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Comparative Study of Enzyme Activity and Heme Reactivity in *Drosophila melanogaster* and *Homo sapiens* Cystathionine β-Synthases

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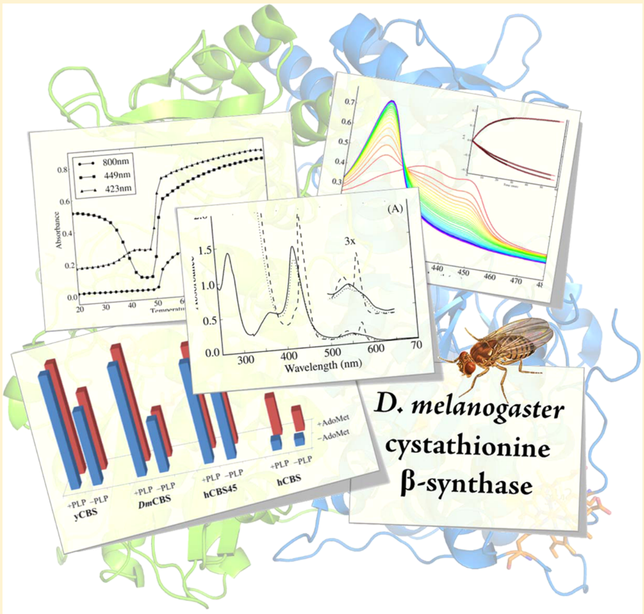
Supporting Information

ABSTRACT: Cystathionine β-synthase (CBS) is the first and rate-limiting enzyme in the transsulfuration pathway, which is critical for the synthesis of cysteine from methionine in eukaryotes. CBS uses 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, the 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 for regulating the enzyme.



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 the synthesis of cysteine from the essential amino acid methionine in eukaryotes. A deficiency in CBS activity 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, reactions 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 are *O*-acetylserine sulfhydrylase and *O*-acetylserine(thio)lyase].

Human CBS (hCBS) is a homotetramer, and each monomer (63 kDa, 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 autoinhibitory 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, 45 kDa) 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

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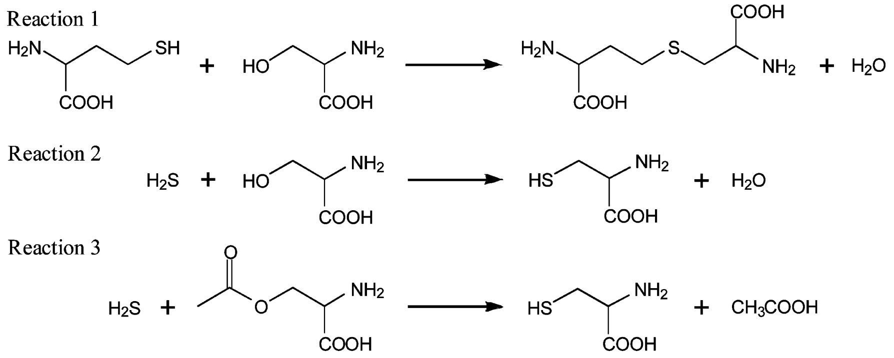
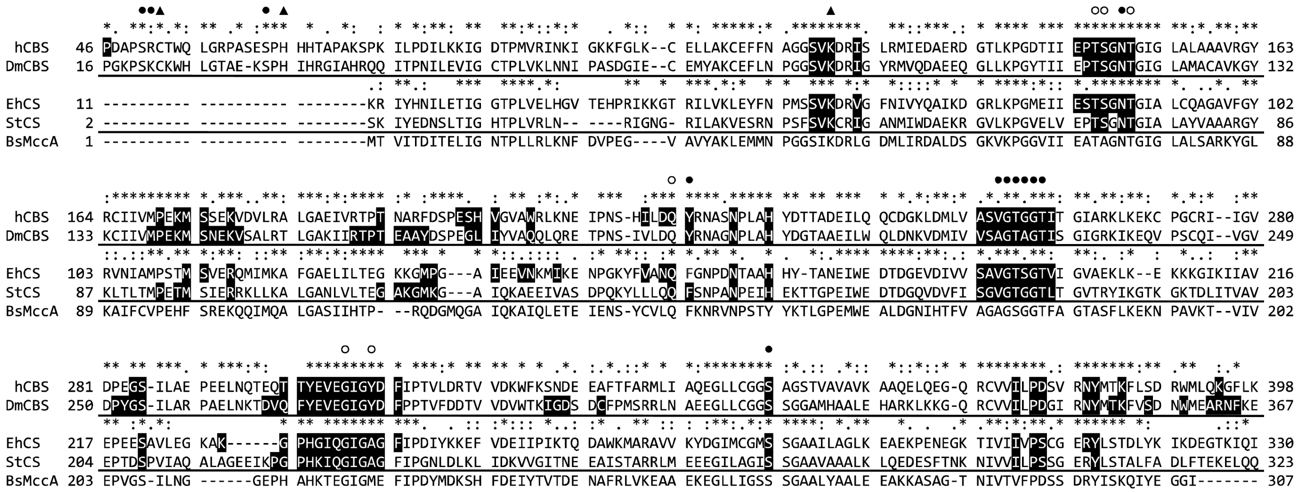
Scheme 1





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           *. **:*.*: ** .* :***  *  . *:  : * *:**: **  **:*::*:*   . *::  * *: *****:* .********.  **::***.: * **** *** ********** **:*.**:**
 hCBS   46 PDAPSRCTWQ LGRPASESPH HHTAPAKSPK ILPDILKKIG DTPMVRINKI GKKFGLK--C ELLAKCEFFN AGGSVKDRIS LRMIEDAERD GTLKPGDTII EPTSGNTGIG LALAAAVRGY 163
DmCBS   16 PGKPSKCKWH LGTAE-KSPH IHRGIAHRQQ ITPNILEVIG CTPLVKLNNI PASDGIE--C EMYAKCEFLN PGGSVKDRIG YRMVQDAEEQ GLLKPGYTII EPTSGNTGIG LAMACAVKGY 132
                                    .: **.:   ***  ****.*:         **  *   ***.*:*  * *  *** *:*  *::::* *  * ****:*:: *.******** *. ..*. **
 EhCS   11 ---------- ---------- --------KR IYHNILETIG GTPLVELHGV TEHPRIKKGT RILVKLEYFN PMSSVKDRVG FNIVYQAIKD GRLKPGMEII ESTSGNTGIA LCQAGAVFGY 102
 StCS    2 ---------- ---------- --------SK IYEDNSLTIG HTPLVRLN-- ----RIGNG- RILAKVESRN PSFSVKCRIG ANMIWDAEKR GVLKPGVELV EPTSGNTGIA LAYVAAARGY  86
BsMccA   1 ---------- ---------- --------MT VITDITELIG NTPLLRLKNF DVPEG----V AVYAKLEMMN PGGSIKDRLG DMLIRDALDS GKVKPGGVII EATAGNTGIG LALSARKYGL  88

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           :********* *.***..**: ***:*:**** :* :****.  : ** :*:.*  ***  :*** ****.***** ** ** ***  * *.*:**:* .*.**.***: **.**:**:  *.*:*  :**
 hCBS  164 RCIIVMPEKM SSEKVDVLRA LGAEIVRTPT NARFDSPESH VGVAWRLKNE IPNS-HILDQ YRNASNPLAH YDTTADEILQ QCDGKLDMLV ASVGTGGTIT GIARKLKEKC PGCRI--IGV 280
DmCBS  133 KCIIVMPEKM SNEKVSALRT LGAKIIRTPT EAAYDSPEGL IYVAQQLQRE TPNS-IVLDQ YRNAGNPLAH YDGTAAEILW QLDNKVDMIV VSAGTAGTIS GIGRKIKEQV PSCQI--VGV 249
           ::.::**.** *:**: ::** :**:*:****  *** *   * *::.:::: . :* **:: :* *.** *.  *  .  *:.**** ****:**:.: *.***.**: **:. :*  :  *..:  :**
 EhCS  103 RVNIAMPSTM SVERQMIMKA FGAELILTEG KKGMPG---A IEEVNKMIKE NPGKYFVANQ FGNPDNTAAH HY-TANEIWE DTDGEVDIVV SAVGTSGTVI GVAEKLK--E KKKGIKIIAV 216
 StCS   87 KLTLTMPETM SIERRKLLKA LGANLVLTEG AKGMKG---A IQKAEEIVAS DPQKYLLLQQ FSNPANPEIH EKTTGPEIWE DTDGQVDVFI SGVGTGGTLT GVTRYIKGTK GKTDLITVAV 203
BsMccA  89 KAIFCVPEHF SREKQQIMQA LGASIIHTP- --RQDGMQGA IQKAIQLETE IENS-YCVLQ FKNRVNPSTY YKTLGPEMWE ALDGNIHTFV AGAGSGGTFA GTASFLKEKN PAVKT--VIV 202

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           ** ** ***. * ***:*:     ********* * ***:* ** ** * * .*. :.*.::* *  *:******** :*.:: .*::  *::*::* * ********.: *******:** .**  :.*
 hCBS  281 DPEGS-ILAE PEELNQTEQT TYEVEGIGYD FIPTVLDRTV VDKWFKSNDE EAFTFARMLI AQEGLLCGGS AGSTVAVAVK AAQELQEG-Q RCVVILPDSV RNYMTKFLSD RWMLQKGFLK 398
DmCBS  250 DPYGS-ILAR PAELNKTDVQ FYEVEGIGYD FPPTVFDDTV VDVWTKIGDS DCFPMSRRLN AEEGLLCGGS SGGAMHAALE HARKLKKG-Q RCVVILPDGI RNYMTKFVSD NWMEARNFKE 367
           ** :*.*:             * ** ******* ***.  . :: :*::: *..: :*  .  ** :: : :**:.*:* ****: *.**    *.  .  .* .**:*:**.* ***** *:          .::*
 EhCS  217 EPEESAVLEG KAK------G PHGIQGIGAG FIPDIYKKEF VDEIIPIKTQ DAWKMARAVV KYDGIMCGMS SGAAILAGLK EAEKPENEGK TIVIIVPSCG ERYLSTDLYK IKDEGTKIQI 330
 StCS  204 EPTDSPVIAQ ALAGEEIKPG PHKIQGIGAG FIPGNLDLKL IDKVVGITNE EAISTARRLM EEEGILAGIS SGAAVAAALK LQEDESFTNK NIVVILPSSG ERYLSTALFA DLFTEKELQQ 323
BsMccA 203 EPVGS-ILNG ------GEPH AHKTEGIGME FIPDYMDKSH FDEIYTVTDE NAFRLVKEAA EKEGLLIGSS SGAALYAALE EAKKASAG-T NIVTVFPDSS DRYISKQIYE GGI------- 307
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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, and small dots indicate semiconservative replacement. Residues with a 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 triangles. The residues hydrogen bonded to the PLP or heme in the crystal structures are marked with filled circles. The residues hydrogen bonded to the substrates or products in the crystal structures are marked with empty circles.

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 *Saccharomyces cerevisiae* and *Trypanosoma 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 maximal activity.[16] Furthermore, in all cases studied, disruption of the bond between the heme and its cysteine thiolate ligand results in a 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 among 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 *S. cerevisiae* on the evolutionary tree of life. *Drosophila melanogaster* is such an organism; therefore, it is valuable to ask whether its CBS contains heme and responds to AdoMet. *D. 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 sequence of the catalytic core region (residues 40−380) of *Dm*CBS is highly similar (85% homologous and 62% identical) to that of 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% homologous and 24% identical); 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 three-dimensional 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 a 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 in 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. On the basis of 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. We prepared ninhydrin reagent by dissolving 0.25 g of ninhydrin in 4 mL of concentrated HCl and 6 mL of glacial acetic acid.

Cloning of *Dm*CBS. The *Dm*CBS sequence was subcloned into a pGEX-6P1 vector following a strategy similar to that used for the human CBS expression construct.[28] The resulting pGEX-6P1-DMCBS allowed 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 the *Dm*CBS polypeptide. Briefly, the *Dm*CBS sequence was amplified by polymerase chain reaction from a first-strand cDNA using a forward primer containing an ApaI site (5′-ctagGGGCCCcaaccgaagccatacgagagg) and a reverse primer containing a NotI site (5′-ctagGCGGCCGCgatcagaactggagaa-cgc). 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 the QIAquick gel extraction kit (Qiagen). Subsequently, *Dm*CBS was ligated into the ApaI- and NotI-linearized pGEX-6P1 vector, from which an internal ApaI site was abolished previously by site-directed mutagenesis.[28] The construct pGEX-6P1-DMCBS was transformed into *Escherichia coli* XL1-Blue cells (Stratagene), and its authenticity was confirmed by DNA sequencing. The 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 via 9% sodium dodecyl sulfate−polyacrylamide gel electrophoresis. The *Dm*CBS-rich fractions were pooled and subsequently concentrated on a YM-30 membrane (Millipore). The buffer was exchanged by pressure dialysis with 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 (reactions 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 buffer (pH 8.6). 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 of 50% (w/v) trichloroacetic acid. The precipitated protein was removed by centrifugation. The supernatant (100 μL) was combined with 100 μL of acetic acid and 100 μL of ninhydrin reagent; the mixture was heated in a boiling water bath for 3 min and immediately cooled in an ice/water mixture. 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 a 5:95 methanol/water mixture. AdoMet-treated *Dm*CBS and hCBS were diluted into and incubated with 0.36 mM AdoMet in 200 mM Tris buffer (pH 8.6) 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 the manufacturer's instructions. Columns were pre-equilibrated with 0.1% acetic acid in a 5:95 methanol/water mixtures, 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 a 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 200000. 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. The 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 and 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 being used. The samples were placed in septum-sealed quartz microcuvettes purged with argon gas. Other reagents were added by injection into a sealed cuvette using a gastight syringe followed by gentle mixing on a vortex mixer. Solutions of sodium dithionite (60 mM $Na_2S_2O_4$) in Ar-degassed Tris buffer

Table 1. Specific Activities of hCBS, hCBS45, *Dm*CBS, and yCBS at pH 8.6 and 37 °C under V_{max} Conditions[a]

	specific activity (units/mg of protein)				AdoMet response	
	without PLP, without AdoMet	without PLP, with AdoMet	with PLP, without AdoMet	with PLP, with AdoMet	without PLP	with PLP
		Serine + Homocysteine → Cystathionine + H_2O (1)				
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
*Dm*CBS	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
		Serine + H_2S → Cysteine + H_2O (2)				
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
		O-Acetylserine + H_2S → Cysteine + CH_3COOH (3)				
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

[a] One unit of activity is defined as the formation of 1 μmol of product in 1 h. 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.

(pH 8.6 and 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 dithionite concentration of 10 mM 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 gastight syringe. Spectra were recorded immediately after the 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 h at pH 6.0.[31,32] Data for 60 min 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 <2 h.

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 and 92 °C at 2 °C intervals. A full spectrum was recorded at each temperature after thermal equilibration for 3 min. A steady decrease in absorbance is followed by a steep increase caused by light scattering of the precipitated protein around the melting point. The absorbance decreases 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 were analyzed to determine the apparent mechanism of CO binding. Briefly, the CO binding reactions were followed spectrophotometrically; complete spectra were recorded at 15 s 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 fit simultaneously using SciPy[33] to the 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 concentration-dependent. b_1 and b_2 are functions of k_1, k_2, and k_3. $b_1 + b_2 = -(k_1 + k_2 + k_3)$; $b_1 b_2 = k_1 k_3$ (see the 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 stimulate *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 (reactions 2 and 3). *Dm*CBS is more active in cystathionine synthesis than hCBS in the absence of AdoMet, exhibiting activity comparable to that of 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 that of 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 because of 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.

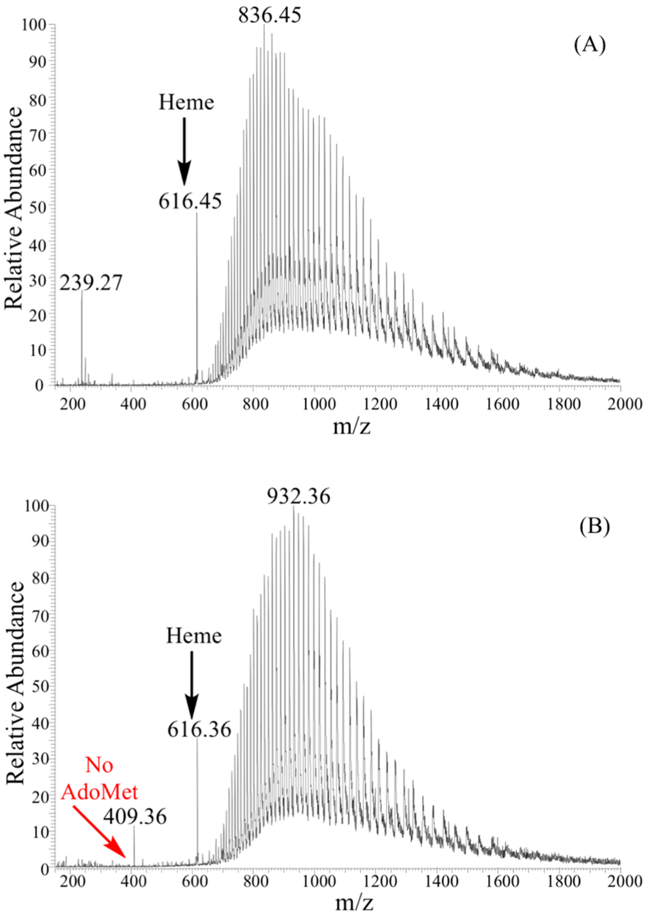

Figure 2. Ion trap mass spectra of as-isolated (A) and AdoMet-treated (B) *Dm*CBS. The mass peak at *m*/*z* 616 is heme. The lack of a mass peak at *m*/*z* 399 indicates the absence of AdoMet. The other low-mass peaks at *m*/*z* 239 and 409 are common plasticizers that were present in the respective background solvent samples.

The FT data reveal a mass of 56877 Da for the *Dm*CBS protein (Figure S1 of the Supporting Information); the calculated mass of the heme-free protein is 56882 Da. Heme dissociates upon ionization and appears as a characteristic mass peak at *m*/*z* 616 (FePPIX calculated mass of *m*/*z* 616.487). Control AdoMet solutions under similar experimental conditions exhibit a peak at *m*/*z* 399 (AdoMet calculated mass of *m*/*z* 398.14). Neither *Dm*CBS sample showed a mass peak at *m*/*z* 399, suggesting that *Dm*CBS does not bind to AdoMet. The same mass spectrometric analysis was performed using hCBS as a control, because hCBS is known to bind AdoMet. The AdoMet mass peak was absent from 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 *Dm*CBS. 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 nm (δ band), 429 nm (Soret or γ band), and 552 nm (α–β bands). Identical heme absorption features are observed at pH 6.0 (Figure 4B, solid

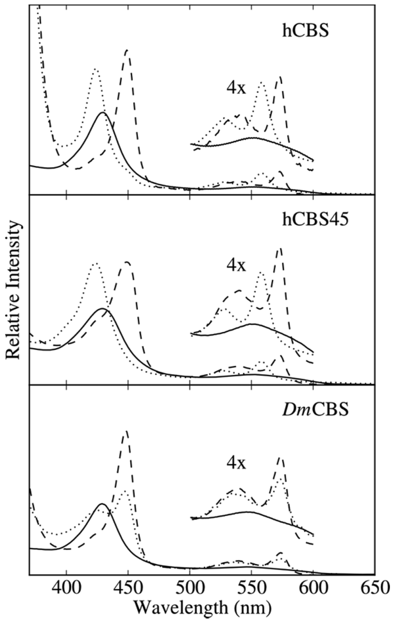

Figure 3. Optical spectra of the Fe(III)- (—), Fe(II)- (–––), and ligand-switched (···) states of hCBS (pH 9.0), hCBS45 (pH 9.0), and *Dm*CBS (pH 8.6). In the case of *Dm*CBS, the ligand switch is incomplete and the spectrum shows a mixture of switched and unswitched species (see the text). Peak positions are summarized in Table 2.

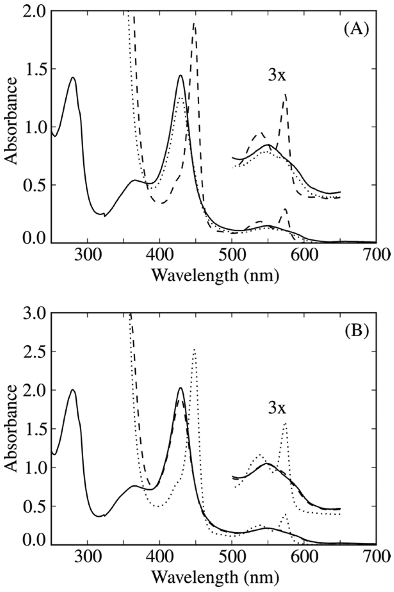

Figure 4. Electronic absorption spectra showing pH-dependent reversible conversion between Fe(III)- and Fe(II)*Dm*CBS. Peak positions are summarized in Table 2: (A) (—) 5 μM Fe(III) *Dm*CBS in 200 mM Tris buffer (pH 8.6), (–––) heme iron reduced to the Fe(II) state by 10 mM sodium dithionite, and (···) heme iron reoxidized to Fe(III) after the pH is lowered to 6.0 via addition of 1.5 M MES buffer (pH 6.0) and (B) (—) 5 μM Fe(III)*Dm*CBS in 200 mM MES buffer (pH 6.0), (–––) heme iron remaining in the Fe(III) state after the addition of dithionite, and (···) heme iron reduced to the Fe(II) state after the pH had been increased to 8.6 by addition of 1.5 M Tris buffer (pH 8.6).

line). The A_{429}/A_{282} ratio is 1.02, which is similar to that of hCBS (1.07). On the basis of the spectral similarity to Fe(III)hCBS,[39] the as-isolated *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, respectively (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 4B, 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 those for 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 4A, dashed line to dotted line). Conversely, when Fe(III)*Dm*CBS at pH 6.0 in the presence of excess dithionite was subjected to a pH shift by addition of concentrated pH 8.6 buffer, the protein was immediately reduced to Fe(II)*Dm*CBS (Figure 4B, 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 of the Supporting Information). These observations suggest that the *Dm*CBS heme behaves like 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 *Dm*CBS. 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 with an unidentified neutral ligand, leading to a blue-shifted Soret band at 424 nm (Figure 3). Fe(II)hCBS and Fe(II)hCBS45 undergo this ligand-switch process with midpoint transition temperatures of 44 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 that proceeds with a midpoint transition temperature of 37 °C (Figure S5 of the Supporting Information). 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, both the newly formed ligand-switched species [Fe(II)*Dm*CBS-424] and the original Fe(II)*Dm*CBS protein denature upon being heated for extended periods (>3 h). Because *Dm*CBS appeared to be denatured 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 and 51 °C, respectively (see the 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 the 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 for *Dm*CBS and 420 nm for 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 that the pH is high enough to stabilize

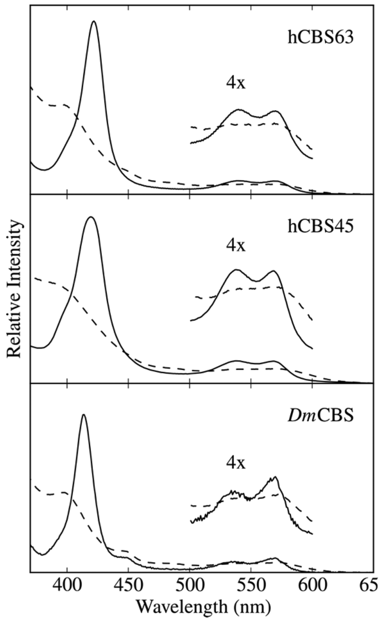

Figure 5. Electronic absorption spectra of the CO adducts (—) and NO adducts (– – –) of hCBS (pH 9.0), hCBS45 (pH 9.0), and *Dm*CBS (pH 8.6). In the case of *Dm*CBS, a small amount of unreacted Fe(II)*Dm*CBS remains present, as evidenced by the shoulder at 450 nm and the greater intensity of the α band.

Table 2. Peak Positions (nanometers) in the Absorption Spectra of *Dm*CBS, hCBS, and hCBS45

	Soret (γ), β, α		
	hCBS	hCBS45	*Dm*CBS
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

the reduced protein: pH 8.6 for *Dm*CBS and 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 order (CO and then dithionite or dithionite and then 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, and 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 ref 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 decreases without a concomitant increase in the magnitudes of other bands.

A kinetic analysis of the absorbance data for these CO binding reactions was conducted as described in Materials and Methods. The results are listed in Table S1 of the Supporting Information. The data for the reaction of Fe(III)hCBS, with dithionite added before CO at pH 9.0, are best fit by a single-exponential equation. This fit gives a k_3 of 0.016 min^{-1}, the rate constant for binding of CO to the reduced CBS heme. This rate is remarkably slow, presumably because the thiolate ligand must dissociate from the heme 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) and Fe(II) oxidation states, and an irreversible gas binding process in which CO binds only to the Fe(II) state. The fact 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 ($k_{\text{obs}} = k_1k_3$) for CO binding is significantly faster at low pH (0.053 min^{-1} for hCBS and 0.094 min^{-1} for *Dm*CBS) than at high pH (0.0064 min^{-1} for hCBS and 0.0015 min^{-1} for *Dm*CBS). These two proteins exhibit 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 band 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 electronic absorption spectra (data not shown) identical to those of the corresponding CO adducts formed from non-ligand-switched, native thiolate-ligated Fe(II) species (Figure 5). Because 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 band at 424 nm) and unconverted Fe(II)*Dm*CBS species (Soret band at 448 nm) (Figure 3). Immediately upon addition of CO, the 424 nm Soret band disappeared with the appearance of a new peak at 414 nm. The remaining unconverted Fe(II)*Dm*CBS (Soret band at 448 nm) 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, five-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 band at 395−397 nm and a broad flat band in the $\alpha-\beta$ region (Table 2 and Figure 5). These spectra are indicative of a five-coordinate heme bearing a single NO axial ligand in each of the three proteins.

■ DISCUSSION

There are several lines of evidence suggesting a correlation among 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 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% of the activity of the wild-type enzyme) and possesses an intact C-terminal regulatory region.[16] These three pieces of evidence plausibly imply a correlation between the presence of heme and the responsiveness to AdoMet. There is also evidence of 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 His/Cys ligation is retained, and the resulting Co(III)hCBS enzyme has activity comparable to that of Fe(III)hCBS. Reduction of Co(III) to Co(II) induces the loss of the cysteine ligand and the loss of enzyme activity.[17]

Other results are inconsistent with a correlation among 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 (*D. 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 sulfur of Cys22.[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 a 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 to be 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 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, for which 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 in which 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 the enzyme is 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 substrates to synthesize cysteine (reactions 2 and 3), whereas cysteine synthase (CS) must use *O*-acetylserine (reaction 3 only). CBS enzymes are approximately 2 orders of magnitude slower than CS enzymes, whose rates are on the order of 10^3−10^4 units/mg of protein.[47−58] Furthermore, a CBS-like protein, *Bs*MccA from *B. 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 reach 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 to influence the kinetics of and specificity toward cystathionine synthesis and serine deamination,[60] and active site variants of hCBS exhibit different effects on cystathionine synthesis with substrates 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 to pH. Among the hemoproteins that bind small molecule ligands for sensing or transport, few are known to exhibit pH-sensitive ligand binding processes. Typically, in these pH-sensitive proteins, the binding rate is faster at high pH.[65−69] There are also cases in which 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 forms only 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 a decreased pH, CBS is not the only example of this. The rates of binding of CO to neuroglobin and *Nassa mutabilis* myoglobin increase with a decrease in pH because of 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 a decrease in the 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 analogues 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 heme-containing 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–iron bond correlates with a 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 in 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. On the basis of 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.

ASSOCIATED CONTENT

Ⓢ Supporting Information

High-resolution FT-IC MS analysis of as-isolated and AdoMet-treated *Dm*CBS (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 (Figures S3–S5), mathematical derivation of the kinetic model for the CBS CO binding reaction, the CO binding process of *Dm*CBS at pH 6.0 monitored by electronic absorption spectroscopy (Figure S6), and apparent rate constants for CO binding reactions of hCBS, hCBS45, and *Dm*CBS (Table S1). This material is available free of charge via the Internet at http://pubs.acs.org.

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Notes

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ABBREVIATIONS

CBS, cystathionine *β*-synthase; hCBS, full-length human CBS enzyme; hCBS45, truncated (Δ414–551) variant of the human CBS enzyme; yCBS, *S. cerevisiae* CBS enzyme; *Dm*CBS, *D. melanogaster* CBS enzyme; CS, cysteine synthase; OASS, *O*-acetylserine sulfhydrylase; AdoMet, *S*-adenosylmethionine; DTT, dithiothreitol; BSA, bovine serum albumin; PLP, pyridoxal 5-phosphate; Tris, tris(hydroxymethyl)-aminomethane; MES, 2-(*N*-morpholino)ethanesulfonic acid; CHES, *N*-(cyclohexyl)-2-aminoethanesulfonic acid.

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