BIOCHEMISTRY

© Copyright 2005 by the American Chemical Society Volume 44, Number 51 December 27, 2005

Articles

The Heme of Cystathionine β-synthase Likely Undergoes a Thermally Induced Redox-Mediated Ligand Switch†

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Received July 7, 2005; Revised Manuscript Received August 31, 2005

ABSTRACT: Cystathionine β-synthase (CBS) is a pyridoxal-5′-dependent enzyme that catalyzes the condensation of homocysteine and serine to form cystathionine. Human CBS is unique in that heme is also required for maximal activity, although the function of heme in this enzyme is presently unclear. The study presented herein reveals that the heme of human CBS undergoes a coordination change upon reduction at elevated temperatures. We have termed this new species "CBS424" and demonstrate that its formation is likely irreversible when pH 9 Fe^{III} CBS is reduced at moderately elevated temperatures (~40 °C and higher) or when pH 9 Fe^{II} CBS is heated to similar temperatures. Spectroscopic techniques, including resonance Raman, electronic absorption, and variable temperature/variable field magnetic circular dichroism spectroscopy, provide strong evidence that CBS424 is coordinated by two neutral donor ligands. It appears likely that the native cysteine(thiolate) heme ligand is displaced by an endogenous neutral donor upon conversion to CBS424. This behavior is consistent with other six-coordinate, cysteine(thiolate)-ligated heme centers, which seek to avoid this coordination structure in the Fe^{II} state. Functional assays show that CBS424 is inactive and suggest that the ligand switch is responsible for eliminating enzyme activity. When this investigation is taken together with other functional studies of CBS, it provides strong evidence that coordination of Cys^{52} to the heme iron is crucial for full activity in this enzyme. We hypothesize that cysteine displacement may serve as a mechanism for CBS inactivation and that second-sphere interactions of the Cys^{52} thiolate with surrounding residues are responsible for communicating the heme ligand displacement to the CBS active site.

Human cystathionine β-synthase (CBS)[1] catalyzes the condensation of serine and homocysteine to yield cystathionine via a β-replacement reaction critical for maintaining minimal intracellular concentrations of homocysteine (*1*). Homocysteine lies at a branch point in methionine metabolism. This toxic metabolite is either conserved by remethylation to methionine via the transmethylation pathway or converted to cysteine in the transulfuration pathway. The reaction catalyzed by CBS is the first committed step in the transsulfuration pathway, which ultimately produces cysteine, a precursor of glutathione, taurine, and possibly hydrogen

† This work was supported by NIH grants R01-HL065217 to J.N.B. and J.P.K. and P01-HD0805 to J.P.K.

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10.1021/bi051305z CCC: $30.25 © 2005 American Chemical Society
Published on Web 12/01/2005

sulfide (H_2S). Two significant pathological conditions are associated with elevated plasma homocysteine: CBS-deficient homocystinuria (CBSDH) and hyperhomocysteinemia. CBSDH is the most frequent cause of homocystinuria and is an autosomal recessive trait resulting from pathogenic mutations in both CBS alleles (*1*). CBSDH is typically accompanied by a variety of clinical and pathological abnormalities, the most extreme of which include mental retardation, dislocated optic lenses, skeletal problems, and thrombotic vascular disease. More than 100 pathological variants of CBS that result in a deficiency of enzyme activity have been described in homocystinuric patients (*2*). Furthermore, individuals with the mildly elevated homocysteine levels characteristic of hyperhomocysteinemia have an increased prevalence of carotid artery, peripheral, and cerebral vascular atherosclerosis (*3*−*5*).

CBS is a unique member of the fold II family of pyridoxal-5′-phosphate (PLP)-dependent enzymes in that it binds a second cofactor, iron protoporphyrin IX (heme *b*) (*6*). The human CBS polypeptide consists of 551 amino acids and forms tetramers of 63-kDa monomers. The CBS polypeptide has a defined organization in which the N-terminal, middle, and C-terminal regions of the protein serve as the heme-binding domain, the catalytic core, and the regulatory domain of the enzyme, respectively (*7*). The C-terminal-autoinhibitory domain (residues 401−551) allosterically regulates CBS activity via a putative conformational change induced by the binding of *S*-adenosylmethionine (AdoMet) (*8*). This C-terminal region can be removed, either by limited proteolysis of the 63-kDa enzyme (*9*) or by expression of a truncation variant in *Escherichia coli* (*10*), resulting in a homodimer of 45-kDa subunits. This truncation also increases the specific activity approximately 2-fold and eliminates AdoMet responsiveness in the 45-kDa form (*9*). The only X-ray crystal structures of CBS available are those of the 45-kDa form of the enzyme (*11, 12*). In the catalytic core region (residues 71−400), the PLP cofactor is bound as a Schiff's base to Lys^{119}. This region is homologous to other members of the fold II class of PLP-dependent enzymes (*13*, *14*). The CBS heme is noncovalently cradled in a hydrophobic pocket formed by an N-terminal loop of ∼15 residues and two adjacent helices. The thiolate of Cys^{52} and the $N_{\epsilon 2}$ atom of His^{65}, both located on the N-terminal loop, axially coordinate the heme cofactor (*11*, *12*). The N-terminal heme-binding region in human CBS is absent from CBS isolated from *Saccharomyces cerevisiae* (*15*, *16*) and *Trypanosoma cruzi* (*17*), neither of which contain heme, suggesting that heme is an evolutionarily new acquisition. Interestingly, the CBS heme was first characterized spectroscopically in "hemoprotein H450," an unusual hemeprotein thought to be closely related to the P450 cytochromes (*18*−*21*). H450 was not identified as CBS until the cDNA sequences of H450 and CBS from *Rattus norvegicus* became available and were compared (*6*).

The role of the heme in CBS remains unclear. It has been demonstrated that the CBS heme serves no direct catalytic role in the synthesis of cystathionine. The heme-free enzyme is catalytically competent, although the specific activity is lower than that of the holoenzyme (*7*, *22*−*24*). Moreover, the β-replacement reaction catalyzed by CBS can be explained in terms of a typical PLP-based mechanism (*9*). Nonetheless, heme is essential for maximal activity in the human enzyme, and activity decreases substantially upon deletion of the heme-binding domain (*7*). Past studies suggest that the heme of CBS behaves in ways comparable to other known heme sensor proteins, such as the NO-sensor soluble guanylyl cyclase (sGC) or the CO-sensor CooA, where the binding of small molecules to a heme center triggers protein conformational changes that significantly modulate protein function. For example, perturbations of the CBS heme axial ligand environment that displace the Cys^{52} ligand (e.g., binding of CO, NO, or cyanide and chelation by $HgCl_2$) result in a considerable decrease in CBS activity (*25*−*27*). Other studies have labeled the CBS heme as a redox sensor, because CBS activity was observed to decrease 2-fold upon reduction of the heme (*28*). Still others have suggested the CBS heme functions as a structural support during protein folding (*24*, *29*). Nonetheless, CBS is capable of unique heme chemistry; a recent study demonstrated a novel pH-dependent redox mechanism by which the CBS heme remains reduced at high pH but spontaneously reoxidizes at low pH (*30*).

The studies presented herein demonstrate that a new, inactive form of CBS can be obtained by heat treatment of Fe^{II} CBS. This species is reminiscent of the inactive forms of cytochrome P450 and chloroperoxidase, i.e., P420 and C420, respectively, and was termed CBS424 for its distinct blue-shifted Soret band. On the basis of absorption, resonance Raman (rR), and magnetic circular dichroism (MCD) spectroscopic data, we propose that CBS424 is coordinated by two neutral donor ligands. It is also observed that the spectral characteristics of Fe^{II} CBS424 are similar to those reported for Fe^{II} Cys^{52} variants and Fe^{II} CBS in the presence of mercuric chloride, a thiol chelator (*26*, *31*, *32*). Thus, the CBS heme appears to lose its native cysteine(thiolate) ligand upon conversion to Fe^{II} CBS424. Formation of CBS424 is also correlated with a complete loss of CBS activity. This observation suggests that the Cys(thiolate)−heme iron interaction must be intact for full enzymatic activity. Similar communication between the heme and active site has been observed in the cases of CO-bound Fe^{II} CBS (*27*), $HgCl_2$-treated Fe^{III} and Fe^{II} CBS (*26*), and the pH dependence of C52S CBS activity (*32*). We suggest here that disruption of several second-sphere interactions of the Cys^{52}(thiolate) are responsible for the observed inactivation of CBS by CO binding, $HgCl_2$ treatment, or formation of CBS424.

[1] Abbreviations: (41−413)CBS, a form of cystathionine β-synthase that spans residues 41−413 whose C and N termini are truncated compared to those of wild-type cystathionine β-synthase; AdoMet, *S*-adenosylmethionine; BSA, bovine serum albumin; C420, an inactive form of chloroperoxidase achieved by incubating Fe^{III} chloroperoxidase at high pH; CBS, cystathionine β-synthase; CBS424, an inactive form of CBS that is obtained by heat treatment of Fe^{II} CBS; CBSDH, CBS-deficient homocystinuria, with homocystinuria resulting from the inactivity of CBS; 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 *Rhodospirillum rubrum*; CPSH, cyclopentanethiol; cyt, cytochrome; cyt *c* M80C, a cytochrome *c* variant in which the methionine heme axial ligand at position 80 has been replaced by a cysteine; DMF, dimethylformamide; H450, hemoprotein 450, a heme protein isolated in 1976 that was later identified as CBS by sequence alignment; KatG, a heme-containing catalase peroxidase; MCD, magnetic circular dichroism; NMR, nuclear magnetic resonance; PLP, pyridoxal 5′-phosphate; rR, resonance Raman; sGC, soluble guanylyl cyclase, an NO-sensing heme protein responsible for the conversion of guanosine 5′-triphosphate to cyclic guanosine 3′,5′-monophosphate; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane; VT/VH, variable-temperature/variable field (MCD); WT, wild type.

MATERIALS AND METHODS

Materials. L-[U-^{14}C]Serine was obtained from Perkin−Elmer Life Sciences and all other chemicals were purchased from Sigma. Chemicals were used as received. Homocysteine was prepared from homocysteine thiolactone as described previously (*33*). Cellulose thin-layer chromatography (TLC) plates were purchased from Selecto Scientific.

Recombinant Human CBS. The cloning of human CBS cDNAs (both the 63- and 45-kDa forms), the expression of the proteins in *E. coli*, and the purification of enzymes to homogeneity were performed as described previously (*10*, *34*). Another form of CBS in which the N and C termini have been truncated, (41−413)CBS, was also utilized in these studies. The amino acid sequence of this double truncate spans residues 41−413 and was prepared by digesting the 63-kDa enzyme with trypsin (*9*). Protein concentrations were determined by the Lowry method (*35*) using bovine serum albumin (BSA) as a standard. Heme content was determined using the pyridine hemochromagen assay (*36*).

CBS Activity Assay. CBS activity was determined by a variation on two previously described radiochemical methods, which utilize [^{14}C]serine as the labeled substrate (*15*, *33*). Final assay mixtures contained 100 mM Tris-HCl at pH 8.6, 250 μM PLP, 0.1 mg/mL BSA, 10 mM [^{14}C]serine (125 cpm/nmol), 10 mM homocysteine, and 1−10 μg of CBS in 0.2 mL of total volume. Select samples also contained additional components, such as 360 μM AdoMet and 10 mM sodium dithionite, as indicated in Table 1. Reactions were initiated by adding substrates, with or without sodium dithionite, to the protein solution at 37 °C and terminated after 10 min by the addition of 50 μL of cold 50% trichloroacetic acid. [^{14}C]Cystathionine was separated from the assay mixture in ~6 h by ascending cellulose thin-layer chromatography in 2-propanol/formic acid/H_2O (80:6:20). TLC plates were dried under a hot air stream and stained with 0.2% ninhydrin in EtOH. The product spot was excised, placed in 10 mL of BioSafe II scintillation cocktail, and quantified by scintillation counting in a Beckman LS-6500 scintillation counter. All assay samples were prepared and assayed for activity in a glovebox under N_2, although all TLC separations were performed aerobically. Heat-treated CBS species, including CBS424, were prepared by heating to 55 °C for ~20 min; these samples were subsequently examined by absorption spectroscopy, put on ice, and assayed immediately. 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.

Electronic Absorption Spectroscopy. Electronic absorption spectra were recorded on a double-beam Cary 4 Bio spectrophotometer equipped with a Peltier temperature controller and set to a spectral bandwidth of 0.5 nm. Spectra of CBS solutions that were 25 mM in boric acid (H_3BO_3) at pH 9.0 and 100 mM in NaCl were recorded at 4 °C, unless stated otherwise. Reactions of Fe^{III} CBS with sodium dithionite were performed anaerobically under an atmosphere of Ar and were initiated by the addition of an Ar-sparged stock solution of reductant to a final concentration of 2 mM. Oxidation of ferrous enzyme was either performed chemically with potassium ferricyanide or performed aerobically by desalting the sample in air using a NAP-5 column (Amersham).

Thermal Characterization. To characterize CBS behavior over a broad temperature range, the absorption spectra of anaerobic samples were monitored between 4 and 70 °C. Fe^{II} CBS was prepared by incubating Fe^{III} CBS with 2 mM sodium dithionite under Ar at 4 °C. Full spectra were recorded after thermal equilibration for 10 min at each temperature. Single wavelength absorbance measurements were made at 449 and 424 nm after each 1 °C increase along a 0.1 °C/min temperature ramp.

Circular Dichroism (CD). CD data were recorded on an Aviv 62A DS spectrometer using a 0.1-cm path-length quartz cell. Wavelength scans were performed over a range of 190−260 nm with a band-pass of 1 nm and an averaging time of 5.0 s/point. CD spectra were recorded at temperature intervals of 5 °C between 25 and 55 °C. Samples were held at the desired temperature (within ±0.2 °C) for 5 min to allow for complete thermal equilibration.

MCD Spectroscopy. MCD spectra were recorded on a Jasco J715 CD spectropolarimeter with the sample compartment modified to accommodate an SM-4000-8 magneto-cryostat (Oxford Instruments). Samples were prepared in 50 mM CHES (pH 9.0) and 100 mM NaCl. Sodium dithionite was introduced anaerobically to 4 °C samples by the addition of an Ar-sparged stock solution to a final concentration of 4 mM. Samples were then heated to 55 °C for ~10 min to induce the formation of Fe^{II} CBS424. The glassing agent ethylene glycol (50%, v/v) was added anaerobically to Fe^{II} CBS424 samples cooled to 4 °C. Addition of ethylene glycol had no effect on the absorption spectrum of any of the samples examined in this study. Samples were transferred to Ar-filled cells via a gastight syringe and frozen in liquid N_2. All MCD spectra were recorded at 7 T. The absorption spectra were unchanged at temperatures at which MCD data were collected. Zero-field CD spectra were subtracted from the MCD spectra for all samples. Variable-temperature/variable-field (VT/VH) data were obtained by ramping the field at 0.7 T/min and recording MCD at 439 nm every second at 2, 4, 10, and 25 K.

rR Spectroscopy. Fe^{II} CBS424 samples were prepared in 5 mm NMR tubes for rR experiments. Solutions were 50 mM in CHES and adjusted to pH 9.0. Fe^{III} CBS solutions (of the 63- or 45-kDa enzymes) were reacted with excess dithionite ion at room temperature under N_2. Reduced samples were then heated to 60 °C in a water bath under inert atmosphere until conversion to Fe^{II} CBS424 was complete (~10 min), as monitored by absorption spectroscopy. Samples were stored on ice until Raman experiments were conducted. rR spectra were obtained at room temperature using 135° backscattering geometry and f_1 collection as described previously (*37*). The 413.1 nm emission from a Kr^+ laser was used for Raman excitation. The laser power was 15 mW at the sample, and a cylindrical lens was used to focus the beam on the sample. The sample was spun at 20 Hz to minimize laser-induced damage to the protein. Absorption spectra were recorded 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.

RESULTS

The Heme of Fe^{II} CBS Undergoes a Thermally Induced Change in Axial Ligation To Form Fe^{II} CBS424. A transition

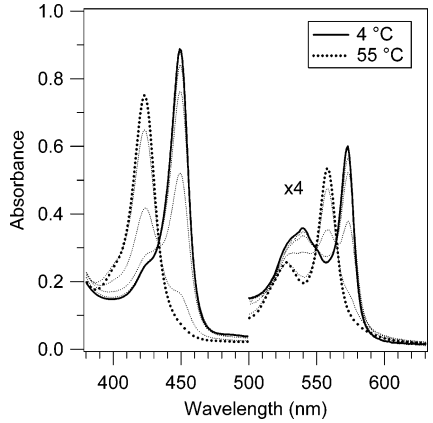

FIGURE 1: Spectral change upon heat treatment of 45-kDa Fe^{II} CBS (7.3 μM in heme, 2 mM sodium dithionite, 25 mM H_3BO_3, and 100 mM NaCl at pH 9.0) as monitored by electronic absorption spectroscopy. Spectra were obtained at 4, 10, 25, 37, 45, 50, and 55 °C with a 10 min equilibration time at each temperature. Spectra at 4 and 55 °C are shown as bold solid (—) and dotted (···) lines, respectively, while intermediate temperatures are shown as thin dotted lines.

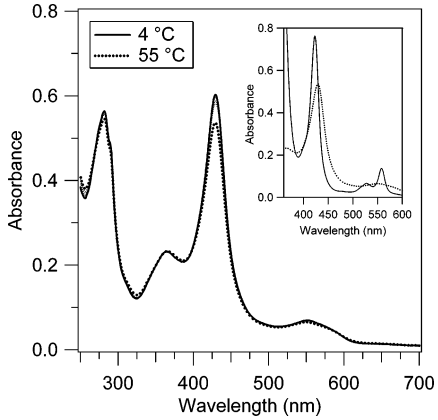

FIGURE 2: Minimal spectral changes upon heat treatment of 45-kDa Fe^{III} CBS (7.4 μM in heme, 25 mM H_3BO_3, and 100 mM NaCl) as monitored by electronic absorption spectroscopy. Spectra were obtained at 4, 10, 25, 37, 45, 50, and 55 °C with a 10 min equilibration time at each temperature. Spectra at 4 and 55 °C are shown as bold solid (—) and dotted (···) lines, respectively, while intermediate temperatures are shown as thin dotted lines. The inset shows 45-kDa Fe^{III} CBS at 55 °C (···) and the resulting spectrum after subsequent addition of sodium dithionite to 2 mM (—).

to a new, spectroscopically distinct species occurs when Fe^{II} CBS is heated at pH 9.0 (Figure 1). At high pH and 4 °C, a solution of Fe^{III} CBS and 2 mM sodium dithionite exists as native Fe^{II} CBS, characterized by electronic absorbance maxima at 449, 540, and 573 nm (*30*). Heating of a 4 °C solution of Fe^{II} CBS at pH 9.0 to 55 °C for ~10 min results in a blue shift of the Soret maximum from 449 to 424 nm. Corresponding shifts of the α- and β-band maxima to 560 and 530 nm, respectively, also occur with heating. In the style of naming heme proteins according to their respective Soret maxima (e.g., cytochrome P450), this new species was termed "Fe^{II} CBS424." Fe^{II} CBS424 can also be obtained by addition of sodium dithionite to a 55 °C solution of Fe^{III} CBS at pH 9.0 (inset of Figure 2). Either reduction of Fe^{III} CBS at or heating Fe^{II} CBS to intermediate temperatures results in absorption spectra consistent with mixtures of Fe^{II} CBS and Fe^{II} CBS424 (Figure 1). The absorption spectrum of Fe^{II} CBS424 suggests that the cysteine(thiolate) is replaced by a neutral donor during conversion to Fe^{II} CBS424. Low-spin heme centers possessing a cysteine(thiolate) ligand in the Fe^{II} state typically exhibit Soret maxima in the range of 440−450 nm (*38*). The 424 nm Soret maximum observed for Fe^{II} CBS424 is more consistent with six-coordinate heme centers possessing two neutral donor ligands (Table S1 in the Supporting Information and references therein). The absorption spectrum of Fe^{III} CBS, characterized by a Soret maximum at 428 nm and a broad α/β absorption at 550 nm, undergoes only a slight reversible decrease in the Soret band intensity when heated to 55 °C for 10 min (Figure 2).

Once Fe^{II} CBS424 is obtained, regeneration of the native CBS heme coordination does not occur. When the temperature is lowered from 55 to 4 °C after complete conversion to Fe^{II} CBS424, this species persists and reversion to Fe^{II} CBS is not observed. The intensity of the 424 nm Soret band ($\epsilon_{424} \approx 115\ cm^{-1}\ mM^{-1}$ at 4 °C) increases as the temperature decreases, suggesting that the Fe^{II} CBS424 heme pocket is stabilized as the temperature is lowered. Upon exposure to oxidants such as air or potassium ferricyanide, Fe^{II} CBS424 converts to yet another new species characterized by a Soret maximum at 412 nm and a broad α/β absorption centered at approximately 530 nm (Figure S1 in the Supporting Information). This species is presumed to be Fe^{III} CBS424. However, the absorption spectrum of this species is ill-defined and likely due to a mixture of species; further spectroscopic investigation was not practical. Addition of dithionite ion to these species regenerates an absorption spectrum very similar to that of Fe^{II} CBS424.

Conversion to Fe^{II} CBS424 is also accompanied by the elimination of the previously reported pH-dependent redox behavior of wild-type CBS (*30*). When Fe^{II} CBS424 at pH 9 is aerobically transferred to pH 6 buffer via a desalting column, the sample oxidizes and exhibits a broad Soret maximum at 412 nm. The addition of 2 mM dithionite ion at 4 °C to this oxidized sample reproduces the Fe^{II} CBS424 spectrum without any apparent reoxidation. This is in contrast to Fe^{III} CBS, which when reduced at 4 °C and pH 6, transiently forms Fe^{II} CBS and then reoxidizes. In further contrast to normal CBS behavior, when concentrated pH 6 buffer is added to Fe^{II} CBS424 at pH 9, the resulting low-pH Fe^{II} CBS424 does not reoxidize. Elimination of the pH-dependent redox behavior is consistent with our proposal of a new coordination environment for the heme in Fe^{II} CBS424.

It is likely that the CBS catalytic core (residues 71−400) alone influences the coordination of Fe^{II} CBS424. Spectral signatures consistent with Fe^{II} CBS424 are observed when solutions of either the full-length (63-kDa), C-terminal truncated (45-kDa), or doubly truncated [(41−413)CBS] forms of CBS are reduced with sodium dithionite at 4 °C and heated to 55 °C for 10 min. As illustrated in Figure 3, the range of A_{449}/A_{424} ratios is nearly identical for both the 63- and 45-kDa forms of the enzyme over the 4−70 °C temperature range. The first derivatives of these temperature curves yield approximate transition temperatures for the Fe^{II} CBS to Fe^{II} CBS424 conversion. The apparent transition temperatures for the truncated and full-length forms of the enzyme are 43 and 44 °C, respectively. The truncate (41−413)CBS behaves only slightly differently, displaying an initial A_{449}/A_{424} ratio of 2.5 and a transition temperature of 40.5 °C (*39*).

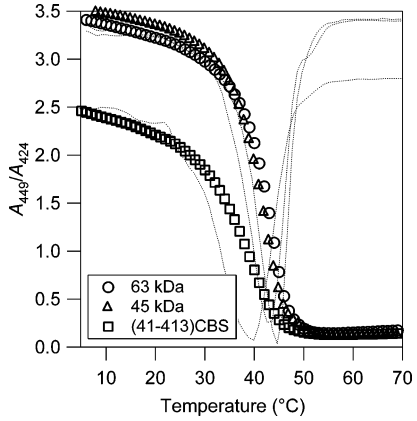

FIGURE 3: Monitoring the absorbance ratio (A_{449}/A_{424}) as a function of temperature for three forms of Fe^{II} CBS: 63-kDa (○), 45-kDa (△), and (41−413) (□). Samples, 3.1, 5.4, and 4.3 μM in heme, respectively, were prepared in 25 mM H_3BO_3, 100 mM NaCl, and 2 mM sodium dithionite at pH 9.0. Samples were heated from 4 to 70 °C at 0.1 °C/min under Ar directly in a quartz cuvette. The first derivatives of the A_{449}/A_{424} versus temperature curves are shown as dotted lines (···); minima of these first-derivative curves are taken to be the transition temperature of the corresponding A_{449}/A_{424} versus temperature curve.

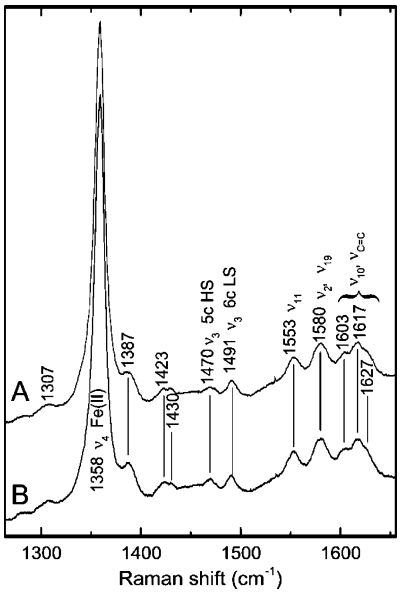

FIGURE 4: rR spectra of 45-kDa (A) and 63-kDa (B) Fe^{II} CBS424. Samples, ~20 μM in heme, were prepared in 78 mM CHES and 6 mM sodium dithionite at pH 9.0 and excited at 413.1 nm. Assignments are based on those for Fe^{II} cytochrome *c* (*39*).

rR and MCD Spectroscopies Suggest that the Heme of Fe^{II} CBS424 Is Coordinated by Two Neutral Donors. A rR spectrum of Fe^{II} CBS424 reveals a mixture of high-spin, five-coordinate and low-spin, six-coordinate ferrous heme in samples prepared from both the 63- and 45-kDa CBS forms (Figure 4). These high-frequency spectra are nearly identical for the 63- and 45-kDa enzyme forms, consistent with the aforementioned absorbance data. When heme protein samples are excited at wavelengths near the Soret transition, the resulting rR spectrum is dominated by bands corresponding to the totally symmetric stretching modes of the heme (*40*). A few of these bands are sensitive to the oxidation and spin state of the heme iron and thus serve as "markers" for these characteristics. Samples, initially verified as Fe^{II} CBS424 by absorption spectroscopy, exhibit ν_4 bands at 1358 cm^{-1}, indicative of ferrous heme. A split ν_3 envelope is observed, consistent with a mixture of high-spin Fe^{II} heme (ν_3 = 1470 cm^{-1}) and low-spin Fe^{II} heme (ν_3 = 1491 cm^{-1}). These frequencies are similar to those of ν_3 bands observed for Fe^{II} sGC (*41*, *42*) and Fe^{II} KatG (*43*), both of which can exist as mixtures of five-coordinate, high-spin and six-coordinate, low-spin heme.

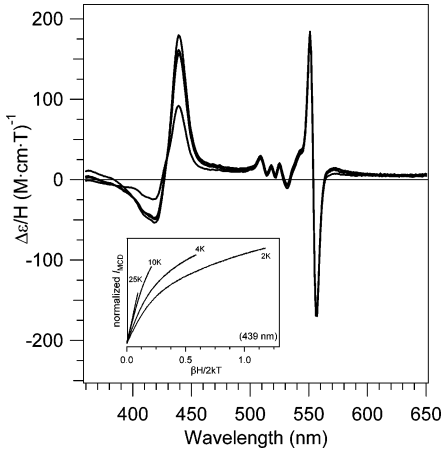

FIGURE 5: Variable-temperature MCD spectra of 45-kDa Fe^{II} CBS424. Samples, 26 μM in heme, contained 25 mM CHES (pH 9.0), 50 mM NaCl, and 2 mM sodium dithionite and were 50% (v/v) ethylene glycol. Spectra were recorded in a magnetic field of 7 T at temperatures of 2.0, 4.0, 10.0, and 25.0 K. Intensity of the MCD bands in the Soret region of the spectrum increased with a decreasing temperature. The inset shows the magnetic saturation behavior of the Soret region *C* term at 439 nm. All VT/VH MCD data were normalized to the highest MCD intensity recorded (2 K and 7 T).

MCD data are consistent with replacement of the cysteine-(thiolate) ligand of Fe^{II} CBS by a neutral donor during conversion to Fe^{II} CBS424. Similar to room-temperature rR results, a mixture of two species is also observed in the low-temperature MCD spectrum of Fe^{II} CBS424 (Figure 5). The spectrum is dominated by an intense, temperature-independent *A* term in the α/β region (crossover at 554 nm) and less intense, temperature-dependent *C* terms in the Soret region (crossover at ~426 nm and maxima at 420 and 439 nm). The *A* term centered at 554 nm arises from the only diamagnetic heme state, low-spin Fe^{II} ($S = 0$). The crossover position of this α band in the low-spin ferrous heme MCD spectrum is diagnostic for the type of ligands bound to heme (*38*, *44*, *45*). Thiolate-ligated Fe^{II} hemes, such as Fe^{II} CBS, typically exhibit an *A* term in the visible region centered at 562−576 nm. The 554 nm α band observed here is more typical of ligation by two neutral donors, such as in Fe^{II} cytochrome b_5 (bis-histidine) or Fe^{II} CooA (His/Pro) (Table S2 in the Supporting Information and references therein). The position of this α band is consistent with our hypothesis that the cysteine(thiolate) is replaced by a neutral donor upon conversion to Fe^{II} CBS424. Temperature dependence of the MCD *C* term originates from the Boltzmann population distribution in the Zeeman-split ground state of a paramagnetic center (*46*). The fact that no Fe^{III} heme was observed in the rR spectra suggests that the *C* terms in the MCD spectrum of Fe^{II} CBS424 arise from high-spin Fe^{II} heme (S = 2). The magnetic saturation behavior of the *C*-term intensity at 439 nm (inset of Figure 4) is consistent with the $S = 2$ assignment, because the observed nesting behavior (nonsuperimposing saturation curves) is a hallmark of

Table 1: Effects of Temperature and Reduction on CBS Activity

treatment pre-assay[a]	during assay[b]	63 kDa (units/mg)	45 kDa (units/mg)
anaerobic		108 ± 12	686 ± 52
	+dithionite	67 ± 6	478 ± 32
	+AdoMet	300 ± 29	
	+dithionite and AdoMet	184 ± 41	
anaerobic and heated to 55 °C for 20 min (cooled before the assay)[c]		183 ± 14	517 ± 61
	+dithionite[d]	136 ± 6	362 ± 27
	+AdoMet	191 ± 11	
	+dithionite and AdoMet	137 ± 6	
anaerobic, reduced, and heated to 55 °C for 20 min (cooled before the assay)[e]		ND[f]	ND

[a] Anaerobic enzyme manipulations prior to the assay were either performed in a glovebox under N_2 or in a sealed quartz cuvette under Ar. [b] All assays were performed anaerobically in a glovebox under N_2 at 37 °C. [c] Heat treatment of Fe^{III} CBS results in a slight decrease in heme Soret intensity (Figure 2). [d] Addition of dithionite to a heat-treated Fe^{III} CBS sample at 37 °C produces ~30−50% Fe^{II} CBS424 (data not shown). [e] These conditions induce the formation of Fe^{II} CBS424. Once Fe^{II} CBS424 was obtained at 55 °C and verified by absorption spectroscopy, the sample was cooled to 37 °C and assayed for activity. [f] ND = not detected.

systems possessing axial zero-field splitting, i.e., $S > {}^1/_2$ (*47*). The oppositely signed pattern of the Soret *C* terms is similar to that of deoxymyoglobin (*48*) and Fe^{II} H93G myoglobin in the presence of CPSH (*49*), suggesting that these signals arise from five-coordinate, high-spin Fe^{II} heme bound by either an imidazole or thiol, respectively. The intensity of the Soret *C* terms indicate that the high-spin, five-coordinate state is not a major component of the Fe^{II} CBS424 sample. Low-temperature MCD spectra of fully high-spin, five-coordinate heme samples, such as hemoglobin and myoglobin (*50*), as well as photolysis products of CO-bound CooA (*51*), are dominated by Soret region *C* terms approximately 10 times more intense than those observed for Fe^{II} CBS424. This is consistent with absorption spectra of Fe^{II} CBS424, in which no high-spin heme could be detected.

CBS Is Inactivated by Fe^{II} CBS424 Formation and Not Heat. Fe^{II} CBS424 is incapable of synthesizing measurable amounts of cystathionine. A summary of the effects of temperature and reduction on CBS activity is presented in Table 1. Both the 63- and the 45-kDa forms of untreated CBS behaved as previously described; activity of the 63-kDa form was stimulated by AdoMet 2−3-fold, while the 45-kDa form was more active than the 63-kDa form but unresponsive to AdoMet (*9*). In agreement with Taoka et al. (*28*), reduction of the 63-kDa form of Fe^{III} CBS at 37 °C resulted in an observed decrease in specific activity. Contrary to previous reports (*26*), reduction of the 45-kDa form of Fe^{III} CBS at 37 °C resulted in a loss in specific activity similar to that observed for the 63-kDa form. In striking contrast, CBS treated with 10 mM dithionite ion and incubated at 55 °C for 20 min to induce formation of Fe^{II} CBS424 exhibited no measurable activity when assayed at 37 °C. Subsequent oxidation of the Fe^{II} CBS424 species via aerobic desalting of dithionite also failed to restore activity.

The order in which reduction and heat treatment are successively applied to Fe^{III} CBS has a profound impact on enzyme activity. As mentioned above, heat treatment of either 63- or 45-kDa Fe^{III} CBS samples containing 10 mM dithionite promoted the formation of Fe^{II} CBS424 and completely abolished activity. Intriguingly, heat treatment of Fe^{III} CBS by a 20 min incubation at 55 °C followed by a separate reduction at 37 °C, conditions which do not promote the full formation of Fe^{II} CBS424, did not completely abolish activity. In fact, both this experiment and one in which dithionite is added to Fe^{III} CBS at 37 °C without prior heat treatment give rise to similar decreases in specific activity. Moreover, incubation of Fe^{III} CBS at 55 °C for 20 min prior to the assay activated the 63-kDa enzyme by ~40% and eliminated its responsiveness to AdoMet, consistent with previous work that attributed this temperature-dependent activation and abolishment of AdoMet response to a thermal disruption of the C-terminal auto-inhibitory, AdoMet-binding domain (*8*). In contrast, heat treatment of the more active 45-kDa Fe^{III} CBS form, in which the auto-inhibitory domain was deleted, showed a decrease in specific activity by ~25%, presumably because of partial enzyme denaturation. When these data are taken together, they show that neither heat treatment nor reduction of Fe^{III} CBS can independently account for the complete loss of activity observed upon conversion to Fe^{II} CBS424. Thus, only when heat and reduction are combined to form Fe^{II} CBS424 is the enzyme fully inactivated.

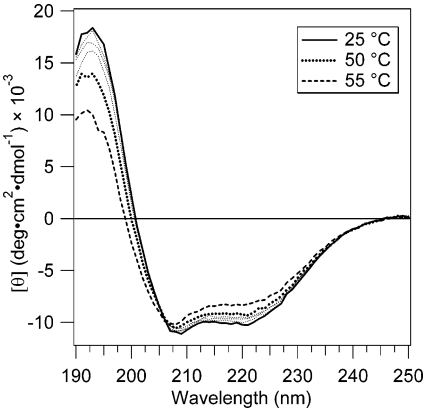

FIGURE 6: CD spectra of 45-kDa Fe^{III} CBS. Samples, 0.1 mg/mL, were prepared in 0.1-cm quartz cells and contained 25 mM H_3BO_3 at pH 9.0. Spectra were recorded at temperatures of 25−55 °C at 5 °C intervals.

CD Spectroscopy Demonstrates that the Thermal Process, which Induces Formation of CBS424, Does Not Appreciably Alter the Native Secondary Structure. Little change is observed in the CD spectrum of Fe^{III} CBS at pH 9.0 over temperatures required to induce the formation of CBS424 (Figure 6). The CD spectrum of Fe^{III} CBS at 25 °C and pH 9.0 is similar to that previously reported (*52*). The negative CD signal intensity at 222 nm ($[\theta]_{222}$), which is correlated with the helix content, decreases only slightly as the temperature is raised from 25 to 50 °C. Thus, the secondary structure of Fe^{III} CBS at the apparent CBS424 transition temperature (~43.5 °C, *vide supra*) is similar to that at room temperature. However, the decrease in $[\theta]_{222}$ as the temperature is raised from 50 to 55 °C is similar to that induced by the 25−50 °C change. This suggests a more significant loss of helix content in Fe^{III} CBS at temperatures above 50 °C, consistent with the previously reported melting temperature of 55 °C for Fe^{III} CBS (*8*). The CD data suggest that the

Fe^{III} CBS sample loses some but not a substantial fraction of its secondary structure when heated to 55 °C for 5 min. This observation is consistent with the aforementioned functional data in which Fe^{III} CBS was heated to 55 °C for 20 min, cooled to 37 °C, and reduced, resulting in only a partial loss of enzyme activity. These CD data also provide further evidence that heat treatment alone does not account for the complete lack of activity observed for Fe^{II} CBS424.

DISCUSSION

This investigation has demonstrated that Fe^{II} CBS undergoes a thermally induced transition to an inactive species that we have termed Fe^{II} CBS424. In contrast, the heme coordination of Fe^{III} CBS is relatively unaffected by temperature over the range that converts Fe^{II} CBS to Fe^{II} CBS424, although Fe^{II} CBS424 can also be produced by reducing Fe^{III} CBS at elevated temperatures. Spectroscopic characterization of Fe^{II} CBS424 suggests that the heme is coordinated by two neutral donors, consistent with the native cysteine ligand being replaced by another endogenous residue. Thus, Fe^{III} CBS most likely undergoes a redox-mediated ligand switch when reduced at moderate temperatures. Formation of Fe^{II} CBS424 also results in a complete loss of CBS activity. We believe that previous spectroscopic investigations of Fe^{II} CBS, which were performed at room temperature and below, may have overlooked Fe^{II} CBS424 because of the elevated temperatures necessary to induce its formation.

Species Exhibiting Spectral Characteristics Similar to Fe^{II} CBS424 Have Been Documented in Previous Studies of CBS and H450. Inquiries into the effects of Cys^{52} perturbation either by site-directed mutagenesis or chelation by mercuric chloride have resulted in the formation of species comparable to Fe^{II} CBS424. Mutation of Cys^{52} to an alanine or serine residue results in variant enzymes, which do not bind a full complement of either PLP or heme (*32*). Although Fe^{II} C52A/S variants display absorption features consistent with low-spin, six-coordinate heme coordinated by two neutral donors, spectral ambiguity prevents unequivocal assignment of these ferrous variants as Fe^{II} CBS424. Addition of $HgCl_2$, a known thiol chelator, to 45-kDa Fe^{II} CBS (or, conversely, dithionite reduction of $HgCl_2$-treated 45-kDa Fe^{III} CBS) results in spectral behavior similar to that observed for the Cys^{52} variants (*26*, *31*). Although slight variations in absorbance maxima are observed when $HgCl_2$-treated Fe^{II} CBS is compared to both the Cys^{52} variants and Fe^{II} CBS424, the rR spectrum of dithionite-reduced $HgCl_2$-treated 45-kDa Fe^{III} CBS is nearly identical to that reported here for Fe^{II} CBS424 (*31*). Thus, the previously reported Fe^{II} Cys^{52} variants and $HgCl_2$-treated Fe^{II} CBS samples are likely to contain hemes in environments similar to that observed for Fe^{II} CBS424. The mutation of a key residue or introduction of a bulky $HgCl_2$ molecule, which are most likely deleterious to the integrity of the heme-binding pocket, reasonably explains the subtle differences in absorption features observed between these species.

The previously reported low-pH species of H450 may actually be Fe^{II} CBS424. The heme protein H450 was first isolated in 1976; however, it was not until 1994 that researchers realized that H450 was CBS (*6*). Initial spectroscopic characterization of dithionite-treated H450 by Omura and co-workers revealed two fully interconvertible pH-dependent forms, termed alkaline and acid (*19*, *20*). Work by Pazicni et al. (*30*) demonstrated that two pH-dependent forms were also characteristic of the 45- and 63-kDa forms of CBS, although the absorption features reported for dithionite-treated H450 at pH 6 were noticeably different from those of dithionite-treated CBS at pH 6. Surprisingly, the pH 6 form of dithionite-treated CBS was identified as Fe^{III} CBS (*30*), while the absorption spectrum of dithionite-treated H450 at pH 6, which exhibited maxima at 425, 530, and 558 nm, was more characteristic of low-spin Fe^{II} heme (*19*, *20*). This study demonstrates that the absorption spectrum of Fe^{II} CBS424 is nearly identical to that reported for the acid form of dithionite-treated H450 and suggests that these are closely related species. The room-temperature MCD spectrum of dithionite-treated H450 at pH 6 is consistent with our conclusion that it may be Fe^{II} CBS424 (*21*, *53*); the reported α-band crossover position for this sample (559 nm) is outside the range typical of thiolate ligation (Table S2 in the Supporting Information) but is close to that obtained for Fe^{II} CBS424 in this study. Thus, in the case of H450, low-pH conditions were sufficient to stimulate the same phenomenon that we were only able to observe in CBS with an increasing temperature at high pH. Intrinsic differences between rat and human CBS could account for this discrepancy; sequence alignment reveals subtle differences between the heme-binding regions of these two enzymes. Intriguingly, absorption spectra identical to the acid form of Fe^{II} H450 were obtained by incubating Fe^{II} H450 at pH 8.0 in 3% *n*-butanol or 2.5% urea, mild protein denaturants, suggesting that a loosened protein backbone allows for the conversion of Fe^{II} H450 to the "acid form" at higher pH values. This behavior is clearly reminiscent of the heat-induced conversion of Fe^{II} CBS to Fe^{II} CBS424. Although we favor the idea that the cysteine ligand is replaced with a neutral donor in CBS424 (*vide infra*), another explanation is plausible for the behavior of H450. The reversibility between the acid and alkaline forms of H450 may arise from a protonation−deprotonation equilibrium, implicating thiol ligation at low pH.

CBS Undergoes a Ligand Switch during Conversion to CBS424. Three equally plausible scenarios could give rise to the blue-shifted Soret band characteristic of Fe^{II} CBS424. These include (1) an elongation of the Fe−S(cysteine) bond, (2) protonation of the native Cys^{52} ligand or the formation of a new hydrogen bond to an adjacent amino acid resulting in a coordinated cysteine(thiol), or (3) a ligand switch resulting in the loss of the native Cys^{52} ligand and coordination by two neutral donors. Intriguingly, the spectral features of thiolate-coordinated heme proteins calculated in theoretical studies were found to be dependent upon the Fe−S(thiolate) bond distance (*54*, *55*). A decrease in the heme iron π back-donation to the porphyrin resulting from an increase in the distance between the iron and the negatively charged thiolate will theoretically produce a blue shift in the Soret band. Although little experimental evidence exists to support these theoretical studies, Fe−S(thiolate) bond elongation is not inconceivable, because the events described in scenario 2 would be expected to weaken the Fe−S interaction. Moreover, distinguishing between coordination by thiols and other neutral donors is difficult (*49*), and all three scenarios could implicate a mixture of five- and six-coordinate species

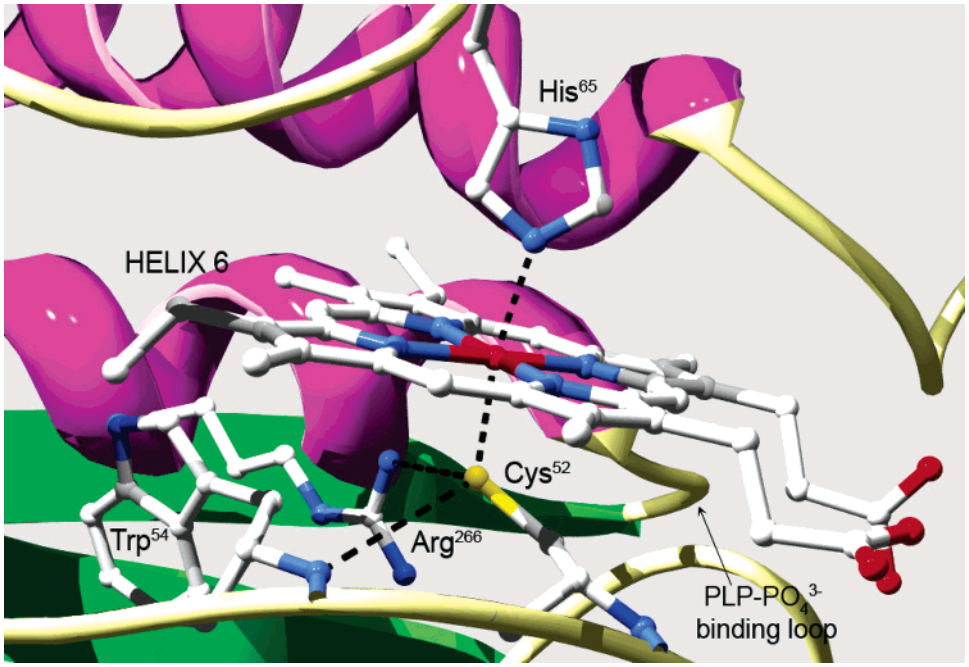

FIGURE 7: View of the immediate heme environment highlighting interactions of the cysteine(thiolate) heme ligand with surrounding residues. The sulfur atom of Cys^{52} forms an ionic interaction with the guanidinium group of Arg^{266} as well as a hydrogen bond with the backbone amide hydrogen of Trp^{54}. This image was generated from PDB file 1JBQ using DeepView/Swiss-PdbViewer 3.7 and rendered in POV-ray 3.6.

because of the loss of the exchanged, protonated, or stretched Fe−S(thiolate) bond. Thus, none of the above scenarios can be ruled out purely on the basis of spectroscopic data.

Two additional lines of evidence add strength to the hypothesis that Cys^{52} is replaced by an endogenous ligand during conversion of CBS to CBS424. The observation that formation of Fe^{II} CBS424 is irreversible regardless of pH, temperature, or redox conditions is most consistent with a ligand-switch scenario. Merely protonating or lengthening the Fe−S(thiolate) bond should be a reversible process. Also, the fact that Fe^{II} CBS424 or a closely related species can be obtained in the absence of Cys^{52} also supports the ligand-switch hypothesis (*26*, *31*, *32*). An adventitious ligand within or near the heme-binding pocket appears to be poised to coordinate the heme iron upon perturbation of Cys^{52} by mutation or chemical modification because the heme remains low-spin and six-coordinate. Similarly, at least one other ligand is able to coordinate to the CBS heme upon conversion to Fe^{II} CBS424. Studies of the truncated CBS forms demonstrate that this putative ligand most likely exists in the catalytic core of the enzyme and not in the C-terminal autoinhibitory domain or within the 41 N-terminal residues. This consideration does not rule out coordination by the N-terminal amino group, as is the case in CooA or cytochrome *f* (*56*, *57*). How heat treatment is able to disrupt cysteine(thiolate) coordination and allow binding of this adventitious ligand is discussed below.

Heat Induces Flexibility within the Peptide Backbone of CBS and Permits the Displacement of Cys^{52} by an Endogenous Adventitious Ligand upon Reduction. Under certain conditions, the CBS heme appears to behave differently than other low-spin, six-coordinate, thiolate-bound heme proteins in response to reduction. It has been documented that heme proteins bearing Cys(thiolate)/(N) coordination, where "N" is any neutral nitrogen donor, when reacted with reducing agents, are either not reduced or undergo a redox-mediated ligand switch to escape the Fe^{II} Cys(thiolate)/(N) state (*30*). Examples include CooA (*57*, *58*) and a variant cytochrome *c* (cyt *c* M80C) (*59*). An example to the contrary is CBS at high pH and low temperature. Thorough spectroscopic characterization of dithionite-treated CBS at pH 9 demonstrates that His/Cys(thiolate) coordination is retained in the Fe^{II} state (*30*). Therefore, the CBS heme is unique among other known Fe^{II} heme centers exhibiting Cys(thiolate)/(N) coordination in that this particular coordination is somehow stabilized in CBS at high pH. The negative charge on the thiolate ligand and the strong electron donation from the histidine imidazole together are expected to stabilize the Fe^{III} state of the heme; therefore, reduction of Fe^{III} heme centers bearing Cys(thiolate)/His coordination may be intrinsically unfavorable. In fact, cyt *c* M80C, which bears Cys(thiolate)/His coordination, exhibits a very low reduction potential of −390 mV relative to SHE (*60*). However, Fe^{III} CBS is reduced at high pH and low temperature. We have demonstrated here that increased temperature induces in Fe^{II} CBS behavior more typical of other Cys(thiolate)/(N) Fe^{II} heme centers. A plausible hypothesis is that heat promotes a slight conformational rearrangement of the CBS polypeptide that breaks putative interactions critical for the stabilization of Fe^{II} Cys(thiolate)/His CBS heme coordination. Loss of these important interactions may permit the CBS heme to employ a ligand switch to escape Fe^{II} Cys(thiolate)/(N) coordination. The identity of these putative interactions and how they may be related to CBS activity are discussed below.

Disruption of Interactions of Cys^{52} with Heme and with Surrounding Residues Appears To Be Deleterious to CBS Activity. These studies have demonstrated that conversion of Fe^{II} CBS to Fe^{II} CBS424 is accompanied by a complete loss of CBS activity. This observation is related closely to previous observations that either binding of CO or treatment with $HgCl_2$ inhibits CBS activity. Taoka et al. demonstrated that binding exogenous CO to the Fe^{II} CBS heme disrupts

native Cys^{52} coordination and fully inhibits enzyme activity at [CO] $\geq$ 60 μM (*27*). A similar result was reported for treatment with $HgCl_2$ (*26*), which coordinates to and displaces Cys^{52}, likely resulting in formation of Fe^{II} CBS424 (*vide supra*). Consideration of our Fe^{II} CBS424 studies and the prior $HgCl_2$ studies together implies that, even though CBS heme retains coordination by two endogenous ligands in Fe^{II} CBS424, enzyme activity is nonetheless compromised. These observations suggest that native Cys^{52} coordination to the heme iron is necessary for full CBS activity. Oddly, while CBS424 is completely inactive, the C52S variant as well as N-terminal deletion variants lacking heme retain a small amount of catalytic competency (*7*, *22*). Thus, other factors besides or in addition to heme coordination by Cys^{52} are essential for catalytic activity in this enzyme.

We favor a hypothesis that implicates disruption or reorganization of second-sphere interactions of the thiolate heme ligand with surrounding residues in the inactivation of CBS by CO binding, Cys^{52} chelation, or conversion to CBS424. As illustrated in Figure 7, the Cys^{52} thiolate likely participates in noncovalent interactions with the guanidinium group of Arg^{266}, located on helix 6, and the backbone amide group of Trp^{54}. Sequence alignments reveal that both Arg^{266} and Trp^{54} are conserved in organisms that possess N-terminal extensions in the CBS protein indicative of heme binding. According to available X-ray structures, secondary interactions with Arg^{266} and Trp^{54} anchor the heme to the catalytic core of the enzyme and are crucial for the integrity of the heme-binding loop, respectively. One or both of these interactions could possibly be stabilizing the negative charge of the cysteine(thiolate) (*vide supra*). Arg^{266} is also likely responsible for the observed communication between the heme and the active-site PLP cofactor, whose phosphate moiety is bound by a loop at the N-terminal end of helix 6. The C52S variant retains a small amount of activity, which may indicate that these putative crucial interactions remain somewhat intact. The observed activity of heme-free CBS variants as compared to inactive CBS424, which retains heme, is plausibly the result of nonspecific folding interactions within the variant enzymes, which give rise to a stable, catalytically competent polypeptide conformation. CO binding, $HgCl_2$ treatment, and conversion of CBS424 inactivate Fe^{II} CBS because specific interactions that serve to connect the heme to the enzyme active site have been interrupted.

Conclusions. This study reveals the surprising ability of the CBS heme to undergo a change in coordination upon reduction at elevated temperatures to the form that we have termed "CBS424." We believe that this process is most likely a redox-mediated ligand switch. Spectroscopic interrogation of Fe^{II} CBS424 suggests that the Cys(thiolate)/His coordination of Fe^{II} CBS is replaced by two neutral donors in Fe^{II} CBS424. The identity of these ligands remains unknown. We have also shown that conversion to Fe^{II} CBS424 is deleterious to CBS function. Intriguingly, neither temperature nor reduction alone inactivates CBS; the heme ligand switch leading to the formation of CBS424 must occur to inactivate the enzyme. This observation, coupled with previous work on CO-bound and $HgCl_2$-treated Fe^{II} CBS, provides strong evidence that the Cys^{52}(thiolate)−Fe bond is crucial for full enzymatic activity. We hypothesize that Cys^{52} displacement is communicated to the active site via disruption of second-sphere interactions involving the Cys(thiolate). Proof of such an allosteric pathway linking the PLP and heme cofactors would serve as striking evidence for a regulatory role for heme in this enzyme.

The experiments outlined in this study do not probe the possible physiological relevance of this redox-mediated heme ligand switch. Although CBS exhibits maximal enzyme activity at pH 8.6 (*28*, *30*), these studies at pH 9 do not address the ligand-switching behavior of the CBS heme at physiologically relevant conditions. Moreover, the observed transition temperature for Fe^{II} CBS424 formation ($\sim$45 °C) is beyond the temperature range experienced by a human cell, suggesting that the process is physiologically irrelevant. However, when this phenomenon is investigated at pH 7.4 and 37 °C, conversion to Fe^{II} CBS424 is indeed observed, albeit at a slower rate (data not shown). Current work in this laboratory is aimed at more fully elucidating the relevance of this redox-mediated heme ligand switch to physiology and to previous work on this intriguing enzyme.

ACKNOWLEDGMENT

The authors are grateful to Professor Thomas Brunold for the use of his MCD spectrometer and to Dr. Darrell McCaslin for his assistance with CD spectroscopy. Funding from NCRR (P20 RR15566), USDA (ND 05299), and the Herman Frasch Foundation (446-HF97) (to K. R. R.) is gratefully acknowledged. We also thank Andrea Lee, Rachel Linck, and Robert Clark for critical reading of the manuscript and helpful discussions.

SUPPORTING INFORMATION AVAILABLE

Figures showing a spectrum of Fe^{III} CBS424 and re-reduced Fe^{II} CBS424 and tables detailing the absorption features and MCD α-band crossover positions for Fe^{II} *b*-type heme centers. This material is available free of charge via the Internet at http://pubs.acs.org.

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BI051305Z