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Comparative study of enzyme activity and heme reactivity in *Drosophila melanogaster* and *Homo sapiens* cystathionine beta-synthases

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Abstract

Cystathionine β-synthase (CBS) is the first and rate-limiting enzyme in the transsulfuration pathway, which is critical for eukaryotes to synthesize cysteine from methionine. CBS uses a coenzyme pyridoxal-5′-phosphate (PLP) for catalysis and S-adenosylmethionine regulates the activity of human CBS, but not yeast CBS. Human and fruit fly CBS contain heme; however, the role for heme is not clear. This paper reports biochemical and spectroscopic characterization of CBS from fruit fly *Drosophila melanogaster* (*Dm*CBS) and the CO/NO gas binding reactions of *Dm*CBS and human CBS. Like CBS enzymes from lower organisms (e.g. yeast), *Dm*CBS is intrinsically highly active and is not regulated by AdoMet. The *Dm*CBS heme coordination environment, reactivity and the accompanying effects on enzyme activity are similar to those of human CBS. The *Dm*CBS heme bears histidine and cysteine axial ligands, and the enzyme becomes inactive when the cysteine ligand is replaced. The Fe(II)heme in *Dm*CBS is less stable than that in human CBS, undergoing more facile reoxidation and ligand exchange. In both CBS proteins, the overall stability of the protein is correlated with the heme oxidation state. Human and *Dm*CBS Fe(II) hemes react relatively slowly with CO and NO, and the rate of the CO-binding reaction is faster at low pH than at high pH. Together the results suggest that heme incorporation and AdoMet regulation in CBS are not correlated, possibly providing two independent means to regulate the enzyme.

Introduction

Cystathionine β-synthase (CBS, EC 4.2.1.22) catalyzes the condensation of homocysteine and serine to cystathionine (Scheme 1, Reaction 1). This reaction is the first and rate-limiting step in the transsulfuration pathway (1), a metabolic pathway critical for eukaryotes to synthesize cysteine from the essential amino acid methionine. Deficiency in CBS activity

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Supporting Information Available

High resolution FT-IC MS analysis of as-isolated and AdoMet-treated DmCBS (Figure S1). Electronic absorption spectra of Fe(III) and Fe(II)*Dm*CBS at pH 7.4 (Figure S2). Thermal denaturation of Fe(II)hCBS, Fe(III)*Dm*CBS and Fe(II)*Dm*CBS (Figure S3–S5). Mathematical derivation of the kinetic model for CBS CO-binding reaction. CO-binding process of *Dm*CBS at pH 6.0 monitored by electronic absorption spectroscopy (Figure S6). Apparent rate constants for CO-binding reactions of hCBS, hCBS45 and *Dm*CBS (Table S1). Supporting materials may be accessed free of charge online at http://pubs.acs.org.

leads to a pathological homocystinuria (1–4). In addition to the canonical cystathionine synthesis reaction, CBS also catalyzes several other β-replacement reactions, including two cysteine synthesis reactions (Scheme 1, Reaction 2 and 3). Reaction 3 is responsible for cysteine biosynthesis in bacteria and plants, which is catalyzed by a related enzyme cysteine synthase (CS, EC 2.5.1.47, other commonly used names: *O*-acetylserine sulfhydrylase, *O*-acetylserine(thio)lyase).

Human CBS (hCBS) is a homotetramer and each monomer (63kDa, 551 amino acids) has a modular structure comprising an N-terminal heme binding domain (residues 1–70), a catalytic core region containing the PLP coenzyme (residues 71–413) and a C-terminal auto-inhibitory domain (residues 414–551). The C-terminal domain of CBS binds the allosteric regulator *S*-adenosylmethionine (AdoMet). AdoMet binding increases the enzyme activity approximately 4-fold (5–7). A truncated dimeric variant of hCBS (hCBS45, residues 1–413, 45kDa) lacking the C-terminal region has been crystallized (8, 9). In the crystal structure, the PLP coenzyme binds to Lys119 in the core region; the heme is coordinated to Cys52 and His65 in the N-terminal domain. The catalytic core is highly conserved among the CBS enzymes and related CS enzymes, and displays significant structural conservation with several other members of the fold-II family of PLP-dependent enzymes (10, 11).

CBS is the only known enzyme to have both PLP and heme cofactors. The catalytic chemistry of CBS can be solely explained by a typical PLP-dependent mechanism (12). In fact, CBS proteins from lower organisms such as *S. cerevisiae* and *T. cruzi* do not have heme and are still highly active (13–15). While the heme does not have a catalytic role, there is clear evidence that changes in the heme environment in hCBS affect enzyme activity. For example, even though heme is not responsible for the catalytic chemistry, hCBS requires heme to achieve maximum activity (16). Furthermore, in all cases studied, disruption of the bond between the heme and its cysteine-thiolate ligand results in loss of enzyme activity (17–22). One plausible hypothesis is that heme and AdoMet regulation act in concert to regulate PLP affinity and enzyme activity. For example, yeast CBS (yCBS) does not bind heme and is not regulated by AdoMet (13–15), whereas hCBS has heme and responds to AdoMet stimulation. Yeast CBS has a significantly lower *in vitro* affinity for PLP than hCBS, and an hCBS variant with the heme binding N-terminal domain deleted exhibits a similarly low affinity for PLP (14, 16). Despite these correlations, the exact function of heme in CBS remains unknown.

It is of interest to know whether there is a connection between heme binding, enzyme activity and AdoMet regulation. One way to investigate this question is to take an evolutionary approach, looking at CBS enzymes from organisms that fall between *Homo sapiens* and *Saccharomyces cerevisiae* on the evolutionary tree of life. *Drosophila melanogaster* is such an organism; therefore it is valuable to ask whether or not its CBS contains heme and responds to AdoMet. *Drosophila melanogaster* CBS (*Dm*CBS) is similar to yet different from hCBS. Unlike hCBS, which is a tetramer, *Dm*CBS is a dimer shown by X-ray crystallography (23) and native gel electrophoresis. The catalytic core region (residues 40–380) of *Dm*CBS exhibits high sequence similarity (85% homology, 62% sequence identity) to hCBS; the C-terminal domain (residues 381–522) of *Dm*CBS is almost the same size as that of hCBS but is less conserved (59% homology, 24% sequence identity); the N-terminal domain (residues 1–39) of *Dm*CBS is significantly shorter, but does contain residues (Cys22 and His34, Figure 1) analogous to those that serve as heme ligands in hCBS. In the recently reported crystal structures of *Dm*CBS, heme indeed is coordinated to these residues. The 3-D structures of the heme-binding domain and the catalytic core of *Dm*CBS are very similar to those of hCBS45.

Here we report the biochemical and spectroscopic characterization of *Dm*CBS and study of the CO and NO gas binding reactions of hCBS, hCBS45 and *Dm*CBS. We demonstrate that *Dm*CBS is active both in the canonical cystathionine synthesis reaction and two alternative cysteine synthesis reactions, but does not respond to AdoMet stimulation. The *Dm*CBS heme exhibits characteristics similar to those of hCBS. Fe(II)*Dm*CBS undergoes pH-mediated redox and thermal ligand switching, but the reduced heme of *Dm*CBS is less stable than that of hCBS. CO binds slowly to Fe(II)*Dm*CBS and to Fe(II)hCBS, with rates that vary with pH. In the CO adducts, the heme-cysteine thiolate bond is disrupted and the enzyme activity is lost. Based on these findings, we conclude that incorporation of heme into CBS and AdoMet regulation of CBS are not correlated, and are characteristics that emerged separately in the course of evolution.

Materials and methods

Materials

Unless otherwise stated, all chemicals were purchased from Sigma-Aldrich or Fluka and were used as received. Nitric oxide (NO) gas was generated *in situ* by reaction of $NaNO_2$, $CuCl_2$ and L-ascorbic acid. Ninhydrin reagent was prepared by dissolving 0.25 g ninhydrin in 4 mL concentrated HCl and 6 mL glacial acetic acid.

Cloning of DmCBS—The *Dm*CBS sequence was subcloned into a pGEX-6P1 vector following a similar strategy to that used for the human CBS expression construct (28). The resulting pGEX-6P1-DMCBS enabled us to express *Dm*CBS as a fusion protein with glutathione-S-transferase (GST), from which GST could be cleaved off with HRV 3C protease (AG Scientific) leaving a single extra residue (Gly) at the N-terminus of *Dm*CBS polypeptide. Briefly, the *Dm*CBS sequence was amplified by PCR from a first strand cDNA using a forward primer containing an ApaI site (5′-ctagGGGCCCcaaccgaagccatacgagagg) and a reverse primer containing a NotI site (5′-ctagGCGGCCGCgatcagaactggagaacgc). After cleavage with ApaI and NotI (NEB Biolabs), the *Dm*CBS fragment was purified and isolated by excision from a 1% agarose gel, and cleaned up using QIAquick gel extraction kit (Qiagen). Subsequently, *Dm*CBS was ligated into the ApaI-NotI linearized pGEX-6P1 vector, in which an internal ApaI site was abolished previously by site directed mutagenesis (28). The construct pGEX-6P1-DMCBS was transformed into *E. coli* XL1-Blue cells (Stratagene) and its authenticity was confirmed by DNA sequencing. Verified plasmid was transformed into *E. coli* Rosetta2 (DE3) expression host cells (Novagen).

Expression and Purification of CBS—Human CBS (hCBS) was expressed and purified as described previously (29). *Dm*CBS was purified in an otherwise identical fashion with the following specific modifications. After cleavage of the fusion protein with HRV 3C protease, GST was removed chromatographically on DEAE Sepharose Fast Flow (GE Healthcare). The column was equilibrated in 15 mM potassium phosphate pH 7.2, 1 mM EDTA, 1 mM DTT and 10% ethylene glycol. Under these conditions, both GST and *Dm*CBS proteins bind to the DEAE Sepharose resin. The separation of the GST from *Dm*CBS was achieved by elution with a linear gradient from 15 to 75 mM potassium phosphate pH 7.2, 1 mM EDTA, 1 mM DTT and 10% ethylene glycol. Unlike hCBS, *Dm*CBS elutes prior to the GST. Protein-containing fractions were analyzed by electrophoresis on 9% SDS–PAGE. The *Dm*CBS-rich fractions were pooled and subsequently concentrated on YM-30 membrane (Millipore). The buffer was exchanged by pressure dialysis to 20 mM HEPES pH 7.4, 1 mM TCEP and 0.01% Tween 20.

CBS Activity Assays—The activities of hCBS, hCBS45, yCBS and *Dm*CBS in the canonical cystathionine synthesis reaction (Reaction 1) were determined using a

radiochemical assay as described previously (13). The activities in the two alternative cysteine synthesis reactions (Reaction 2 and 3) were measured using a colorimetric reaction described by Gaitonde (30) with the following modifications. To monitor cysteine synthesis, the reaction mixture (600 μL) contained: 0.5 mg/mL BSA, 10 mM DTT, 0.0168 mg/mL enzyme, 24 mM L-serine or *O*-acetyl-L-serine, 25.5 mM Na_2S and optionally 0.5 mM PLP and/or 0.36 mM AdoMet in 200 mM Tris (pH 8.6) buffer. The reaction temperature was 37 °C and the reaction was initiated by adding the enzyme. At five different time points 100 μL of the reaction mixture was taken and mixed with 20 μL 50% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation. The supernatant (100 μL) was combined with 100 μL acetic acid and 100 μL ninhydrin reagent; the mixture was heated in a boiling water bath for 3 minutes and immediately cooled in ice water. The absorbance at 560 nm was measured to determine the amount of cysteine produced. A standard curve was generated by the same method using cysteine solutions of known concentration and containing all other reagents except enzyme. Under these conditions, the enzymes were fully saturated with substrates ([substrate]>10K_m, data not shown).

Mass Spectrometry—As-isolated and AdoMet-treated *Dm*CBS and hCBS were analyzed by mass spectrometry for the presence of AdoMet. As-isolated *Dm*CBS and hCBS were diluted directly into 0.1% acetic acid in 5:95 methanol/water. AdoMet-treated *Dm*CBS and hCBS were diluted into and incubated with 0.36 mM AdoMet in 200 mM Tris (pH 8.6) buffer at 37°C for 20 min. All samples were then desalted at room temperature using two consecutive detergent removal spin columns (Pierce, Rockford, IL) according to manufacturer instructions. Columns were pre-equilibrated with 0.1% acetic acid in 5:95 methanol/water, and the CBS samples were eluted off the column in the same solvent. Prior to mass spectrometry analysis, the CBS samples were diluted with acetic acid and methanol to achieve 1% acetic acid in 50:50 methanol/aqueous sample. Mass spectrometry data were acquired using a 7T linear trap/FT-ICR (LTQ FT Ultra) hybrid mass spectrometer (Thermo Scientific Inc., Bremen, Germany) equipped with an automated chip-based nano-ESI source (Triversa NanoMate, Advion BioSciences, Ithaca, NY). The resolving power of the FT-ICR mass analyzer was set at 200,000. The spray voltage was 1.3–1.5 kV relative to the inlet of the mass spectrometer.

Electronic Absorption Spectroscopy—Electronic absorption spectra were recorded on a Cary 4 Bio spectrophotometer. The spectral bandwidth was set to 0.5 nm. Temperature was controlled by a Peltier temperature controller at 20 °C, unless otherwise stated. CBS samples (200 μL, 5 μM heme) were prepared in 200 mM buffers unless otherwise indicated. Tris buffer (pH 8.6, 9.0) and MES buffer (pH 6.0) were used in these experiments. All buffer solutions contained an additional 100 mM NaCl and were degassed with argon before use. The samples were placed in septum-sealed quartz microcuvettes purged with argon gas. Addition of other reagents was done by injecting the reagents into a sealed cuvette using a gas-tight syringe followed by gently mixing on a vortex mixer. Solutions of sodium dithionite (60 mM $Na_2S_2O_4$) in Ar-degassed Tris buffer (pH 8.6, 9.0) or MES buffer (pH 6.0) were prepared and used within 20 min of preparation. These dithionite solutions were added to Fe(III)CBS to achieve a final concentration of 10 mM dithionite in the cuvette. CO was supplied in the form of CO gas-saturated buffer (100 μL); the appropriate pH buffer was deoxygenated with argon prior to saturation with CO. NO gas (100 μL) was injected to the headspace of the cuvette using a gas-tight syringe. Spectra were recorded immediately after addition and mixing of reagents. Absorbance data were corrected for the concentration change caused by dilution when appropriate. At the concentration used (10 mM $Na_2S_2O_4$), the dithionite ion concentration is expected to remain essentially unchanged for at least 2 hours at pH 6.0.(31, 32) Data for 60 minutes of CO binding were analyzed at this low pH,

and were sufficient for the reaction to go to completion. All other experiments that utilized dithionite solutions at pH 6.0 were completed in less than 2 hours.

Thermal Characterization—To characterize the thermal stability of CBS proteins, the absorption spectra of an anaerobic protein sample (200 μL, 5 μM) in 200 mM Tris buffer (pH 8.6) were monitored between 20°C and 92°C at 2°C intervals. A full spectrum was recorded at each temperature after thermal equilibration for 3 minutes. A steady decrease in absorbance is followed by a steep increase due to light scattering of precipitated protein around the melting point. The absorbance drops again after the precipitates fall to the bottom of the cuvette. The T_m was defined as the inflection point when absorbance was plotted against temperature, averaged from three independent experiments. Errors in the observed T_m are determined by the temperature interval used and are no more than ±2°C.

Kinetic Analysis—Electronic absorption spectral data for the CO binding reactions was analyzed to determine the apparent mechanism of CO binding. Briefly, the CO binding reactions were followed spectrophotometrically; complete spectra were recorded at 15 sec intervals until no further changes were observed. Because Fe(III)CBS, Fe(II)CBS and Fe(II)CBS-CO have distinct Soret absorption maxima, changes in their Soret absorbances over time were fitted simultaneously using SciPy (33) to a biexponential equation $\Delta A = a_1\exp(b_1 t) + a_2\exp(b_2 t) - (a_1+a_2)$ derived from the following model unless otherwise stated:

$$\text{Fe(III)CBS} \underset{k_2}{\overset{k_1}{\rightleftharpoons}} \text{Fe(II)CBS} \xrightarrow{k_3} \text{Fe(II)CBS-CO}$$

The rate constants k_1 and k_2 are pH-dependent; k_3 is [CO]-dependent. b_1, b_2 are functions of k_1, k_2, k_3 and $b_1+b_2 = -(k_1+k_2+k_3)$, $b_1 b_2 = k_1 k_3$ (see Supporting Information for details).

Protein Sequence and Structure Analysis—Protein sequences were obtained from UniProt (34). Multiple sequence alignment was done using Clustal Omega (35) and edited in JalView (36). Crystallographic data were obtained from the Protein Data Bank (37) and visualized in PyMol (38).

Results

Enzyme Activity—The activity of *Dm*CBS is higher than or comparable to that of hCBS in the three different reactions; however, AdoMet does not simulate *Dm*CBS activity. Table 1 compares the activity of *Dm*CBS and hCBS in the classical cystathionine synthesis reaction (Reaction 1) and the two alternative cysteine synthesis reactions (Reaction 2 and 3). *Dm*CBS is more active in cystathionine synthesis than hCBS in the absence of AdoMet, exhibiting activity comparable to yCBS and the hCBS45 variant lacking the C-terminal autoinhibitory domain when exogenous PLP is present. In cysteine synthesis, *Dm*CBS is less active than yCBS or hCBS45, with activity comparable to hCBS in the absence of AdoMet. Unlike hCBS, *Dm*CBS does not respond to AdoMet stimulation; the activity of *Dm*CBS in each of the three different reactions was not altered upon addition of a 1200-fold molar excess of AdoMet (0.36 mM). Even at 5.0 mM AdoMet (17000-fold excess), no stimulation of *Dm*CBS activity was observed. *Dm*CBS is somewhat more active than hCBS in catalyzing Reaction 3, possibly due to better accommodation of the *O*-acetylserine substrate.

To test whether *Dm*CBS binds to AdoMet, as-isolated and AdoMet-treated *Dm*CBS samples were analyzed by mass spectrometry. Ion trap mass spectra of *Dm*CBS, with and without preincubation with AdoMet, are shown in Figure 2. The FT data reveal a mass of 56877 Da for the *Dm*CBS protein (Figure S1); the calculated mass of the heme-free protein is 56882

Da. Heme dissociates upon ionization and appears as a characteristic mass peak at 616 (FePPIX calculated mass 616.487). Control AdoMet solutions under similar experimental conditions exhibit a peak at m/z 399 (AdoMet calculated mass 398.14). Neither *Dm*CBS sample showed a mass peak at 399, suggesting that *Dm*CBS does not bind to AdoMet. The same mass spectrometric analysis was performed using hCBS as a control, since hCBS is known to bind AdoMet. The AdoMet mass peak was absent in the as-isolated hCBS spectrum, but present in the AdoMet-treated hCBS spectrum (data not shown). These results show that *Dm*CBS and hCBS were isolated free of AdoMet; upon incubation with AdoMet, hCBS bound AdoMet but *Dm*CBS did not.

pH-dependent Redox Behavior of DmCBS

The *Dm*CBS heme exhibits spectral features and pH dependent redox behaviors that are similar to those of hCBS and hCBS45 (Figure 3). The electronic absorption spectrum of as-isolated *Dm*CBS at pH 8.6 is shown as a solid line in Figure 3, *Dm*CBS panel, with absorption bands at 367 (δ band), 429 (Soret or γ band) and 552nm (α–β bands). Identical heme absorption features are observed at pH 6.0 (Figure 4(B), solid line). The A_{429}/A_{282} ratio is 1.02, which is similar to that of hCBS (1.07). Based on the spectral similarity to Fe(III)hCBS (39), the asisolated *Dm*CBS contains a low-spin Fe(III) heme with one cysteine thiolate ligand and additional neutral donor ligand, presumably histidine. At pH 8.6, Fe(III)*Dm*CBS is readily reduced by sodium dithionite to give a new, Fe(II)*Dm*CBS species with a spectrum similar to that of Fe(II)hCBS. Upon reduction, the Soret band sharpens and red shifts to 448 nm and the broad α/β absorption envelope sharpens, to give two unsymmetrical α and β bands at 574 and 540 nm (dashed line in Figure 3, *Dm*CBS panel) indicative of a six-coordinate, low-spin, thiolate-ligated heme. At pH 6.0, Fe(III)*Dm*CBS did not appear to react with dithionite, as there was no change observed in the electronic absorption spectrum (Figure 4(B), solid line and dashed line). This result suggested that the protein remained in the Fe(III) state even in the presence of excess reducing agent. This result is in contrast to hCBS; transient formation of Fe(II)hCBS was observed followed by pH-dependent reoxidation to Fe(III)hCBS (39). When Fe(II)*Dm*CBS at pH 8.6 and in the presence of excess dithionite was subjected to a pH shift by addition of concentrated pH 6.0 buffer, the protein immediately reoxidized to Fe(III)*Dm*CBS (Figure 4(A), dashed line to dotted line). Conversely, when Fe(III)*Dm*CBS at pH 6.0 and in the presence of excess dithionite was subjected to a pH shift by addition of concentrated pH 8.6 buffer, the protein immediately reduced to Fe(II)*Dm*CBS (Figure 4(B), dashed line to dotted line). At an intermediate pH (7.4), the electronic absorption spectrum in the presence of excess dithionite showed a mixture of Fe(III) and Fe(II) species (Figure S2). These observations suggest that the *Dm*CBS heme behaves similarly to the hCBS heme, with pH-dependent redox processes that are fully reversible (39). The difference is that *Dm*CBS never appears to be reduced at low pH, presumably because the reoxidation process is very fast and the equilibrium position favors the reoxidized species.

Thermal stability of DmCBS

Fe(III)*Dm*CBS is as thermally stable as Fe(III)hCBS, but the heme of Fe(II)*Dm*CBS is significantly less stable than that of Fe(II)hCBS. Two different thermal stabilities were examined: 1) the thermal stability of the heme ligation environment, and 2) the thermal stability of the entire protein (thermal unfolding melting temperature). The reduced CBS heme undergoes a thermally induced ligand-switch process in which the cysteine thiolate is replaced by an unidentified neutral ligand, leading to a blue-shifted Soret at 424 nm (Figure 3). Fe(II)hCBS and Fe(II)hCBS45 undergo this ligand-switch process with midpoint transition temperatures of 44°C and 43°C, respectively; the same process occurs very slowly at 37°C (18, 40). Fe(II)*Dm*CBS showed a similar ligand-switch process which proceeds with a midpoint transition temperature of 37°C (Figure S5). When held at 37°C, the rate of ligand

switching is significantly faster for Fe(II)*Dm*CBS than for Fe(II)hCBS. Even at 30°C, a full, clean conversion cannot be achieved because during the ligand switch process, as both the newly formed ligand switched species (Fe(II)*Dm*CBS-424) and the original Fe(II)*Dm*CBS protein denature upon extended heating (>3 hr). Because *Dm*CBS appeared to denature before the ligand switch process was complete, we compared the melting temperature of *Dm*CBS to that of hCBS to identify differences in the protein stabilities. Thermal denaturation was measured by light scattering at 800 nm; the unfolding is irreversible and the protein visibly precipitates. The melting temperatures (T_m) for Fe(II)*Dm*CBS and Fe(II)hCBS are comparable – the transition midpoints occur at 52°C and 51°C, respectively (see Supporting Information). The melting temperature of Fe(III)*Dm*CBS is 59°C, which is slightly higher than the reported T_m of 55°C for Fe(III)hCBS (41). These observations suggest that the overall stabilities of human and *Dm*CBS proteins are influenced by the heme oxidation state. When the heme is oxidized, *Dm*CBS is more stable than hCBS; however, the when the heme is reduced the two proteins exhibit fairly comparable stabilities. In contrast, the heme environment stabilities are comparable in the Fe(III) state, but differ in the reduced, Fe(II) state, where reoxidation and ligand switching are more facile for *Dm*CBS.

Gas-binding Reactions of CBS

Fe(II)*Dm*CBS, Fe(II)hCBS and Fe(II)hCBS45 react with CO in a pH dependent manner. Three proteins, *Dm*CBS, hCBS and hCBS45 were reacted with CO at high and low pH. None showed appreciable gas binding in the oxidized, Fe(III) state; no changes were observed in the electronic absorption spectra after addition of CO. When dithionite was present, all three proteins reacted with and bound CO on time scales of hours. The CO-bound CBS proteins show no detectable activity. For each of the three proteins, under all pH conditions, the CO-bound product was the same regardless of the order of addition of dithionite and CO; the spectra of the final CO-adducts are characteristic of low-spin Fe(II) CO-bound hemes with blue-shifted Soret peaks and symmetrical, flattened α and β bands (Figure 5 and Table 2). The Soret peak positions (414 nm, *Dm*CBS; 420 nm, hCBS) indicate that the cysteine thiolate ligand is no longer bound to the heme iron in these CO adducts (40). The rate of CO binding was monitored at high pH, where the hemes appeared to be fully reduced, and at low pH, where the hemes appear to be oxidized even in the presence of dithionite. The high pH condition was chosen so as to be high enough to stabilize the reduced protein: pH 8.6 for *Dm*CBS, pH 9.0 for hCBS and hCBS45. The electronic absorption changes were monitored as a function of time, after addition of dithionite and CO in either addition order (CO, dithionite; dithionite, CO). At high pH, *Dm*CBS, hCBS and hCBS45 were all reduced immediately upon addition of dithionite; the product Fe(II) species then bound CO at a slower rate. This two-phase process was observed regardless of the order of addition of the reagents and for all three proteins (*Dm*CBS, hCBS, hCBS45). The CO binding process was different at low pH (6.0). None of the proteins were ever present in a fully reduced state, even when dithionite was added first (*vide supra*, and see (39)). When CO was added, and regardless of the order of addition, *Dm*CBS and hCBS reacted with CO to form the same low-spin Fe(II)-CO-bound product species seen at high pH (identical visible spectra); however, the rate of CO binding was faster at low pH than at high pH. Although the dominant species observed spectroscopically are the Fe(III) and Fe(II)CO forms of *Dm*CBS and hCBS, it is evident from the presence of a small amount of Fe(II) that the reaction must proceed via the reduced species. In contrast with *Dm*CBS and hCBS, the CO adduct of hCBS45 appears to be unstable at pH 6.0; the intensity of the Soret absorption due to the Fe(III) species diminishes without a concomitant increase in other bands.

A kinetic analysis of the absorbance data for these CO binding reactions was carried out as described in the Experimental section. The results are tabulated in Table S1. The data for the reaction of Fe(III)hCBS, with dithionite added before CO at pH 9.0, are best fitted by a single exponential equation. This fit gives k_3 = 0.016 min^{-1}, the rate constant for CO binding to the reduced CBS heme. This rate is remarkably slow, presumably due to the fact that the thiolate ligand must dissociate from the heme in order for CO to bind. In all other cases, a biexponential equation is necessary to fit the data. The biexponential equation models a system consisting of a reversible redox process, between the Fe(III)/Fe(II) oxidation states, and an irreversible gas binding process where CO binds only to the Fe(II) state. That this model is required to fit the data implies that even after addition of excess dithionite and with no visual evidence in the electronic absorption spectrum, a portion of the protein remains in the Fe(III) state. Consistent with a requirement for protonation and loss of the thiolate ligand prior to CO binding, the observed rate constant ($k_{obs}=k_1k_3$) for CO binding is significantly faster at low pH (hCBS 0.053 min^{-1}, *Dm*CBS 0.094 min^{-1}) than at high pH (hCBS 0.0064 min^{-1}, *Dm*CBS 0.0015 min^{-1}). These two proteins show fairly comparable behavior in the absolute rates of CO binding. In contrast, hCBS45 reacts more rapidly with CO at high pH (0.29 min^{-1}) than hCBS and *Dm*CBS, and the Fe(II)hCBS45-CO adduct is not stable at low pH. No effect of the order of addition of dithionite and CO is seen with any of the proteins, suggesting that the gas-binding step is rate-limiting.

The ligand-switched, thiolate-free forms Fe(II)*Dm*CBS-424, Fe(II)hCBS-424 and Fe(II)hCBS45-424 bind CO gas at a very high rate, much faster than the Fe(II) forms that bear the native thiolate ligands. The 424 nm Soret of the ligand-switched species disappeared immediately after CO-saturated buffer was added, and the binding process was too fast to follow on a conventional spectrophotometer. The product CO adducts had identical electronic absorption spectra (data not shown) to the corresponding CO adducts formed from non-ligand switched, native thiolate-ligated Fe(II) species (Figure 5). Since the ligand-switching process for *Dm*CBS did not go to completion, CO was added to a mixture of converted, Fe(II)*Dm*CBS-424 (Soret 424 nm) and unconverted, Fe(II)*Dm*CBS species (Soret 448 nm) (Figure 3). Immediately upon addition of CO, the 424 nm Soret disappeared with the appearance of a new peak at 414 nm. The remaining unconverted Fe(II)*Dm*CBS (448 nm Soret) also bound CO to give the same product, but at a much slower rate. These observations strongly support the conclusion that it is the presence of the thiolate ligand that impedes CO binding in native, thiolate-coordinated CBS proteins.

The three proteins, *Dm*CBS, hCBS, and hCBS45 reacted with NO to form similar, 5-coordinate heme-NO adducts. The same NO adducts were obtained regardless of whether the starting point was the Fe(III) or dithionite-generated Fe(II) species. The resulting NO adducts exhibit poorly resolved spectra with the Soret at 395–397 nm and a broad flat band in the α–β region (Table 2 and Figure 5). These spectra are indicative of a 5-coordinate heme with bearing a single NO axial ligand in each of the three proteins.

Discussion

There are several lines of evidence suggesting correlation between heme binding, AdoMet regulation, PLP affinity and enzyme activity in CBS. The yeast CBS enzyme does not contain heme and does not respond to AdoMet, while the human CBS does contain heme and is activated approximately 4-fold by AdoMet. Yeast CBS is always highly active, while hCBS reaches a high activity level only in the presence of heme and AdoMet. The hemeless Δ1-70 hCBS variant is not AdoMet-responsive, although this protein is active (with ~20% activity relative to the wild-type enzyme) and possesses an intact C-terminal regulatory region (16). These three pieces of evidence plausibly implicate correlation between the presence of heme and responsiveness to AdoMet. There is also evidence for a connection

between heme and PLP affinity. The affinity of hCBS for the PLP coenzyme *in vitro* is higher than that of yeast CBS, and a variant human enzyme with the heme-binding N-terminal domain deleted exhibits a low affinity for PLP (14, 16). Further evidence points to a structural role for heme in maintaining enzyme activity. The heme in hCBS bears a cysteine thiolate ligand, which must remain bound for the enzyme to be active. Small molecules such as CO and NO bind to the hCBS heme, replacing the cysteine ligand, and the gas-bound forms of the enzyme are inactive (19, 42). The hCBS cysteine thiolate ligand can be replaced by another neutral ligand, or removed with $HgCl_2$ and the resulting enzymes are inactive (17, 18, 21, 43). The Fe(III) in the heme can be replaced with Co(III) while retaining the His/Cys ligation and the resulting Co(III)hCBS enzyme has comparable activity to that of Fe(III)hCBS. Reduction of Co(III) to Co(II) induces loss of the cysteine ligand and loss of enzyme activity (17).

Other results are inconsistent with a correlation between heme, AdoMet, PLP and enzyme activity. AdoMet is known to interact with the C-terminal domain of hCBS (44); specifically, the CBS1 and CBS2 domains in the C-terminus are identified as the AdoMet binding sites (45, 46). When an hCBS fragment composed of residues 416−551 is expressed in *E. coli*, the pair of C-terminal CBS domains alone are sufficient to bind AdoMet (46), an observation inconsistent with a role for the heme in AdoMet binding. Furthermore, yeast CBS, which does not contain heme, binds AdoMet with very high affinity, yet the enzyme does not respond to AdoMet with a change in activity (13, 14). Given these inconsistencies, we chose to further explore the biochemical consequences of the heme in CBS through comparative studies of CBS proteins from human and fruit fly (*Drosophila melanogaster*).

*Dm*CBS contains heme but does not respond to AdoMet stimulation, suggesting that these two characteristics are not correlated with one another. *Dm*CBS is isolated as a heme-containing protein; the crystal structure revealed that His34 and Cys22 are ligands to the heme, with Arg235 and Trp24 within hydrogen bonding distance of the Cys22 sulfur (23). This coordination sphere and associated hydrogen bonding network, as well as the spectral characteristics of Fe(III)*Dm*CBS reported herein, are essentially identical to those of Fe(III)hCBS. *Dm*CBS is active in all three reactions tested, but the enzyme does not respond to AdoMet stimulation in catalyzing any of these reactions. In this regard, *Dm*CBS is more like yeast CBS, which also does not respond to AdoMet stimulation. The lack of response to AdoMet, combined with the fact that *Dm*CBS contains heme, suggests that heme and AdoMet are not functionally or evolutionarily connected.

It seems likely that *Dm*CBS does not bind AdoMet. There are multiple reasons why a CBS enzyme may appear unresponsive to AdoMet stimulation: it may be unable to bind AdoMet, it may be locked in a conformation that cannot be activated even though AdoMet binds, or it may have a high affinity for AdoMet such that AdoMet is always bound and it therefore does not respond to exogenous AdoMet. For example, the C431S variant of hCBS is not activated by AdoMet because it does not bind AdoMet (45). On the other hand, the hCBS S466L variant binds AdoMet, but because this variant enzyme is constitutively activated, its activity does not increase in response to AdoMet binding (41). Yeast CBS is similar to S466L hCBS, where constitutively high activity is paired with high AdoMet binding affinity but no AdoMet-dependent activation (13, 14). There is no example yet known of a CBS protein where AdoMet is tightly bound at all times. No evidence of AdoMet was seen in the *Dm*CBS crystal structure (23); furthermore, when an AdoMet molecule was docked into the C-terminal domain of the protein, there was insufficient space to accommodate the guest molecule (23). Thus, *Dm*CBS is not crystallized with a tightly bound AdoMet molecule and the crystallography implies that AdoMet may not bind at all. Our mass spectrometry experiment confirmed that *Dm*CBS is isolated free of tightly-bound AdoMet. In addition, we see no evidence of AdoMet-binding when *Dm*CBS is incubated with AdoMet. These

results suggest that the high activity of *Dm*CBS is an intrinsic property of the enzyme and not a consequence of the enzyme being saturated by AdoMet when isolated. The mass spectroscopy results suggest that *Dm*CBS does not respond to AdoMet stimulation because the enzyme cannot bind AdoMet; however, we cannot rule out low affinity binding that does not survive the sample preparation procedures.

The CBS enzymes, yCBS, hCBS, hCBS45 and *Dm*CBS, can utilize both serine and *O*-acetylserine as substrate to synthesize cysteine (Reactions 2 and 3), whereas cysteine synthase (CS) must use *O*acetylserine (Reaction 3 only). CBS enzymes are approximately two orders of magnitude slower than CS enzymes, whose rates are on the order of 10^3–10^4 U/mg protein (47–58). Furthermore, a CBS-like protein *Bs*MccA from *Bacillus subtilis*, catalyzes a cystathionine synthesis reaction analogous to the CBS canonical reaction (Reaction 1) using *O*-acetylserine and homocysteine (59). CBS and CS enzymes have very similar active site structures in the vicinity of the PLP coenzyme in both the resting state and the intermediate states; the structure of the mouth of the channel shows greater variability in size and shape (8, 23–26). Differences in the active site pocket and the channel through which the substrates get to this pocket may affect whether and how a certain substrate binds. The presence or absence of specific residues as general acid/base catalysts may also influence the reaction rate and substrate selectivity. Consistent with this model, multiple residues in the yCBS active site were shown influence kinetics of and specificity toward cystathionine synthesis and serine deamination (60) and active site variants of hCBS exhibit differential effects on cystathionine synthesis from serine and cysteine (61).

The CBS heme was postulated to be a gas sensor because the gas-bound enzyme is inactive (19, 62); however, the slow rate and complexity of the gas binding reactions suggest that such a role is less likely. When the heme iron is reduced, all three CBS proteins (hCBS, hCBS45 and *Dm*CBS) bind CO and NO on time scales of hours. For comparison, the CO sensor CooA and NO sensor sGC, which undergo significant conformational changes in response to gas binding, react completely within minutes with CO and NO, respectively (63, 64). The gas binding process in CBS is pH-dependent; therefore, were the heme to function as a gas sensor, sensing would be coupled with pH. Among the hemoproteins that bind small molecule ligands for sensing or transport, few are known to exhibit pHsensitive ligand binding processes. Typically, in these pH-sensitive proteins the binding rate is faster at high pH (65–69). There are also cases reported where the ligand dissociation rate is faster at high pH (65, 69–73). The CBS proteins are distinct in that the apparent binding rate is faster at low pH and the rate decreases as the pH increases. Mechanistic considerations and kinetic analysis of CO binding suggest that the CO-Fe bond only forms after the cysteine thiolate ligand has departed; therefore, the binding rate is limited by the rate of thiolate dissociation. The departing thiolate ligand must be protonated, a process that is faster at low pH. This explanation is consistent with our observation that the apparent CO binding rate of ligand-switched CBS, in which the thiolate ligand is absent, is exceedingly fast. Although it is rare for the ligand binding rate to increase with decreased pH, CBS is not the only example. The rates of CO binding to neuroglobin and *Nassa mutabilis* myoglobin increase with decreasing pH due to protonation of histidine ligands that are replaced by CO (74, 75). Protonation of a heme propionate group is proposed to account for decreases in the binding rate with decreasing pH of Cl^- to *Tokunagayusurika akamusi* hemoglobin (76) and CO to heme-H and heme-GH model complexes (77).

Previous studies suggested that the heme is critical for human CBS folding and assembly. Improper folding and degradation of hCBS enzyme are observed in heme-deficient expression systems (78) and addition of heme to heme-deficient CBS after expression cannot restore either the activity or the absorption spectrum (79). Expression of human CBS in heme biosynthesis-deficient systems can be rescued by addition of heme analogs or a

chemical chaperone (78) resulting in normal enzyme activity and AdoMet-responsiveness (17). When a CBS-GST fusion protein was expressed in heme-biosynthesis-deficient *E. coli* cells, it was possible to isolate modest amounts of a heme-free fusion protein. This heme–free protein was deficient in PLP and exhibited 3% of the activity of the hemecontaining fusion protein (78). Together, these results support a proposed role for heme in establishing CBS structure (80, 81). While heme may be necessary for proper folding during expression, our results also indicate that the global stabilities of properly folded CBS enzymes (both human and *Drosophila*) are affected by changes at the heme. It is a phenomenological observation that disruption of the cysteine thiolate-to-iron bond correlates with complete loss of enzyme activity. In cases where it was shown that this bond was broken, no CBS activity was observed (17–19, 21, 42, 43). This observation suggests communication between the heme-binding domain and the catalytic PLP; however, the nature of this communication is not known. The results herein show that, just like human CBS, *Dm*CBS loses activity once the cysteine thiolate ligand is removed from the heme, further strengthening the postulated correlation between heme and enzyme activity. It is possible that heme serves multiple roles: first in maintaining protein structure during translation and folding and then in regulating stability and activity in the mature protein.

Conclusion

We conclude that in human and *Drosophila* CBS, heme and AdoMet regulation are not correlated. Like yeast CBS, *Dm*CBS is active both in the canonical cystathionine synthesis reaction and two alternative cysteine synthesis reactions and does not respond to AdoMet stimulation. At the same time, *Dm*CBS is a heme protein, with spectroscopic characteristics similar to those of human CBS. Fe(II)*Dm*CBS undergoes pH-mediated redox and thermal ligand switching, but the reduced heme in *Dm*CBS is less stable than that in hCBS. CO binds slowly to Fe(II)*Dm*CBS and to Fe(II)hCBS, with rates that vary with pH. In the CO adducts, the heme-cysteine thiolate bond is disrupted and the enzyme activity is lost. Based on these findings, we conclude that the incorporation of heme and AdoMet regulation in CBS are unrelated, and presumably emerged separately in the course of evolution.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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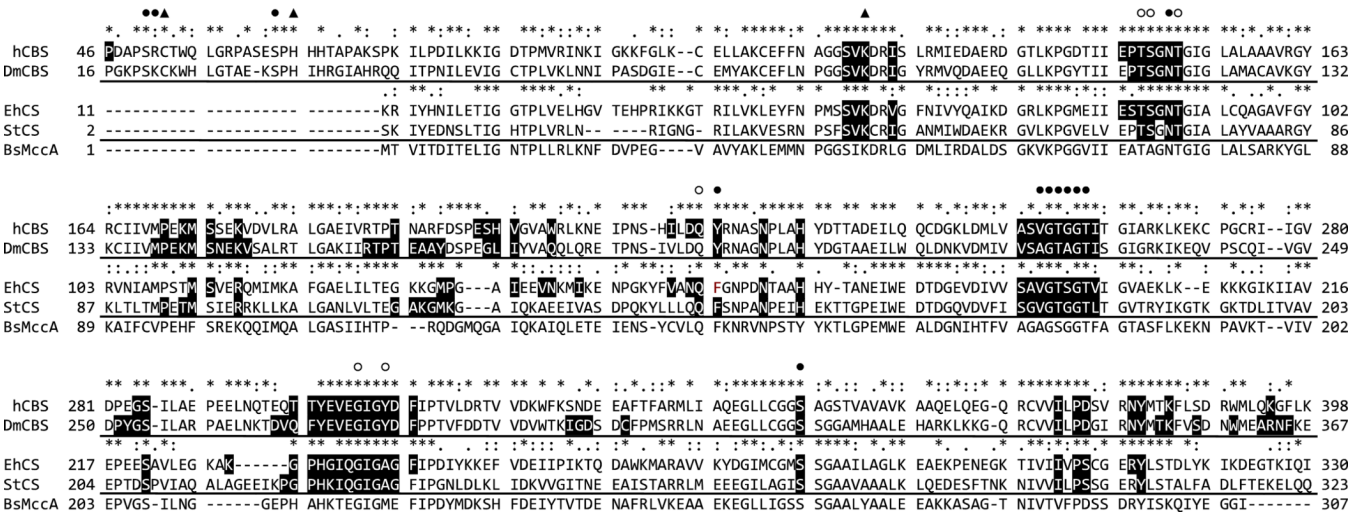


Figure 1.

Sequence alignment of hCBS, *Dm*CBS, *Entamoeba histolytica* cysteine synthase (*Eh*CS), *Salmonella typhimurium* cysteine synthase (*St*CS) and an *O*-acetylserine-dependent CBS-like protein from *Bacillus subtilis* (*Bs*MccA). Asterisks indicate identical amino acids, small dots indicate semi-conservative replacement. Residues in black background form the active site pocket in the crystal structures (8, 23–26) as identified by castP (27). The PLP and heme binding residues are marked with ▲. The residues hydrogen bonded to the PLP or heme in the crystal structures are marked with ●. The residues hydrogen bonded to the substrates/products in the crystal structures are marked with ○.

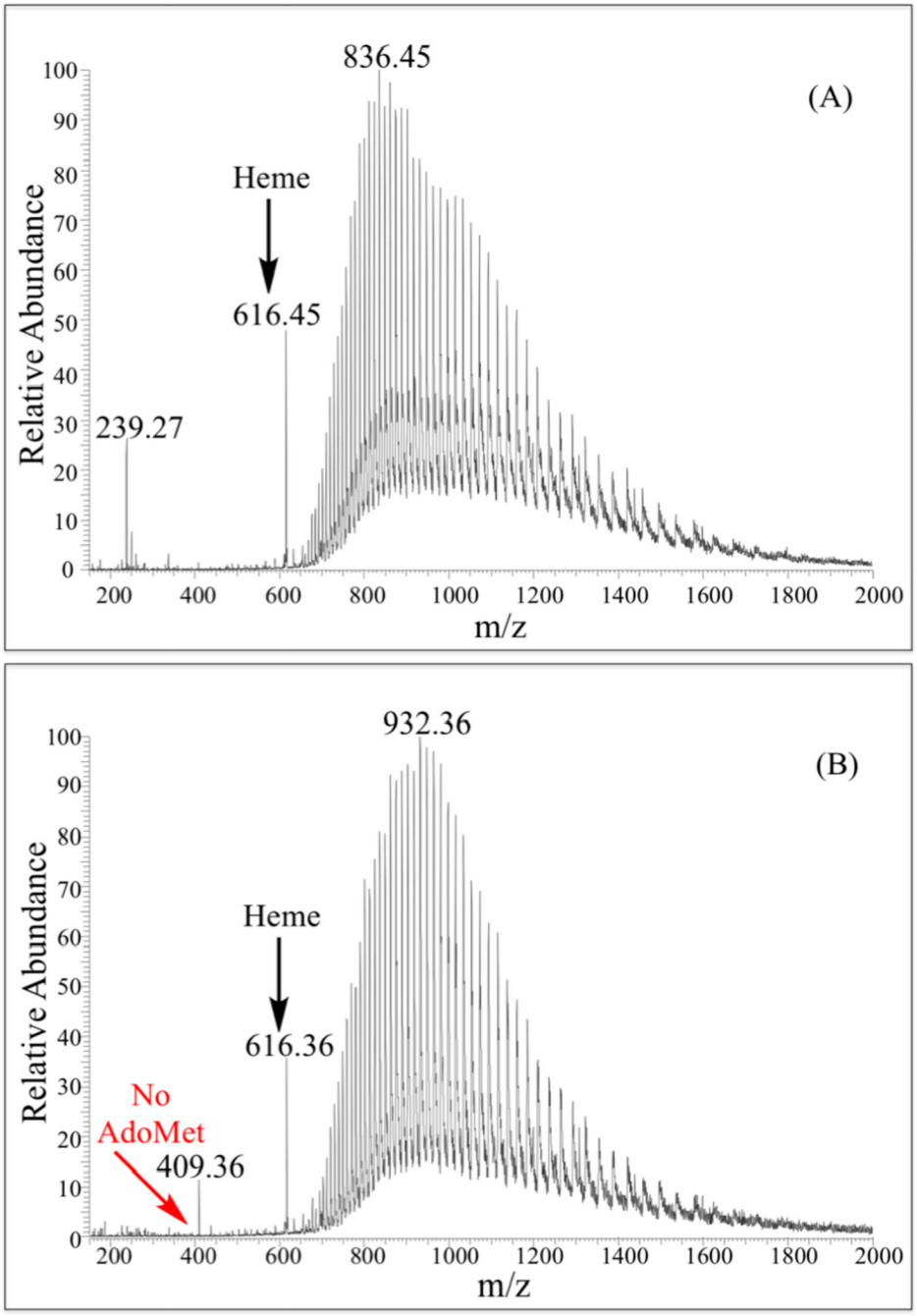


Figure 2.

Ion trap mass spectra of as-isolated (A) and AdoMet-treated (B) *Dm*CBS. The mass peak at 616 is heme. Lack of a mass peak at 399 indicates the absence of AdoMet. The other low mass peaks at 239 and 409, are common plasticizers that were present in the respective background solvent samples.

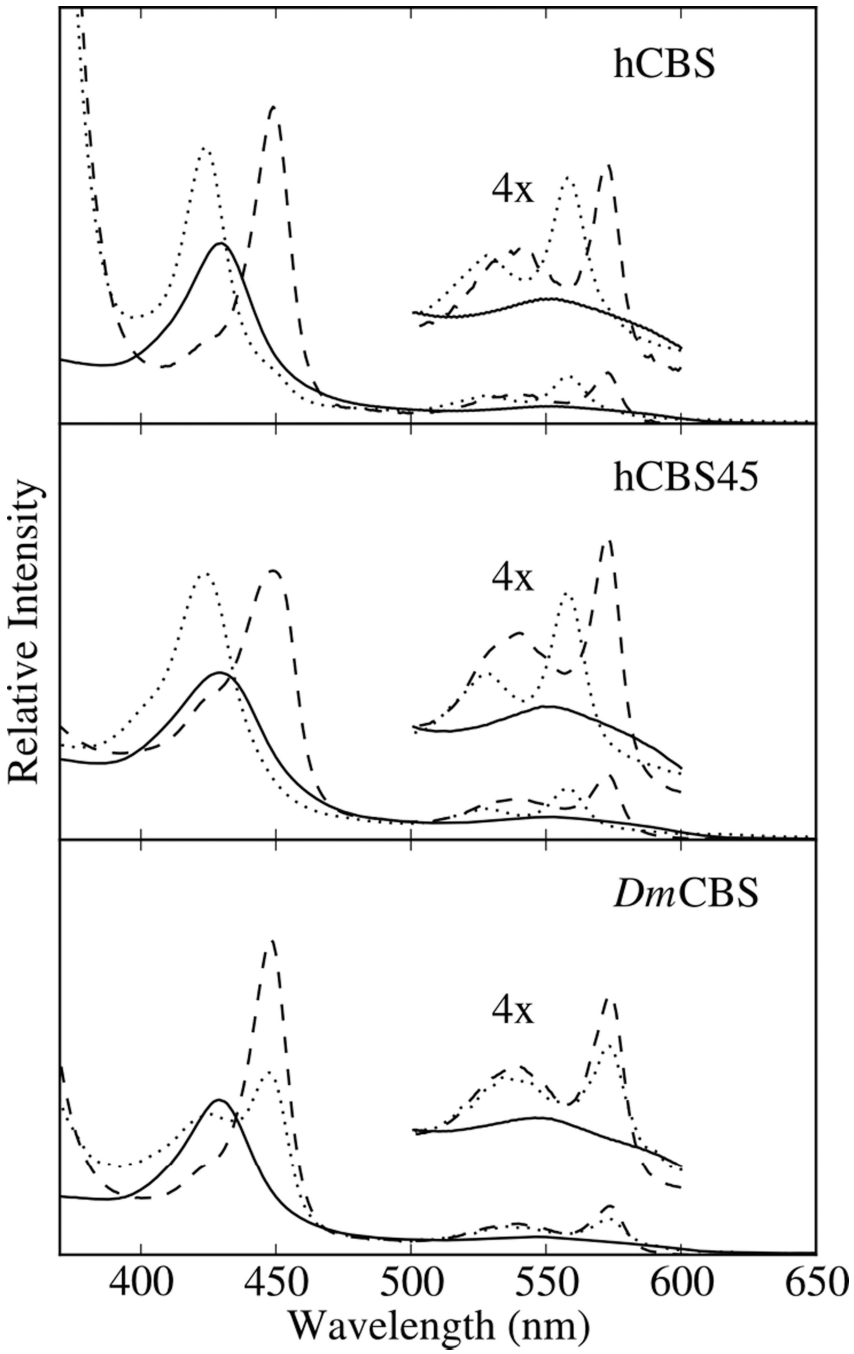


Figure 3.

Optical spectra of the Fe(III)- (solid line), Fe(II)- (dashed line) and ligand-switched (dotted line) states of hCBS (pH 9.0), hCBS45 (pH 9.0) and *Dm*CBS (pH 8.6). In the case of *Dm*CBS, the ligand-switch is incomplete and the spectrum shows a mixture of switched and unswitched species (see text). Peak positions are summarized in Table 2.

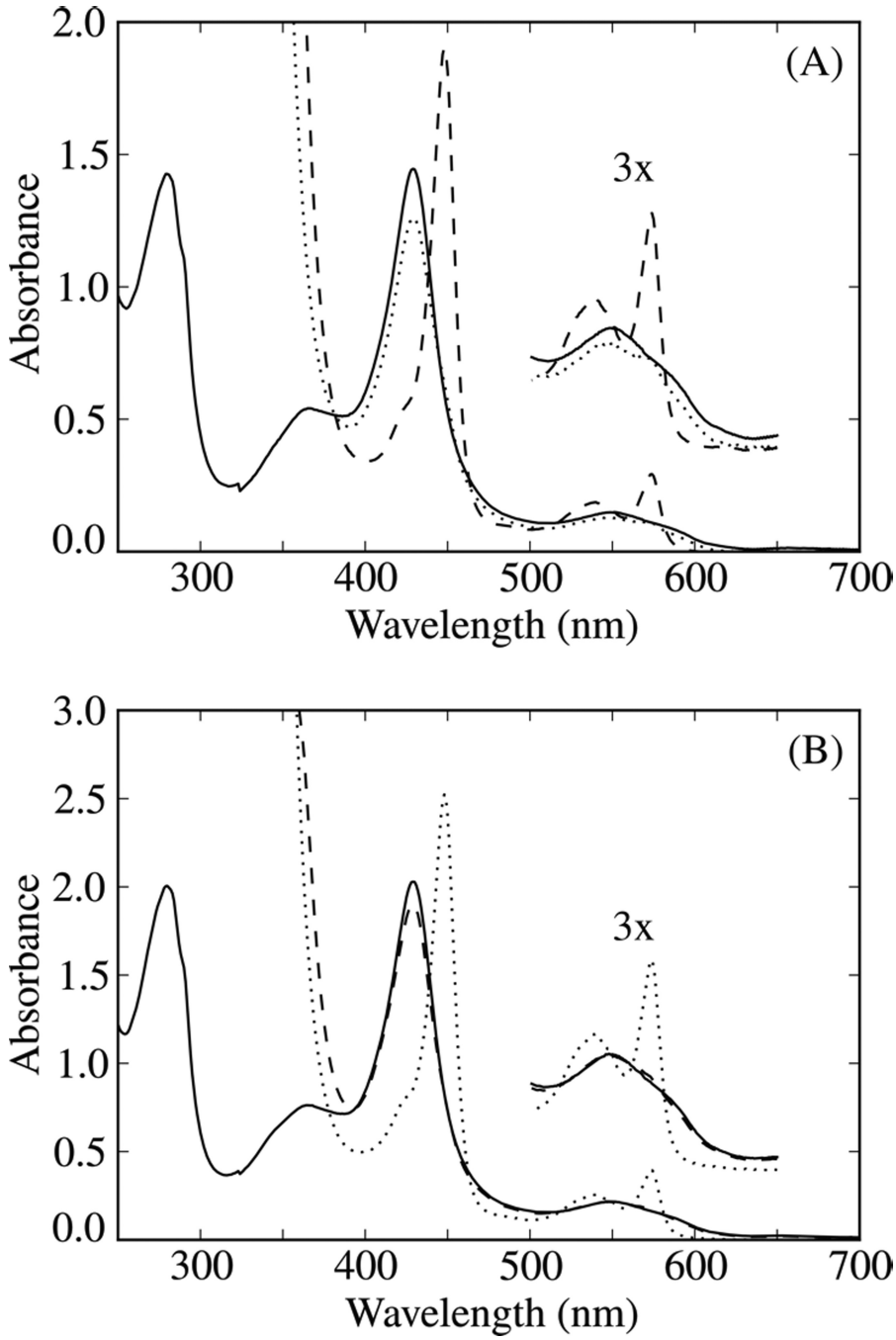


Figure 4.

Electronic absorption spectra showing pH-dependent reversible conversion between Fe(III)- and Fe(II)*Dm*CBS. Peak positions are summarized in Table 2. (A) solid line: 5 μM Fe(III)*Dm*CBS in 200 mM pH 8.6 Tris buffer; dashed line: the heme iron is reduced to Fe(II) state by 10 mM sodium dithionite; dotted line: the heme iron is reoxidized to Fe(III) after lowering the pH to 6.0 by adding 1.5M pH 6.0 MES buffer. (B) solid line: 5 μM Fe(III)*Dm*CBS in 200 mM pH 6.0 MES buffer; dashed line: the heme iron stays in Fe(III) state after adding dithionite; dotted line: the heme iron is reduced to Fe(II) state after raising the pH to 8.6 by adding 1.5 M pH 8.6 Tris buffer.

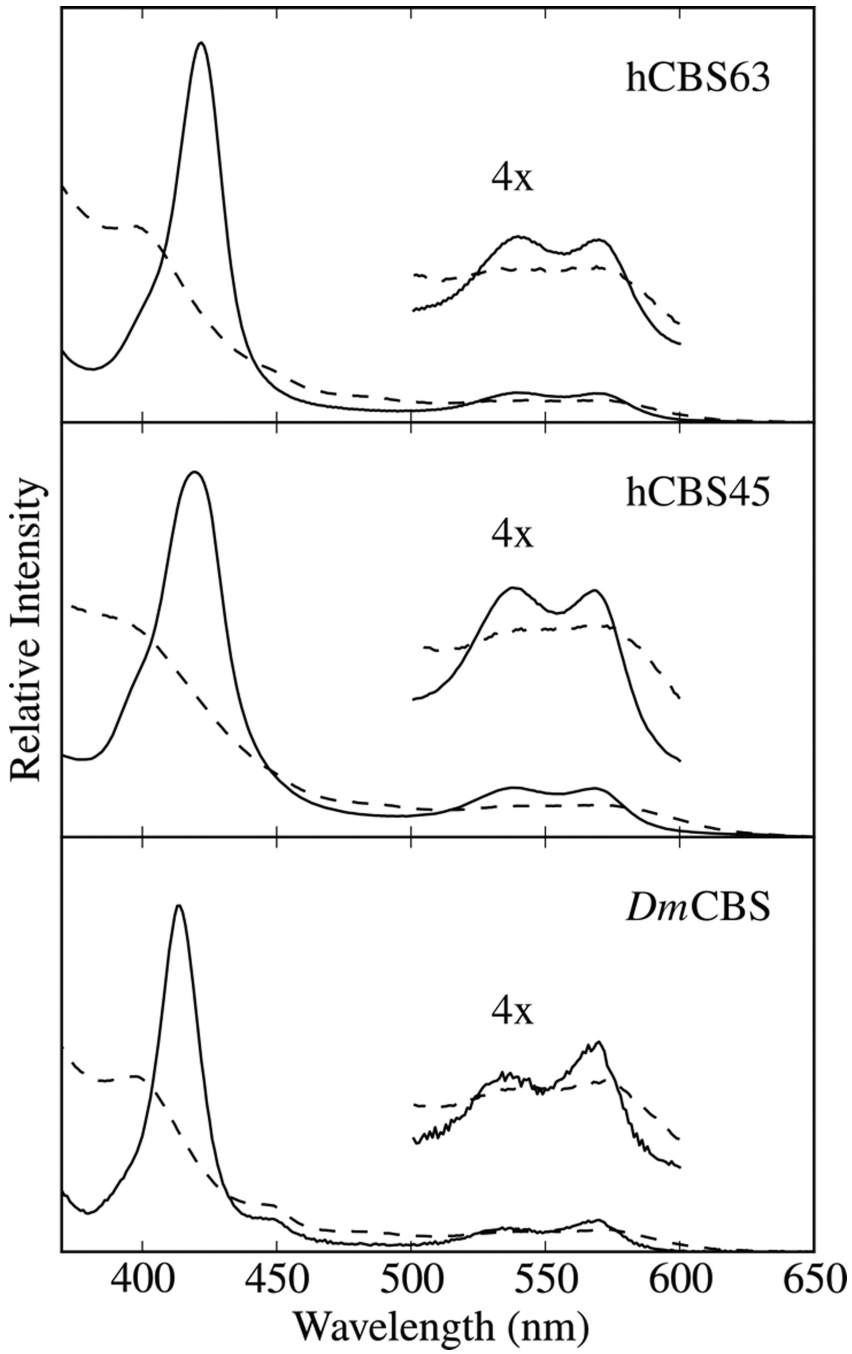
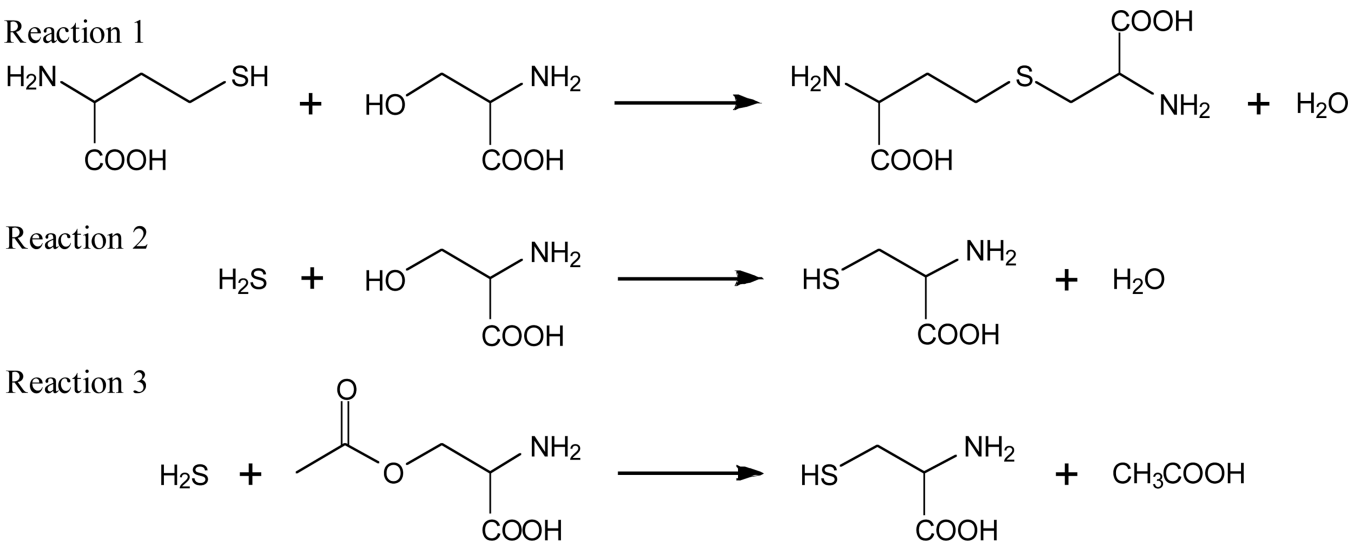


Figure 5.
Electronic absorption spectra of the CO adducts (solid lines) and NO adducts (dashed lines) of hCBS (pH 9.0), hCBS45 (pH 9.0) and *Dm*CBS (pH 8.6). In the case of *Dm*CBS, a small amount of unreacted Fe(II)*Dm*CBS remains present, as evidenced by the shoulder at 450 nm and greater intensity in the α band.



Scheme 1.

Table 1

Specific activities of hCBS, hCBS45, *Dm*CBS and yCBS at pH 8.6, 37 °C (U/mg of protein) under V_{max} conditions. One unit of activity is defined as formation of 1 μmol product in 1 hour. The assay mixture (in 200 mM Tris buffer, pH 8.6) contained: 0.5 mg/mL BSA, 10 mM DTT, 0.0168 mg/mL enzyme, 10 mM L-serine and 10 mM L-homocysteine for Reaction 1, 24 mM L-serine or *O*-acetyl-L-serine and 25.5 mM Na_2S for Reactions 2 and 3, and when indicated, 0.5 mM PLP and/or 0.36 mM AdoMet. The AdoMet response is defined as the ratio of specific activities with and without AdoMet, respectively.

	Specific Activity (U/mg of protein)				**AdoMet Response**	
PLP	−	−	+	+	**−PLP**	**+PLP**
AdoMet	−	+	−	+		
Reaction 1: serine + homocysteine → cystathionine + H_2O						
hCBS	81±9	228±16	106±4	424±27	2.8±0.4	4.0±0.3
hCBS45	980±51	1070±25	1104±38	1152±29	1.09±0.06	1.04±0.04
DmCBS	576±5	529±17	1227±94	1279±33	0.92±0.03	1.04±0.08
yCBS	831±45	894±39	1245±155	1260±170	1.08±0.07	1.0±0.2
Reaction 2: serine + H_2S → cysteine + H_2O						
hCBS	37±2	60±4	31±4	109±1	1.6±0.1	3.5±0.4
hCBS45	136±15	140±0	200±5	196±3	1.0±0.1	0.98±0.03
*Dm*CBS	38±2	36±2	42±1	41±3	0.94±0.08	0.98±0.08
yCBS	226±6	219±14	223±3	219±15	0.97±0.07	0.98±0.07
Reaction 3: O-acetylserine + H_2S → cysteine + CH_3COOH						
hCBS	13±1	21±1	14±1	32±1	1.6±0.1	2.3±0.2
hCBS45	104±1	107±3	140±5	137±3	1.03±0.03	0.98±0.04
*Dm*CBS	31±2	30±2	36±1	37±3	1.0±0.1	1.03±0.09
yCBS	148±3	152±3	148±1	145±1	1.02±0.03	0.98±0.01

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Table 2

Peak positions (nm) in the absorption spectra of *Dm*CBS, hCBS and hCBS45.

	hCBS	**hCBS45**	***Dm*CBS**
	Soret/γ, β, α	**Soret/γ, β, α**	**Soret/γ, β, α**
Fe(III) form	430, 553	429, 552	429, 552
Fe(II) form	449, 540, 573	449, 540, 573	448, 540, 574
Ligand switched form	424, 528, 559	424, 529, 558	424 (only partially converted)
CO adduct	420, 539, 570	419, 537, 568	414, 539, 570
NO adduct	397, 539, 566	395, 540, 570	397, 540, 573