Characterization of the Heme and Pyridoxal Phosphate Cofactors of Human Cystathionine β-Synthase Reveals Nonequivalent Active Sites[†]

Shinichi Taoka, Matthew West, and Ruma Banerjee*

Department of Biochemistry, University of Nebraska, Lincoln, Nebraska 68588-0664

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ABSTRACT: Cystathionine β-synthase is an unusual enzyme that requires the cofactors heme and pyridoxal phosphate (PLP) to catalyze the condensation of homocysteine and serine to generate cystathionine. This transsulfuration reaction represents one of two major cellular routes for detoxification of homocysteine, which is a risk factor for atherosclerosis. While the β-replacement reaction catalyzed by this enzyme suggests a role for the pyridoxal phosphate, the role of the heme is uncertain. In this study we have examined the effect of changing one of the ligands to the heme on the activity of the enzyme. Binding of carbon monooxide results in the displacement of a thiolate ligand to the ferrous heme, and is accompanied by complete loss of cystathionine β-synthase activity. Furthermore, inhibition by CO is competitive with respect to homocysteine, providing the first indication that the homocysteine binding site is in the proximity of heme. Binding of both CO and cyanide to ferrous cystathionine β-synthase occurs in two distinct isotherms and indicates that the hemes are nonequivalent. We have employed fluorescence spectroscopy to characterize the bound PLP and its interaction with serine. PLP bound to cystathionine β-synthase is weakly fluorescent and exists as a mixture of the protonated and unprotonated tautomers. Reaction with hydroxylamine releases the oxime and greatly enhances the associated fluorescence. Binding of serine is accompanied by a shift to the unprotonated tautomer of the external aldimine as well as the appearance of a new fluorescent species at ~400 nm that could be due to the aminoacrylate or to a gemdiamine intermediate. These data provide the first characterization of the PLP bound to cystathionine β-synthase. Treatment of cystathionine β-synthase with hydroxylamine releases two PLPs after 1 day and results in complete loss of activity. Incubation for an additional 3−4 days results in the release of two more PLPs. These data lead us to revise the PLP stoichiometry to 4 per tetramer, and to the conclusion that the heme and PLP sites in cystathionine β-synthase are nonequivalent.

Cystathionine β-synthase (CBS)[1] is a unique enzyme that is dependent on both heme and pyridoxal phosphate for activity. It catalyzes the condensation of homocysteine and serine to generate cystathionine. This transsulfuration reaction is the first step in the catabolic removal of homocysteine from mammalian cells. Elevated levels of homocysteine are correlated with cardiovascular diseases [reviewed in (*1*)] and neural tube defects (*2*). Mutations in CBS are the most common cause of homocystinuria that results in severely elevated homocysteine levels with attendant cardiovascular diseases and other complications.

Human CBS is a homotetrameric enzyme with a subunit molecular mass of 63 kDa. The gene encoding human CBS has been cloned and overexpressed (*3*−*5*). The recombinant protein has kinetic properties that are indistinguishable from the wild-type enzyme (*6*). The stoichiometries of bound heme and PLP are controversial, and 2 (*6*) or 4 (*7*) mol of each cofactor per mole of tetramer has been reported.

Substitution of the hydroxyl group of serine with the thiol of homocysteine is a β-replacement reaction of the type that is commonly catalyzed by PLP enzymes. The role of PLP can therefore be surmised in analogy with enzymes such as tryptophan synthase and *O*-acetylserine sulfhydrylase (Figure 1). In contrast, the chemistry of the reaction catalyzed by CBS provides no obvious rationalization for the role of heme. Indeed this cofactor was discovered only 4 years ago (*7*), and remains poorly characterized. Based on EPR spectroscopy of the rat (*8*) and human (Ojha and Banerjee, unpublished results) CBS and on magnetic circular dichroism studies on the rat enzyme (*9*), the ligands to the heme appear to be cysteine and imidazole, respectively. In both the ferric and ferrous states, the heme is six coordinate and low spin.

Alternative roles that can be considered for the heme in CBS include structural, catalytic, and regulatory. Its presence is essential for CBS since the level of heme saturation apparently controls the level of PLP saturation (*7*). However, it is unclear whether the heme plays a direct role in catalysis. Recent studies from our laboratory have indicated that under in vitro conditions, the activity of CBS is modulated by the ambient redox potential, and that this is correlated with oxidation state changes in the heme (*6*).

All other homocysteine utilizing enzymes employ an active site zinc to enhance the nucleophilicity of homocysteine [pK_a

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* To whom correspondence should be addressed. Email: rbanerje@unlinfo2.unl.edu. FAX: 402-472-7842.

[1] Abbreviations: CBS, cystathionine β-synthase; BSA, bovine serum albumin; DTNB, dithiobis(nitrobenzoic acid); DTT, dithiothreitol; PLP, pyridoxal phosphate.

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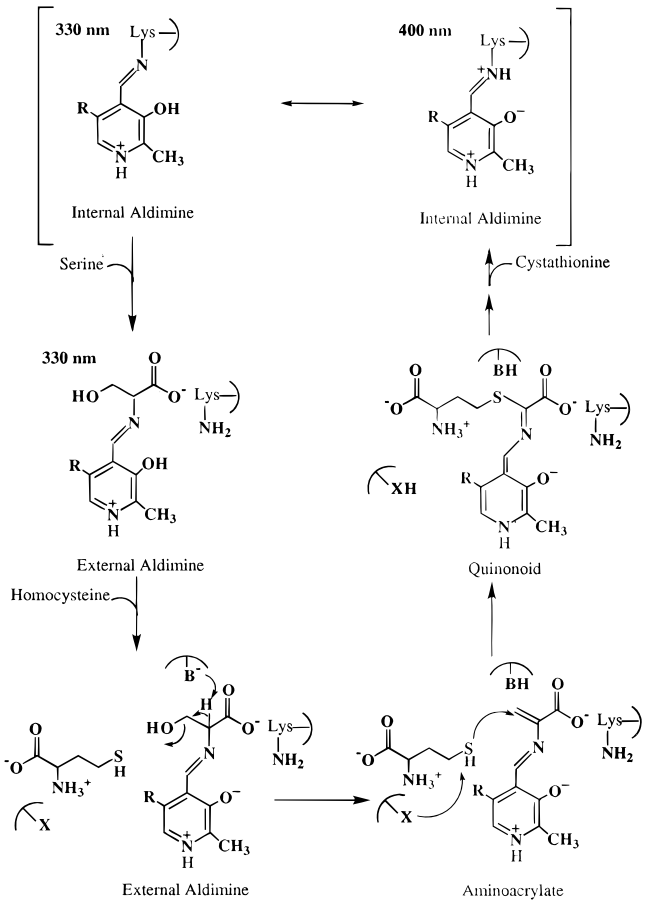

FIGURE 1: Postulated structures of PLP intermediates in the reaction catalyzed by CBS. The mechanism of activation of homcysteine is unknown. X could be either heme or an active site base.

= 10 (*10*)]. In cobalamin-independent (*11*) and cobalamin-dependent (*12*) methionine synthases, it has been demonstrated that homocysteine coordinates directly to the zinc; in betaine homocysteine methyltransferase (*13*), this interaction is presumed. CBS lacks zinc, and the mechanism by which it activates homocysteine is unknown. A role that has been proposed for the heme is that it functions in activation of homocysteine (*6*).

In this study we have characterized the binding of ferrous heme ligands, CO and cyanide, to the heme in CBS, and have examined their effect on catalysis. Since the heme absorption spectrum dominates the UV−visible spectrum of the enzyme, we have employed fluorescence spectroscopy to characterize the bound PLP, and to monitor its interaction with serine. Our studies reveal that the hemes in the CBS tetramer are nonequivalent and display very different affinities for ligands. Similarly, removal of only half the PLPs results in complete loss of CBS activity. Together, these data suggest nonequivalent binding sites for both cofactors. Furthermore, blocking of the cysteine ligation site by coordination of CO to heme results in complete loss of CBS activity. Inhibition by CO is reversible and competitive with respect to homocysteine. These data provide the first characterization of the serine and homocysteine binding sites on CBS.

MATERIALS AND METHODS

Materials. The following chemicals were purchased from Sigma: BSA, DTNB, DTT, Tris, homocysteine thiolactone, pyridoxal 5′-phosphate, hydroxylamine, sodium dithionite. Potassium cyanide and ammonium hydroxide were from Fisher Scientific Co., and CO was from the BOC Group Inc. (Murray Hill, NJ). L-[U-^{14}C]serine (158 mCi/mmol) was purchased from Amersham Life Science. AdoMet 1,4-butane disulfonate was a generous gift from Knoll Farmaceutici Spa (Milano, Italy). Titanium chloride was purchased from Aldrich.

L-Homocysteine was synthesized from L-homocysteine thiolactone as described previously (*14*). The concentration of homocysteine was determined by titration with DTNB, and calculated using an ϵ_{412} of 13.6 mM^{-1} cm^{-1}. Titanium(III) citrate was prepared from titanium chloride and sodium citrate at pH 7 as described previously. (*15*).

Enzyme Isolation and Assay. Recombinant human cystathionine β-synthase was purified and assayed as described previously (*6*). The recombinant strain expressing human CBS was a generous gift from Dr. Warren Kruger (Fox Chase Cancer Institute). Protein concentration was determined using the Bradford reagent (Bio-Rad), with BSA as standard.

Removal of PLP from the Protein. PLP was removed by incubation of CBS (0.5 mg) in either 100 mM Tris (pH 8.6) or potassium phosphate (pH 7.2), containing 5 mM hydroxylamine, for 1−4 days at 4 °C. For activity measurements, unbound PLP was removed by dialysis against 2 × 1 L of 50 mM Tris (pH 8.6) containing 2.5 mM DTT at 4 °C. For quantitation of PLP that was released from the enzyme by treatment with hydroxylamine, a fluorimetric method was employed as described previously (*6*). For this, the PLP oxime was separated from CBS by Centricon filtration.

Inhibition Kinetics. Inhibition of CBS activity was measured under anaerobic conditions as described previously (*6*). CBS (3.8 μg) was reduced by addition of 5 mM titanium citrate. Anaerobic potassium cyanide (dissolved in distilled water) was added to the reaction mixture to achieve final concentrations ranging from 0 to 50 mM.

CO inhibition of CBS activity was assayed under anaerobic conditions by modification of the previously described procedure (*6*). To avoid diffusion of CO from the liquid to the gas phase, the reaction was carried out in a 1 mL plastic syringe as follows. Components of the reaction mixture were added to the syringe in a Coy anaerobic chamber (with a 95:5 atm of N_2:H_2). CO-saturated anaerobic water (1 mM CO concentration at 25 °C) was employed as a stock, and the desired aliquot was added to give final concentrations ranging from 0 to 100 μM. After all the components were added, the position of the plunger was adjusted to eliminate the gas phase, and the nozzle of the syringe was sealed with a rubber septum. The syringes containing the assay mixtures were incubated at 37 °C for 30 min, and the reaction was terminated as described previously. To determine the pattern of inhibition of CO with respect to homocysteine, enzyme activity was monitored at a given CO concentration in the presence of varying concentrations of homocysteine as indicated in the figure legend.

Absorption Spectroscopy of CBS in the Presence of Cyanide or CO. Binding of ligands to ferrous CBS was monitored under anaerobic conditions in a cuvette. CBS (0.24 mg/mL) was reduced by 2−5 mM reductant (either titanium citrate or dithionite), and aliquots of anaerobic cyanide or CO solutions were added. Since spectral changes were

observed during the first 1−2 min following addition of the ligand, the spectrum at each ligand concentration was recorded after 5 min. The concentration range for cyanide and CO varied from 0 to 100 mM and from 0 to 270 μM, respectively. For the CO titration, the headspace in the cuvette was kept to a minimum.

Fluorescence Spectroscopy. Fluorescence spectra were recorded at 25 °C in a Perkin-Elmer Luminescence spectrometer LS 50. Spectra were scanned at 1 nm/s, and 6 nm slit widths were employed for excitation and emission. A solution containing CBS (0.3 mg/mL) in 0.25 M Tris, pH 8.6, was employed for these studies. Excitation and emission spectra were recorded using conditions described in the figure legends. The fluoresecence intensity of the buffer was subtracted. The K_d for serine was determined by measuring enhancement (at 463 nm) or quenching (at 494 nm) of fluorescence emission as a function of serine concentration.

Equilibrium Dialysis. Equilibrium dialysis was performed to determine the K_d for serine. Solution-containing enzyme (3.4 μM CBS in 75 μL of 0.1 M potassium phosphate buffer, pH 7.2) was placed in the cap of an inverted 0.5 mL microcentrifuge tube and sealed with dialysis membrane as described previously (*16*). The upper chamber contained 75 μL of [^{14}C]-L-serine (ranging in concentration from 2 to 33 μM, 50 000 dpm/μmol) in 0.4 M Tris at pH 8.6. The two solutions were allowed to equilibrate for 3 days at 4 °C. Radioactivity was measured in the two compartments, and the data were subjected to Scatchard analysis.

RESULTS

Characterization of CO Binding and Inhibition. We have examined the binding of CO to ferrous CBS. Reduction of CBS results in a red shift of the Soret band from 428 to 450 nm. Addition of CO to reduced enzyme results in conversion to a species with a Soret absorption maximum at 422 nm with an isosbestic point at 434 nm (Figure 2A). At concentrations of CO that were readily attainable for titration, partial conversion of the 450 nm ferrous form to the 422 nm ferrous CO form is observed. When reduced CBS was added to CO-saturated anaerobic buffer, full conversion to the ferrous CO form was observed (Figure 2A, inset). Binding of CO to CBS was analyzed in an Eadie−Hofstee plot (Figure 2B). Two distinct binding isotherms are observed and correspond to K_d's of 1.5 ± 0.1 and 68 ± 14 μM, respectively. The amplitudes associated with the two binding isotherms are approximately equivalent.

Binding of CO to ferrous CBS results in inhibition of enzyme activity (Figure 3). In the steady-state assay, complete loss of enzyme activity is observed at a CO concentration of 60 μM, and yields a K_i of 5.6 ± 1.9 μM. CO is a reversible inhibitor and competitive with respect to homocysteine (Figure 3, inset). The K_i obtained from this analysis is similar, 5.0 ± 1.6 μM.

Characterization of Cyanide Binding and Inhibition. Binding of cyanide to ferrous CBS causes a blue shift in the Soret maximum from 450 to 435 nm (Figure 4A). An Eadie−Hofstee analysis of the data reveals two distinct slopes, yielding K_d's of 3.3 ± 0.6 and 54 ± 22 mM, respectively (Figure 4B). The amplitudes associated with the two phases are approximately equal. Inhibition of ferrous CBS with cyanide yields a K_{i_1} of 2.3 ± 0.7 mM (Figure 5). The fits to the data using one or two inhibition constants are comparable and therefore do not permit distinction between the two alternatives. The same value for K_{i_1} within experimental error (2.5 ± 0.7 mM) and a value of 60 ± 14 mM for K_{i_2} are obtained when the data are modeled using two ligand binding sites. Since cyanide is known to react with PLP, inhibition of ferric CBS by cyanide was examined. In the oxidized state, the heme in CBS does not bind cyanide (Taoka and Banerjee, unpublished data), so inhibition under these conditions is expected to be independent of a mechanism involving heme. Cyanide inhibits ferric CBS with a K_i of 5.1 ± 1.2 mM, which is similar albeit slightly higher than that observed for ferrous CBS (Figure 5).

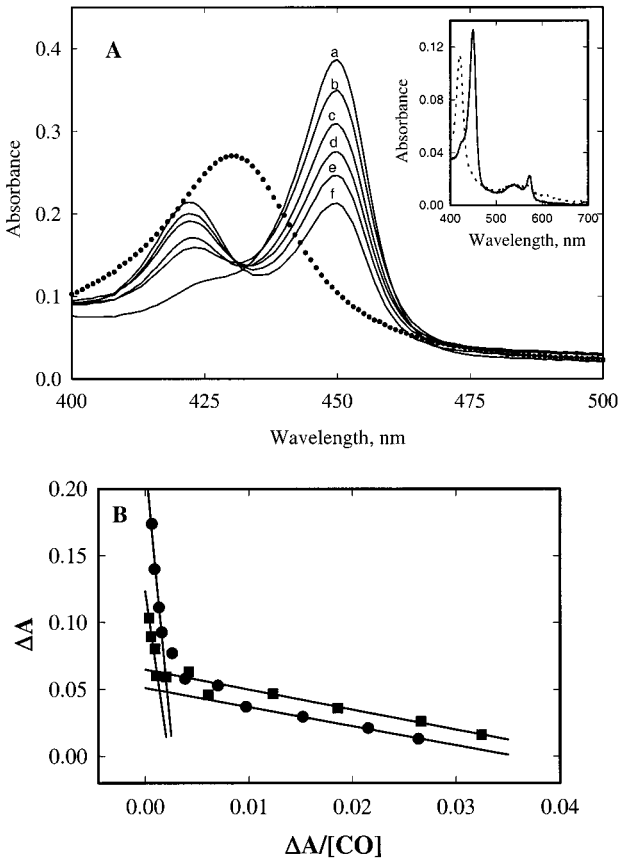

FIGURE 2: Spectral analysis of CO binding to ferrous CBS. (A) To an anaerobic solution of dithionite-reduced CBS (0.24 mg/mL) in 0.6 mL of 0.25 M Tris buffer, pH 8.6, were added aliquots of CO-saturated water, and the absorption spectra were recorded. The dotted line represents the spectrum of ferric CBS prior to dithionite reduction. The different traces were recorded in the presence of the following CO concentrations: (a) 0 μM, (b) 3.8 μM, (c) 30 μM, (d) 84 μM, (e) 160 μM, and (f) 270 μM. Inset: Comparison of spectra of ferrous (—) and ferrous−CO CBS in ~1 mM CO (•••). (B) Eadie−Hofstee analysis of CO binding data at two wavelengths: 450 nm (●) and 422 nm (■).

Fluorescence Spectroscopy of CBS. The PLP in CBS is very weakly fluorescent. Excitation spectra show peaks at 330 and 400 nm, when emission wavelengths are set at 450 and 500 nm, respectively (Figure 6). These are likely to correspond to the two tautomers of the internal aldimine, respectively (Figure 1). Treatment of CBS with hydroxylamine, which reacts with the internal aldimine to form a PLP oxime, results in the enhancement of the excitation and emission fluorescence at 350 and 450 nm, respectively (data not shown). Fluorescence emission at 500 nm decreased upon addition of hydroxylamine. The amplitude's of fluorescence

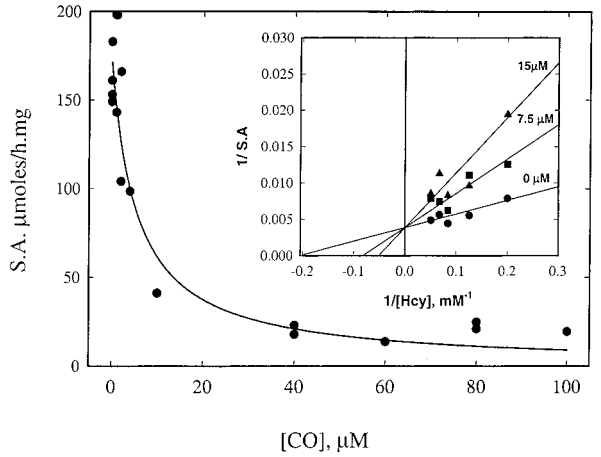

FIGURE 3: Inhibition of CBS by CO. The specific activity of CBS was monitored in the anaerobic assay at varying concentrations of CO. The concentrations of homocysteine and serine were 15 and 30 mM, respectively. Circles represent the experimental data, whereas the line represents a fit to the data using the equation: $SA = [SA]_o/(1 + [CO]/K_i)$, where SA_o is the specific activity in the absence of inhibitor. A K_i of 5.6 ± 2.0 μM is obtained from this analysis. Inset: Steady-state initial velocity inhibition kinetics. Activity of ferrous CBS was monitored in the presence of a fixed concentration of CO and varying concentrations of homocysteine. The lines represent a global fit to the data set using the equation: $V = V_{max}[S]/(K_m(1 + [I]/K_i) + [S])$ for competitive inhibition. The kinetic constants obtained from this fit are $K_i = 5.0 \pm 1.7$ μM for CO, $K_{m_{Hcy}} = 4.9 \pm 1.8$ mM, and $V_{max} = 260 \pm 32$ μmol h^{-1} (mg of protein)$^{-1}$. In contrast, a statistically poorer fit was obtained when the data were treated for noncompetitive inhibition: $K_{i_1} = 14 \pm 12$ μM and $K_{i_2} = 18 \pm 11$ μM, $K_{m_{Hcy}} = 6 \pm 2.3$ mM, and $V_{max} = 280 \pm 39$ μmol h^{-1} (mg of protein)$^{-1}$.

enhancement and diminution are dependent on hydroxylamine concentration. This confirms that the 450 and 500 nm emission spectra in CBS are due to PLP.

Addition of serine results in changes in the fluorescence spectrum of CBS. Whereas an enhancement of fluorescence emission is observed at ~450 nm, quenching is seen at 500 nm (Figure 6). These changes are presumably due to conversion of the internal aldimine to the external aldimine of PLP with serine. In addition, a new fluorescence emission feature is observed at ~400 nm, and its intensity is enhanced by increasing concentrations of serine. Analysis of the quenching data at 494 nm as a function of serine concentration yields a K_d of 7.5 ± 1.7 μM. A similar value is obtained from the data for fluorescence enhancement at 463 nm. In contrast, when serine is added to PLP-free CBS, generated by extensive dialysis of hydroxylamine-treated enzyme, no change in the fluorescence spectrum is observed at either 463 or 494 nm (data not shown). This confirms that the observed serine-dependent changes are due to the interaction of the substrate with PLP. The K_d for serine was verified independently by equilibrium dialysis using radiolabeled serine. A stoichiometry of 4.7 ± 0.6 serines per tetramer was obtained (data not shown).

PLP Stoichiometry. In light of the above data indicating nonequivalent hemes and four serine binding sites per tetramer, we have reexamined the PLP stoichiometry. We have previously reported that two PLPs are bound per tetramer in recombinant human CBS (*6*). However, as we had noted earlier, this was surprising based on the homology between tryptophan synthase and CBS that suggests that

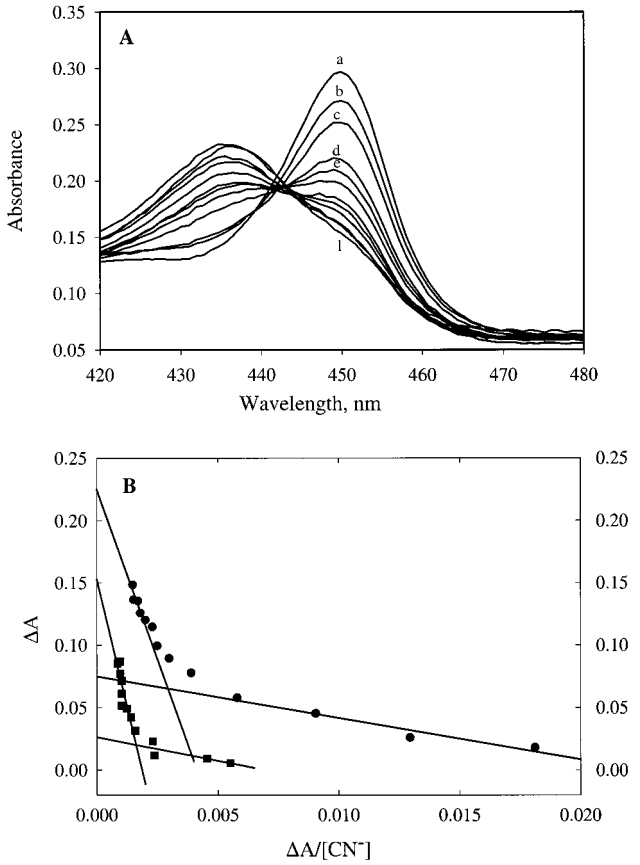

FIGURE 4: Spectral analysis of cyanide binding to ferrous CBS. To an anaerobic solution of dithionite-reduced CBS in 0.25 M Tris·HCl buffer, pH 8.6, were added aliquots from a stock potassium cyanide solution, and the absorption spectra were recorded. The different traces were recorded in the presence of the following cyanide concentrations: (a) 0 mM, (b) 1 mM, (c) 2 mM, (d) 5 mM, (e) 10 mM, (f) 30 mM, (g) 40 mM, (h) 50 mM, (i) 60 mM, (j) 70 mM, (k) 80 mM, and (l) 100 mM. (B) Eadie−Hofstee analysis of the cyanide binding data at two wavelengths: 450 nm (●) and 435 nm (■).

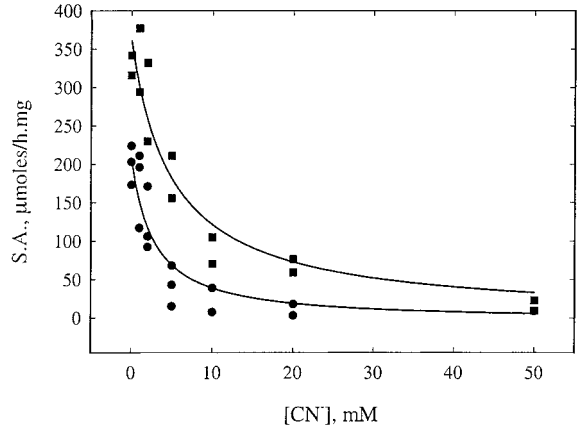

FIGURE 5: Inhibition of CBS by cyanide. The specific activity of CBS was monitored in the anaerobic or aerobic assay at varying concentrations of cyanide. The concentration of homocysteine was 15 mM, and that of serine was 30 mM. Squares represent the experimental data from the aerobic assay using ferric CBS, and circles from the anaerobic assay using ferrous CBS. The lines represent fits to the data using the equation described in the Figure 3 legend.

lysine 119 in CBS forms the internal aldimine with PLP. Since CBS is a homotetramer, it was unexpected that only two PLPs appeared to be bound. We have thus redetermined the PLP content of human CBS, using hydroxylamine to

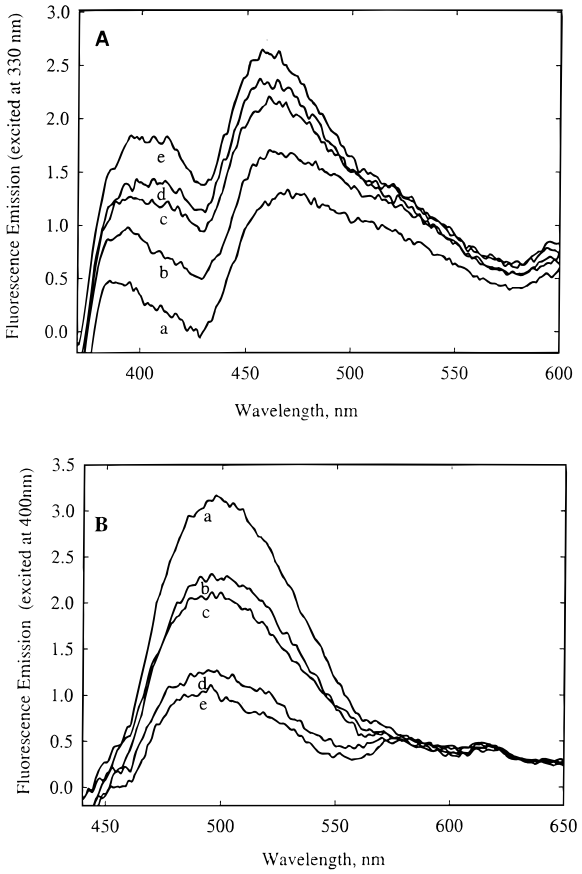

FIGURE 6: Fluorescence changes in CBS-bound PLP induced by serine. The excitation wavelengths are indicated in the figures. (A) Enhancement of fluorescence emission by serine at ~400 and 450 nm. The concentrations of serine in successive spectra are as follows: (a) 0 μM, (b) 10 μM, (c) 21 μM, (d) 26 μM, and (e) 31 μM. (B) Quenching of fluorescence emission by serine at 500 nm. The concentrations of serine in successive spectra are as follows: (a) 0 μM, (b) 5.2 μM, (c) 10 μM, (d) 16 μM, and (e) 52 μM.

Table 1: Correlation between PLP Content and CBS Activity

treatment[a]	PLP/tetramer[b]	SA[c] (μmol^{-1} h^{-1} mg^{-1})
none	4	321 ± 60 (−PLP) 329 ± 32 (+PLP)
5 mM NH_2OH, 4 °C, 24 h	2	17.7 ± 6.5 (−PLP) 112 ± 23 (+ PLP)
5 mM NH_2OH, 4 °C, 72 h	0	0 (−PLP) 3.9 ± 2.8 (+ PLP)

[a] Following treatment with hydroxylamine, CBS was dialyzed extensively as described under Materials and Methods. [b] PLP was determined using a fluorometric method as described under Materials and Methods. [c] The assays were conducted in the presence or absence of added PLP, and the specific activities represent the average of three experiments.

liberate bound PLP as an oxime that is separated from the protein by Centricon filtration. Hydroxylamine treatment releases two PLPs per tetramer after 1 day at 4 °C, as reported previously, and is accompanied by almost complete loss of activity. Prolonged incubation (3−4 days) results in the release of two additional PLPs (Table 1). Based on these results, the PLP content of CBS is revised to be four per tetramer. Addition of PLP to hydroxylamine-treated enzyme results in partial (34%) recovery of enzyme activity when two of the four PLPs were initially removed, but no recovery if all four were removed. The reason for this difference is presently unclear. Since the loss of only two of the four PLPs

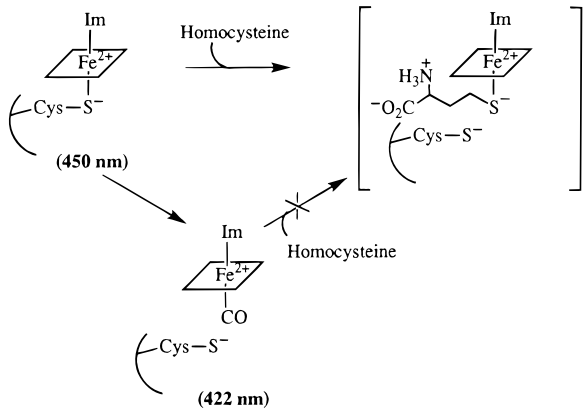

FIGURE 7: Postulated mode of CO binding to ferrous CBS. The ligands to the CBS heme are predicted to be imidazole and cysteine, respectively. Binding of CO results in displacement of the thiol ligand as indicated by changes in the visible absorption spectrum. A hypothetical ES species in indicated in parentheses in which homocysteine displaces the ligating cysteine.

results in loss of 94% of the enzyme activity, it indicates that like the hemes, the PLPs associated with the CBS tetramer are nonequivalent.

DISCUSSION

Cystathionine β-synthase is one of two key mammalian enzymes that is responsible for the management of cellular homocysteine levels. It has been estimated that approximately 50% of the homocysteine is catabolized via the transsulfuration pathway, whereas the remaining 50% is salvaged via remethylation (*17*). Hence, understanding the mechanism and regulation of CBS is key to understanding cellular homocysteine metabolism. Superficially, the reaction catalyzed by CBS resembles that of other β-replacement reactions catalyzed by PLP-dependent enzymes. In this mechanism, binding of serine results in a transschiffization reaction in which the internal aldimine is replaced by an external aldimine (Figure 1). The external aldimine then undergoes proton abstraction at the α-carbon followed by elimination to generate an aminoacrylate intermediate. Borscok and Abeles reported that a low level of tritium is released from the enzyme−[α-^{3}H]serine complex, and that this was enhanced in the presence of homocysteine (*18*). Thus, the aminoacrylate may be formed preferentially in the ternary complex in which homocysteine is bound. Activation of homocysteine to generate a thiolate is required for nucleophilic attack on the aminoacrylate species. Reprotonation at Cα and transschiffization generate the product, cystathionine.

In this study, we have characterized two aspects of the mechanism depicted in Figure 1. First we have examined the potential role of heme in the reaction by evaluating whether changes in the ligands to heme affect enzyme activity. We have proposed previously that the heme could, in principle, serve to activate homocysteine by direct coordination, in which the cysteine ligand is replaced by homocysteine (Figure 7). To test this hypothesis, we have examined the effect of blocking the thiol binding site with heme ligands on the activity of the enzyme. Second, we have used fluorescence spectroscopy and equilibrium dialysis to characterize the enzyme-bound PLP and the interaction between PLP and serine.

CBS as isolated has two low-spin six-coordinate hemes per tetramer (*6*), with a red-shifted Soret absorption maximum at 428 nm. The oxidized heme shows no affinity for common ferric ligands such as azide, cyanide, and fluoride (Taoka and Banerjee, unpublished data). Reduction of the CBS-bound heme with titanium citrate results in a shift in the Soret band to 450 nm, and an ~2-fold decrease in enzyme activity (*6*). In the reduced state, two ligands, CO and cyanide, bind to the heme and bring about significant blue shifts of the Soret band to 422 and 435 nm, respectively. The spectrum of CO-treated CBS resembles that of several ferrous−CO complexes of heme proteins including the heme sensor CooA (*19*), horseradish peroxidase (*20*), and indoleamine 2,3-dioxygenase (*21*). This indicates that CO binding displaces the cysteine ligand but that the imidazole ligand is retained (Figure 7). Similarly, the cyano−CBS complex is similar to that of other heme proteins that bind cyanide in the ferrous state, including indoleamine 2,3-dioxygenase (*21*) and horseradish peroxidase (*20*). Binding of CO to the heme in CBS has been reported previously for the rat enzyme (*8*) that was described as H450 prior to the recent discovery that H450 is CBS (*7*). However, characterization of CO binding and its effect on enzyme activity have not been reported previously, nor has the characterization of cyanide binding to ferrous CBS.

Binding of CO to CBS occurs in two distinct isotherms, and an ~45-fold difference in affinities separates the two binding sites. Due to the technical difficulty of performing titrations at high concentrations of CO using a 1 mM CO-saturated stock solution, the standard deviation for the second K_d is large. Changes in the UV−visible spectrum are consistent with the loss of the cysteine ligand and retention of the imidazole ligand, to give six-coordinate low-spin ferrous heme as shown in Figure 7. Binding of CO results in complete loss of enzyme activity and yields a K_i of 5.6 ± 1.9 μM, which is slightly higher than the K_{d1} for CO binding (1.5 ± 0.1 μM). Thus, blocking of the ligation site for cysteine results in complete loss of CBS activity and argues for a role for the heme in catalysis. This is further supported by the competitive inhibition kinetics displayed by CO with respect to homocysteine. These data provide the first indication that the homocysteine binding site is in the proximity of heme.

Binding of cyanide to ferrous CBS similarly displays biphasic behavior, yielding K_d's that differ by a factor of ~16-fold. Compared to CO, the affinity for cyanide is much lower, being in the millimolar rather than micromolar range. The low affinity for the second cyanide results in a high value for K_{d_2} (54 ± 22 mM). Thus, as with CO binding, the cyanide data indicate that the hemes have very different affinities for ligands reflecting differences in their microenvironments and/or accessibility. Although cyanide inhibits ferrous CBS with a K_i (2.7 ± 1.0 mM) that is similar to K_{d_1} (3.3 ± 0.6 mM), these data are not simple to interpret. The correspondence between K_i and K_{d_1} for cyanide binding to ferrous heme may be purely coincidental since cyanide is known to interact with PLP. Indeed, cyanide inhibits ferric CBS with a K_i of 5.1 ± 1.2 mM, similar to the K_i measured for ferrous CBS. These data suggest that inhibition of CBS by cyanide is due to its interaction with PLP since it is observed even under conditions where cyanide does not bind to the heme. Nevertheless, these data provide further evidence for the nonequivalence of the hemes bound to CBS.

Fluorescence spectroscopy provides the first information on the characteristics of PLP in CBS. Since the visible spectrum is dominated by heme absorption, it is difficult to directly monitor PLP-specific changes. The fluorescence spectra result from absorption features centered at ~330 and 400 nm that give rise to emission maxima at ~450 and 500 nm, respectively. Treatment of CBS with hydroxylamine results in a strong fluorescence emission at 450 nm, consistent with the formation of PLP oxime, and confirms that the weak fluorescence observed with CBS is due to bound PLP. We assign the absorption features to the two tautomeric forms of the internal aldimine in which the imine nitrogen is either unprotonated (330 nm) or protonated (400 nm). Similar absorption maxima have been observed in other PLP-dependent enzymes including ornithine decarboxylase [335 and 420 nm (*22*)], aminolevulinate synthase [330 and 420 nm (*23*)], and tryptophan synthase [410 nm (*24*)]. The low fluorescence yield from the PLP bound to CBS could be due to energy transfer to heme; however, the distance between the two cofactors is not known.

Addition of serine results in interesting changes in the fluorescence spectrum. Serine causes enhancement of emission at ~450 nm, and quenching at 500 nm. In addition, a new emission maximum is observed at ~400 nm which increases in intensity with increasing concentration of serine, and corresponds to an excitation maximum at ~330 nm. The reciprocal changes at 450 and 500 nm suggest that formation of the external aldimine of serine is accompanied by a change in the distribution of the two tautomers, with the 330 nm or unprotonated species predominating. The origin of the 400 nm emission is unknown. Two candidates that can be considered are the aminoacrylate species shown in Figure 1 or a gemdiamine intermediate formed during the transschiffization reaction. The absorption maxima of both these species can be at low wavelengths, e.g., ~350 nm for the aminoacrylate and 310−340 nm for the gemdiamine (*24*). Removal of PLP from CBS by hydroxylamine treatment followed by dialysis results in the loss of the fluorescence emission at 450 and 500 nm, and insensitivity of the fluorescence spectrum to addition of serine.

The K_d for serine obtained from the fluorescence data (7.5 ± 1.7 μM) is significantly lower than its K_m [2.0 ± 0.3 mM (*6*)]. Equilibrium dialysis measurements confirm the binding data from fluorescence measurements and yield a stoichiometry of ~4 serines per tetramer. Unlike binding of heme ligands to CBS, binding of serine to PLP is monophasic.

We have redetermined the PLP stoichiometry. Our results confirm that using conditions described in the literature for removal of PLP (*7*), only two PLPs are removed as we have described previously (*6*). This is accompanied by the complete loss of enzyme activity. Based on these results, we had erroneously assumed that all the PLPs had been removed. However, prolonged incubation of CBS with hydroxylamine results in the release of two additional PLPs. Thus, CBS has four PLPs per tetramer, consistent with four serine binding sites.

In summary, our studies on the binding of cyanide and CO to CBS as well as the release of PLP from the enzyme indicate nonequivalent binding sites. Plasma emission spectroscopy and pyridine hemochrome analyses indicate the presence of two hemes per tetramer (*6*). These hemes have

very different affinities for the ligands, CO and cyanide. Similarly, two of the four PLPs are released more readily. The binding and inhibition data together indicate that only two of the four active sites are involved in catalysis; i.e., CBS exhibits half-of-sites activity. In addition, the weak fluorescence of CBS-bound PLP suggests proximity of the two cofactors although alternative explanations are also possible. Our fluorescence data provide the first characterization of PLP bound to CBS.

Inhibition of CBS activity by the heme ligand, CO, supports a role for the heme in catalysis. Alternatively, a conformational change could account for the inhibition, although it should be noted that neither a spin state change nor a coordination number change accompanies binding of CO to CBS. It is interesting that the heme in CBS resembles the heme in the CO-sensing transcriptional activator, CooA, from *Rhodospirillum rubrum*. This protein as-isolated has a six-coordinate, low-spin heme in which cysteine is one of the ligands. The thiolate ligand is apparently exchange-labile and is replaced by an imidazole in the ferrous state (*19*). CO containing CooA resembles CBS in that imidazole and CO are the fifth and sixth ligands, respectively (*25*). Binding of CO to the heme results in a conformational change that activates CooA as a transcriptional regulator. While our results indicate that ligand exchange in the heme modulates activity of CBS, they do not as yet allow distinction between a catalytic versus a regulatory role.

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