Binding of Pyridoxal 5′-Phosphate to the Heme Protein Human Cystathionine β-Synthase†

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ABSTRACT: Cystathionine β-synthase (CBS), a pyridoxal 5′-phosphate (PLP) dependent enzyme, catalyzes the condensation of serine and homocysteine to form cystathionine. Mammalian CBS was recently shown to be a heme protein. While the role of heme in CBS is unknown, catalysis by CBS can be explained solely by participation of PLP in the reaction mechanism. In this study, treatment of CBS with sodium borohydride selectively reduced the Schiff base but did not affect the heme. Purification and sequencing of the PLP-cross-linked peptide from a trypsin digest of the reduced enzyme revealed the evolutionarily conserved Lys119 to be the residue forming the Schiff base. Serine and hydroxylamine form an α-aminoacrylate and an oxime with PLP in CBS, respectively. The sulfhydryl-containing substrate, homocysteine, disturbs the heme environment but does not interact with PLP. In contrast to other PLP-dependent enzymes, CBS emits no PLP-related fluorescence when excited at 296 or 330 nm. PLP but not heme dissociates from the enzyme in the presence of hydroxylamine. The dissociation of PLP is a multistage process involving a short ∼500 s lag phase, followed by a rapid inactivation and a slower PLP−oxime formation. PLP-free CBS exhibits a decrease of secondary structure as well as loss of CBS activity that can be only partially restored by PLP. This study constitutes the first comprehensive investigation of PLP interaction with a heme protein.

Pyridoxal 5′-phosphate (PLP)[1] is a cofactor in numerous transformations of amino acids such as transamination, β-elimination, β,γ-replacement, decarboxylation and racemization (*1, 2*). In general, the cofactor acts as an "electron sink", withdrawing electrons from the substrate and forming imines with amino groups of substrates. In all of the PLP-containing enzymes studied so far, the carbonyl group of the coenzyme binds to an ε-amino group of a lysine residue in the active site to form an internal aldimine (*1*). In the course of catalysis, the amino group of the substrate replaces the enzyme lysine in the Schiff base to form an external aldimine. The lysine residue thus plays an essential role in reactions catalyzed by PLP-dependent enzymes. The unstable Schiff base in the PLP−lysine complex can be reduced by sodium borohydride to form a chemically stable secondary amine. This chemical cross-linking of PLP to the enzyme facilitates identification of the essential lysine in the PLP-binding site in PLP-dependent enzymes (*3*).

Cystathionine β-synthase (EC 4.2.1.22, CBS) catalyzes the PLP-dependent replacement of the β-OH group of serine by homocysteine to form cystathionine, an essential intermediate in transsulfuration. Human and rat CBS are homotetramers of 63 kDa subunits (*4*) and associate into higher oligomers in solution (*5*). Both enzymes are activated by *S*-adenosyl-L-methionine (AdoMet)—a principal regulator of sulfur flux through the transsulfuration pathway (*6*). Deficiency in CBS is the most common cause of homocystinuria, an inherited autosomal recessive metabolic disease. About half of all patients with homocystinuria respond to pharmacological doses of pyridoxine, a precursor of PLP (*7*).

We recently showed that heme is also an essential cofactor for mammalian CBS (*8*). The presence of heme in mammalian CBS is striking because the mechanism of the β-replacement reactions catalyzed by the enzyme can be explained using PLP as the only cofactor (*9, 10*). All the other structurally and functionally related enzymes such as bacterial and plant *O*-acetylserine sulfhydrylase, OASS, EC 4.2.99.8 (*11, 12*), tryptophan synthase, EC 4.2.1.20 (*13*), and serine, EC 4.2.1.13, and threonine, EC 4.2.1.20 dehydratases (*14*), contain PLP as the only cofactor. The β-replacement reaction catalyzed by the above PLP-dependent enzymes proceeds according to the general mechanism shown in Scheme 1[2] (*15*). Changes in the status of PLP intermediates are concomitant with corresponding changes in the spectral

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[1] Abbreviations: AdoMet, *S*-adenosyl-L-methionine; CBS, cystathionine β-synthase; CD, circular dichroism; OASS, *O*-acetylserine sulfhydrylase; PLP, pyridoxal 5′-phosphate; TBS, Tris buffered saline (10 mM Tris·HCl, 150 mM NaCl, pH 8.6); TPCK, N^{α}-tosyl-L-phenylalanine chloromethyl ketone.

[2] The original of the Scheme 1 was kindly provided by Dr. Paul F. Cook. Reprinted from: (1995) *Biochemistry 34*, 12152−12160 (©1995, American Chemical Society).

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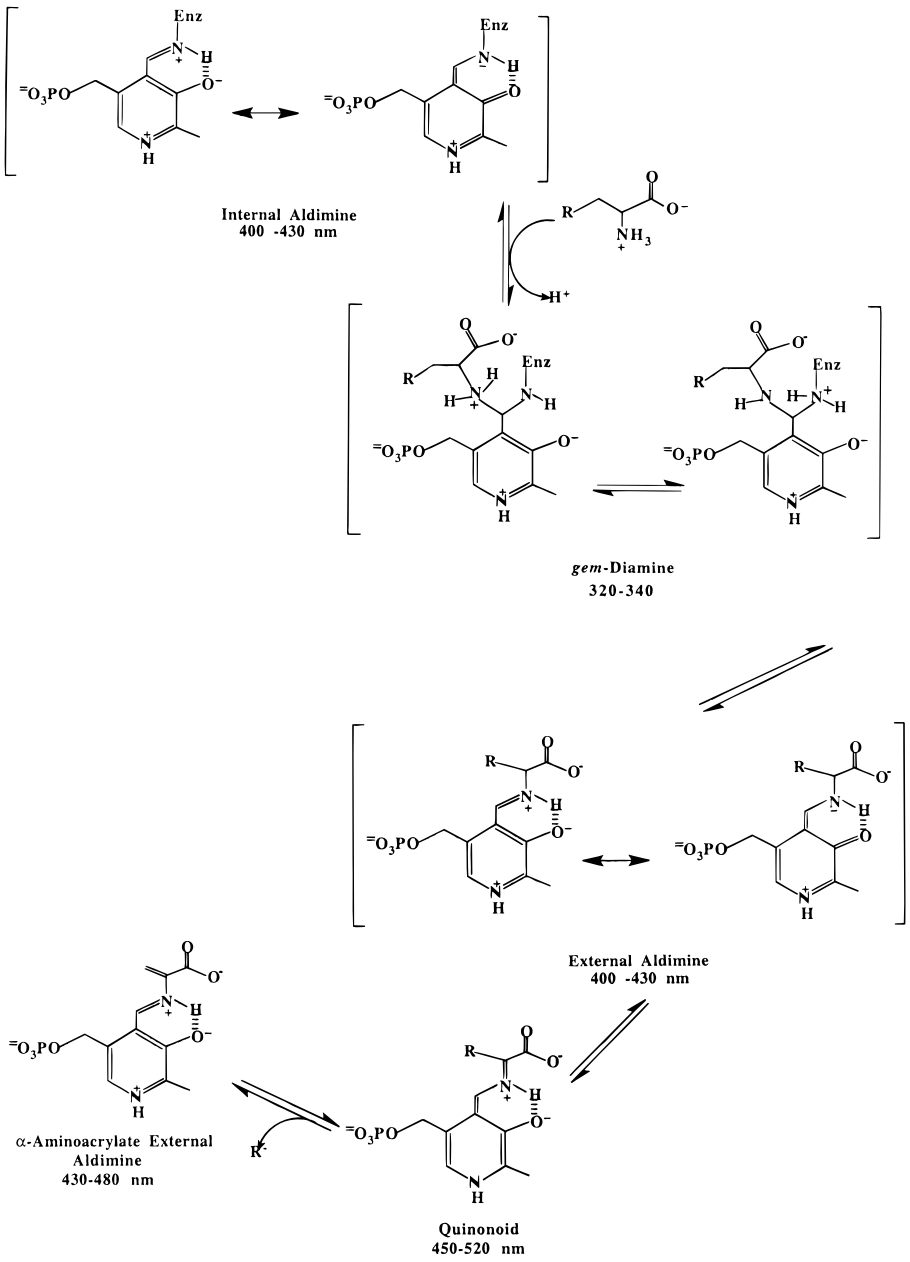
Scheme 1



properties of PLP. This fact allows investigation of various intermediates formed in the reaction pathway.

Trypsinolysis of the human recombinant CBS into a dimer of 45 kDa subunits was recently studied in detail (*16*). This 45 kDa CBS consists of an evolutionarily conserved sequence, is twice as active as the full length CBS, and is not sensitive to AdoMet regulation. The lack of formation of higher oligomers by the 45 kDa CBS facilitates the study of the enzyme in solution by physical methods.

Amino acid sequence comparisons between the rat CBS and other related PLP-dependent enzymes (*17*) indicated that there are two highly conserved regions in CBS that may include the essential lysine residue involved in the formation of Schiff base with PLP. One is the region containing the SVK motif around Lys116 in rat CBS (Lys119 in human CBS) that aligns with a lysine−PLP residue in bacterial and plant OASS, bacterial tryptophan synthase, and various serine and threonine deaminases (*18*). Another region is the motif SK*NAGLKCE at Lys94 in rat CBS (Lys97 in human CBS) with six residues identical to the PLP binding site in aspartate aminotransferases from seven sources (*17*), suggesting that Lys89 may be the residue binding PLP in rat CBS (Lys92 in human CBS).

This paper describes number of experiments designed to determine the mechanism of PLP dissociation of CBS and the molecular properties of this hemeprotein in the presence and absence of PLP.

MATERIALS AND METHODS

Chemicals. Amino acids, dithiotreitol, *S*-adenosyl-L-methionine, N^{α}-tosyl-L-phenylalanine chloromethyl ketone (TPCK, specific inhibitor of chymotrypsin), trypsin (protein sequencing quality), and urea were obtained from Sigma. Acetonitrile and trifluoroacetic acid were purchased from Fluka, L-[^{14}C]-serine was obtained from NEN Life Sciences Products.

Human Recombinant CBS. The cloning of human liver CBS cDNA in the expression vector pAX5$^-$ (U. S. Biochemical Corp.), transformation of *E. coli* XL1-Blue MR (Stratagene) with the plasmid construct, and purification of the enzyme to homogeneity were performed as described previously (*19*). The CBS active core (a dimer of 45 kDa CBS subunits) was prepared by limited proteolytic cleavage with trypsin of the β-galactosidase−CBS fusion protein and was isolated from the crude *E. coli* extract as previously described (*20*). Further purification followed the procedure previously developed for the full length CBS.

Cross-Linking of PLP to CBS and Purification of the Cross-Linked Peptide. The 45 kDa CBS, 2 mg (44.4 nmol), was dissolved in 100 μL of 0.1 M Tris·HCL buffer, pH 8.6. The sample was then incubated in three changes of sodium borohydride for 20 min each. The reduced protein was separated from borohydride on Sephadex G-25 fine, equilibrated in ammonium bicarbonate buffer, 100 mM, pH 8.0. The filtrate was then denatured with 8 M urea, reduced with dithiothreitol, carboxymethylated, and cleaved with TPCK-treated trypsin (1:25 w/w for 24 h at 37 °C) according to the procedure of ref *21*. The mixture of peptides was then separated on a C_8 column (Vydac 228TP104, 5 × 200 mm) in a gradient of 0−80% acetonitrile (v/v) in 0.1% trifluoroacetic acid at a flow rate of 1 mL/min. A peak was collected eluting at 3−4 min which exhibited the spectra of the reduced PLP−lysine at 325 nm. This fraction was vacuum-dried, dissolved in 100 μL of 10 mM triethylamine, and further purified on a C_{18} column (Vydac 218TP54, 5 × 200 mm) in a gradient of 0−80% acetonitrile in 10 mM triethylamine·HCl buffer, pH 6.5. Fractions eluted at 41−42 min having a distinct peak at 325 nm were pooled, vacuum-dried, dissolved in 100 μL of ammonium bicarbonate, and cleaved overnight at 4 °C with 1:100 w/w of Glu C endoproteinase. The cleaved mixture of peptides was rechromatographed on the C_{18} column as described above. The final peptide absorbing at 325 nm, eluted at 38 min, was used for NH_2-terminal peptide sequencing on a 477A protein sequencer (Applied Biosystems) using standard Edman chemistry.

Spectral Measurements. UV−vis spectra were measured on a diode array model 8453 UV−vis spectrophotometer (Hewlett-Packard). Fluorescence measurements were performed on a RF-5301PC (Shimadzu) in TBS, pH 8.6, at room temperature using excitation and emission slits of 5 nm. To ensure equimolar amounts of CBS, the concentrations of the purified enzymes were adjusted to give a heme absorbance value of 0.025 at 428 nm. Circular dichroism spectra were measured with an Aviv 62DS spectrometer (Lakewood, NJ) equipped with a thermoelectric temperature control unit at 25 °C in 1 mm and 1 cm quartz cells for far-UV and visible regions, respectively, at protein concentrations of 0.1 and 1 mg/mL, respectively. The protein concentrations were determined from the combined heme and PLP absorbance at 428 nm using $\epsilon_{428} = 107$ mM^{-1}·cm^{-1} and $\epsilon_{428} = 81$ mM^{-1}·cm^{-1} for 63 and 45 kDa CBS, respectively. The extinction coefficients were calculated from at least 3 different enzyme preparations, >90% pure, having a ratio A_{428}/A_{280} of 1.3. Protein concentrations in the determination of the extinction coefficient were determined by the method of ref *22*. BSA was assayed spectrophotometrically using $E^{1\%}_{280} = 6.6$. The value of ϵ_{428} for the full length protein is somewhat higher than our previously published values (81.5 and 82.6 mM^{-1}·cm^{-1} in refs *8* and *16*, respectively). This discrepancy is most likely due to the incomplete saturation of the protein with heme which may have affected the previous estimates.

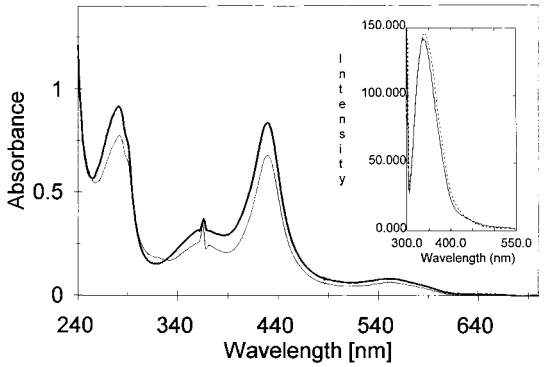


FIGURE 1: Reduction of human recombinant CBS by sodium borohydride. The native 63 kDa CBS (thick line), 2 mg in 100 μL of Tris·HCl buffer, pH 8.6, was incubated with 100 mM $NaBH_4$, and the spectra of the reduced enzyme were scanned (—). Inset: Fluorescence spectra of 100× diluted native (—) and the borohydride-reduced (- - -) CBS at the excitation wavelength of 296 nm.

CBS Assay. CBS activity was determined by a previously described radioisotope assay using [^{14}C]serine as the labeled substrate (*5*). One unit of activity is defined as the amount of CBS that catalyzes the formation of 1 μmol of cystathionine in 1 h at 37 °C. Concentrations of both homocysteine and serine substrates were equimolar at 5 mM. For measurements of CBS inactivation due to hydroxylamine, 1 mM pyridoxal was used to quench the hydroxylamine in the enzyme assay mix.

Preparation of PLP-Free CBS. PLP-free CBS was prepared by dialyzing the CBS solution against Tris·HCl buffer, pH 8.0, containing 5 mM hydroxylamine for 6 h at 4 °C, followed by dialysis of the sample against Tris·HCl buffer, 10 mM, pH 8.6, containing 150 mM sodium chloride (TBS) overnight at 4 °C. The PLP-free CBS typically retained 5−10% of the original activity indicating that a small residual amount of PLP remains bound to the PLP-free enzyme. This was in agreement with our determination of residual PLP performed by the method of ref *23*.

Reconstitution of PLP-Free CBS with PLP. PLP-free CBS, 0.1 mg/mL, preincubated in 0.1 M Tris·HCl, pH 8.0, for 30 min with 10 different PLP concentrations from 0 to 50 μM was added at a final concentration of 0.01 mg/mL to the enzyme assay mix. Catalytic activity of the reconstituted enzyme was determined as described above.

RESULTS

Sodium Borohydride Selectively Reduces PLP in Human CBS. Comparison of the UV−vis absorption spectra of native 63 kDa CBS and the enzyme reduced with sodium borohydride in Figure 1 shows about a 15% decrease in the absorbance at 280, 360, and 428 nm for the reduced enzyme. Reduction of heme with sodium dithionite, on the other hand, causes a shift of the Soret peak of the CBS heme from 428 to 448 nm (*8*, *24*, *25*). The shift was not observed when CBS was reduced with sodium borohydride. In addition, no α and β peaks at 540 and 573 nm appeared, indicating that heme was not reduced (*8*). These results indicate that the iron in

Table 1: NH_2-Terminal Sequencing of the PLP Cross-Linked Peptide

amino acid	Cys	Glu	Phe	Phe	Asn	Ala	Gly	Gly	Ser	Val	ND[c]	Asp	Arg
yield[a] (pmol)	522	229	278	252	236	278	243	206	87	179		118	68
position[b]	109	110	111	112	113	114	115	116	117	118	119	120	121

[a] Yield of amino acid after each cycle of Edman degradation. Loaded 660 pmol of the peptide (an estimate from the peak at 325 nm using ϵ_{325} = 6250 $M^{-1}\cdot cm^{-1}$). [b] Position in the amino acid sequence of human CBS. [c] ND = amino acid not detected.

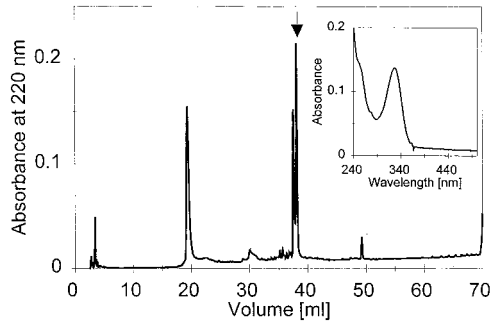


FIGURE 2: Last step of the isolation of the PLP-containing peptide derived from trypsin cleavage of the sodium borohydride reduced CBS on a reverse phase C_{18} HPLC column (see Materials and Methods for details). The arrow marks the peptide exhibiting a distinct peak of pyridoxamine in the visible spectrum at 325 nm shown in the inset.

the heme moiety was not reduced; however, we cannot rule out a reduction of the porphyrin ring.

According to ref *26*, the protonated and unprotonated Schiff bases of PLP absorb at 420 and 350 nm, respectively. Therefore, the observed spectral effects reflect a selective reduction of the PLP−lysine internal aldimine. The small increase in absorbance at 325 nm is most likely due to formation of the reduced Schiff base (*26*). The reduced enzyme exhibited no enzymatic activity. Identical spectral changes were observed for the 45 kDa CBS treated with sodium borohydride (results not shown).

Human CBS contains 8 tryptophan, 8 tyrosine, and 15 phenylalanine residues per 63 kDa subunit (*27*). The fluorescence spectrum of CBS excited at 296 nm arises mostly from tryptophan emission (*16*). The fluorescence spectrum of the enzyme before and after the sodium borohydride reduction is shown in the inset of Figure 1. It seems clear that the reduction does not significantly effect the environments of the tryptophan residues. This suggests that the reduction of the lysine−PLP aldimine complex in CBS does not significantly affect the overall conformation of the enzyme. Finally, no PLP-related fluorescence in the region of 450−500 nm was observed.

Lys 119 Is the Essential Lysine Residue in the Active Site of Human CBS. The full length CBS is prone to aggregation and adsorption to surfaces. To identify the lysine residue involved in the formation of the Schiff base with PLP, we used the 45 kDa active core, which lacks these undesirable properties. After sodium borohydride reduction, the enzyme was denatured, carboxymethylated, and cleaved with trypsin and the PLP cross-linked peptide was purified by reverse-phase HPLC (Figure 2). The absorption spectrum of the purified peptide from fraction 39 (Figure 2, inset) clearly indicates the presence of the reduced Schiff base, with absorption maximum at 325 nm. Lack of absorption at 280 nm in this peptide indicates that there is no contribution from tyrosine or tryptophan residues to the spectrum.

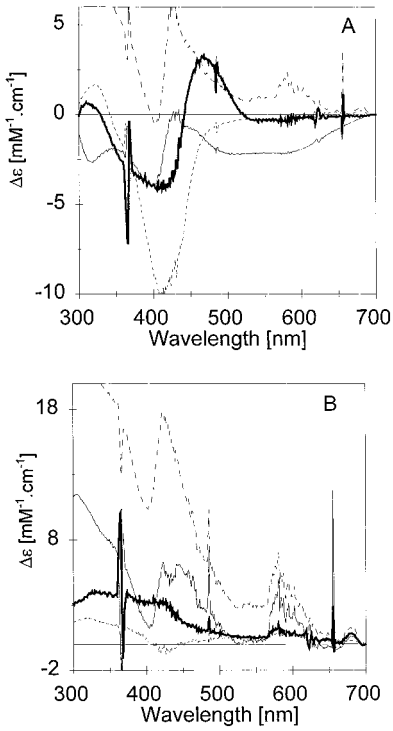


FIGURE 3: (A) Difference visible spectra of the 45 kDa holo-CBS complexes with serine (thick line), serine + homocysteine (—), homocysteine (− −), and hydroxylamine (- - -). (B) Difference visible spectra of the 45 kDa PLP-free CBS complexes with serine (thick line), serine + homocysteine (—), homocysteine (− −), and hydroxylamine (- - -). Difference spectra were obtained by subtracting the spectra of the enzyme−ligand complexes from the free enzyme spectrum.

Amino-terminal sequencing of the peptide provided the unambiguous single sequence starting at Cys109 following the trypsin cleavage site after Lys108 (Table 1). The sequence ended at the second trypsin cleavage site at Arg121. There was a single gap in the sequence between Val118 and Asp120, at a position corresponding to Lys119. Presumably, the elution volume of the lysine residue changed due to its cross-linking with PLP.

Interaction of the Substrates and Hydroxylamine with PLP. The presence of heme, which absorbs light at the same wavelength as PLP, makes it difficult to distinguish the effect of ligands on PLP from the effects on heme. Since a change in the oligomerization state complicated the spectral analysis of 63 kDa CBS, the nonaggregating 45 kDa CBS was used to measure interaction of substrates with PLP.

Difference spectra of the 45 kDa CBS complexes with these same ligands are shown in Figure 3A. Interaction of serine with the enzyme induces an increase of absorbance at 466 and 312 nm with isosbestic points at 329 and 442 nm, respectively. According to studies of D-serine dehydratase (*28*), OASS (*29*), and tryptophan synthase (*30*), serine-induced absorption in the region between 430 and 480

nm corresponds to the formation of the α-aminoacrylate−PLP Schiff base (Scheme 1). Both peaks at 312 and 466 nm in CBS−serine complex were abolished by the addition of an equimolar amount of homocysteine, indicating that, indeed, the α-aminoacrylate is a reaction intermediate. A decrease in the absorbance at 405 nm induced by serine represents transchiffization between the internal and external aldimines. This change, however, was only about 50% of that observed when hydroxylamine was added.

The PLP−oxime maximum is shifted from 333 nm (free PLP−oxime) to 321 nm (45 kDa CBS−oxime). Homocysteine, on the other hand, exhibits a sharp positive peak at 429 nm preceded by a sharp minimum at 401 nm. The model spectrum of homocysteine−PLP complex does not exhibit these features (results not shown). It is likely that the observed spectral effects are due to an interaction of homocysteine with heme.

To distinguish the heme contribution to the difference absorption spectra, the interaction of ligands with PLP-free CBS was measured under the same conditions (Figure 3B). While serine and hydroxylamine did not show any significant changes in the visible spectra after mixing with the PLP-free enzyme, the homocysteine−PLP-free CBS complex showed the same spectral pattern as that of measured for the homocysteine−holo-CBS complex, except that this time the intensity difference was 3-fold greater. The region around 300 nm is also more intense, indicating that this region does not arise from homocysteine−PLP interactions in holo-CBS. Furthermore, these results suggest that, while serine and hydroxylamine interact with PLP in CBS, homocysteine does not. Presumably, the sulfhydryl substrate changes the heme environment, but the origin of the effect is unknown. A small spectral effect of homocysteine at 580 nm, the region where only heme absorbs, supports this suggestion.

In model spectra of serine, homocysteine, and hydroxylamine with PLP in TBS, formation of serine−PLP aldimine does not alter the PLP spectrum. There is only a small increase in absorbance and a shift from 410 to 415 nm. Conversely, addition of homocysteine and hydroxylamine completely abolished the PLP maximum at 410 nm with the concomitant appearance of new peaks at 323 and 333 nm, respectively (results not shown).

PLP, but Not Heme, Dissociates from CBS Due to Treatment with Hydroxylamine. Hydroxylamine is a carbonyl-specific reagent known to induce reversible dissociation of PLP from CBS and other PLP-dependent enzymes (*31*). We treated CBS with 5 mM hydroxylamine, followed by dialysis of the hydroxylamine−PLP complex from the enzyme, to prepare the PLP-free enzyme. The comparison of the difference spectrum of the 63 kDa PLP-free enzyme with that of the enzyme reduced by sodium borohydride and the enzyme after 6 h incubation with 1 mM of hydroxylamine is shown in Figure 4. It is clear that the spectral changes in the visible region caused by the reduction are quite similar to those induced by hydroxylamine and dialysis. Both treatments cause about a 15−20% decrease in absorbance at 428 and 350 nm, which corresponds to the disappearance of protonated and unprotonated Schiff base, respectively. The difference spectra for the 45 kDa enzyme are identical to those of the full length enzyme (results not shown).

Virtually no loss of heme was detected by the pyridine−hemochromogene method (*32*). Indeed, the visible spectrum

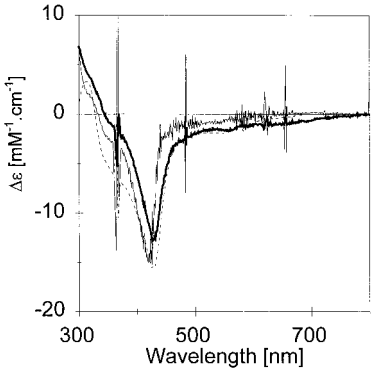


FIGURE 4: Difference visible spectra of the 63 kDa CBS: Apo- and holoenzymes (thick line); holo-CBS and the holo-CBS preincubated with 1 mM hydroxylamine for 6 h at room temperature (—); holo-CBS and borohydride-reduced holo-CBS (- - -). The spectra were measured in TBS at 25 °C.

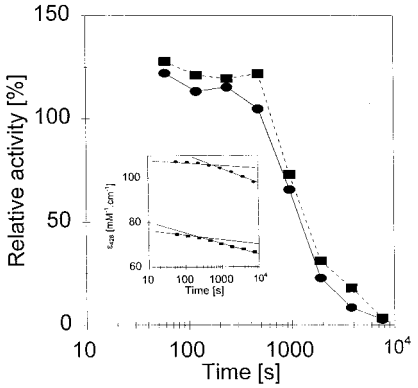


FIGURE 5: Inactivation of the 63 kDa (●—●) and 45 kDa (■- -■) CBS by 1 mM hydroxylamine in TBS, pH 8.6, at 25 °C. Inset: Decrease of the molar absorbance at 428 nm for 63 kDa (●—●) and 45 kDa (■- -■) CBS in the same experiment.

of the PLP-free enzyme still contains the Soret peak of heme at 428 nm with a shoulder at 363 nm, similar to the borohydride-reduced CBS (Figure 1). The Soret peak of the PLP-free enzyme shifts to 448 nm upon reduction with sodium dithionite (results not shown). Taken together, our data indicate that hydroxylamine induces specific dissociation of PLP from CBS, while it leaves the heme unaffected.

Dissociation of PLP from CBS is a Multistep Process. The inset in Figure 5 shows the time dependence of the spectral changes at 428 nm induced by 1 mM hydroxylamine. The data show a decrease of the protonated form of the PLP−lysine internal aldimine in both 63 and 45 kDa CBS. The measurement suggests that the amount of protonated PLP−lysine aldimine decreases slowly after a 500 s lag phase, followed by pseudo-first-order decay kinetics, with the rate constant of 0.036 and 0.022 h^{-1} for 63 and 45 kDa CBS, respectively.

Compared to the hydroxylamine-induced decrease of the internal PLP−lysine aldimine, inactivation of the enzyme with 1 mM hydroxylamine proceeds about 50 times faster, with a pseudo-first-order rate constant of 1.67 and 1.47 h^{-1} for 63 and the 45 kDa CBS, respectively. The lag time for the inactivation is also about 500 s (Figure 5).

The dependence of enzyme inactivation on the concentration of hydroxylamine indicates that at least 10 μM hydroxylamine is needed to inactivate CBS. At 1 mM hydroxylamine the enzyme loses at least 90% of its activity after 1 h of

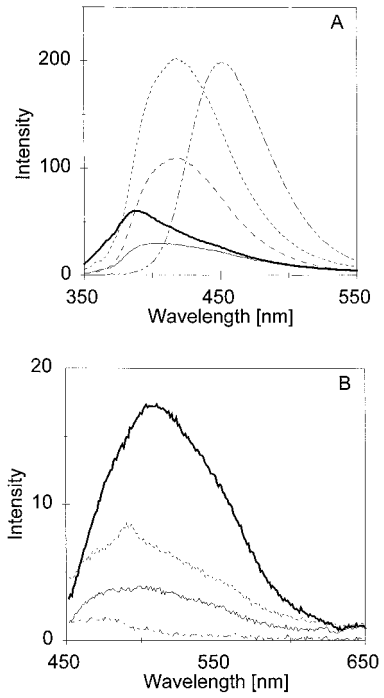


FIGURE 6: (A) Fluorescence spectra of the 45 kDa CBS (2 μM) excited at 330 nm and (B) the spectra excited at 420 nm: 45 kDa holo-CBS (thick line); PLP-free CBS (—); 45 kDa holo-CBS after adding 1 mM hydroxylamine and incubation for 2 h at 25 °C (— —); 45 kDa holo-CBS incubated with 1 mM hydroxylamine for 6 h at 25 °C (- - -); complex of 100 μM PLP with 20 mM hydroxylamine incubated for 10 min at 25 °C, 50 times diluted for measurement (— - -).

incubation. No difference in the hydroxylamine-induced inactivation of CBS was observed when Tris-buffered saline, pH 8.6, was replaced by a potassium phosphate buffer (0.1 M, pH 8.0) (results not shown).

Addition of 2 mM PLP to CBS inactivated by 1 mM hydroxylamine did not restore enzymatic activity (results not shown). This observation suggests that the PLP−oxime moiety is tightly bound to CBS and cannot be replaced by PLP, unless it is removed from the enzyme by dialysis.

Fluorescence Spectra. To demonstrate the formation of the PLP−oxime species, fluorescence spectra of the 45 kDa CBS, excited at 330 nm, were measured in the presence and absence of hydroxylamine (Figure 6A). Fluorescence from 45 kDa CBS is weak and appears at the wavelength of 388 nm. The 63 kDa CBS does not show any emission at all (spectrum not shown). The low quantum yield of PLP-related fluorescence indicates that the PLP fluorescence in CBS is quenched by surrounding amino acid residues as well as by the inner filter effect of heme. While the free PLP−oxime exhibits a strong emission maximum at 451 nm, the enzyme-bound PLP−oxime maximum is blue shifted to 418 nm. In the PLP free enzyme this 418 nm peak is reduced by about 90 % indicating nearly complete removal of the enzyme-bound oxime.

When the sample is excited at 420 nm, the wavelength at maximal absorbance of protonated internal Lys119−PLP aldimine, a weak fluorescence at 508 nm is observed in 45 kDa CBS (Figure 6B). The full length CBS exhibits a fluorescence maximum at the same wavelength but with about half of the intensity of 45 kDa CBS (results not shown). Formation of the oxime completely quenches the fluorescence of free PLP and almost completely quenches the PLP-related fluorescence in 45 kDa CBS.

Structural Changes of CBS after Dissociation of PLP. Far-UV circular dichroism spectra of the PLP-free enzyme, relative to the holoenzyme, suggest that dissociation of PLP causes a decrease in α-helix content. These changes are only partially reversible, as only 10−20% of the signal is recovered after addition of PLP. Similarly, the CD spectra of holo- and PLP-free CBS in the visible region indicate that the native structure of the enzyme is disrupted by PLP removal and cannot be recovered simply by addition of the cofactor to the PLP-free enzyme (results not shown).

Reconstitution of Active CBS from PLP-Free CBS with PLP. Reconstitution of the active holoenzyme was determined by measurement of enzyme activity of both the 63 and 45 kDa PLP-free CBS which had been preincubated for 30 min at room temperature with different concentrations of PLP.

When the enzyme activity was plotted against the concentration of PLP in the reaction mixture, a typical hyperbolic saturation pattern was observed (results not shown). Using nonlinear regression, K_{PLP} values of 0.7 ± 0.1 and 0.3 ± 0.02 μM were calculated for 63 and 45 kDa CBS species, respectively. These findings indicate that the 45 kDa CBS binds PLP tighter than the full length enzyme.

The yield of active holo-CBS was between 20 and 40%. The reconstitution of CBS is not pH dependent in the range between 6.5 and 9.5 in Tris·acetate buffers. The yield also did not depend on the presence of heme, 1−160 mM NaCl, 0−15 mM β-mercaptoethanol, or 0−16% glycerol. The presence of 1% bovine serum albumin, however, increased the recovery by ≈20%. Conversely, phosphate and carbonate buffers inhibited the reconstitution of the active enzyme in the pH range 6.0−7.0 and 9.0−9.5, respectively, indicating that phosphate and carbonate anions may compete with PLP for binding at the PLP-binding site.

DISCUSSION

PLP and Heme in Human CBS Exhibit Different Susceptibilities to Reduction. To our knowledge, CBS is the first enzyme containing both heme and PLP−two very distinct prosthetic groups (*8*). While the role of PLP has been previously described and is sufficient to explain the mechanism of CBS catalysis (*9, 33*), the role of heme is yet to be determined.

In CBS, borohydride at pH 8.6 reduces only the PLP cofactor, leaving the heme intact. This is understandable because while the sodium borohydride reduction is an obligate two electron process, reduction of ferric to ferrous heme is a one electron process. Heme in CBS can be reduced only by a strong reducing reagent such as sodium dithionite ($E^{\circ\prime} = -0.527$ mV for $SO_3^{2-}/S_2O_4^{2-}$ couple (*34*)). The borohydride-reduced CBS has no activity, indicating that the reduction of the internal lysine−PLP aldimine is sufficient to inactivate the enzyme. The tryptophan fluorescence spectra suggest that little, if any, change in the enzyme conformation occurs upon reduction of the PLP cofactor.

Essential Lys119 of PLP in the Binding Site of CBS Is Highly Conserved. CBS belongs to the β-family of PLP-dependent enzymes catalyzing β-elimination or β-replacement reactions (*35*). In this family, the motif SVK containing

the essential lysine is absolutely conserved in 12 bacterial and plant OASSs (*36*). These authors mutated the lysine in this motif in all of these enzymes and expressed the mutants in an *E. coli* cysteine auxotroph. The lack of growth provided the experimental evidence that the lysine in the SVK motif is essential for the function of OASS. In ref *37* it was found that the corresponding Lys43 in bacterial OASS, Lys108 in spinach chloroplast, and Lys49 in spinach cytosolic OASS are the lysine residues which form the internal aldimine with PLP. Recently, in ref *18* it was found by sequence alignment that the β-replacement catalyzing enzymes form a structurally distinct category of PLP dependent enzymes. These authors named this subgroup the "fold type II" enzymes. Members of this group are distinguished by two domains of mixed α/β structure with the PLP bound between them. The lysine residue forming the Schiff base is conserved in more than 50 β-replacement catalyzing enzymes of this type including human CBS.

In this work, we provide experimental evidence that Lys119 in human CBS is the lysine essential for the formation of the internal aldimine with PLP confirming the unambiguous assignment of CBS to the fold type II enzymes. Thus, the other possible motif, $S^{91}K$*NAGLKCE, which contains six residues identical to those in the PLP-binding site in seven aspartate aminotransferases, was excluded.

Formation of External Aldimine and Oxime with PLP in Human CBS. The β-replacement reaction proceeds via multiple steps, characterized by distinct spectral changes arising from PLP intermediates, as described in Scheme 1. Like OASS with *O*-acetylserine (*38, 39*) and tryptophan synthase with L-serine in the presence of α-glycerol 3′-phosphate (*40*), CBS forms an α-aminoacrylate intermediate with L-serine. Formation of this intermediate is concomitant with increases in the absorbance at 466 and 312 nm (Figure 3). This observation is in agreement with model measurements for D-serine dehydratase (*28*). The isosbestic point at 442 nm matches that for D-serine dehydratase (*28*) and OASS (*29*), suggesting similarities in the environments of the substrate−PLP external aldimines. The immediate disappearance of the peaks at 466 and 312 nm in CBS upon addition of the sulfhydryl substrate, homocysteine, results in conversion of the serine α-aminoacrylate into the final product−cystathionine. Previous CD measurements of the serine−CBS complex suggested that substrate-induced conformational changes occur upon serine binding (*20*).

The decrease of absorbance at 405 nm due to the decrease of the internal aldimine induced by serine, however, is only about half of that induced by hydroxylamine. This suggests that the formation of the external α-aminoacrylate in CBS is not complete even at >1600-fold excess of serine over CBS. This is in agreement with the measurements of Borcsok and Abeles (*9*), who showed that in the absence of homocysteine, the elimination of the β-substitutent is much slower than the α-proton abstraction. Most likely, there exists a significant thermodynamic barrier to α-aminoacrylate formation from serine in the absence of the second substrate in CBS.

Homocysteine itself does not appear to interact with PLP in the enzyme, although it may disturb the heme environment in CBS (Figure 3). One of the axial coordination positions of heme in CBS is a thiolate (*25, 41*), and a possible mechanism of the interaction of homocysteine with heme is replacement of the axial thiolate. Our preliminary EPR measurements, however, did not indicate any measurable effect of homocysteine on the EPR spectra of heme in CBS. Therefore, the nature of the interaction of homocysteine with CBS requires further investigation.

Formation of oximes in PLP-dependent enzymes catalyzing β-replacement has not been extensively studied. Hydroxylamine forms an oxime with PLP, absorbing at 333 nm at pH 8.6, in agreement with model compounds (*42*). The oxime in CBS exhibits a blue-shifted maximum at 321 nm. Formation of the oxime in CBS can be followed spectrophotometrically as there is a decrease in the amount of the internal aldimine, as seen by the decrease in the absorbance at 428 nm (Figure 3) or the increase in fluorescence at 418 nm (Figure 6). The spectrum of the PLP−oxime in CBS is quite different from the spectrum of the PLP−oxime in aspartate aminotransferase, in which the formation of the oxime is accompanied by a red shift of the PLP maximum from 357 to 370 nm (*43*). The differences in both PLP and oxime spectra suggest a difference in the PLP binding pocket of the two enzymes, possibly affecting the ionization state of the internal PLP−aldimines. While the PLP−aldimine in aspartate aminotransferase is in the unprotonated form at pH 8.2 (λ_{max} = 360 nm) (*2*), the PLP−aldimine in CBS is mostly in the protonated form at pH 8.6 (λ_{max} = 428 nm).

Dissociation and Association of PLP in Human CBS. In the early seventies the first studies on dissociation of PLP from semipurified rat CBS were published (*44, 45*) without knowing that the enzyme was a heme protein. Both groups used hydroxylamine to induce the PLP dissociation from the enzyme and provided the initial data on reactivation of the enzyme by PLP, although they did not study the dissociation or the reconstitution of the enzyme in any detail. These authors determined that the reconstitution of the native active holoenzyme from the PLP-free enzyme results in 51−84% recovery. The PLP dissociation constant for rat CBS was found to be 1 μM (*45*).

Our value of 0.7 μM for K_{PLP}, estimated from the steady-state kinetic measurements of 63 kDa human CBS, is close to the dissociation constant for the rat enzyme. This suggests a strong affinity for PLP of mammalian CBS. This high affinity for PLP places mammalian CBS in a group of β-replacement catalyzing enzymes with strong PLP binding (*10*).

Dissociation of PLP from human CBS is a multiple step process, in which a 500 s long lag phase is followed by a fast inactivation of the enzyme and by a slower formation of an oxime with hydroxylamine. The 50-fold difference between the rates of the enzyme inactivation and formation of the oxime suggest that hydroxylamine induces changes in the enzyme molecule resulting in its inactivation before the formation of the oxime. One possible explanation is a mechanism in which a small molecule of hydroxylamine replaces a water molecule frequently found in the neighborhood of the PLP binding site in PLP-dependent enzymes (e.g. ref *46*). To answer the question whether the rate of oxime formation is influenced by the CBS substrates, we measured the rate of oxime formation using the increase of fluorescence at 418 nm. We did not observe any rate increase of oxime formation in the presence of the substrates (our unpublished measurements).

Structural changes in CBS due to the dissociation of PLP from the enzyme were studied by circular dichroism spectroscopy. CD spectra in both the far UV and visible regions indicate a loss of secondary structure as well as localized changes around the PLP-binding pocket and possibly also the heme-binding region. These structural changes can only be partially reversed by adding PLP to the PLP-free enzyme, consistent with the yields of active CBS reconstituted from the PLP-free enzyme by PLP. Lower degree of reconstitution of native CBS, compared to that of reconstitution of semipurified CBS or CBS in crude cell extracts, indicates that the purified recombinant enzyme is more prone to irreversible inactivation. Our observation of a 20% increase in CBS reconstitution in the presence of 1% BSA supports this suggestion.

Fluorescence Spectra of CBS. The intrinsic fluorescence of the internal aldimine is a useful tool for the study of PLP-dependent enzymes. In the structurally and functionally closest relatives of CBS, OASS (*47*) and tryptophan synthase (*48*), excitation at 298 nm induces tryptophan fluorescence at 337 nm and a PLP-based emission of the aldimine at 500 nm. In contrast to this observation the human 63 kDa CBS holoenzyme shows no sign of PLP-related fluorescence when excited at 296 nm. The environment of the tryptophans in CBS (λ_{max} = 345 nm) appears to be more polar than the tryptophan environment in bacterial OASS (λ_{max} = 326 nm) and in bacterial tryptophan synthase (λ_{max} = 327 nm) (*48*).

Many PLP-dependent enzymes exhibit distinct fluorescence in the region of 420−500 nm when excited at 325−330 nm (*48*−*50*). While 63 kDa CBS exhibits no fluorescence in this region, a weak emission appears in 45 kDa CBS, but it is blue shifted to 388 nm. The lack of PLP-related fluorescence in CBS is most likely due to the inner effect of heme. However, the presence of strong quenchers close to the PLP-binding pocket, possibly removed during the trypsin cleavage of the 63 kDa CBS into 45 kDa CBS, could be another explanation.

The PLP-related emission peak at 380 nm in 63 kDa CBS appears only after reduction of the enzyme with borohydride. The emission of reduced PLP in CBS is blue-shifted by 100 nm compared to the fluorescence of reduced PLP in tryptophan synthase (*48*), suggesting that there are significant differences in the PLP environment of the two enzymes. The differences in the PLP visible spectra of the two enzymes support this suggestion.

To interpret a weak fluorescence band of the 45 kDa CBS at 388 nm excited at 330 nm as well as a weak fluorescence of both 45 and 63 kDa CBS at 508 nm excited at 420 nm, it is necessary to consider the formation of tautomers of the internal Schiff base. Enolimine and ketoenamine represent two tautomers of the Schiff base distinguished by hydrogen bonding exhibiting two distinct fluorescence bands at 365 and 490 nm, respectively (*51*). Substituted aldamine with no double bond from the nitrogen of the lysine amino group with emission at 390 nm and an excitation at around 330 nm represent the third spectrally distinct species (*52*). Fluorescence measurements of tryptophan synthase under physiological conditions showed the presence of both tautomeric forms (*48*). On the other hand, the emission maximum at 388 nm in 45 kDa CBS is presumably due to the presence of PLP in the substituted aldamine form whereas the emission at 508 nm can most likely be assigned to the protonated ketoenamine, apparently the most abundant form of PLP.

The fluorescence spectra of CBS are quite different compared to the spectra of other structurally related PLP-dependent enzymes. The presence of heme as well as the polar PLP-binding pocket are most likely responsible for these differences. Investigating if there is any interaction between these two cofactors and delineating the role of heme in CBS is a new facet to the study of PLP-dependent enzymes and catalysis.

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