpha-\beta$ regions and is dominated by the intense temperature-independent

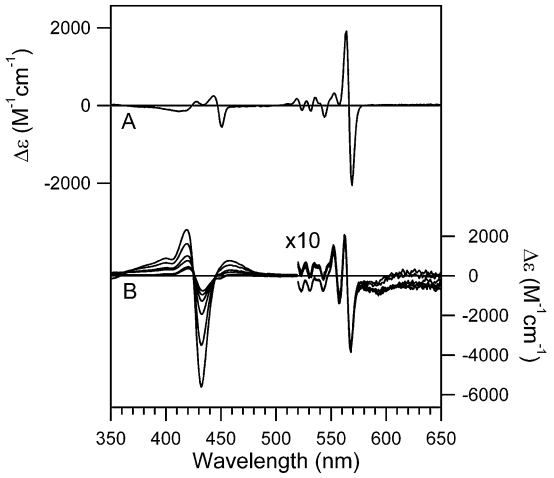

FIGURE 6: MCD spectra of Fe(III) Δ1−40 CBS-45 treated with sodium dithionite at high and low pH. All samples contained 45 mM buffer with 45 mM NaCl and 55% (v/v) glycerol. Excess solid sodium dithionite was added prior to freezing. Panel A shows CBS-45, 21 μM in heme, in CHES, pH 9.0. The spectrum was recorded at a temperature of 4.0 K in a magnetic field of 7.0 T. Panel B shows CBS-45, 21 μM in heme, in MES, pH 6.0. Spectra were recorded at temperatures of 4.0, 8.0, 15.0, 25.0, 50.0, and 85.0 K in a field of 7.0 T. Intensity of the MCD bands in the Soret region of spectrum B increased with decreasing temperature.

A-term present in the $\alpha-\beta$ region with a crossover point at 566 nm (Figure 6a). The temperature independence of the MCD spectrum of this alkaline species is consistent with an assignment of an $S = 0$, low spin, ferrous heme (*49*, *52*). At low pH, the MCD spectrum is dominated by intense signals in the Soret region centered at 425 nm and also contains substantially weaker signals in the $\alpha-\beta$ region (Figure 6b). Although the MCD spectrum of the alkaline species is fully temperature-independent, the Soret signals of the low-pH spectrum display strong temperature dependence, a hallmark of spectra dominated by the *C*-term. The weak, derivative-shaped features in the $\alpha-\beta$ region are temperature-independent and suggest that a small population of two low-spin ferrous species are present in this sample. The sharp α-band at 566 nm lies in a region typical of thiolate ligation (562−567 nm) (*48*, *49*, *53*), consistent with the presence of residual Fe(II) CBS. The α-band at 555 nm, however, is blue-shifted and appears in a region suggestive of another axial ligand environment, possibly bis(histidine) (*53*). A mixture of temperature-dependent and -independent features is observed at pH values between 6.0 and 9.0 (data not shown). A reasonable assignment of the low-pH species might be a high-spin, $S = 2$, ferrous heme. However, a magnetic saturation plot is most consistent with an $S = {}^1/_2$ spin state, suggesting that the acid species contains low-spin, *ferric* heme. Intriguingly, the MCD spectra of the acid species and those of Fe(III) CBS (Figure 2) appear to be very similar, particularly in the Soret region containing the temperature-dependent *C*-terms. Thus, MCD spectroscopy suggests that the acid species is Fe(III) CBS, even in the presence of excess reductant.

Resonance Raman Spectroscopy Reveals the Presence of Fe(III) CBS in Dithionite-Treated Samples at Low pH. Addition of reductant fully reduces Fe(III) CBS at high pH. At pH 9.2, 441.6 nm-excited resonance Raman spectra for CBS-45 and CBS-63 in the presence of excess dithionite exhibit ν_4 bands at 1356 cm^{-1}, indicative of ferrous heme

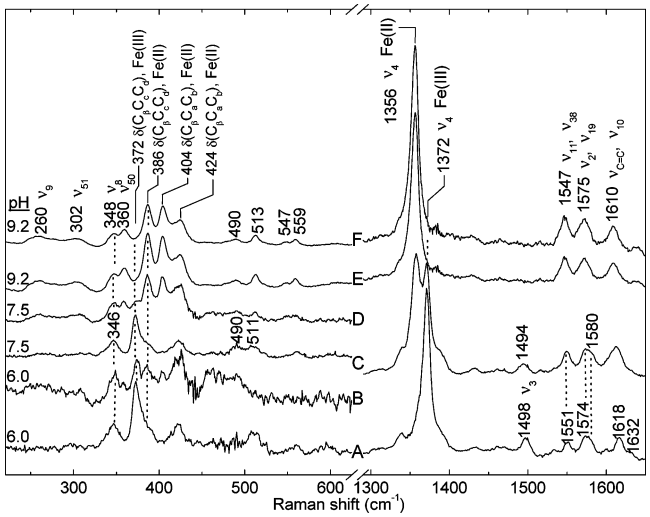

FIGURE 7: Resonance Raman spectra of Fe(III) CBS treated with sodium dithionite as a function of pH. The final dithionite ion concentration fell in the range of 3−6 mM: (A) CBS-45 in 50 mM MES, pH 6.0, 100 mM NaCl, 413.1 nm excitation; (B) CBS-45 in 50 mM MES, pH 6.0, 100 mM NaCl, 441.6 nm excitation; (C) CBS-45 in 50 mM MOPS, pH 7.5, 100 mM NaCl, 413.1 nm excitation; (D) CBS-45 in 50 mM MOPS, pH 7.5, 100 mM NaCl, 441.6 nm excitation; (E) CBS-45 in 50 mM CHES, pH 9.2, 100 mM NaCl, 441.6 nm excitation; (F) CBS-63 in 50 mM CHES, pH 9.2, 100 mM NaCl, 441.6 nm excitation. CBS-45 and CBS-63 samples were 20 and 25 μM, respectively. Assignments are based on those for ferro- and ferricytochrome *c* (*45*).

(Figure 7). These high-frequency spectra are similar to that reported for CBS-45 in 100 mM Tris buffer at pH 8.6 (*46*).

In contrast to the high-pH spectra, ferric heme features dominate the resonance Raman spectra of Fe(III) CBS samples treated with dithionite ion at low pHs. At pH 6.0, it becomes more difficult to obtain a resonance Raman spectrum with 441.6-nm excitation as seen by the decreased signal-to-noise ratio in Figure 7B. This difficulty is attributable to poor resonance enhancement of Raman scattering by the dominant ferric form with 441.6 nm excitation (Soret λ_{max}= 428 nm, Figure 4). The 413.1 nm-excited spectrum of CBS-45 treated with sodium dithionite at pH 6.0 is dominated by ferric heme, as judged by the lack of ferrous heme features in the high-frequency spectrum and its overall similarity to the Fe(III) CBS-45 spectra in Figure 3. The rR data show that, in alkaline solution, the product of CBS and dithionite ion is six-coordinate, low-spin, ferrous heme. Under acidic conditions, however, the rR spectra reveal that the equilibrium population of ferrous heme is low. Reduced heme is converted to six-coordinate, low-spin, ferric heme without significant build up of the ferrous heme at steady state.

At physiological pH, the heme exists in both Fe(II) and Fe(III) forms in the presence of excess dithionite. Figure 7 shows that at pH 7.5, two ν_4 bands are observed for the dithionite-treated solution, one at 1356 cm^{-1}, corresponding to Fe(II) CBS, and one at 1372 cm^{-1}, characteristic of Fe-(III) CBS. The ν_3 envelope is broadened and centered at 1494 cm^{-1}, consistent with the presence of multiple species. Since the ν_3 band is observed at 1498 cm^{-1} for Fe(III) CBS (Figure 3) and at 1493 cm^{-1} for Fe(II) CBS (*46*), we attribute this envelope to overlapping ν_3 bands from Fe(III) and Fe(II) CBS. The low-frequency spectrum of dithionite-treated CBS-45, pH 7.5, obtained with 441.6 nm excitation is very similar

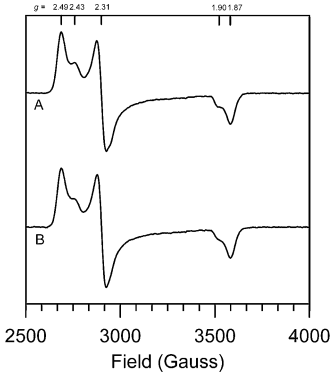

FIGURE 8: A comparison of the X-band EPR spectrum of Fe(III) CBS-63 treated with sodium dithionite with that of Fe(III) CBS-63: (A) CBS-63 in 100 mM MES, pH 6.0, 100 mM NaCl, 4.4 mM sodium dithionite; (B) CBS-63 in 100 mM MES, pH 6.0, 100 mM NaCl. Sample A was reacted with dithionite ion for 30 min, and both samples were approximately 140 μM in heme. Spectra were recorded at 20 K, 9.360 GHz microwave frequency, 0.51 mW microwave power, 4.00×10^5 receiver gain, 8.3 G modulation amplitude, and 0.66 s time constant. Spectra represent added scans (16 scans for sample A and 8 scans for sample B) and comprise 1024 data points.

to that recorded at pH 9.2 because the ferric component of the CBS mixture is poorly enhanced with 441.6-nm excitation. By virtue of its similarity with the pH 9.2 spectrum, the low-frequency spectrum clearly shows the presence of ferrous heme at pH 7.5. However, the 413.1-nm excited spectrum is comparable to that of dithionite-treated CBS-45 at pH 6, because the ferric component of the mixture is preferentially enhanced at this excitation wavelength.

Electron Paramagnetic Resonance Spectroscopy Confirms the Identity of the Acid Species as Fe(III) CBS. CBS-63 treated with sodium dithionite for 30 min at pH 6.0 exhibits EPR spectral features identical to those of Fe(III) CBS-63. No other signals were observed in the EPR spectrum, other than those of the low-spin, ferric heme. The dithionite-treated sample displays two overlapping sets of rhombic signals (Figure 8) with g values (major $g_z = 2.49$, $g_y = 2.31$, and $g_x = 1.8$; minor $g_z = 2.43$, $g_y = 2.31$, and $g_x = 1.90$) identical to those observed in our study of the truncated enzyme (vide supra). The major component accounts for approximately 75% of the total integrated intensity in the spectra of Fe(III)- and dithionite-treated CBS-63. Additionally, the absolute signal intensities of the two samples were comparable, although the total intensity of the reductant-treated sample was slightly less than that of untreated Fe(III) CBS, due to the presence of residual ferrous heme. It is this small fraction of ferrous heme that gives rise to the ferrous heme bands in the pH 6 rR spectra of Figure 7. Taken together, the MCD, rR, and EPR spectra provide conclusive evidence that the acid (428 nm) species observed by electronic absorption spectroscopy is Fe(III) CBS.

DISCUSSION

The impetus for this investigation was to determine whether a connection exists between the CBS heme, the function of which is currently unclear, and the striking pH dependence of CBS activity over the physiological pH range. The hypothesis that the CBS heme might regulate activity through pH modulation of its coordination environment stems from the early work on H450, which demonstrated that the H450 heme exhibits pH-dependent behavior in the Fe(II) state (*19*−*21*). Our experimental approach was to (a) determine whether the structural basis for the pH dependence of activity is localized within the catalytic or the regulatory domain of CBS and (b) determine whether any correlation exists between the pH-dependent behaviors of the heme and the enzyme activity. Our data reveal that although the catalytic domain is the sole determinant of pH-dependent enzyme activity, no correlation exists between the pH-dependent behaviors of the heme and the enzyme activity. Specifically, we found no evidence for changes in coordination number, identity of the axial ligands, or protonation states of the axial ligands for either the ferric or the ferrous form of CBS. However, the stability of Fe(II) CBS exhibits a steep pH dependence that extends into the physiological pH range. Remarkably, when steady state is achieved after treatment of CBS with reducing agents at pH 7.4, Fe(III) CBS is the dominant species. The discussion below highlights alternative explanations for the prominent pH dependence of enzyme activity, as well as further implications for the observed pH-dependent redox behavior of the CBS heme.

Axial Ligation of Fe(III) CBS Is Insensitive to pH; Therefore, pH-Dependent Enzyme Activity Is Not Directly Mediated by the Ferric Heme Moiety. The pH dependence of Fe(III) CBS activity is localized to ionizations occurring in the catalytic core of the enzyme. CBS-45 is a truncation variant of CBS-63 in which the C-terminal autoinhibitory domain has been deleted, leaving the catalytic core, comprising the heme- and PLP-binding domains. Thus, comparison of pH versus activity profiles for Fe(III) CBS-45 and Fe(III) CBS-63 may locate the source of the pH dependence to either the catalytic or regulatory regions of the enzyme. Our functional assays reveal that both Fe(III) CBS-63 and Fe(III) CBS-45 are highly pH-dependent through the physiologically relevant pH range of 6.0−8.0, in agreement with previous studies of the full-length enzyme (*50*). The fact that the pH-dependent profile of enzyme activity is nearly identical for the full-length and truncated enzymes implies that the catalytic core of the enzyme governs the pH dependence of CBS activity. This evidence allows us to conclude that the pH profile derives from pH-dependent changes in the heme axial ligand field, the PLP cofactor and its immediate environment, or both.

Although heme is required for maximal enzyme activity (*5*), there is little evidence to date that suggests a plausible role for this cofactor in CBS. Only higher eukaryotes possess the N-terminal heme-binding domain; CBS from *Saccharomyces cerevisiae* and *Trypanosoma cruzi* do not contain heme but do contain PLP (*25*−*28*). Previous work has precluded a catalytic role for the heme (*9*, *24*, *25*, *27*, *46*), and regulatory or structural roles are currently being considered as possible functions of the heme moiety (*5*, *13*, *50*, *54*, *55*). Hemes are now known to regulate protein function by acting as sensors, in addition to their well-established roles in oxygen transport, electron transfer, and catalytic transformations. In systems such as the enzyme soluble guanylyl cyclase (*56*−*58*), the bacterial transcription factor CooA (*59*, *60*), and the rhizobial FixL protein (*61*−*63*), the binding of small molecules (NO, CO, and O_2, respectively) to a heme center triggers protein conformational changes that significantly modulate protein function. The recent observation that CBS activity decreases 2-fold upon reduction of the heme

suggested that the CBS heme may act as a redox sensor, modulating enzyme activity though redox changes at the heme center (*50*). Few other ways in which the CBS heme could be regulating the activity of this enzyme have been explored, however. Considering that the pH dependence of CBS activity is localized to the catalytic domain, another reasonable hypothesis is that a proton transfer that affects the ligand environment of the heme may regulate CBS. Although subtle changes with pH are observed in the resonance Raman and EPR spectra of Fe(III) CBS-45 and Fe(III) CBS-63, the electronic absorption and MCD spectra of the two enzyme forms remain unaltered, indicating that the first coordination sphere of the ferric heme is not greatly affected by varying pH. Therefore, pH-dependent modulations of the ferric heme are not responsible for changes in catalytic activity, implying the Fe(III) heme is not a pH-dependent regulator of CBS enzyme activity, because no significant modifications occur to the ferric heme over the pH range at which activity is most pH-dependent.

In light of our spectroscopic and functional characterization of Fe(III) CBS, a likely explanation is that the pH dependence of CBS activity results from ionizations involving residues in the vicinity of the PLP, the cofactor itself, or PLP intermediates formed during the course of the catalytic cycle. That pH-dependent enzyme activity may be controlled by the PLP moiety or amino acids within the vicinity of the cofactor is consistent with the behavior of other members of the β-family of PLP enzymes, including *O*-acetylserine sulfhydrylase and tryptophan synthase from *Salmonella typhimurium*. In these PLP-containing enzymes, the pH dependence of enzyme activity is apparently linked to specific ionizations within the PLP-binding domain (*64*, *65*). In the case of tryptophan synthase, recent studies have suggested that the deprotonation of βLys^{87} is required for competent catalytic turnover. The specific ionizations occurring within the PLP-binding domain of human CBS that may give rise to the strong pH-dependent activity profile remain to be identified.

Low-Spin, Six-Coordinate, Cysteine-Ligated Ferrous Hemes, Such as That of CBS, May Be Thermodynamically Unstable. This investigation has revealed that Fe(III) CBS is favored at low pH, even in the presence of excess reductant. Addition of reductant to samples of Fe(III) CBS at high- and low-pH produced two spectrally distinct products, termed the alkaline (449 nm) and acid (428 nm) species, respectively. The alkaline species was unambiguously characterized as Fe(II) CBS, but the acid species was unexpectedly found to be Fe(III) CBS. At low pH, absorption spectroscopy revealed a transient reduction of the ferric heme followed by reoxidation of the ferrous heme under anaerobic, reducing conditions. Thus, a net reduction of Fe(III) CBS is not achieved at lower pH values. This behavior unfortunately prevents the accurate determination of the pH dependence of Fe(II) CBS activity, because the heme would reoxidize over the course of the assay.

Although it has not been discussed in the literature, resistance of ferric heme to reduction, presumably due to the instability of ferrous, six-coordinate, thiolate-ligated heme centers, has been documented for other proteins (Table 1). Cheesman et al. report that the Cys(thiolate)/His-ligated "heme-1" of SoxAX from *Rhodovulum sulfidophilum* remains in the Fe(III) state even in the presence of excess dithionite ion at pH 7 (*14*). Similarly, at pH 7, only a small amount of Fe(II) CBS is present at equilibrium, as judged by a small absorption maximum at 449 nm in the electronic absorption spectrum (data not shown). Cyt *c* M80C, a cytochrome *c* variant that exhibits Cys(thiolate)/His coordination, has also been reported to exhibit unusual behavior in the presence of reductants (*66*). Another subset of heme proteins are incapable of attaining the ferrous state while retaining Cys(thiolate)/(N) ligation (where "N" represents any endogenous nitrogen-donating ligand). Upon addition of reductant, both C420, an inactive form of chloroperoxidase, and the bacterial CO-sensor CooA from *Rhodospirillum rubrum* undergo ligand switches in which the Cys(thiolate) is displaced (*67*−*69*). Furthermore, recent work by Perera et al. has demonstrated that cysteine(thiol) is a reasonable ligand in ferrous heme complexes, suggesting that heme proteins that are presumed to lose cysteine(thiolate) coordination upon reduction may actually retain a neutral cysteine as a ligand in the Fe(II) state (*70*). Thus, no literature precedent for a ferrous heme displaying Cys(thiolate)/(N) coordination exists. These observations suggest that at physiological pH, ferrous Cys(thiolate)/(N)-ligated heme is unstable and, to maintain the Fe(II) state, requires the substitution of another residue for the Cys(thiolate) ligand. The reduction of Fe(III) CBS at high pH, however, does not appear to conform to this axial ligand exchange behavior. The 449 nm Soret maximum for Fe(II) CBS suggests that it retains its coordination environment upon reduction at high pH. Consequently, we suspect that pH must affect the stability of the ferrous Cys(thiolate)/(N)-ligated heme in CBS.

Table 1: Effect of Adding Reductants to Various Heme Proteins Exhibiting Cys(thiolate)/(N) Coordination

protein	axial ligands	effect of reductant	ref
CBS (pH 6)	Cys(thiolate)/His	transiently reduced	this work
SoxAX (heme 1)	Cys(thiolate)/His	not reduced	*14*
cyt *c* M80C	Cys(thiolate)/His	a	*66*
C420	Cys(thiolate)/His	Cys → His	*67*
CooA	Cys(thiolate)/Pro(N)	Cys → His	*68*, *69*

[a] Smulevich et al. report that reduction by sodium dithionite was sluggish and spectroscopic samples prepared in this manner were unsatisfactory.

Inconsistencies with Previous Work on H450 Can Be Explained by Implicating a Partially Denatured Form of CBS. Although previous spectroscopic studies on Fe(II) H450 also show pH-dependent behavior, these early experiments may have been plagued by a partially denatured form of the enzyme. The heme protein H450 was isolated from pig liver in the late 1970s, and nearly 2 decades passed before researchers realized that the protein was in fact CBS. Whether H450 was isolated as CBS-63 or as CBS-45 remains unclear, though the reported gel electrophoresis experiments suggest that H450 was actually a mixture of the proteolytically cleaved and full-length enzymes (*18*). Initial electronic absorption experiments by Omura and co-workers on H450 revealed two pH-dependent forms, alkaline and acid, of the dithionite-treated enzyme (*19*, *20*), similar to those we report here for CBS-45 and CBS-63. The alkaline species of H450 and CBS are identical, both exhibiting absorption maxima at 449, 540, and 573 nm. In contrast, the acid species of H450 was characterized by absorbance maxima at 425, 530, and 558 nm, significantly different from the acid species of

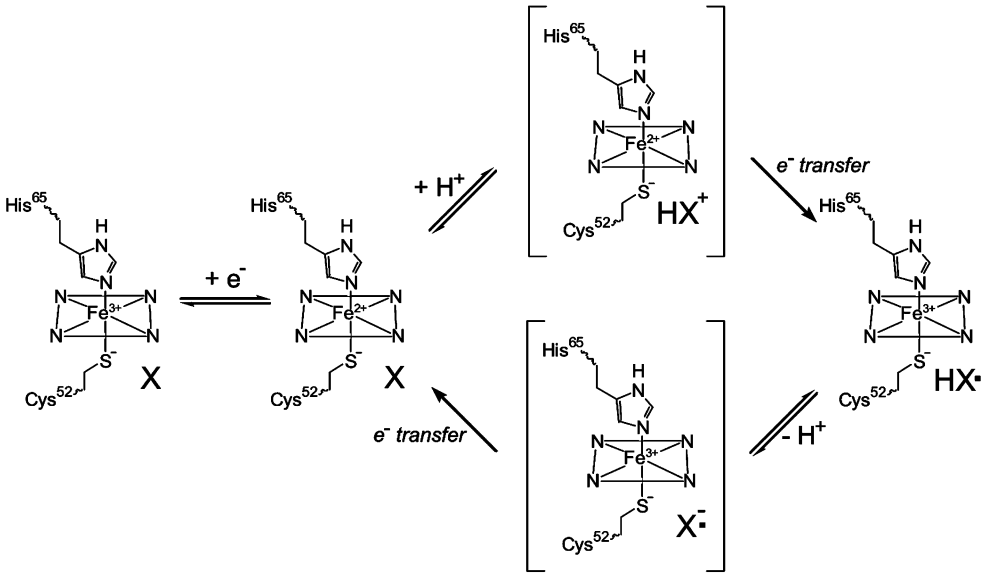

FIGURE 9: Proposed proton-gated electron-transfer process involving the CBS heme. "X" represents a remote site which, when protonated, accepts an electron transferred from the CBS heme. Hypothetical intermediates are contained in brackets.

CBS that we report herein. Subsequent room-temperature MCD work by Svastits et al. lead to the hypothesis that the low-pH H450 species was the result of protonation and possible displacement of the cysteine thiolate to yield either a six-coordinate, cysteine(thiol)/histidine-bound heme or a five-coordinate, histidine-bound heme (*21*). We have evidence that the acid form of H450 was actually partially denatured. Omura and co-workers reported that the spectrum of the 425 nm species could also be attained by the addition of either *n*-butanol or urea, known protein denaturants, to a solution Fe(II) H450 at high pH. We have reproduced the spectrum of the H450 acid species by thermally denaturing Fe(II) CBS at pH 9 (Figure S3). We therefore suspect that the manner in which H450 was isolated may account for the difference between the low-pH species of H450 and CBS. Intriguingly, the activity of CBS-63 has been shown to increase upon partial thermal denaturation of the enzyme, a fact previously attributed to heightened flexibility of the protein matrix and displacement of the autoinhibitory C-terminal domain from the catalytic site (*6*). Displacement of a heme ligand upon denaturation of CBS may also provide a mechanism by which a conformational change within the enzyme increases activity. Current work in our laboratory is aimed at elucidating the nature of this partially denatured form of CBS.

The Novel Heme Redox Chemistry of CBS Suggests That Redox Regulation of This Protein Must Be Coupled to pH. The transient formation of the alkaline species that is observed upon addition of reductant to Fe(III) CBS indicates that the CBS heme is initially reduced by the addition of excess reductant and then is reoxidized in an intramolecular electron-transfer process. As discussed earlier, ferrous Cys-(thiolate)/(N)-ligated heme appears to be unstable near physiological pH and is either resistant to oxidation or undergoes a ligand switch to accommodate the change in heme redox state. We have demonstrated, however, that Fe(II) CBS is indeed the dominant species for a short time at low pH and ultimately reoxidizes, even under strictly anaerobic conditions. Thus, it is not that the ferric Cys(thiolate)/(N)-ligated heme of Fe(III) CBS at low pH is simply resistant to reduction but rather that the ferrous Cys(thiolate)/(N)-ligated heme of Fe(II) CBS is reoxidized and remains ferric even in the presence of excess reductant but would rereduce by an intramolecular electron transfer if the pH were raised. We hypothesize that Fe(II) CBS reoxidizes to escape the ferrous Cys(thiolate)/(N)-ligated state. Similarly unstable systems employ a ligand switch; however, a ligand switch is unlikely for CBS because no other suitable nearby residue appears poised to replace Cys^{52}. The pH-dependence of the rate of Fe(II) CBS reoxidation suggests that an ionizable site within the enzyme is controlling this process. A reasonable model explaining these observations is a proton-gated electron transfer (Figure 9), in which protonation of a remote site in the enzyme (apparent $pK_A \approx 6.5$) triggers an electron transfer from the heme to the protonated site or another electron acceptor located elsewhere within the enzyme. Thus, the location of the electron is determined by pH; at high pH, the electron resides at the heme, while at lower pHs, the electron resides at the electron receptor, represented by a radical species in Figure 9. Formation of a radical at the electron receptor is speculation because no evidence of such a species has been observed. A radical is also not the only possible scenario, because a multiple-electron reduction is possible if the CBS heme is serving as an electron conduit.

At this time, the identity of the reducible site within the enzyme remains unknown. The fact that DTT does not affect the reoxidation phenomenon suggests that accessible disulfide linkages are not involved. The electron acceptor may be the PLP cofactor, because this moiety has been shown to form radicals both in solution (*71*) and during the course of enzymatic reactions (*72*), as well as undergo a two-electron reduction with sodium borohydride when bound to CBS (*22*). An explanation of the greater stability of the ferrous Cys-(thiolate)/(N)-ligated heme of Fe(II) CBS at high pH also remains elusive. What is certain, however, is that the pH dependence that governs the redox behavior of this enzyme is distinct from the ionizations that mediate CBS activity, because the apparent pK_A's for these processes are quite different.

The findings presented here have implications for the recent suggestion that the heme of CBS serves as a redox sensor, where the redox state of the heme influences the catalytic rate of the enzyme (*50*). The fact that the CBS heme reoxidizes in the presence of excess reductant at physiological pH and cannot be rereduced after one reduction/reoxidation cycle suggests that a simple redox-sensing role for the heme is unlikely. Moreover, a redox-sensing function for the CBS heme is less plausible based on comparisons made to other Cys(thiolate)/(N)-ligated heme proteins, because this particular axial ligand pair does not appear to be stable to heme reduction at physiological pH.

In conclusion, this study reveals that a connection between the heme of CBS and pH does exist but surprisingly is not related to the strong pH dependence of enzyme activity. Rather, the connection between pH and the heme is associated with the redox behavior of the heme itself. We have demonstrated that the CBS heme favors the ferric state at lower pH and, in the presence of reductants, attains the ferric state via transient formation of the ferrous state. Fe(II) CBS and Fe(III) CBS are apparently interconverted not by a simple ferric/ferrous redox couple but by what appears to be a proton-controlled internal electron transfer process. Finally, our results suggest that a straightforward redox mechanism of regulation in this enzyme is unlikely, because only a small amount of Fe(II) CBS exists at equilibrium after addition of reductant at physiological pH. If redox regulation is occurring in this enzyme, it must be coupled to the pH of the cellular microenvironment.

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SUPPORTING INFORMATION AVAILABLE

Figures showing the thiolate sulfur-to-iron charge-transfer bands of Fe(III) CBS and a comparison of charge-transfer band positions of various cysteine-ligated heme centers, the pH-dependence of Fe(III) CBS EPR spectra, and the spectral changes that occur upon thermal denaturation of Fe(II) CBS-45. This material is available free of charge via the Internet at http://pubs.acs.org.

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