Ferrous Human Cystathionine β-Synthase Loses Activity during Enzyme Assay Due to a Ligand Switch Process†

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ABSTRACT: Cystathionine β-synthase (CBS) is a pyridoxal-5′-phosphate-dependent enzyme that catalyzes the condensation of serine and homocysteine to form cystathionine. Mammalian CBS also contains a heme cofactor that has been proposed to allosterically regulate enzyme activity via the heme redox state, with Fe^{II} CBS displaying approximately half the activity of Fe^{III} CBS *in vitro*. The results of this study show that human Fe^{II} CBS spontaneously loses enzyme activity over the course of a 20 min enzyme assay. Both the full-length 63-kDa and truncated 45-kDa form of CBS slowly and irreversibly lose activity upon reduction to the Fe^{II} form. Additionally, electronic absorption spectroscopy reveals that Fe^{II} CBS undergoes a heme ligand exchange to Fe^{II} CBS424 when the enzyme is incubated at 37 °C and pH 8.6. The addition of enzyme substrates or imidazole has a moderate effect on the rate of the ligand switch, but does not prevent conversion to the inactive species. Time-dependent spectroscopic data describing the conversion of Fe^{II} CBS to Fe^{II} CBS424 were fitted to a three-state kinetic model. The resultant rate constants were used to fit assay data and to estimate the activity of Fe^{II} CBS prior to the ligand switch. Based on this fit it appears that Fe^{II} CBS initially has the same enzyme activity as Fe^{III} CBS, but Fe^{II} CBS loses activity as the ligand switch proceeds. The slow and irreversible loss of Fe^{II} CBS enzyme activity *in vitro* resembles protein denaturation, and suggests that a simple regulatory mechanism based on the heme redox state is unlikely.

Mammalian cystathionine β-synthase (CBS[1]) is the only known PLP-dependent enzyme that also contains a heme cofactor. CBS catalyzes the condensation of homocysteine with serine to form cystathionine as the first committed step in the transsulfuration pathway. Inborn errors that cause a deficiency in CBS activity allow an accumulation of homocysteine known as homocystinuria and are associated with skeletal and vascular abnormalities, eye lens dislocation, and mental retardation (*1*). Over 100 pathogenic mutations have now been identified in the gene that codes for human CBS (*2*), with 140 listed on a continuously updated CBS website at http://www.uchsc.edu/cbs/cbsdata/cbsmain.htm. Because homocysteine is a toxic metabolite, even a small increase in plasma homocysteine levels constitutes a condition known as hyperhomocysteinemia, which is associated with an increased risk for Alzheimer's, Parkinson's, and cardiovascular disease, as well as stroke and neural tube defects (*3*−*6*). Although about 50% of patients with CBS deficiency can be treated by administration of vitamin B_6 (a PLP precursor), the nonresponsive remainder are often more severely afflicted and require a methionine-restricted diet to minimize the accumulation of homocysteine (*1*, *7*). Understanding the biochemical behavior of CBS, including its regulation, could help to suggest new treatment strategies for patients with CBS deficiency.

CBS is a homotetramer of 63-kDa monomers, each of which contains a PLP and heme *b* cofactor (iron protoporphyrin IX) in relatively close proximity, with the closest approach between the heme edge and the phosphate at 14 Å (*8*). Although PLP is required to perform the standard β-replacement reaction which generates cystathionine, the heme cofactor does not participate directly in the enzymatic mechanism (*9*−*12*). CBS from lower organisms, such as yeast and *Trypanosoma cruzi*, does not contain heme but accomplishes the same catalytic reaction (*13*−*15*). Full-length human CBS consists of 551 amino acids that comprise an N-terminal heme-binding domain, a central catalytic core, and a C-terminal autoinhibitory domain (*16*). Two protein folding motifs within the autoinhibitory domain have been termed "CBS domains" and are found in a number of other proteins unrelated to CBS (*17*, *18*). Binding of AdoMet to the autoinhibitory domain allosterically regulates CBS by

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[1] Abbreviations: AdoMet, *S*-adenosylmethionine; BSA, bovine serum albumin; CBS, cystathionine β-synthase, independently identified as the heme protein H450; CooA, a CO-sensing heme protein in *Rhodospirillum rubrum* that regulates the transcription of a CO-oxidation system; Fe^{II} CBS424, an inactive form of Fe^{II} CBS that has undergone a ligand switch; Fe^{II} CBS449, a fully-active form of Fe^{II} CBS present immediately after heme reduction; FixL, an O_2-sensing heme protein in rhizobia that regulates gene expression associated with N_2 fixation; MES, 2-(*N*-morpholino)ethanesulfonic acid; PLP, pyridoxal 5′-phosphate; sGC, soluble guanylyl cyclase, an NO-sensing heme protein that catalyzes the conversion of GTP to cGMP; TLC, thin-layer chromatography; Tris, tris(hydroxymethyl)aminomethane.

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increasing the enzyme activity about 3-fold (*19*, *20*). A truncated 45-kDa form of CBS (1−413) lacking the autoinhibitory domain has been expressed in *Escherichia coli* and successfully crystallized (*21*, *22*), yielding an X-ray crystal structure that strongly resembles *O*-acetylserine sulfhydrylase, another member of the fold type II family of PLP enzymes (*23*). Unlike full-length CBS, the 45-kDa form is a homodimer with about 3-fold greater enzyme activity and no responsiveness to AdoMet binding (*24*, *25*).

In the absence of an O_2-binding, electron transport, or catalysis function (*26*), allosteric regulation seems the most probable role for the CBS heme. Fe^{III} CBS does not bind exogenous ligands and appears to be a poor target for regulation (*9*, *27*, *28*), but Fe^{II} CBS is able to bind CO, NO, CN^-, and various isonitriles (*9*, *28*−*30*). Several studies have revealed that changes at the heme site generated by gaseous ligand binding do affect CBS activity. CO or NO binding to Fe^{II} CBS causes a complete loss in enzyme activity (*9*, *30*). In both the Fe^{II}−CO and −NO species the native Cys^{52} ligand has been displaced, suggesting that disruption of the Fe−Cys^{52}(thiolate) bond is ultimately responsible for the loss of enzyme activity. Scission of the Fe−Cys^{52} bond can also be achieved by $HgCl_2$ chelation of the cysteine, creating a similarly inactive enzyme (*31*). Clearly changes at the heme site are communicated to the PLP active site and modulate enzyme activity, which supports an allosteric role for the CBS heme.

The observation that Fe^{II} CBS displays only half-maximal enzyme activity has led to the hypothesis that CBS is regulated by the heme redox state (*32*). This hypothesis is particularly appealing because homocysteine sits at the junction between two metabolic pathways where the flux is determined by the cellular redox potential. Under reducing conditions methionine synthase is active and converts homocysteine into methionine as part of the transmethylation cycle (*33*). Conversely, under oxidizing conditions methionine synthase becomes inactive (*33*) and CBS is fully active, allowing the competing transsulfuration pathway to dominate. Cysteine, generated as the final product in the transsulfuration pathway, is a key metabolite in the synthesis of glutathione, one of the primary determinants of cellular redox potential (*34*, *35*). Therefore, when the cellular redox potential becomes too oxidizing homocysteine is diverted toward a pathway which generates more glutathione and restores a reducing cellular potential. Deactivation of CBS via heme reduction could provide another mechanism that helps favor the transmethylation pathway under reducing conditions. Several studies using cultured liver cells support this hypothesis and have shown that cystathionine synthesis is increased in the presence of cellular oxidants and decreased in the presence of cellular antioxidants (*34*, *36*−*38*). However, it has not been shown whether decreased cystathionine production is due to reduction and inactivation of Fe^{III} CBS, or depletion of the homocysteine pool by fully activated methionine synthase.

We have previously reported that the reduction behavior of CBS is more complicated than initially anticipated, demonstrating both pH- and temperature-dependence. At pH 6.5 and in the presence of excess sodium dithionite, CBS is initially reduced to Fe^{II} CBS but subsequently reoxidizes to an equilibrium mixture of the Fe^{II} and Fe^{III} redox states (*39*). The position of the equilibrium is reversibly controlled by pH. At pH 9 the protein appears fully reduced, but acidification to pH 6.5 causes the protein to reoxidize to Fe^{III} CBS. Heating Fe^{II} CBS to 55 °C at pH 9 enables a ligand switch that blue-shifts the heme Soret band maximum from 449 nm to 424 nm and irreversibly destroys enzyme activity (*40*). The change in the Soret band maximum indicates that the native Cys^{52} ligand has been replaced by some other neutral donor ligand, which may explain the loss of enzyme activity. This 424 nm species (termed Fe^{II} CBS424) cannot be converted into the original 449 nm species by altering the temperature, pH, or redox state.

We report herein that Fe^{II} CBS undergoes a time-dependent loss of activity at pH 8.6 and 37 °C, most likely through spontaneous conversion to the inactive Fe^{II} CBS424 species. Spectroscopic observation of Fe^{II} CBS under pseudo-assay conditions reveals a slow and irreversible ligand switch to the 424 nm species which can be modestly affected by the presence of enzyme substrates or heme ligands. Through kinetic analysis of both the enzymatic and spectroscopic data we have determined that the initial 449 nm Fe^{II} CBS species has the same enzymatic activity as Fe^{III} CBS. It is the subsequent ligand switch process that causes the gradual loss of enzyme activity that is observed on the assay time scale. The odd behavior of Fe^{II} CBS suggests that allosteric regulation by the heme is unlikely to be accomplished by a simple redox-controlled mechanism.

MATERIALS AND METHODS

Materials. L-[U-^{14}C]Serine (150 mCi/mmol) was purchased from Perkin-Elmer. Titanium(III) chloride was purchased from Acros Organics. Sodium dithionite (99.9+ % purity) was purchased from Fluka. All other chemicals were purchased from Sigma-Aldrich and used as obtained. Homocysteine was prepared from homocysteine thiolactone by base hydrolysis, after which the solution pH was adjusted to 8.6 (*41*). Cellulose thin-layer chromatography (TLC) plates were purchased from Selecto Scientific. Yeast CBS and the 63- and 45-kDa isoforms of recombinant human CBS were overexpressed in *E. coli* and purified to homogeneity as previously reported (*14*, *19*, *42*). The protein concentration of each purified CBS preparation was determined by the Lowry method (*43*), using bovine serum albumin (BSA) as a standard, and the heme concentration in the human CBS isoforms was measured using the pyridine hemochromagen assay (*44*).

CBS Enzymatic Assays. Anaerobic assays of human or yeast CBS enzyme activity were performed in a glovebox under N_2 as previously described (*40*), with a few modifications. For time-dependent assay experiments, 40-μL aliquots were removed from a 440-μL assay mixture at 2 min intervals and added to a new tube containing 10 μL of ice-cold 50% trichloroacetic acid to precipitate the protein and stop the reaction. All assay mixtures contained 100 mM buffer (Tris at pH 8.6 or triethanolamine at pH 7.4), 250 μM PLP, 0.1 mg/mL BSA, 5−25 μg/mL CBS, 10 mM [^{14}C]-serine (400 cpm/nmol), and 10 mM homocysteine. Fe^{II} CBS samples utilized 10 mM sodium dithionite as a heme reductant. Select samples also contained 150 μM AdoMet or 50−150 mM imidazole, as described in the figure legend for each experiment. All nonenzyme assay components, including substrates, effectors, and sodium dithionite, were

combined and preincubated at 37 °C for 5 min before addition of CBS to start the assay. Assay mixtures containing 10 mM sodium dithionite formed Fe^{II} CBS within seconds when the human enzyme was added. Cystathionine was isolated from the assay mixture by ascending cellulose TLC in 2-propanol/formic acid/H_2O (80:6:20), conducted outside of the glovebox. The product spot was excised and counted in 10 mL of BioSafeII scintillation cocktail on a Beckman LS-6500 scintillation counter. All assays were performed in triplicate, and the standard deviation of each set is reported as error. One unit of activity is defined as the amount of CBS required to catalyze the formation of 1 μmol of cystathionine in 1 h at 37 °C.

Electronic Absorption Spectroscopy. The time- and temperature-dependent conversion of Fe^{II} CBS to the Fe^{II} CBS424 species was followed spectroscopically on a double-beam Cary 4 Bio spectrophotometer equipped with a Peltier temperature controller and set to a spectral bandwidth of 0.5 nm. Protein solutions were prepared in Ar-sparged buffer (100 mM Tris at pH 8.6 or 100 mM triethanolamine at pH 7.4) with various combinations of AdoMet, substrate, and imidazole added. The samples were placed in septum-sealed quartz microcuvettes with the headspace subsequently purged by Ar flow before equilibration to 37 °C. Spectra were recorded at 30 s intervals started immediately after the anaerobic injection of aqueous sodium dithionite to 2 mM via gastight syringe. Experiments run for more than 120 min were reduced with 10 mM dithionite and were scanned at longer intervals. The CBS concentration in the spectroscopic samples was generally 5−10 times greater than that used for the enzymatic assays and was kept low (1−5 μM heme) to more closely approximate assay conditions. BSA was included to maintain a constant overall protein concentration (0.12 mg/mL). Complete conversion to the Fe^{II} CBS424 species was accomplished at the end of each kinetic experiment by heating the reduced sample at 55 °C for 10 min and equilibrating back to 37 °C for 8 min before recording the final spectrum.

Single-Wavelength Fits of the Kinetic Data. The rate at which Fe^{II} CBS was transformed into Fe^{II} CBS424 was fitted by a single-wavelength method which utilized the Solver function in Microsoft Excel 2002 (a linear least-squares approach) to find values for the rate constants that generated minimal residuals between the predicted and experimental absorbance measurements. The decreasing absorbance at 449 nm, corresponding to the loss of the Fe^{II} CBS449 species, was fitted using a triexponential decay eq 1. The absorbance at time infinity (Abs_∞) was supplied experimentally using spectra of Fe^{II} CBS424 forced to full conversion by heat-treatment (*40*). Values α, β, and γ are collections of constants that include the absorbance at time zero, the rate constants, and the extinction coefficient of each species.

$$Abs_t = Abs_\infty + \alpha \cdot e^{-k_1 t} + \beta \cdot e^{-k_2 t} + \gamma \cdot e^{-k_3 t} \quad (1)$$

Calculated Fit of Fe^{II} CBS Specific Activity. The specific activity of each Fe^{II} CBS species present during Fe^{II} CBS424 formation was estimated by fitting the time-dependent assay data with an equation derived from the kinetic analysis of the Fe^{II} CBS spectroscopic data. Since the time-dependent assay experiments were conducted for 20 min, only Fe^{II} CBS449 and the two intermediates (I_1 and I_2) were expected to be present and contributing to cystathionine production. Rate equation 2 describes the μmol of cystathionine produced per minute based on the concentration of each species at time t, the sample volume (V), and the specific activity of Fe^{II} CBS449 (SA_0), I_1 (SA_1), and I_2 (SA_2). The specific activity of each Fe^{II} CBS species is a constant of unknown magnitude to be determined by the fit.

$$\text{rate} = [Fe^{II}\ CBS449]_t \cdot SA_0 \cdot V + [I_1]_t \cdot SA_1 \cdot V + [I_2]_t \cdot SA_2 \cdot V \quad (2)$$

$$[Fe^{II}\ CBS449]_t = [Fe^{II}\ CBS449]_0 \cdot e^{-k_1 t} \quad (3)$$

$$[I_1]_t = [Fe^{II}\ CBS449]_0 \cdot \frac{k_1}{k_2 - k_1}(e^{-k_1 t} - e^{-k_2 t}) \quad (4)$$

$$[I_2]_t = [Fe^{II}\ CBS449]_0 - [Fe^{II}\ CBS449]_t - [I_1]_t \quad (5)$$

$$\text{micromoles of cystathionine at time } t = \int_0^t \text{rate} = [Fe^{II}\ CBS449]_0 \cdot V \cdot \left(\frac{SA_0 + SA_1 \cdot \frac{k_1}{k_2 - k_1} - SA_2 - SA_2 \cdot \frac{k_1}{k_2 - k_1}}{k_1}(1 - e^{-k_1 t}) + \frac{k_1(SA_1 - SA_2)}{k_2(k_2 - k_1)}(e^{-k_2 t} - 1) + SA_2 \cdot t \right) \quad (6)$$

Equations 3−5 describe the concentration of each Fe^{II} CBS species at time t (*45*). No significant accumulation of the final species is expected over 20 min, so k_3 and the concentration of Fe^{II} CBS424 were disregarded to simplify the equation. Rate equation 2 was then integrated over the 0 → t time interval to yield eq 6, which calculates the amount of cystathionine produced at a specific time point, accounting for the changing concentration of each Fe^{II} CBS species as Fe^{II} CBS converts to Fe^{II} CBS424. The integrated equation was then fitted to the assay data to solve for the specific activity of each Fe^{II} CBS species, using the Solver function in Microsoft Excel 2002 to minimize the residuals created by the difference between the predicted and measured amount of cystathionine produced at each time point. The specific activity of Fe^{II} CBS449, I_1, and I_2 were successfully fitted either without constraints or by setting the activity of Fe^{II} CBS449 equal to that of Fe^{III} CBS. The specific activities reported for Fe^{II} CBS424, I_1, and I_2 are an average between the values calculated by these two methods, with the average deviation shown as the error. Other constraints (such as assuming I_1 and I_2 have equal activity) did not produce viable fits.

RESULTS

The 63- and 45-kDa Forms of Fe^{II} CBS Show a Time-Dependent Loss of Enzyme Activity under a Variety of Experimental Conditions. Time-dependent assay experiments performed at pH 8.6 and 37 °C revealed that Fe^{II} CBS gradually loses activity, as shown by a decrease in cystathionine production over time (Figure 1). At the CBS concentrations used in this study, the amount of cystathionine produced by Fe^{III} CBS is linear with time over 20 min when the activity is measured aerobically or anaerobically. Fe^{II}

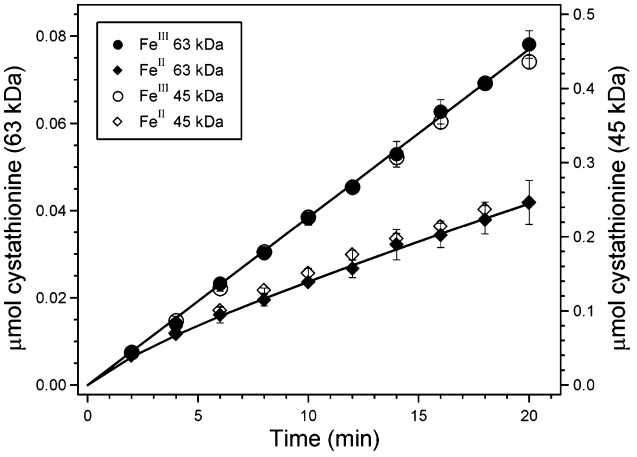


FIGURE 1: Comparison of cystathionine production by Fe^{II} and Fe^{III} CBS, measured by anaerobic enzyme assay. Cystathionine production by Fe^{II} CBS decreases over time; when the activities of the 63- and 45-kDa forms are plotted on the left and right axes, respectively, it becomes apparent that the fractional loss of Fe^{II} CBS activity is identical for the two proteins. No loss of cystathionine production occurs for Fe^{III} CBS of either form. Fe^{II} CBS was generated at the start of the assay by addition of enzyme (11 μg/mL 63-kDa CBS or 7.7 μg/mL 45-kDa CBS, final concentration) to an assay mixture (buffer, PLP, BSA, and substrates, as described in Materials and Methods) containing 10 mM sodium dithionite at pH 8.6 and 37 °C. Error bars were calculated from the standard deviation of three replicate measurements; if no bars appear, the magnitude of the error is smaller than the plotted point. Solid lines represent calculated fits to the 63-kDa CBS data.

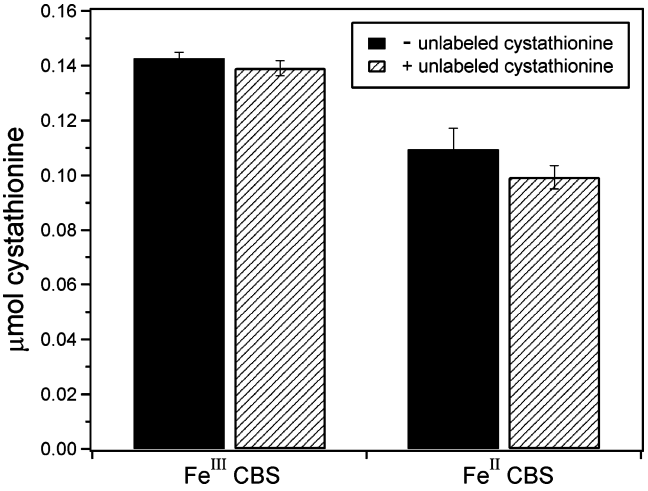


FIGURE 2: Product inhibition of 63-kDa CBS by unlabeled cystathionine has less effect on enzyme activity than reduction of the CBS heme from Fe^{III} to Fe^{II}. The inclusion of 0.050 μmol (250 μM) unlabeled cystathionine with either Fe^{III} or Fe^{II} CBS caused a minimal decrease in ^{14}C-labeled cystathionine production, as compared to the loss of activity caused by heme reduction. Fe^{II} CBS was generated at the start of the assay by addition of enzyme (11 μg/mL 63-kDa CBS, final concentration) to an assay mixture (buffer, PLP, BSA, and substrates) containing 10 mM sodium dithionite and 0 or 250 μM unlabeled cystathionine at pH 8.6. Assays were conducted for 20 min at 37 °C. Error bars were calculated from the standard deviation of three replicate measurements.

CBS, prepared by addition of Fe^{III} CBS to an assay solution containing 10 mM dithionite, initially produces cystathionine at the same rate as the Fe^{III} enzyme, but cystathionine production falls off over the 20-min assay. Plotting cystathionine production by the 63- and 45-kDa enzyme forms on separate *y*-axes effectively overlays the assay data, revealing an identical fractional loss in Fe^{II} CBS activity for both enzyme forms. This result indicates that the C-terminal AdoMet-binding domain of CBS does not play a role in the spontaneous loss of Fe^{II} CBS enzyme activity.

The loss of Fe^{II} CBS activity is due to reduction of the CBS heme, as confirmed by a series of control experiments. Fe^{III} CBS assayed in the presence of excess salt (NaCl or Tris) did not lose activity over time, suggesting that the effect is specific to dithionite. The loss of Fe^{II} CBS activity was independent of the dithionite concentration, providing there was enough reductant present to keep the heme reduced. Assays performed with 2 or 10 mM dithionite showed the same time-dependent loss of Fe^{II} CBS activity (data not shown). However, assay samples with only 2 mM dithionite occasionally showed some heme reoxidation, as determined by electronic absorption spectroscopy. The addition of 10 mM dithionite to yeast CBS (no heme) caused only a small loss in activity over time (Supplemental Figure 1, Supporting Information). Dithionite or its oxidation products may directly affect CBS activity to a minor extent, but the significant loss in activity seen when dithionite is added to human CBS requires heme reduction. Several experiments were also conducted using titanium(III) citrate as the heme reductant (*46*), but the significant inhibition observed with yeast CBS (Supplemental Figure 1) led us to suspect that the titanium ion may interfere with the assay, making it a poor choice for use as a heme reductant in these experiments. Consequently, all further studies were carried out with dithionite as the reductant.

The loss of Fe^{II} CBS enzyme activity cannot be explained by increased sensitivity to product inhibition. Unlabeled cystathionine was added to Fe^{III} and Fe^{II} CBS assay samples to determine the extent of product inhibition, as determined by a reduction in the amount of ^{14}C-labeled cystathionine produced during a 20 min assay. Product inhibition was observed when 0.050 μmol (250 μM) of unlabeled cystathionine was included in the assay of 63-kDa CBS. The Fe^{III} and Fe^{II} forms each display a small decrease in ^{14}C-labeled cystathionine production when unlabeled cystathionine is included. However, reduction of the heme from Fe^{III} to Fe^{II} CBS causes a comparatively greater loss in cystathionine production, regardless of whether unlabeled cystathionine is included (Figure 2). The 45-kDa form of CBS was also assayed in the presence of unlabeled cystathionine, and no significant difference in product inhibition was detected between the two isoforms (data not shown). These data suggest the Fe^{III} and Fe^{II} forms of CBS may have slightly different sensitivities to product inhibition, but not enough to account for the significant decrease in activity observed when the CBS heme is reduced from Fe^{III} to Fe^{II}.

Activation of 63-kDa CBS by AdoMet did not prevent the loss of Fe^{II} CBS activity. The addition of 150 μM AdoMet to the 63-kDa form of CBS increased the activity of both the Fe^{III} and Fe^{II} protein by $\sim$ 3-fold, but the same time-dependent loss of Fe^{II} CBS activity was observed over the course of the experiment (Supplemental Figure 2, Supporting Information). This result reveals that activation of 63-kDa CBS by AdoMet does not prevent the spontaneous loss of Fe^{II} CBS enzyme activity.

Fe^{II} CBS assayed at pH 7.4 shows a more severe loss of enzyme activity over time than Fe^{II} CBS assayed at pH 8.6.

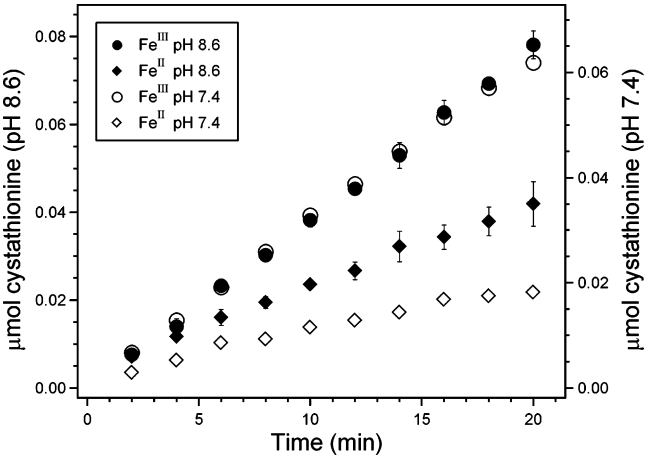


FIGURE 3: 63-kDa Fe^{II} CBS exhibits a greater loss of activity at pH 7.4 (right axis) than pH 8.6 (left axis). No loss of cystathionine production occurs over time for Fe^{III} CBS assayed at either pH. Fe^{II} CBS was generated at the start of the assay by addition of enzyme to an assay mixture (buffer, PLP, BSA, and substrates) containing 10 mM sodium dithionite at 37 °C. The pH 8.6 and pH 7.4 samples each contained 11 μg/mL 63-kDa CBS.

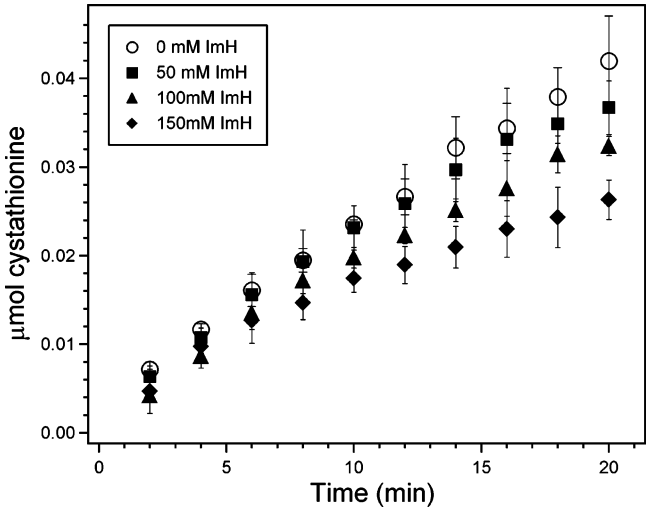


FIGURE 4: Exogenous imidazole accelerates the loss of 63-kDa Fe^{II} CBS enzyme activity in a concentration-dependent manner. Fe^{II} CBS was generated at the start of the assay by addition of enzyme (11 μg/mL final concentration) to an assay mixture (buffer, PLP, BSA, and substrates) containing 10 mM sodium dithionite and 0, 50, 100, or 150 mM imidazole at pH 8.6 and 37 °C.

The exact pH of the cytosolic microenvironment in which CBS functions is not known, although the pH is likely to be significantly lower than 8.6; therefore, pH 7.4 was chosen to represent generic physiological conditions. Time-dependent Fe^{III} and Fe^{II} CBS assays conducted at pH 7.4 show the same overall behavior observed at pH 8.6, with cystathionine production by Fe^{III} CBS linear over time while cystathionine production by Fe^{II} CBS gradually falls off (Figure 3). Since CBS enzyme activity is highly pH-dependent, assays are typically conducted at pH 8.6, the point of maximum activity. Although pH 7.4 may be more physiologically relevant, CBS assayed at pH 7.4 displays only half the maximal activity (*39*). When the pH 8.6 and pH 7.4 assay data are overlaid using separate *y*-axes, the Fe^{III} data for the two pH regimes overlaps, effectively canceling out the effect of pH on the PLP active-site of CBS. It becomes clear upon this analysis that Fe^{II} CBS loses activity faster at pH 7.4. There are several possible explanations for the accelerated loss of Fe^{II} CBS activity at pH 7.4. Fe^{II} CBS at pH 7.4 may undergo the same time-dependent loss of Fe^{II} CBS activity previously described at pH 8.6, albeit slightly faster due to some effect of the lower pH. Alternately, there may be an additional process that contributes to the loss of pH 7.4 enzyme activity over time (*vide infra*).

Reoxidation of Fe^{II} CBS to Fe^{III} CBS significantly decreases enzyme activity. We previously observed that Fe^{II} CBS undergoes spontaneous heme reoxidation to Fe^{III} CBS at pH values less than 8, even in the presence of excess reductant under inert atmosphere (*39*). As the solution pH decreases, the rate of reoxidation increases, along with the amount of Fe^{III} CBS present when the process reaches equilibrium. To determine the effect of heme reoxidation on CBS enzyme activity, Fe^{III} CBS was transiently reduced by 2 mM dithionite and allowed to reoxidize to Fe^{III} CBS in 25 mM MES at pH 6.5 and 4 °C. As determined spectroscopically, the reoxidation process reached equilibrium after 20 min and ~85% of the protein was reoxidized to Fe^{III} CBS. The reoxidized protein was then assayed at pH 6.5 along with untreated Fe^{III} CBS at the same pH. Comparison between untreated and reoxidized Fe^{III} CBS showed that the reoxidized protein lost more than 75% of its original activity (data not shown), demonstrating that the spontaneous reoxidation process damages CBS enzyme activity. At pH 7.4 the reoxidation is slow and only a small fraction of the protein would be expected to reoxidize during a 20 min assay, but it may be enough to explain the faster loss of Fe^{II} CBS enzyme activity measured at pH 7.4. To avoid this complication, all further studies were conducted at pH 8.6, where minimal reoxidation occurs.

Imidazole Accelerates the Loss of Fe^{II} CBS Enzyme Activity in a Concentration-Dependent Manner. Imidazole, a good Fe^{II} heme ligand, was included in several enzyme assays to determine whether heme ligation plays a role in the loss of Fe^{II} CBS enzyme activity. The results revealed that exogenous imidazole accelerates the loss of Fe^{II} CBS activity. When CBS is reduced at pH 8.6 and 37 °C in the presence of 50−150 mM imidazole, the cystathionine production falls off more rapidly than in the absence of added heme ligand (Figure 4). Furthermore, increasing the imidazole concentration increases the rate at which Fe^{II} CBS activity is lost. Addition of an extra 150 mM Tris to time-dependent Fe^{II} CBS assay samples did not reproduce the imidazole results, nor did it affect the loss of Fe^{II} CBS enzyme activity over time. The imidazole effect was also unique to Fe^{II} CBS. The enzyme activity of Fe^{III} CBS was unaffected by the inclusion of 150 mM imidazole, and a plot of cystathionine production versus time displays the same linear behavior observed in the absence of imidazole (data not shown). To determine the full extent of Fe^{II} CBS inhibition by imidazole, Fe^{II} CBS was incubated with 100 mM imidazole at pH 8.6 and 37 °C for 2 h prior to assay, allowing the time-dependent inhibition process to reach a conclusion. Subsequent assay revealed that the imidazole-treated enzyme was completely inactive. Due to the instability of the imidazole-treated protein, the effect of removing the exogenous imidazole by dialysis or size-exclusion chromatography was not tested. Taken together, these results imply that imidazole causes loss of Fe^{II} CBS

activity via ligation to the Fe^{II} heme, rather than a hydrophobic interaction or salt effect.

In order to better understand what happens to Fe^{II} CBS that causes it to gradually lose enzyme activity, we conducted a series of time-dependent spectroscopic experiments under pseudo-assay conditions. Changes in the characteristic heme absorption bands were used to interrogate heme coordination and provide clues as to the mechanism by which Fe^{II} CBS loses enzyme activity. Previous studies in our lab have shown that Fe^{II} CBS is particularly temperature sensitive and undergoes a ligand switch to a new species, called Fe^{II} CBS424, when briefly heated to 55 °C (*40*). In this process the native cysteinate heme ligand is replaced by a neutral donor ligand from within the protein and the Soret band absorption maximum shifts from 449 nm to 424 nm. The new heme ligand is supplied by the region spanning residues 41−413 (*40*), although the identity of the new ligand is not known and there are no good ligand candidates within 10 Å of the heme face. His^{232}, Lys^{267}, Lys^{271}, and possibly the N-terminal proline (present in both the 63- and 45-kDa forms of CBS) are the closest potential ligands (*21*). Since the new Fe^{II} CBS424 species is completely inactive, we chose to investigate whether Fe^{II} CBS424 could also be formed at 37 °C and thus could account for the loss of Fe^{II} CBS enzyme activity over time.

Electronic Absorption Spectroscopy Reveals That Fe^{II} CBS449 Spontaneously Converts to Fe^{II} CBS424 at 37 °C. Electronic absorption spectroscopy was used to follow the conversion of Fe^{II} CBS to Fe^{II} CBS424 over time at pH 8.6 and 37 °C. Reduction of the Fe^{III} CBS heme by injection of 2 mM sodium dithionite takes place immediately, generating an Fe^{II} CBS species with a Soret band maximum at 449 nm; this species is termed Fe^{II} CBS449 for clarity. Spectra of Fe^{II} CBS recorded at specific time intervals reveal a progressive loss of the 449 nm Soret peak and growth of the new 424 nm Soret peak as the ligand switch takes place and Fe^{II} CBS424 is formed (Figure 5). The nearly isosbestic conversion of Fe^{II} CBS449 to Fe^{II} CBS424 can be followed for up to 48 h before protein degradation becomes a problem and spectral intensity is lost at all peak positions (data not shown). However, even 48 h is insufficient for the conversion to reach completion or equilibrium at 37 °C. Identical spectroscopic behavior was observed for both the 63- and 45-kDa isoforms of CBS, indicating that the conversion process is not dependent on the C-terminal AdoMet-binding domain or the quaternary structure of the protein. The ligand switch is unique to Fe^{II} CBS, as spectra of Fe^{III} CBS recorded over several hours at pH 8.6 and 37 °C show no change.

Temperature and pH affect the rate of Fe^{II} CBS424 formation. The conversion of Fe^{II} CBS449 to Fe^{II} CBS424 is highly temperature-dependent, as has been noted previously (*40*). At 4 °C no Fe^{II} CBS424 is observed, whereas at 55 °C the ligand switch is largely complete even before the sample can equilibrate to the new temperature. The effect of pH is more complicated; recall that Fe^{II} CBS slowly undergoes spontaneous heme reoxidation to Fe^{III} CBS at pH 7.4 (*39*). Fe^{II} CBS reduced at pH 7.4 and 37 °C displayed spectral features indicative of simultaneous Fe^{II} CBS424 formation *and* reoxidation to Fe^{III} CBS, with the reoxidized form eventually dominating (data not shown). After reduction, the initial 449 nm Soret peak was replaced by a broad band around 426 nm formed by a mixture of the 424 and

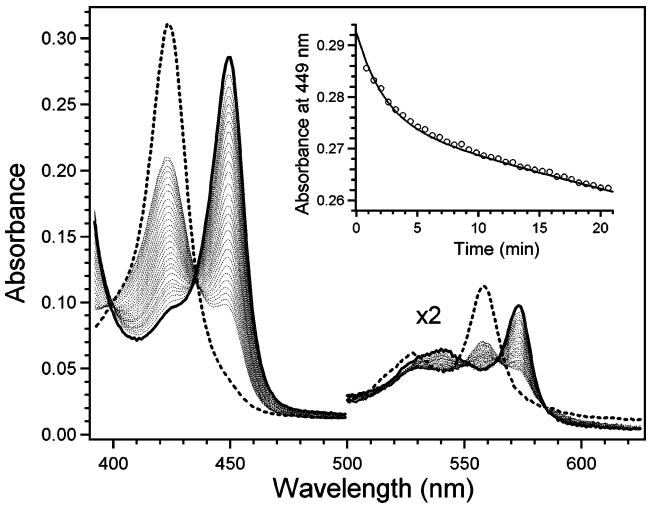


FIGURE 5: Fe^{II} CBS undergoes a ligand switch at pH 8.6 and 37 °C. Electronic absorption spectroscopy of 63-kDa CBS collected over 48 h reveals that Fe^{II} CBS449 is slowly replaced by Fe^{II} CBS424. The spectroscopic sample was 2.6 μM in CBS heme and contained 100 mM Tris at pH 8.6 and 13 μg/mL BSA. Upon anaerobic injection of sodium dithionite to 10 mM, spectra were recorded every 36 s for the first 1.5 h, then every 15 min for 22.5 h and every 30 min for 24 h. The first spectrum recorded is of Fe^{II} CBS449 and is shown as a bold solid line (—). Traces collected over time are shown as dotted lines (•••). Fe^{II} CBS424 was prepared separately, as described previously (*40*), and is shown for comparison as a bold dashed line (- - -). Inset: The absorbance at 449 nm was plotted against time and fitted to a triexponential rate equation using the kinetic parameters listed in Table 1. The individual data points are shown as open circles (○), and the fit is a solid line (—). Only the initial 20 min of absorbance data are shown, although the full 48 h plot can be seen in Supplemental Figure 3.

428 nm Soret peaks of Fe^{II} CBS424 and reoxidized Fe^{III} CBS, respectively. The 500−600 nm (α, β) region also revealed a mixture of oxidized and reduced species. The loss of 449 nm absorbance observed for Fe^{II} CBS at pH 7.4 occurred faster than at pH 8.6, strikingly similar to the accelerated loss of Fe^{II} CBS enzyme activity measured at pH 7.4. To simplify spectroscopic analysis, all remaining experiments were conducted at pH 8.6, where the reoxidation rate is negligible.

Unlike pH and temperature, other general solution conditions had little effect on the conversion of Fe^{II} CBS449 to Fe^{II} CBS424. The formation of Fe^{II} CBS424 was independent of buffer type (Tris or borate), as well as CBS, salt, or overall protein concentration. BSA was included to mimic assay conditions and appeared to stabilize CBS: identical samples lacking BSA displayed an inconsistent rate of Fe^{II} CBS424 formation. Varying the BSA concentration did not otherwise influence the rate of conversion of Fe^{II} CBS to Fe^{II} CBS424.

CBS Enzyme Substrates Modulate the Fe^{II} CBS424 Formation Rate. Serine and homocysteine affect the rate of Fe^{II} CBS424 formation, as observed by the loss of 449 nm absorbance over time. The addition of 10 mM serine (the first substrate to bind in the ping-pong enzyme mechanism) slows the loss of the 449 nm species, whereas 10 mM homocysteine (the second substrate) accelerates the loss (Figure 6). The addition of both 10 mM serine and 10 mM homocysteine to create turnover conditions, as in the activity assay, shows an intermediate effect on the ligand switch as the opposing effects of the two substrates compete, making

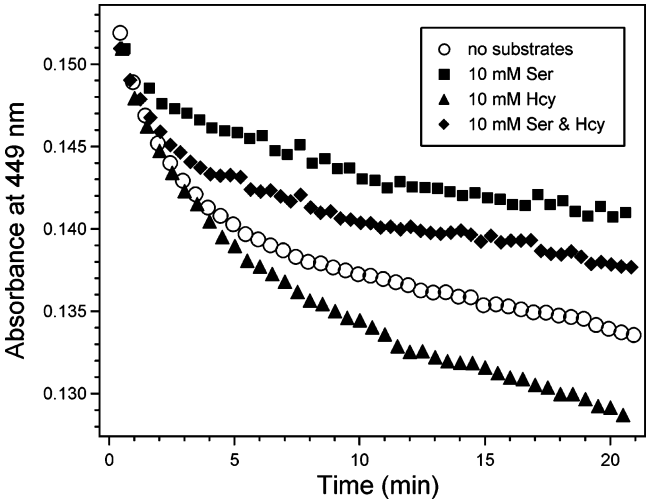


FIGURE 6: CBS enzyme substrates alter the rate of conversion of Fe^{II} CBS449 to Fe^{II} CBS424. Plotted is the loss of 449 nm absorbance over time. 63-kDa CBS (1.2 μM heme) was incubated at 37 °C with 100 mM Tris (pH 8.6), 71 μg/mL BSA, and either no substrate, 10 mM serine, 10 mM homocysteine, or 10 mM serine and 10 mM homocysteine. Spectra were recorded from 420 to 454 nm every 30 s for 20 min after anaerobic injection of sodium dithionite to 2 mM.

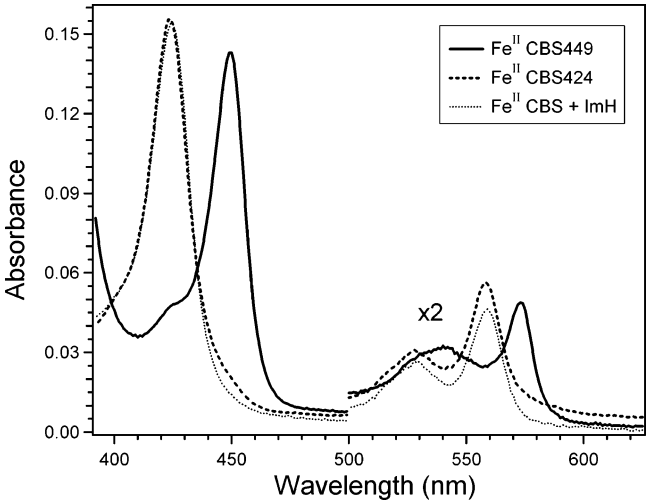


FIGURE 7: Fe^{II} CBS forms a 424 nm species in the presence of imidazole. 63-kDa CBS (1.3 μM by heme) in 100 mM Tris (pH 8.6), 71 μg/mL BSA, 10 mM serine, 10 mM homocysteine, and 150 mM imidazole (pH 8.6) was reduced by anaerobic injection of sodium dithionite to 2 mM and incubated at 37 °C for 30 min. To complete the conversion to the 424 nm species the sample was incubated at 55 °C for 10 min and re-equilibrated to 37 °C prior to recording the final spectrum. The 424 nm species formed in the presence of imidazole is shown as a dotted line (···). Fe^{II} CBS449 (solid line, —) and Fe^{II} CBS424 (dashed line, - - -) generated in the absence of exogenous imidazole are shown for comparison.

the loss of 449 nm absorbance only slightly slower than in the absence of either substrate. Lower substrate concentrations, such as 0.7 mM serine and/or 0.6 mM homocysteine, generate the same results seen with 10 mM serine and/or 10 mM homocysteine, indicating that the exact serine or homocysteine concentration is less important than the identity of the added substrate. Taken together, these results show that substrate binding at the PLP active site of CBS influences the ligand switch at the heme site. In all cases where substrates were included, the Fe^{II} CBS424 species eventually showed some instability, with all the characteristic absorption features decreasing in intensity. For this reason, subsequent kinetic experiments were run for only 20 min (the length of a time-dependent assay). Additionally, the inclusion of AdoMet with the 63-kDa form of CBS slightly accelerates the loss of the 449 nm species and concomitant gain of the 424 nm species. The effect of including excess PLP could not be determined, as the PLP interacted with other solution components and obscured the heme Soret absorption.

Exogenous Imidazole Accelerates the Conversion of Fe^{II} CBS to a 424 nm Species. Spectra of Fe^{II} CBS were followed in the presence of imidazole to determine whether imidazole accelerates the loss of Fe^{II} CBS enzyme activity by displacing the native Cys(thiolate) heme ligand. Unfortunately, since imidazole is a neutral donor ligand, the Fe^{II} CBS species formed when imidazole displaces Cys^{52} and binds to the heme is expected to generate a spectrum virtually identical to that of Fe^{II} CBS424. However, if imidazole provides a more readily accessible heme ligand than the protein-derived ligand in Fe^{II} CBS424, the rate at which Fe^{II} CBS449 disappears may be accelerated in a concentration-dependent manner. Spectra of CBS reduced in the presence of 150 mM imidazole showed the formation of a 424 nm species indistinguishable from Fe^{II} CBS424 (Figure 7). A plot of 449 nm absorbance versus time indicates that Fe^{II} CBS449 disappears faster in the presence of 50 mM imidazole than when no ligand is added (Figure 8). Increasing the imidazole

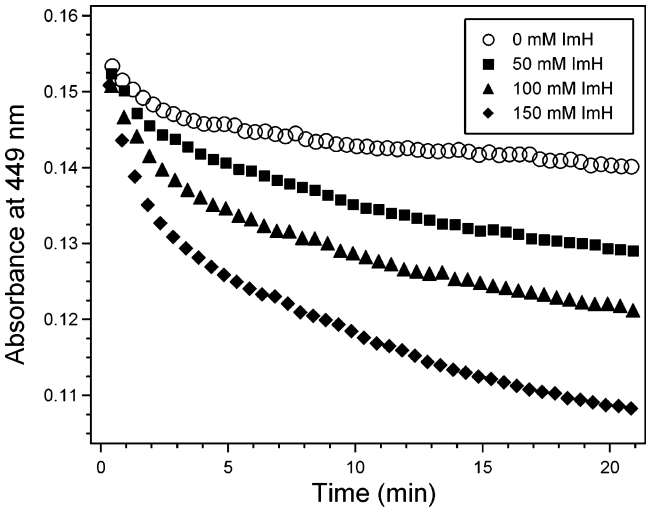


FIGURE 8: Exogenous imidazole accelerates the loss of Fe^{II} CBS449 in a concentration-dependent manner. 63-kDa CBS (1.3 μM heme) was incubated at 37 °C with 100 mM Tris (pH 8.6), 71 μg/mL BSA, 10 mM serine, 10 mM homocysteine, and either 0, 50, 100, or 150 mM imidazole (pH 8.6). Spectra were recorded from 420 to 454 nm every 30 s for 20 min upon anaerobic injection of sodium dithionite to 2 mM.

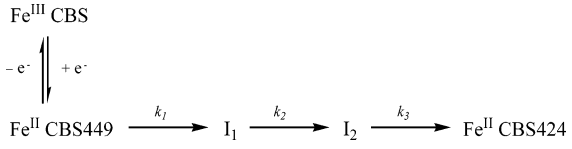
concentration from 50 to 150 mM further accelerates the loss of the 449 nm species. At concentrations ≤ 10 mM, imidazole had no effect on the process, similar to a previous report indicating that 10 mM imidazole is insufficient to interact with Fe^{II} CBS (*28*). Control experiments conducted with NaCl or extra Tris (which has a roughly similar conjugate acid/base distribution as imidazole) did not reproduce the rate effects observed with imidazole: the loss of Fe^{II} CBS449 was not accelerated. The inclusion of 250 mM L-histidine (pH 8.6) also had no effect. When CBS was reduced in the presence of 10 mM serine, 10 mM homocysteine, and 100 mM imidazole, the conversion to the

Table 1: Kinetic Parameters for the Conversion of Fe^{II} CBS449 to Fe^{II} CBS424[a]

step	amplitude	k (min^{-1})	$t_{1/2}$ (min)
1	(α) 0.02	0.4	2
2	(β) 0.07	0.01	7×10
3	(γ) 0.2	0.0003	2×10^3

[a] From the triexponential fit to the loss of 449 nm absorbance collected for 63-kDa Fe^{II} CBS over 48 h at 37 °C. Experimental data depicted in Figure 5.

Scheme 1



424 nm species was noticeably slower than with 100 mM imidazole alone (data not shown). Additionally, the final 424 nm species generated by CBS reduced in the presence of ≥50 mM imidazole appears to be less stable than Fe^{II} CBS424 and loses spectral intensity over the course of 60 min, consistent with the exchange of an endogenous ligand for imidazole.

Conversion of Fe^{II} CBS449 to Fe^{II} CBS424 Generates Triphasic Kinetics. To interrogate the mechanism by which Fe^{II} CBS449 spontaneously converts to Fe^{II} CBS424, we performed a kinetic analysis of the time-dependent spectroscopic data and generated a model that provides a framework for understanding the Fe^{II} CBS ligand switch. Plotting the loss of 449 nm absorbance versus time for the 48 h experiment in which Fe^{II} CBS449 is converted to Fe^{II} CBS424 generates a curve which is best fitted by a triexponential decay equation (Figure 5, inset, or Supplemental Figure 3, Supporting Information). The fit requires three unique rate constants (Table 1) and is first order with respect to the CBS concentration. Varying the CBS concentration did not change the calculated rate constants, which would have invalidated the model and indicated a need to test higher order rate equations. The triexponential best-fit suggests that the ligand switch is intramolecular and takes place in three consecutive, irreversible steps with two unique intermediates (sequential model, Scheme 1). Three-step parallel models increased the complexity of the system but did not improve the overall fit. Any attempt to fit the data to a model with reversible steps, including cooperative models, increased the degrees of freedom in the system such that many solutions were possible, although none improved the quality of the fit over the simple sequential model. In agreement with the sequential model, the ligand switch to Fe^{II} CBS424 is totally irreversible when induced by incubation at 55 °C (*40*).

Fit of the Assay Data Suggests That Fe^{III} CBS and Fe^{II} CBS449 Have Identical Activity. Since Fe^{II} CBS449 undergoes a spontaneous ligand switch that generates additional Fe^{II} species, including Fe^{II} CBS424 and possibly two intermediates, measuring the true activity of Fe^{II} CBS is not trivial. We employed a computational approach to determine the enzyme activity of each species, relying upon the kinetic model generated from the spectroscopic data in combination with the time-dependent assay data. This approach assumes that the loss of Fe^{II} CBS enzyme activity measured over time is directly related to the slow conversion of Fe^{II} CBS449 to Fe^{II} CBS424 seen spectroscopically. Using eq 6, which describes the amount of cystathionine generated over time by the interconverting Fe^{II} CBS species, we successfully fitted the time-dependent assay data of Fe^{II} CBS (fit lines in Figure 1). This successful fit suggests that the odd enzymatic and spectroscopic behaviors observed for Fe^{II} CBS are linked and enables us to calculate the enzyme activity of Fe^{II} CBS449 and the two predicted intermediates, I_1 and I_2 (Table 2). Previous work in our lab has shown that fully converted Fe^{II} CBS424 is completely inactive (*40*). The best-fits to the assay data indicate that the specific activity of Fe^{II} CBS449 and Fe^{III} CBS are essentially equal. This result can be easily observed by noting the similar amount of cystathionine produced by Fe^{III} and Fe^{II} CBS at the 2 min point of an assay, before significant conversion of Fe^{II} CBS449 to the first intermediate has occurred (Figure 1). The intermediates are predicted to be substantially less active than Fe^{III} CBS, with the second intermediate expected to have little or no enzyme activity. Unlike the calculated specific activity of Fe^{II} CBS449, the activities of I_1 and I_2 should be considered rough estimates as they show some variability based on the model used to fit the spectroscopic and assay data. The conversion of Fe^{II} CBS449 to the first intermediate could be largely responsible for the observation that Fe^{II} CBS is less active than Fe^{III} CBS over a 10−30-min assay (*32*, *40*), although the identity of the predicted intermediates remains unknown at this time.

Table 2: Measured and Calculated Specific Activities for Fe^{III} and Fe^{II} CBS

species	63 kDa (units/mg)	63 kDa + AdoMet (units/mg)	45 kDa (units/mg)
Fe^{III} CBS[a]	109 ± 4	307 ± 26	589 ± 7
Fe^{II} CBS449[b]	115 ± 7	363 ± 79	650 ± 87
I_1[b]	54 ± 4	148 ± 14	336 ± 16
I_2[b]	29 ± 39	0 ± 0	0 ± 0
Fe^{II} CBS424[c]	nd[d]	nd	nd

[a] Specific activities are the average of three measurements with the standard deviation shown as error. [b] Specific activities of Fe^{II} CBS449 and the two predicted intermediates (I_1 and I_2) were fitted using time-dependent assay data and spectroscopically measured kinetic parameters. Calculated results were averaged for the two best models, one which fitted the ferrous species independently and one that assumed that Fe^{III} CBS and Fe^{II} CBS449 have identical activity. The spread between the two models is represented as the error in the calculated activity. [c] Fe^{II} CBS424 was generated as previously described (*40*). [d] Not detected.

DISCUSSION

To date, heme cofactors within proteins are expected to function in one of four roles: catalysis, O_2 binding, electron transport, or allosteric regulation. Several studies have confirmed that the CBS heme is not necessary for catalytic turnover (*10*−*12*), and CBS in lower organisms such as yeast does not utilize heme (*13*, *14*). In addition, the CBS heme iron is unable to bind O_2 (*26*, *27*) and has no obvious electron transport role. Although proper heme incorporation is important for correct protein folding and full CBS enzyme activity (*8*), a purely structural role for a biologically expensive molecule like heme would be unexpected. The remaining possibility is allosteric regulation. While the Fe^{III} form of the CBS heme is unreactive toward exogenous ligands including imidazole and pyridine derivatives, azide,

fluoride, cyanide, and various isonitriles (*9*, *27*, *28*), Fe^{II} CBS displays decreased enzyme activity that can be further inhibited by CO, NO, and cyanide (*9*, *30*).

Allosteric regulation based on the heme redox state has been hypothesized as a role for the CBS heme. Since CBS sits at the junction between the transmethylation and transsulfuration pathways, a loss in CBS activity due to heme reduction could potentially function as a switch between the competing pathways (*32*). To date the activity of Fe^{II} CBS has been in dispute; in one study the 63-kDa full-length protein lost activity after reduction (*32*), while in another study no loss in activity was detected for either the 63- or 45-kDa form of Fe^{II} CBS (*16*). Our observation that both forms of Fe^{II} CBS slowly lose activity in a time-dependent fashion may help to rationalize these differences, since assays performed for different lengths of time may yield disparate results. Although we support the hypothesis that the CBS heme most likely serves an allosteric role, the data presented herein suggest that a purely redox-controlled mechanism is unlikely, as the *in vitro* behavior of Fe^{II} CBS resembles protein instability more than allosteric regulation.

Through the combined application of enzyme assay and optical spectroscopy, we discovered that Fe^{II} CBS shows unique time-dependent behavior, entirely unlike that of Fe^{III} CBS. The 63- and 45-kDa forms of Fe^{II} CBS gradually lose activity during enzyme assay at pH 8.6 and 37 °C, while under similar spectroscopic conditions Fe^{II} CBS undergoes a ligand switch to the inactive Fe^{II} CBS424 species. The loss of Fe^{II} CBS activity appears to be a direct result of the ligand switch process, and is not a result of product inhibition, a salt effect, or inhibition by dithionite or its oxidation products. The identity of the new ligand in the Fe^{II} CBS424 species is unknown, although it is most likely an endogenous neutral donor from within the region spanning amino acids 41−413 that replaces the original Cys^{52}(thiolate) ligand (*40*). There are no naturally occurring mutations or recombinant variants associated with the original heme ligands or nearby residues that give any further indication of what the identity of the new heme ligand might be. Since the rate of the ligand switch is not dependent on the CBS concentration or other solution conditions, the process is intramolecular, with the heme and its new ligand residing in the same molecule. Although Fe^{II} CBS clearly undergoes this ligand switch under pseudo-assay conditions, the physiological relevance of this behavior is unclear.

Heme-Based Redox Regulation Is Unlikely To Be the Method of Allosteric Control in CBS. In order to translate a biological signal into the appropriate physiological response, heme sensor proteins tend to respond selectively, rapidly, and reversibly to molecular signals. To date there are no established heme sensor proteins controlled solely by heme redox state. Full-length sGC, CooA, and FixL, containing both the heme and active domains, are selectively regulated by the binding of the correct gas molecule (NO, CO, and O_2, respectively). For example, Fe^{II} sGC is activated more than 100-fold by NO binding and about 3-fold by CO binding, but does not bind or otherwise respond to the presence of O_2, which is available in much greater concentrations (*47*). CooA is only activated by CO binding in the Fe^{II} state; the presence of O_2 causes heme oxidation (*48*, *49*). FixL binds NO and CO more tightly than O_2, but only O_2 binding elicits maximal enzyme inactivation (*50*−*52*). In addition, all three heme sensors respond rapidly to the presence of their respective signal molecule, with an "immediate" response on the standard assay time scale. NO photolyzed from a caged derivative activated sGC in under 20 ms, faster than the operational limit of the method (*53*). Despite the relatively slow ligand association rates measured for CooA and FixL (*54*, *55*), no lag time has been reported for the response of CooA or FixL upon ligand binding. Finally, each heme sensor responds to its signal molecule reversibly, despite differences in the speed and complexity of the ligand dissociation mechanism. Regardless of whether ligand binding causes activation or deactivation, the protein reverts to its original state once the signal molecule is released (*47*−*49*, *55*). If CBS is truly a heme sensor protein, it might be expected to behave similarly and respond selectively, rapidly, and reversibly to its molecular signal. However, the slow and irreversible loss of CBS activity seen upon heme reduction does not resemble other heme sensor proteins, which may suggest that the loss of enzyme activity seen for Fe^{II} CBS is not an indication of a redox-controlled regulatory mechanism.

The slow conversion of Fe^{II} CBS449 to Fe^{II} CBS424 more closely resembles protein unfolding than the regulatory behavior of heme sensor proteins like sGC, CooA, and FixL. Some proteins switch heme ligands as part of the unfolding process; for example, the native Met^{80} heme ligand in Fe^{II} horse ferricytochrome *c* is replaced by lysine and then histidine in increasingly denaturing conditions (*56*). Similarly, Fe^{II} P450 exchanges a Cys(thiolate) ligand for a neutral donor ligand to form P420 when the protein is exposed to increased temperature, pressure, salt concentration, or denaturant concentration (*57*−*60*). Fe^{II} CBS may be an unstable species created only *in vitro*, as it has not been shown whether Fe^{II} CBS can form *in vivo*. The CBS heme is expected to have a very low reduction potential, similar to the Cys/His-ligated heme in M80C cytochrome *c*, measured at −390 mV (*61*). In reduced Fe^{II} CBS there may be unfavorable interactions between the anionic Cys^{52}(thiolate) ligand and the neutral Fe^{II} porphyrin, which may lengthen the axial iron−ligand bonds and distort the heme pocket. Intermediates I_1 and I_2 could represent such conformational distortions, which are communicated to the PLP active site and cause decreased enzyme activity. Ultimately, Fe^{II} CBS must undergo structural movement to replace Cys^{52} with another endogenous heme ligand, since there are no good ligand candidates within 10 Å. The slow and irreversible conversion of Fe^{II} CBS449 to Fe^{II} CBS424 suggests that this movement is more likely partial denaturation than an allosteric conformational change.

The CBS Heme May Serve an Alternate Allosteric Function. Although the data presented in this study suggest that regulation of CBS solely in response to heme reduction is unlikely, an allosteric function is still the most probable role for the CBS heme. Heme sensor proteins are dependent upon their ability to relay a signal from the heme to the functional domain and thereby modify protein activity. Reciprocal communication between heme and functional sites of heme sensor proteins has been documented. In sGC, NO binding to the heme allosterically stimulates cyclization of GTP to cGMP. Conversely, GTP or cGMP and PP_i binding at the active site appears to affect the rate of NO association and dissociation (*62*−*65*). Additionally, a laser photolysis study of the transcriptional activator CooA showed faster CO

rebinding in the presence of target DNA versus nonspecific or no DNA (*66*). Sensitive communication between the heme and active site is essential for function in heme sensor proteins.

CBS clearly demonstrates reciprocal communication between the heme and enzyme active sites, which is consistent with an allosteric regulatory role for the CBS heme. Any displacement of the native Cys^{52}(thiolate) ligand causes a substantial decrease in CBS enzyme activity (*9*, *30*, *31*), although the lack of specificity in this response makes it an implausible regulatory mechanism. Here we report the first evidence where binding of substrates at the PLP site affects the heme site of CBS. Serine and homocysteine, either independently or in combination, altered the rate of the Fe^{II} ligand switch (Figure 6). Substrate binding did not simply stabilize the CBS protein structure: homocysteine accelerated the ligand switch while serine slowed it. AdoMet binding at the C-terminal autoinhibitory domain also slightly accelerated the rate of the ligand switch, which may indicate communication from the autoinhibitory domain directly to the heme site, or indirectly through stimulation of the PLP active site. Regardless, the heme and PLP sites of CBS demonstrate reciprocal communication similar to that observed in sGC and CooA.

Human CBS Could Require a Protein Binding Partner. Although the discovery that human CBS contains a heme cofactor was made over a decade ago (*8*), the CBS heme function has remained a topic for speculation. The ambiguous nature of the CBS heme may be a result of artificial *in vitro* conditions; the physiological heme function could require additional binding partners. CBS isolated from liver often copurifies with other proteins (*27*, *67*, *68*). Additionally, human CBS interacts with several sumoylation proteins and contains a potential sumoylation site near the heme (*69*). The crystal structure of 45-kDa CBS reveals a solvent-exposed heme (*21*, *22*) which is not predicted to be covered by the AdoMet-binding domain in the 63-kDa form (*70*). Although heme cofactors in sensor proteins need to be somewhat solvent exposed in order to facilitate response to environmental conditions, the degree of heme exposure in CBS is unusual. It is plausible that the exposed heme could be poised to interact with another protein. The crystal structure of 45-kDa CBS also reveals a solvent-exposed CXXC motif of unknown function only 18 Å from the heme, although this loop may be buried in the 63-kDa form (*71*). CBS proteins isolated from yeast and *Trypanosoma cruzi* lack both heme and the CXXC motif (*13*, *15*, *21*), which may suggest that the function of these two redox-active sites is related. Further studies are needed to identify the proteins that copurify with CBS and determine whether they alter the enzymatic or spectroscopic properties of CBS. The results of such studies may prove useful in ascertaining the true role of the CBS heme.

CONCLUSIONS

The data presented herein reveal that Fe^{II} CBS unexpectedly loses activity over the course of a standard enzyme assay, and that this loss of activity can be correlated spectroscopically to the time-dependent formation of inactive Fe^{II} CBS424. The loss of Fe^{II} CBS activity appears to be caused by the Fe^{II} CBS424 ligand switch process, whereby the native Cys^{52} heme ligand is replaced by an endogenous neutral donor ligand that has yet to be identified. The slow inactivation of Fe^{II} CBS observed *in vitro* does not resemble other heme sensor proteins and suggests that simple redox regulation is an unlikely role for the CBS heme. However, in the absence of a catalytic or electron transport role, the reciprocal communication between the heme and active sites of CBS suggests that the heme does serve some type of allosteric function.

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SUPPORTING INFORMATION AVAILABLE

Figure which compares the loss of yeast CBS activity caused by sodium dithionite versus titanium(III) citrate (Supplemental Figure 1). Figure which overlays the Fe^{III} and Fe^{II} assay data for 63-kDa CBS in the presence and absence of AdoMet (Supplemental Figure 2). Full 48 h of the 449 nm absorbance versus time data from Figure 5, including both a bi- and a triexponential fit (Supplemental Figure 3). This material is available free of charge via the Internet at http://pubs.acs.org.

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