Evidence for Heme-mediated Redox Regulation of Human Cystathionine \(\beta\)-Synthase Activity*

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Human cystathionine \(\beta\)-synthase catalyzes the first step in the catabolic removal of the toxic metabolite, homocysteine. It is unique in being dependent on both pyridoxal phosphate (PLP) and heme for activity. The reaction involves condensation of serine and homocysteine to give cystathionine. Although the role of PLP can be rationalized in analogy with other PLP-dependent enzymes that catalyze \(\beta\)-replacement reactions, the role of the heme is unknown. In this study, we have purified and characterized the recombinant human enzyme and have examined the effect of heme oxidation state on enzyme activity. We find that under reducing conditions, generated by addition of titanium citrate, the enzyme exhibits a 1.7-fold lower activity than under oxidizing conditions. Reoxidation of the ferrous enzyme with ferricyanide results in alleviation of inhibition. This redox-linked change in enzyme activity correlates with changes in heme oxidation state monitored by UV-visible spectroscopy. Dithiothreitol, which does not reduce the enzyme-bound heme, does not perturb enzyme activity. These studies provide the first evidence for redox-linked regulation of cystathionine \(\beta\)-synthase which is heme-dependent.

Cystathionine \(\beta\)-synthase (EC 4.2.1.22, CBS)† is one of two key mammalian enzymes that metabolizes cellular homocysteine. The latter is a toxic metabolite, formed by the breakdown of \(S\)-adenosylhomocysteine, and its elevated levels are correlated with an increased risk for cardiovascular diseases (1–4). In most tissues, homocysteine is diverted via two major pathways into innocuous products (Fig. 1). Thus, transmethylation, catalyzed by methionine synthase, converts it to methionine, while trans-sulfuration, catalyzed by CBS, yields cystathionine (Reaction 1). Catastrophic mutations in CBS result in severe hyperhomocysteinemia and is an inborn error of metabolism that displays an autosomal recessive inheritance (2). Early and aggressive occlusive arterial diseases are a dominant clinical phenotype in these patients. Cystathionine \(\beta\)-synthase is a tetramer of identical subunits and is allosterically regulated by AdoMet (5). The early literature on this enzyme is fraught with conflicting claims of the size and subunit composition of the native enzyme that stems from the proteolytic sensitivity of its subunits. Cleavage of the 63-kDa subunit at a hypersensitive site results in a 48-kDa product that exists as a homodimer (6, 7). The recent cloning of the cDNA encoding human CBS (8, 9) and its expression in Escherichia coli have made available larger amounts of native \(\alpha_\alpha\) enzyme, which is not degraded to the \(\alpha_\beta\) species by E. coli proteases (10).

CBS is unique in being dependent on two cofactors, heme and PLP, for activity (11). Overall, the reaction catalyzed by CBS involves \(\beta\)-replacement in which the hydroxyl group of the substrate, serine, is exchanged for the thiolate of homocysteine (Reaction 1).

Enzymatic reactions involving either \(\beta\)-replacement or \(\beta\)-elimination reactions (viz. tryptophan synthase and tryptophanase) are characteristically PLP-dependent and generally believed to share a common mechanism. For CBS, it is predicted that an external aldimine is formed between the serine and PLP (12). The elimination and replacement reactions confer overall retention of configuration at the \(\beta\)-carbon of cystathionine (12). This mechanism does not include an obvious role for the heme, and its function is presently unknown.

The heme in CBS is iron protoporphyrin IX, as judged by the pyridine hemochrome assay (11, 13), but appears to be in an unusual environment in the protein. The spectrum of the ferric and dithionite-reduced ferrous hemes have Soret absorption maxima at 428 and 450 nm, respectively (13, 14). Unlike the cytochrome P450s, which derive their name from the 450 nm absorption maximum of the CO-ferrous heme complex, the 450 nm absorption in CBS is observed in the absence of CO. Treatment of ferrous CBS with CO results in the Soret band shifting to 420 nm (14). Whereas the role of the heme in CBS is unknown, it may be required for PLP binding, since the extent of PLP saturation is apparently determined by the heme content of the enzyme (11). In nature, heme cofactors function in electron transfer, as reversible oxygen carriers, in catalyzing oxygenation chemistry or serve regulatory roles as in the soluble guanylate cyclase. Based on the condensation chemistry catalyzed by CBS, electron transfer, oxygenation, and oxygen binding roles for the heme in CBS would appear to be unlikely.

In this study, we have examined the effect of heme reduction on CBS activity. We demonstrate that the redox state of the heme modulates enzyme activity. Thus, reduction of heme by titanium citrate lowers activity by a factor of ~2, and its reoxidation by ferricyanide restores the original activity. Based on these results, we postulate that the heme in CBS is redox-active and reversibly regulates the activity of the enzyme.

**EXPERIMENTAL PROCEDURES**

**Materials**

The following materials were obtained from Sigma: bovine serum albumin, 5,5'-dithiobis(nitrobenzene), \(L\)-homocysteine thiocarbamoyl, leupetin, aprotinin, pepstatin, benzamidine, TLCK, dithiothreitol, PLP, dithionite, thiombin, potassium ferricyanide, DEAE-Sepharose, \(\delta\)-ami-

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†The abbreviations used are: CBS, cystathionine \(\beta\)-synthase; PLP, pyridoxal phosphate; TLCK, \(N\,^p\)-tosyl-L-lysine chloromethyl ketone; AdoMet, \(S\)-adenosylmethionine; CHAPS, 3-(\(3\)-[\(3\,\text{cholamidopropyl})\]di-methylammonio)-1-propanesulfonic acid; CHES, 2-(cyclohexylamino)ethanesulfonic acid.
nolevulinic acid, and glutathione. [14C]Serine (158 mCi/mmol) was purchased from Amersham Life Sciences Inc. Glutathione S-transferase-Sepharose and Q-Sepharose (fast flow) were purchased from Pharmacia Biotech. Homocysteine was prepared from homocysteine thiolactone as described previously (15). AdoMet, 1,4-butane disulfonate was a generous gift from Knoll Farmaceutici Spa (Milano, Italy).

**Expression and Purification of CBS**

Human CBS was purified from a recombinant expression system (pGEX4T1/hCBS) described previously (16) which produces a fusion protein with glutathione S-transferase at the N terminus. Limited proteolysis by thrombin separates glutathione S-transferase from CBS and generates a N-terminal extension containing the residues Gly-Ser-Pro-Glu-Phe-Pro-Gly-Arg-Pro-Ala-Gly preceding the initiating methionine. E. coli containing pGEX4T1/hCBS was grown in Luria broth medium containing ampicillin (75 μg/ml) at 30 °C to an A600 of 0.4. Expression of the recombinant protein was induced with isopropyl-1-thio-β-D-galactopyranoside (100 μM) in the presence of 75 μl/day β-aminolevulinic acid, and the cells were allowed to grow for an additional 21 h. E. coli cells (~30 g wet weight obtained from 6 liters of culture) were suspended in ~200 ml of buffer A containing 50 mM Tris, pH 8.0, 10 mM EDTA, 1.3 mM benzamidine HCl, 14 mM β-mercaptoethanol, 0.05 mM TLCK, 1 mg/liter leupeptin, 1 mg/liter aprotinin, and 1 mg/liter pepstatin. The cells were disrupted with a Heat Systems Ultrasonic Processor XL, operated at an output setting of 7 for 6 min with 3-min intervals by titration with the thiol reagent, dithiobis(nitrobenzene), and bovine serum albumin as standard. Two independent samples including the common transition metal ions. Two independent samples were sent for analysis along with the appropriate buffer blanks.

**Metal Analysis**

Enzyme preparations were analyzed for their total metal content by plasma emission spectroscopy (15) at the Chemical Analysis Laboratory, University of Georgia, Athens. This method detects 20 metal ions including the common transition metal ions. Two independent samples were sent for analysis along with the appropriate buffer blanks.

**Determination of Heme and PLP Content**

Heme concentration was determined by the pyridine hemochrome assay as described previously (19). PLP was measured fluorimetrically using a modification of a published procedure (20). PLP was released from the enzyme by treating 0.5 mg of CBS in 1.5 ml of 0 mM phosphate buffer, pH 7.2, with 5 mM hydroxylamine at 4 °C for 16 h followed by centrifugation in a Centricon concentrator (P30, Amicon). The absorption spectrum of the filtrate (containing PLP) and the retentate (containing CBS and heme) were recorded. The filtrate contained no heme and was employed for the fluorimetric analysis. Fluorescence emission of the PLP oxime was detected at 446 nm following excitation at 353 nm. A standard curve was generated using eight PLP samples of
known concentrations, which had been determined spectrophotometrically using $\epsilon_{280} = 5.1 \text{ m}^{-1} \text{ cm}^{-1}$ in 0.1 N HCl.

Total Amino Acid Analysis

A Beckman amino acid analyzer, model 6300, was employed to determine the total amino acid composition of CBS. The sample was prepared by dialyzing CBS overnight against distilled water. Amino acid analysis was performed at the protein sequencing facility at the University of Nebraska Medical Center in Omaha.

RESULTS AND DISCUSSION

Enzyme Purification—We have used a 3-step protocol to purify recombinant human CBS to $\sim 95\%$ purity and in 40% overall yield (Table I). The subunit molecular mass of the protein is 63 kDa as reported previously (10). We did not observe formation of the proteolytically truncated 48-kDa product. The specific activity of the enzyme obtained from this procedure is $\sim 280 \mu$mol/h/mg protein at 37 °C and is comparable to the specific activity of 200 to 300 $\mu$mol/h/mg protein reported previously (10). Purified CBS eluted as a single peak from a size exclusion column with a retention time consistent with it being a tetramer$^2$ (data not shown).

$\text{pH}$ and Kinetic Studies—Expression of human CBS in E. coli and yeast using conventional expression systems has been problematic, yielding very low amounts of protein (9, 21). This has been circumvented recently by the generation of fusion proteins with subsequent release of CBS from its fusion partner by limited proteolysis. In both systems, the resulting recombinant CBS has modifications at the N terminus (10, 16), an outcome that is now fairly common in proteins expressed using this strategy. In order to ascertain whether or not the N-terminal modification impacts the kinetic properties of the enzyme, the $K_m$ values for the two substrates (Table II) were determined in the presence and absence of the allosteric effector, AdoMet (5). The $K_m$ for homocysteine is $4.8 \pm 0.5$ mM ($-\text{AdoMet}$) and $5.0 \pm 0.9$ mM (+AdoMet). The $K_m$ for serine in the presence and absence of AdoMet were both $2.0 \pm 0.3$ mM.

The $K_m$ for homocysteine has been reported to range from $0.3$ to $25$ mM from independent determinations. The reported $K_m$ values at $7.9 \pm 0.2$ and $8.8 \pm 0.2$ (Fig. 2). The pH profile of the recombinant enzyme. It displays a bell-shaped dependence with a pH optimum of $\sim 8.5$ and reveals two titratable $pK_a$ values at 7.9 $\pm$ 0.2 and 8.8 $\pm$ 0.2 (Fig. 2). The pH profile is identical in the anaerobic assay in the presence of titanium citrate, conditions under which the heme is reduced to the ferrous state (see below). Thus, the pH dependence and kinetic parameters of recombinant human CBS are indistinguishable from those of the wild type enzyme.

Metal Analysis and Homocysteine Activation—CBS, like other homocysteine-utilizing enzymes such as the $B_12$-dependent and $B_{12}$-independent methionine synthases, needs to activate the nucleophile, homocysteine. This is expected to involve deprotonation of homocysteine ($pK_a = 10$ (25)) to generate the thiolate anion. The other two enzymes appear to use a common strategy in which homocysteine is activated via coordination to an active site zinc (26, 27). To test whether or not CBS also contains zinc, plasma emission spectroscopy was employed. Metal analysis revealed the presence of two irons per $\epsilon_4$ tetramer. No other metal ions were found. Hence, human CBS, unlike the $B_{12}$-dependent and -independent methionine synthases, does not utilize zinc to activate homocysteine.

Characterization of Heme—The iron stoichiometry obtained from plasma emission spectroscopy is surprising since CBS is a homotetramer. It raises the possibility that the heme is bound at the subunit interface of dimers or that the enzyme exhibits half of site activity. It contradicts a stoichiometry of 1 heme/monomer reported recently for the recombinant human enzyme (11). In order to resolve this discrepancy, we repeated the pyridine hemochrome analysis on CBS and redetermined the extinction coefficients of the ferrous and ferric states.

The heme spectrum of CBS analyzed by the pyridine hemochrome method is typical of iron protoporphyrin IX, with $\alpha$ and $\beta$ absorption maxima at 557 and 525 nm, respectively. Based on the reported extinction coefficients (19), and the amount of protein used in the assay, we estimate that recombinant CBS has $2.2 \pm 0.3$ hemes/tetramer, the average obtained from six independent determinations.

The extinction coefficient for CBS-bound heme was estimated on protein samples taken from the same enzyme preparations that had been subjected to plasma emission spectroscopy and on which we had an independent estimation of the iron content. Based on our analysis, the extinction coefficient for ferric CBS at 428 nm is $166 \pm 7$ $\text{mM}^{-1} \text{cm}^{-1}$ at pH 8.6. This value is 2-fold higher than that reported recently by Kraus and co-workers (11) for recombinant human CBS.

It is possible that during expression of the recombinant human enzyme, heme is limiting, and the CBS that we purify is only half-saturated with heme, accounting for the discrepancies between Kraus’s (11) preparation and ours. At least two lines of evidence rule out this possibility. First, the UV-visible absorption spectrum of our enzyme is comparable to that of

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$^2$ CBS was separated on a calibrated Superose 12 FPLC column (Pharmacia Biotech) using 50 mM Tris, pH 8.6, containing 150 mM KCl, 5 mM dithiothreitol, and 10 $\mu$M AdoMet.

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**TABLE I**

| Procedure         | Volume ml | Total protein mg | Total units | Specific activity $\mu$mol/h/mg | Yield % |
|-------------------|-----------|------------------|-------------|---------------------------------|---------|
| Sonoicte          | 230       | 2,507            | 16,400      | 5.4                             | 6.54    |
| Q-Sepharose       | 410       | 915              | 14,090      | 15.4                            | 86      |
| GST-Sepharose     | 106       | 69               | 12,890      | 186                             | 79      |
| Thrombin          | 54        | 55               | 11,140      | 204                             | 68      |
| DEAE-Sepharose    | 18        | 23               | 6,505       | 278                             | 10      |

**TABLE II**

| Parameter                                      | Recombinant CBS | Native CBS$^a$ |
|------------------------------------------------|-----------------|----------------|
| Specific activity                              | 295 $\mu$mol/h/mg | 160–340 $\mu$mol/h/mg |
| $K_m$(Ser) + AdoMet                            | 2.0 $\pm$ 0.3 mM | 1 $\mu$m |
| $K_m$(Ser) – AdoMet                            | 2.0 $\pm$ 0.3 mM | 0.7–8.3 mM |
| $K_m$(Hcy) + AdoMet                            | 5.0 $\pm$ 0.9 mM | 0.7 mm |
| $K_m$(Hcy) – AdoMet                            | 4.8 $\pm$ 0.3 mM | 1–25 mm |
| $K_m$(AdoMet)                                  | 0.96            | 0.76 or 1.93   |
| Hemes/tetramer                                 | 2               | 2 or 4         |
| PLP/tetramer                                   | 2               | 4              |
| pH optimum                                     | 8.5             | 8.3–8.6        |

$^a$The references from which these values are taken are reported in the text.
both wild type and recombinant CBS purified by Kraus and co-workers (10). The ratio of the absorbances at 280 and 428 nm is 0.76 for the liver (28) and 0.89 for the recombinant (29) human enzymes, respectively. Protein purified in our laboratory exhibits a ratio of 0.96, comparable to 1.03 reported for the native pig enzyme by Kim and Deal (13). These authors had also concluded that 2.1 mol of heme are bound per mol of tetramer. Hence, the heme to protein ratio of all these preparations is similar. The extinction coefficient estimated by Kraus and co-workers (10) ($\epsilon_{280,\text{nm}} = 81.5 \text{ m}^{-1} \text{ cm}^{-1}$ at pH 8.6) appears to have been based on an assumed stoichiometry of 4 hemes/tetramer. Instead, a value of 2 hemes/tetramer, consistent with the metal and pyridine hemochrome analyses, would yield the same extinction coefficient as determined by us (i.e. $166 \text{ m}^{-1} \text{ cm}^{-1}$ at pH 8.6).

Second, specific activities of our preparations are comparable to those reported for the native (160–340 units/mg(4) (12, 28, 30)) and recombinant (200–300 units/mg (11)) enzymes. If there was a 2-fold difference in the heme content between our preparations and those reported in the literature, a 2-fold lower specific activity would have been expected based on the direct correlation between specific activity and heme saturation in CBS (11). Hence, based on the spectroscopic, metal analysis, and activity data, we conclude that the recombinant human enzyme is indistinguishable from the native enzyme in its heme content and binds two hemes per tetramer.

**Characterization of PLP—** We have reexamined the PLP content of CBS using a fluorimetric method in which the cofactor is selectively separated from the enzyme by treatment with hydroxylamine, followed by filtration. The heme remains bound to the enzyme, as evidenced by the absorption spectra of the filtrate (which has PLP) and the retentate (which has heme). Based on this analysis, 1.92±0.2 PLP per tetramer was observed, the average from four independent determinations. Thus, heme and PLP appear to be bound to the tetramer in a ratio of 1:1. Since CBS is a homotetramer, the presence of one heme and one PLP per dimer suggests that the minimum requirement for the active site is the dimer.

In order to eliminate the possibility of an error in the protein determination that could account for the observed cofactor to tetramer ratio, we subjected the purified protein to total amino acid analysis to obtain an independent measure of the protein concentration. The recovered protein mass (8.7 ± 1.8 µg) was within 87% of the expected value (10 ± 0.3 µg) estimated using the Bradford method with bovine serum albumin as a standard. This rules out a discrepancy between the estimated and actual protein concentration and confirms a 1:1 heme to PLP ratio per dimer.

The PLP stoichiometry is surprising due to the homology between CBS and the PLP binding domain of tryptophan synthase (31), which suggests that lysine 119 in CBS forms the internal aldime with PLP. Since four such lysines are available per tetramer, it is surprising that only two PLPs appear to be bound. We can rule out partial saturation of PLP in CBS in our preparations since the enzyme activity in the presence and absence of exogenous PLP is virtually the same. We cannot, however, rule out the possibility that half of the enzyme that we and others (11, 28, 30) have isolated (with a specific activity of ~300 units/mg) is inactive. While comparison of the UV-visible absorption spectrum allow us to conclude that preparations of CBS reported in the literature have the same heme content as ours, comparison of the specific activities of the various CBS preparations lead us to infer that they also have a similar PLP content.

**Redox Effects on CBS Activity—** Homocysteine is a branch point metabolite and can be diverted into transmethylation or trans-sulfuration pathways (Fig. 1). By using an in vitro system that contained enzyme and substrate concentrations approximating in vivo conditions in rat liver, Finkelstein and Martin (32) have demonstrated that the flux through the transmethylation and trans-sulfuration pathways is almost equal. Both methionine synthase and CBS, the enzymes operating at this junction, have redox active cofactors, cobalamins in the former and heme in the latter. It is well known that methionine synthase is redox-sensitive, since the intermediate cob(I) alamin is labile to oxidation, and this leads to inactive enzyme. Thus, a lower redox potential enhances activity of methionine synthase (33). In order to examine whether or not redox changes in the heme cofactor have any effect on CBS activity, we compared spectroscopic changes accompanying reduction with changes in enzyme activity.

The enzyme as isolated has low spin ferric heme with a Soret absorption maximum at 428 nm and a broad absorption peak at 550 nm. This spectrum is unaffected by deoxygenation to give an anaerobic solution (Fig. 3). The heme can be reduced using a variety of reagents including dithionite and titanium citrate. Upon addition of 5 mM reductant, the spectrum of the enzyme changes, with the Soret band shifting to 450 nm, and the $\alpha$ and $\beta$ bands sharpen at 571 and 540 nm respectively (Fig. 3), similar to that noted previously with dithionite (13, 14).

In order to measure enzyme activity under comparable conditions, we have modified the standard aerobic assay into an anaerobic one as described under “Experimental Procedures.” Under these conditions, the activity of CBS is routinely enhanced 1.4-fold (Table III). It is not known whether the activation observed under anaerobic conditions is due to protection of the substrate, homocysteine, from oxidation during the assay or results from the oxidative lability of the enzyme itself. Addition of 5 mM titanium citrate routinely decreased activity by ~1.7-fold. This inhibition of enzyme activity was reversed by oxidation back to ferric heme on addition of 5 mM potassium.

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3 These authors had estimated a native molecular mass of 218 kDa (similar to the 252 kDa that is expected from an $\alpha_4\beta_4$ tetramer of 63-kDa subunits). However, they had erroneously attributed the oligomeric structure to $\alpha_2\beta_2$, based on their observation of two different subunit sizes of 61 and 45 kDa, respectively. From what is now known about the proteolytic sensitivity of the liver enzyme, it is likely that they had a mixture of truncated dimers and native tetramers, with the latter predominating to yield a molecular weight estimate close to that expected for the $\alpha_4$ species.

4 These purifications yielded the truncated dimer that is reported to have a $V_{\text{max}}$ that is similar to that of the tetramer (Fig. 5 in Ref. 7).
ferricyanide (Table III and Fig. 3). The observed inhibition of CBS activity could be due to oxidation state changes in the heme cofactor or in the protein (viz. disulfide reduction). In order to distinguish between the two, we have examined the effect of 5 mM potassium ferricyanide on the spectral properties of heme and on CBS activity under anaerobic assay conditions. Dithiothreitol does not reduce the ferric heme (data not shown) nor the dimer interface. The heme cofactor is redox active. Reversible modulation of CBS activity is correlated with the redox state of the heme with ferrous CBS being approximately 2-fold less active than the ferric enzyme. This redox sensitivity may be of significance in the regulation of homocysteine flux through the trans-sulfuration pathway under more reducing conditions. The action of γ-cystathionase converts cystathionine to cysteine, a precursor of the major cellular redox buffer, glutathione. Under oxidizing conditions, an increase in CBS activity could lead indirectly to an increase in the antioxidant, glutathione. These studies raise the possibility that under in vivo conditions, redox potential may mediate reciprocal regulation at a metabolic junction crucial to removal of the toxic metabolite, homocysteine.

Conclusions—Human CBS is a tetrameric protein composed of identical subunits. It is unique in having both PLP and heme as cofactors. In this study, the stoichiometry of bound PLP and heme to the tetramer has been carefully established. One equivalent each of heme and PLP appear to bound per dimer. This suggests that the active site is constructed at the dimer interface. The heme cofactor is redox active. Reversible modulation of CBS activity is correlated with the redox state of the heme with ferrous CBS being approximately 2-fold less active than the ferric enzyme. This redox sensitivity may be of significance in the regulation of homocysteine flux through the trans-sulfuration pathway in response to changes in the ambient redox status of the cell (Fig. 1).

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