Domain Architecture of the Heme-Independent Yeast Cystathionine β-Synthase Provides Insights into Mechanisms of Catalysis and Regulation†

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ABSTRACT: Cystathionine β-synthase from yeast (*Saccharomyces cerevisiae*) provides a model system for understanding some of the effects of disease-causing mutations in the human enzyme. The mutations, which lead to accumulation of L-homocysteine, are linked to homocystinuria and cardiovascular diseases. Here we characterize the domain architecture of the heme-independent yeast cystathionine β-synthase. Our finding that the homogeneous recombinant truncated enzyme (residues 1−353) is catalytically active and binds pyridoxal phosphate stoichiometrically establishes that the N-terminal residues 1−353 compose a catalytic domain. Removal of the C-terminal residues 354−507 increases the specific activity and alters the steady-state kinetic parameters including the K_d for pyridoxal phosphate, suggesting that the C-terminal residues 354−507 compose a regulatory domain. The yeast enzyme, unlike the human enzyme, is not activated by *S*-adenosyl-L-methionine. The truncated yeast enzyme is a dimer, whereas the full-length enzyme is a mixture of tetramer and octamer, suggesting that the C-terminal domain plays a role in the interaction of the subunits to form higher oligomeric structures. The N-terminal catalytic domain is more stable and less prone to aggregate than full-length enzyme and is thus potentially more suitable for structure determination by X-ray crystallography. Comparisons of the yeast and human enzymes reveal significant differences in catalytic and regulatory properties.

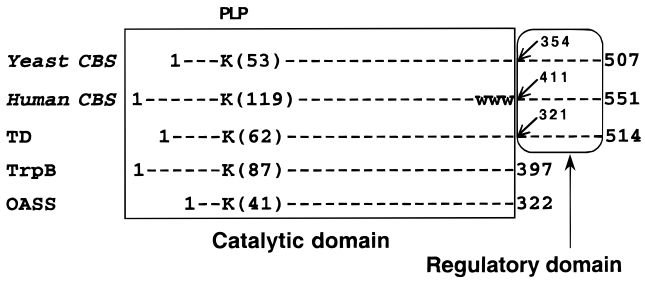
Mutations in the gene encoding human cystathionine β-synthase (CBS)[1] are the major cause of homocystinuria and lead to the accumulation of L-homocysteine, which is a risk factor for coronary heart disease and other human diseases (*1*−*4*). CBS (EC 4.2.1.22) catalyzes the pyridoxal phosphate (PLP) dependent β-replacement reaction of L-homocysteine with L-serine to form L-cystathionine (eq 1).

$$\text{L-homocysteine} + \text{L-serine} \rightarrow \text{L-cystathionine} + H_2O \quad (1)$$

The deduced sequences of CBS from man (*5*, *6*), rat (*7*), and yeast (*Saccharomyces cerevisiae*) (*8*, *9*) exhibit extensive homology (Scheme 1).

The N-terminal domain of CBS has been obtained by proteolysis of the rat and human enzymes (*10*−*12*) or by a mutation that eliminates C-terminal residues 410−551 or 411−551 of human CBS (*13*) (Scheme 1). The N-terminal domain is more active than the full-length enzyme (*11*−*13*), suggesting that the C-terminal domain regulates enzyme activity. The sequences of the N-terminal CBS domains from rat, human, and yeast exhibit significant homology with the sequences of PLP-dependent enzymes that belong to the β-family (*14*) or Fold II (*15*). Related enzymes in Fold II

Scheme 1: Organization of the Catalytic and Regulatory Domains of Five PLP Enzymes in Fold II (*15*)[a]



[a] The scheme shows the residue numbers for the lysine residues (K) that bind PLP and for the C-termini of yeast CBS (*8*, *9*), human CBS (*5*, *6*), TD [biosynthetic threonine deaminase from *E. coli* (*17*)], TrpB [tryptophan synthase β subunit from *S. typhimurium* (*45*, *46*)], and OASS [*O*-acetylserine sulfhydrylase from *S. typhimurium* (*47*, *48*)]. The arrows indicate the residues that initiate the C-terminal regulatory domain of yeast CBS (see Results), human CBS (*13*), and threonine deaminase (17). The "www" in human CBS indicates three tryptophan residues (408−410). Two mutations in human CBS cDNA derived from patients introduce stop codons at positions encoding W409 or W410 that lead to C-terminal truncation (*13*). The sequences of the N-terminal domains of human and yeast CBS exhibit significant homology to each other and to TrpB, OASS, and the catalytic domain of TD (see the introduction). The sequences of the C-terminal domains of human and yeast CBS exhibit homology to each other but not to the C-terminal domain of TD.

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[1] Abbreviations: CBS, cystathionine β-synthase; F-CBS, full-length CBS; T-CBS, truncated CBS; pF-SEC, expression plasmid for F-CBS; pT-SEC, expression plasmid for T-CBS; PLP, pyridoxal phosphate; bicine, *N*,*N*-bis(2-hydroxyethyl)glycine; MOPS, 3-morpholinopropanesulfonic acid; SAM, *S*-adenosyl-L-methionine; MBP, MOPS−bicine−proline; PAGE, polyacrylamide gel electrophoresis.

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include the β subunit of bacterial tryptophan synthase, bacterial and plant *O*-acetylserine sulfhydrylase, and the catalytic domain of bacterial threonine deaminase (see refs *6*, *7*, *12*, and *16*, the electronic appendix to ref *15*, and Scheme 1). Crystallographic results demonstrate that biosynthetic threonine deaminase from *Escherichia coli* (*17*) is composed of an N-terminal catalytic domain and a C-terminal regulatory domain, consistent with earlier genetic studies (*18*). The combined sequence analyses and activity results suggest that CBS, like threonine deaminase, is composed of an N-terminal catalytic domain and a C-terminal regulatory domain (Scheme 1).

S-Adenosyl-L-methionine (SAM) is reported to activate rat, human, and yeast CBS (*19*−*21*). The discovery that either a mutation (D444N) in the C-terminal domain of human CBS (*22*) or a deletion of the C-terminal domain eliminates SAM activation (*13*) or binding (*23*) suggests that SAM is an allosteric activator that binds to the C-terminal regulatory domain.

At least 92 different disease-associated mutations have been identified in the CBS gene of patients with homocystinuria [see mutation update (*2*) and CBS website at http://www.uchsc.edu/sm/cbs]. About one-half of the patients respond to high doses of pyridoxine, the precursor of PLP (*2*). Deletion of the C-terminal regulatory domain corrects some, but not all, disease-causing CBS mutations in man (*13*, *24*), suggesting that a mutation in the catalytic domain can alter allosteric communication with the regulatory domain. Thus, CBS is an excellent system for correlating changes in activity with changes in structure, which is a fundamental goal of research on allosteric enzymes.

Both rat and human CBS bind heme in addition to PLP and require both cofactors for activity (*25*, *26*). Heme may bind or activate L-homocysteine (*26*−*28*) and may regulate CBS activity by changes in oxidation state (*26*). The presence of heme in rat and human CBS is surprising because heme is not found in other PLP enzymes including yeast CBS (*29*, *30*). Because heme largely masks the spectroscopic properties of the PLP coenzyme in rat and human CBS, the yeast enzyme is more useful for spectroscopic studies of reactions with PLP at the catalytic site and for investigations of the effects of allosteric ligands on the reactions of substrates with PLP. Our mechanistic studies using absorption and circular dichroism spectroscopy identified key intermediates in the reaction of the heme-independent yeast CBS (*29*). Here we examine the domain architecture of the heme-independent yeast CBS by comparing the biochemical properties of the full-length enzyme with those of the N-terminal catalytic domain.

EXPERIMENTAL PROCEDURES

Chemicals, Enzymes, and Buffers. 5,5′-Dithiobis(2-nitrobenzoic acid), lead acetate, ferric chloride, L-cystathionine, L-serine, and hemin were from Fluka. Sodium sulfide was from Aldrich. SAM, δ-aminolevulinic acid, and L-homocysteine thiolactone were from Sigma. L-[U-^{14}C]Serine (160 mCi/mmol) was from NEN Life Science Products. Gigapite was from Seikagaku, Japan. L-Homocysteine was prepared from L-homocysteine thiolactone as described (*31*). The L-homocysteine concentration was determined immediately before use in the enzyme assay by titration with 5,5′-dithiobis(2-nitrobenzoic acid) using $\epsilon_{412} = 13\,600\ M^{-1}\ cm^{-1}$ (*32*). Buffer B was 50 mM Na/bicine containing 1 mM EDTA and 1 mM DTT at pH 7.8; 0.02 mM PLP was added when indicated. Buffer K was 10 mM potassium phosphate containing 1 mM EDTA and 1 mM DTT at pH 7.5; 0.02 mM PLP was added when indicated. MBP buffer (*33*) contained 50 mM 3-morpholinopropanesulfonic acid, 50 mM bicine, and 50 mM proline. The pH was raised with sodium hydroxide to 11.2; the solution was then back-titrated with HCl to the desired pH.

Construction of pT-SEC, a Vector for Overexpression of T-CBS. To overexpress T-CBS from yeast (*S. cerevisiae*), we used PCR to insert two nonsense codons (TAA and TAG) after the site encoding the residue 353 amino acid of the F-CBS gene on the pF-SEC, which was previously designated pSEC[2] (*29*). Two PCR primers were used, where base changes are shown in bold: forward, 5′-GACTCTTC-AAAGCTG**T**A**A**GCTT**A**GACGACAAAATACGCTG-3′; reverse, 5′-CAGCGTATTTTGTCGTC**T**AAGC**TT**A**C**AG-CTTTGAAGAGTC-3′. The Quick Change site-directed mutagenesis kit and *pfu* DNA polymerase (Stratagene) were used for PCR as described in the Stratagene catalog. pT-SEC was isolated, and the DNA sequence (1059 bp) was confirmed by DNA sequence analysis by the University of Maryland Biopolymers Core Facility. *E. coli* XL-1 blue transformed with pT-SEC was used for overexpression of T-CBS as described for F-CBS (*29*).

Enzyme Purification. F-CBS was purified in the presence of protease inhibitors (1 mM PMSF, 0.1 mM TPCK, 0.1 mM TLCK, 1 mg/L aprotinin, 2 mg/L leupeptin, 2 mg/L pepstatin, and 1 mM benzamidine hydrochloride) as described (*29*). T-CBS was purified by the same general procedures and on the same scale with the following modifications. Ammonium sulfate fractionation was between 30% and 65% saturation. Protease inhibitors were used until DEAE-Sephacel chromatography. The active fractions after Gigapite column chromatography were collected and concentrated by ultrafiltration. Ammonium sulfate was added to 30% saturation. The enzyme solution was loaded onto a 2.5 × 20 cm butyl-Sepharose 4B column equilibrated with buffer K (+PLP). The column was then washed with 300 mL of buffer K (+PLP). The enzyme was eluted with a 1 L linear gradient from 30% to 0% ammonium sulfate in buffer K (+PLP). The active T-CBS fractions, which eluted at 20% ammonium sulfate, were concentrated to 20 mg/mL, dialyzed against buffer B (+PLP), and stored at −85 °C. Approximately 300 mg of homogeneous T-CBS was obtained from a 1 L culture.

Preparation of Apoenzymes. T-CBS and F-CBS (1−5 mg/mL) in buffer B were incubated for 10 min with 20−100 mM hydroxylamine in the presence of 0.2 M NaCl. The hydroxylamine solution was freshly prepared in buffer B from hydroxylamine hydrochloride and adjusted with NaOH to pH 8. The PLP oximes were removed from the apoenzymes by gel filtration on a PD-10 column (Amersham Pharmacia Biotech) using buffer B. Both apoenzymes exhibited less than 1% residual activity. Because apo-F-CBS

[2] pSEC was constructed from PSTR4-2 (Cys4), which was a generous gift from Dr. Yolande Surdin-Kerjan, Centre de Genetique Moleculaire du Centre National de la Recherche Scientifique, 91198 Gif-sur-Yvette Cedex, France.

Table 1: Molar Absorption Coefficients at 280 nm for CBS

enzyme	MW	Trp	Tyr	cystine	ϵ_{280}(calc) GuHCl	ϵ_{280} buffer	$A_{280}^{0.1\%}$ buffer
apo-F-CBS	55 900	4	16	0	43 300	48 130	0.86
holo-F-CBS	55 900	4	16	0		52 550	0.94
apo-T-CBS	38 700	4	12	0	38 160	40 900	1.06
holo-T-CBS	38 700	4	12	0		44 500	1.15

was more susceptible to proteolysis and aggregation than holo-F-CBS, all analyses of apo-F-CBS were carried out within a few hours after removal of PLP.

Determination of Molar Absorption Coefficients for CBS. Molar absorption coefficients (ϵ) for apoenzyme forms of F-CBS (apo-F-CBS) and T-CBS (apo-T-CBS) were determined by the Edelhoch method (*34*−*36*) (Table 1). This method assumes that the ϵ values for model compounds for Trp, Tyr, and cystine in 6 M GuHCl are approximately equal to the ϵ values for the same chromophores of a protein unfolded in 6 M GuHCl. Molar absorbance coefficients of the holoenzyme forms of F-CBS and T-CBS were determined after addition of a stoichiometric amount of PLP to a solution of the apoenzyme of known concentration and absorbance (see fluorescence titrations in Figure 4). The specific absorbance of a 0.1% solution ($A_{280}^{0.1\%}$) of each enzyme in buffer was determined from the calculated ϵ_{280} values and the molecular weights (Table 1).

Enzyme Assays, Protein Determination, Gel Electrophoresis, and Sequence Analysis. (A) Assay Method 1. CBS was assayed by a modification of a standard chromatographic method (*10*) using L-[U-^{14}C]serine as described (*29*). One unit of activity is the formation of 1 μmol of cystathionine/h at 37 °C. Standard assay conditions utilized 5 mM L-serine and 5 mM L-homocysteine. The effects of SAM on enzyme activity were determined by preincubating the enzyme with various concentrations of SAM (0.5, 1, 2, 5, 10, 30, 50, 100, or 1000 μM) at 37 °C for either 5 min or 1 h before addition of L-serine and L-homocysteine at the following respective concentrations: 5 and 5 mM, 20 and 5 mM, or 5 and 0.1 mM. Steady-state kinetic analyses utilized a range of concentrations of L-serine (0.25−25 mM) and L-homocysteine (0.25−25 mM). Effects of pH on enzyme activity were assayed between pH 6 and pH 10 in the MBP buffer (*33*) using 5 mM L-serine and 5 mM L-homocysteine. The effect of PLP concentration on CBS activity was determined by preincubating freshly prepared apo-F-CBS and apo-T-CBS with PLP at 30 °C for 30 min before assay of specific activity. The preincubation mixture contained 200 mM Tris-HCl (pH 8.6), 5 mM L-serine, 0.1 μg of apo-CBS, 5 μg of BSA, and 0−10 μM PLP in 18 μL. The assay was initiated by addition of 2 μL of L-homocysteine (50 mM). Both apoenzymes exhibited full recovery of activity after incubation with PLP.

(B) Assay Method 2. To monitor the purification of CBS, the catalytic activity in the reaction shown in eq 2 was

$$\beta\text{-mercaptoethanol} + \text{L-cysteine} \rightarrow S\text{-hydroxyethyl-L-cysteine} + H_2S \quad (2)$$

followed by a modification of a published method (*37*). The reaction of H_2S with lead acetate to form lead sulfide was monitored continuously in a spectrophotometer by the increase in absorbance at 390 nm. The reaction mixture contained 30 mM L-cysteine, 30 mM β-mercaptoethanol, 0.4 mM lead acetate, 50 μM PLP, and 200 mM Tris-HCl, pH 8.6, in 1 mL, and was incubated at 37 °C for 5 min. The reaction was initiated by addition of CBS (1−5 μg), and the initial rate of increase at 390 nm was followed for up to 3 min.

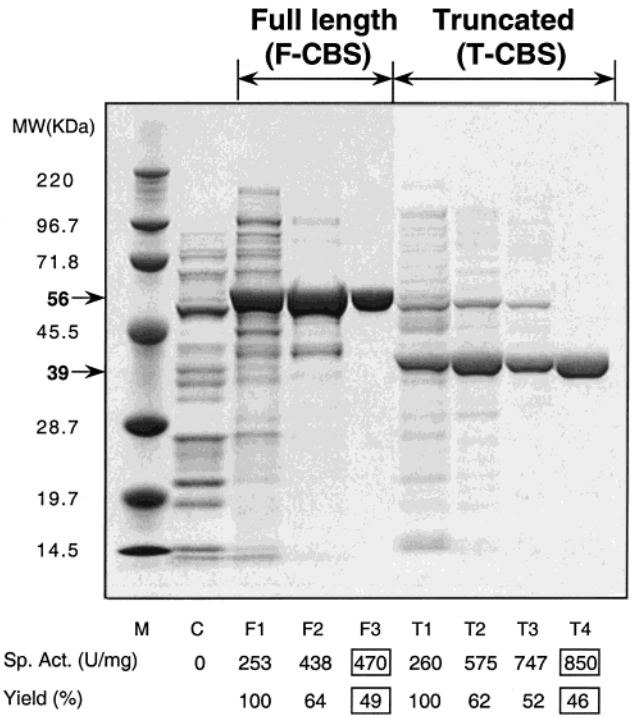


FIGURE 1: Purification of F-CBS and T-CBS followed by SDS−PAGE and activity measurements. Lanes: M, molecular mass standards (molecular mass in kDa in parentheses) myosin (220), phosphorylase *b* (96.7), bovine serum albumin (71.8), ovalbumin (45.5), carbonic anhydrase (28.7), β-lactoglobulin (19.7), and lysozyme (14.5); C, cell-free extracts of *E. coli* XL-1 blue/pF-SEC (no induction of F-CBS); F1, cell-free extract of *E. coli* XL-1 blue/pF-SEC (induction of F-CBS); F2, F-CBS after DEAE-Sephacel column chromatography; F3, F-CBS after Gigapite column chromatography; T1, cell-free extracts of *E. coli* XL-1 blue/pT-SEC (induction of T-CBS); T2, T-CBS after DEAE-Sephacel column chromatography; T3, T-CBS after Gigapite column chromatography; T4, T-CBS after butyl-Sepharose column chromatography. About 10 μg of protein was applied per lane. The specific activity (units/mg) and percent yield for each step are shown at the bottom of the figure; boxed values are for the final preparations. Details of the purification of F-CBS have been reported (*29*).

Protein concentrations of crude extracts were determined by the Coomassie blue protein assay reagent (Pierce, USA) using bovine serum albumin as a standard. The concentrations of purified F-CBS and T-CBS were determined from the specific absorbance at 280 nm (Table 1). SDS−PAGE utilized 10% gels (Novex). Native PAGE utilized 8% Tris−glycine gels (Novex). Active protein bands in native gels were detected by soaking the gel (8.5 × 7.5 cm) in 30 mL of the assay mixture (method 2) for 5 min at 37 °C, followed by transfer of the gel into 5% acetic acid (*38*). SDS and native gels were also stained with Coomassie blue. N-Terminal amino acid sequences of protein bands from SDS gels were determined by Perkin-Elmer Biosystems.

Spectroscopic Methods. Absorption spectra were measured using a Hewlett-Packard 8452 diode array spectrophotometer at 25 °C. Fluorescence spectra were measured using a PTI dual excitation spectrofluorometer at 25 °C. Fluorescence

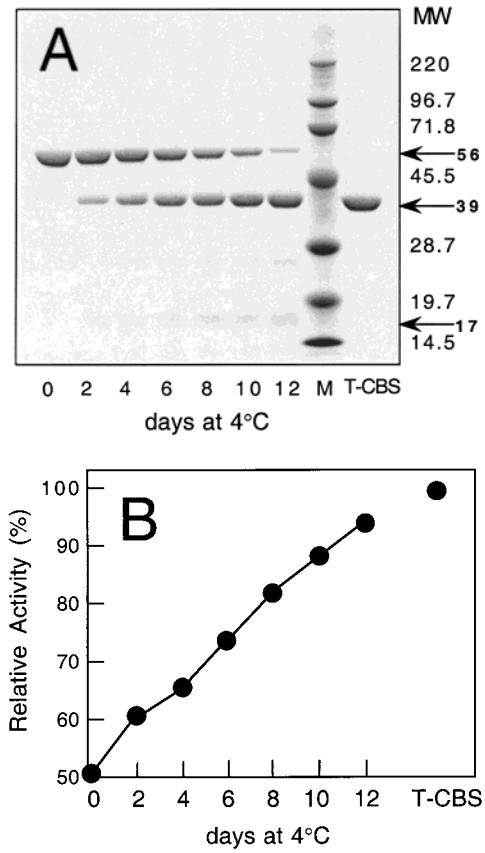


FIGURE 2: Proteolysis of F-CBS increases activity. F-CBS was stored at 4 °C in 100 mM potassium phosphate buffer, pH 7.0, containing 0.2 M NaCl after the final purification shown in Figure 1. Aliquots were analyzed every 2 days by SDS−PAGE (A) and assays of specific activity (method 1) (B). T-CBS was purified from *E. coli* XL-1 blue/pT-SEC (Figure 1). Lane M: molecular mass standards as in Figure 1.

titrations were used to determine L-serine binding. Holo-F-CBS (1.0 μM) and holo-T-CBS (1.0 μM) were titrated with aliquots of L-serine to give final concentrations of 10−100 μM. The increase in fluorescence emission at 540 nm (excitation at 460 nm) was determined at 100 s after each addition of L-serine. The apparent K_d values for L-serine were calculated from $\Delta F = \Delta F_{max}[Ser]/K_d + [Ser]$. The species with maximum emission at 540 nm is attributed to the aldimine of aminoacrylate (see Results).

RESULTS

During our initial studies of yeast CBS (*29*), we observed, but did not report, that the enzyme tended to aggregate and to be cleaved to an active, lower molecular weight species. These results suggest that the yeast enzyme may be composed of subdomains. To investigate the domain architecture of yeast CBS, we engineered a truncated form of yeast CBS, termed T-CBS, and compared the biochemical properties of T-CBS with those of the full-length enzyme, F-CBS.

Design, Overexpression, and Purification of T-CBS. To determine a suitable site for engineering a truncated form of CBS (T-CBS), we obtained the N-terminal sequences of the major products of cleavage of F-CBS, which had molecular masses of about 39 and 17 kDa (see below and Figure 2A). Whereas the N-terminal sequence of the 39 kDa band was the same as that of F-CBS, the N-terminal sequence of the 17 kDa band was EASTTKYAD, which corresponds

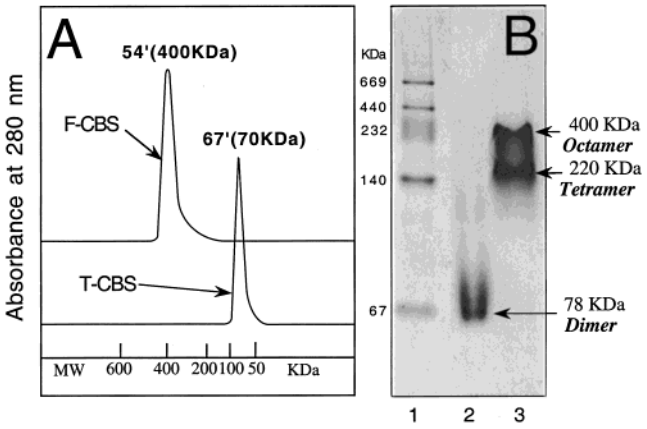


FIGURE 3: Oligomeric states of T-CBS and F-CBS. (A) Gel filtration elution profiles of T-CBS and F-CBS. Samples (250 μL at 20 mg/mL) were diluted to 5 mg/mL with 750 μL of 100 mM potassium phosphate buffer, pH 7.0, containing 0.2 M NaCl, and were applied to a Spherogel TSK 3000 SW column (21.5 × 300 mm; Beckmann) using an LKB HPLC system. The molecular masses were determined from molecular mass standards (molecular mass in kDa in parentheses): thyroglobulin (669), ferritin (440), catalase (232), lactate dehydrogenase (140), and bovine serum albumin (67). (B) Native PAGE of purified T-CBS (lane 2) and F-CBS (lane 3). The sizes of molecular mass markers in lane 1 (see above) are indicated. All bands of T-CBS and F-CBS that stained with Coomassie blue (78, 400, 220 KDa) exhibited enzyme activity upon activity staining on duplicate gels (see Experimental Procedures).

to residues 354−362 of yeast CBS (*6*, *16*). E354 corresponds closely in sequence alignments with human CBS to the beginning of the C-terminal domain of human CBS that results from a stop codon that leads to termination at W409 or W410 (*13*) or from tryptic cleavage at R413 (*12*) (see Scheme 1). Consequently, we chose residues 1−353 for the N-terminal domain of CBS, termed T-CBS. We engineered the overexpression vector for T-CBS, termed pT-SEC (see Experimental Procedures), by introducing two nonsense codons into pF-SEC, the overexpression vector for F-CBS, which was previously designated pSEC (*29*). T-CBS was overexpressed by *E. coli* XL-1 blue transformed with pT-SEC as described for F-CBS (*29*). Purification of T-CBS by a modification of the method for F-CBS (*29*) (see Experimental Procedures and Figure 1) produced ∼300 mg of homogeneous T-CBS per 1 L culture in 46% yield. The specific activity of pure T-CBS (850 units/mg) was significantly higher than that of F-CBS (470 units/mg) (Figure 1).

Cleavage of F-CBS. Purified F-CBS was gradually cleaved over a period of 12 days at 4 °C to yield a major 39 kDa band and a minor 17 kDa band on SDS gels (Figure 2A). The 39 kDa band appeared identical to that of purified T-CBS. The relative activity of the enzyme increased over 12 days and approached that of T-CBS (Figure 2B). We later found that the cleavage of this preparation was prevented by a combination of four protease inhibitors (1 mM PMSF, 0.1 mM TPCK, 0.1 mM TLCK, and 1 mg/L aprotinin) and that two other preparations of F-CBS were not cleaved in either the presence or absence of protease inhibitors. We conclude that the cleavage observed in Figure 2 resulted from a trace contaminant of a protease in that preparation.

Oligomeric States of T-CBS and F-CBS. Gel filtration elution profiles (Figure 3A) showed that T-CBS eluted as a single sharp peak with a molecular mass of ∼70 kDa

Table 2: Comparison of Human and Yeast CBS

	human CBS			yeast CBS		
	F-CBS	T-CBS	ref	F-CBS	T-CBS	ref
no. of amino acids	551	~410	*5*, *12*	507		*8*, *16*
				507	353	this work
molecular mass (kDa)	63	45	*44*	55		*21*
				56	39	this work
no. of subunits	4[a]	2	*12*	4		*21*
				4−8	2	this work
PLP/subunit	1.0	1.0	*12*, *23*, *25*	1.0	1.0	this work
K_d(PLP) (μM)	0.7	nd	*27*	2.7 ± 0.47	0.9 ± 0.1	this work
Hb/subunit	1.0	1.0	*12*, *25*	none		*29*
	0.5	1.0	*23*		none	this work
regulation by SAM	yes	no	*12*, *13*, *20*, *23*	yes		*21*
				no	no	this work
pH optimum	~8.5		*26*	~8.5	~8.5	this work
specific activity (μmol mg^{-1} h^{-1})	220	538	*12*	73		*21*
	295	nd	*26*	470	850	this work
V_{max} (μmol mg^{-1} h^{-1})				1115 ± 51	1492 ± 86	this work
k_{cat} (s^{-1})	6	6.4	*12*	7.6		*21*
	11		*23*	17.3 ± 0.8	16.0 ± 0.96	this work
k_{cat} (s^{-1}) + SAM	20.5	6.7	*12*	17.3 ± 0.8	16.0 ± 0.96	this work
	21		*26*			
K_m(Ser) (mM)	3	2.7	*12*	2.2		*21*
	2.7	18	*23*	11.23 ± 0.92	5.34 ± 0.64	this work
K_m(Hcys) (mM)	1.5	0.5	*12*	2.2		*21*
	5	10	*23*	0.615 ± 0.051	0.445 ± 0.059	this work
K_d(Ser) (μM)	7.5	23	*23*	18.6 ± 0.33	14 ± 0.25	this work

[a] Higher multimers were also observed (*12*).

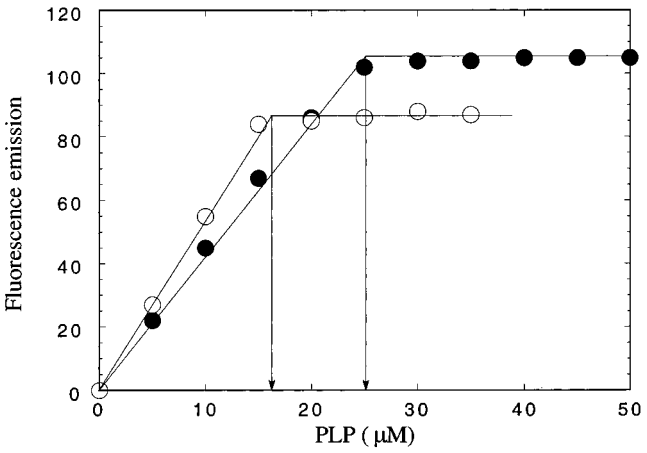


FIGURE 4: Determination of the stoichiometry of PLP binding by fluorescence titrations. Apo-F-CBS (○, 17.9 μM) and apo-T-CBS (●, 25.8 μM) were titrated with aliquots of PLP to give the indicated final concentrations (5−50 μM). The fluorescence emission at 514 nm (excitation at 412 nm) was determined 3 min after each addition of PLP. The plots exhibited sharp breaks at 18 μM (F-CBS) and 26 μM (T-CBS), demonstrating a 1:1 stoichiometry of PLP per subunit.

corresponding to a dimer, whereas F-CBS eluted as a broader peak with a molecular mass of ~400 kDa corresponding to an octamer. Upon electrophoresis in native gels, T-CBS appeared mainly as a dimer, whereas F-CBS appeared as a mixture of tetramer and octamer (Figure 3 and Table 2).

Catalytic Properties of F-CBS and T-CBS. F-CBS and T-CBS both exhibited maximal activity at pH 8.5 and similar pH optima at pH ~8.5 (Table 2). The specific activity of both forms of the enzyme was unaffected by the addition of SAM (0.5−1000 μM) under assay conditions described under Experimental Procedures (Table 2). The apoenzymes of both F-CBS and T-CBS showed no visible absorbance and exhibited full recovery of activity after incubation with PLP. The activities of F-CBS and T-CBS were absolutely dependent upon the addition of PLP. The determination of the effects of PLP concentration on CBS activity as described under Experimental Procedures yielded K_d values for PLP of 0.9 ± 0.1 μM for T-CBS and 2.7 ± 0.47 μM for F-CBS (Table 2). The stoichiometry of PLP binding obtained by fluorescence titrations of apo-F-CBS and apo-T-CBS with PLP was 1:1 (Figure 4 and Table 2). Addition of L-serine to yeast CBS results in the appearance of an absorbance band centered at 460 nm that is attributed to the aldimine of aminoacrylate (*29*). This species exhibits a fluorescence emission maximum centered at 540 nm upon excitation at 460 nm. Measurements of fluorescence emission at 540 nm as a function of L-serine concentration (see Experimental Procedures) yielded K_d values for L-serine of 18.6 μM for F-CBS and 14 μM for T-CBS (Table 2).

Figure 5A−D shows the initial rates of L-cystathionine formation by F-CBS and T-CBS as a function of L-serine or L-homocysteine concentration at fixed concentrations of the other substrate. Although the rates increased hyperbolically with increasing low concentrations of either substrate, some substrate inhibition occurred at higher concentrations of L-homocysteine, but not of L-serine. The lines represent fits of the full data sets for F-CBS and T-CBS to eq 6 for model 3 (see Scheme 2 and Discussion). Kinetic parameters derived from the fits to model 3 are listed in Table 2 and in the Figure 5 legend. Panels E and F of Figure 5 show double reciprocal plots of the data for T-CBS as a function of low L-serine or L-homocysteine concentration (0.25−2 mM) at fixed concentrations of the other substrate (0.25−2 mM) (*39*). The parallel lines obtained by fits of the data to eq 3 are indicative of a ping-pong mechanism.

DISCUSSION

Our results allow comparison of some structural, catalytic, and regulatory properties of yeast CBS with those of human

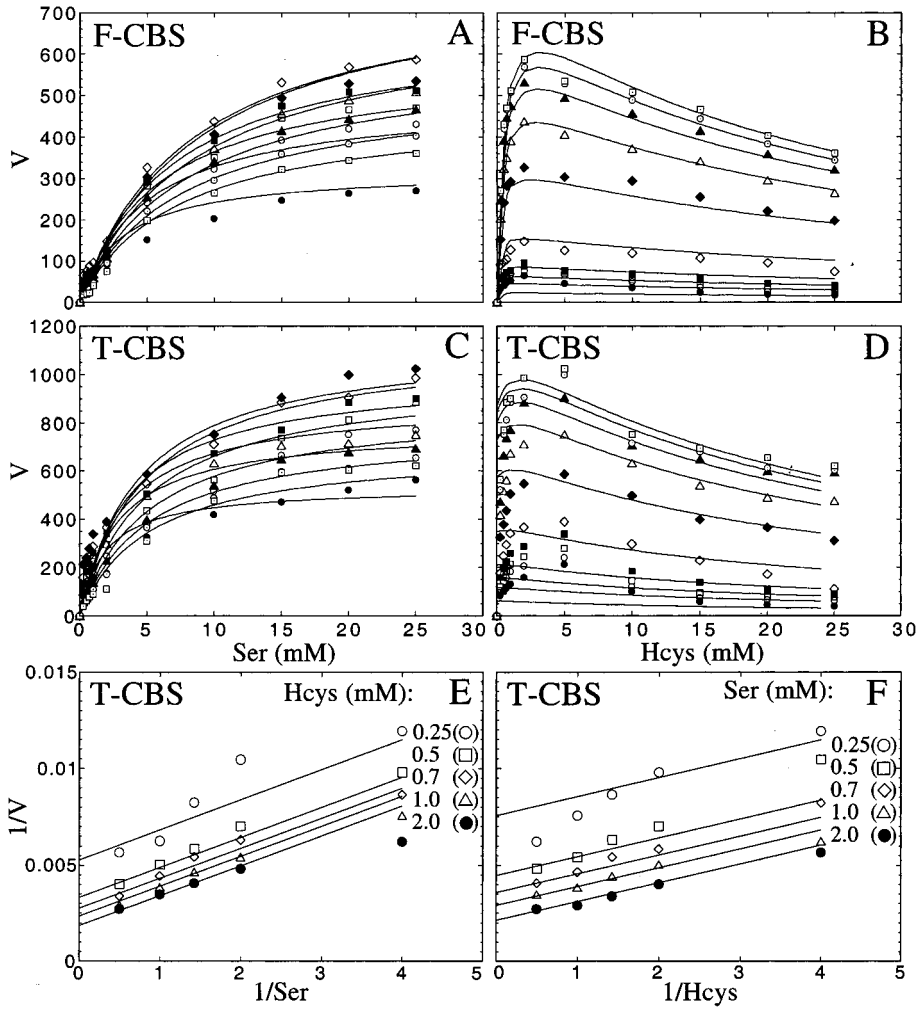


FIGURE 5: Steady-state initial velocity kinetics. (A−D) Effects of L-serine concentration at different fixed L-homocysteine concentrations on the initial velocity of F-CBS (A) and T-CBS (C). Effects of L-homocysteine concentration at different fixed L-serine concentrations on the initial velocity of F-CBS (B) and T-CBS (D). The concentrations of L-serine and L-homocysteine in (A−D) are 0.25 mM (●), 0.5 mM (○), 0.7 mM (□), 1 mM (■), 2 mM (◇), 5 mM (◆), 10 mM (△), 15 mM (▲), 20 mM (⊙), and 25 mM (⊡). (E, F) Double reciprocal plots of the data for T-CBS as a function of low L-serine or L-homocysteine concentration (0.25−2 mM) at fixed concentrations of the other substrate (0.25−2 mM). The parallel lines in (E) and (F) obtained by fits of the data to eq 3 are indicative of a ping-pong mechanism (see Discussion). The solid lines in (A−D) show the fits of the entire data sets to eq 6 (model 3) for L-homocysteine inhibition (see Scheme 2). The kinetic parameters obtained by analysis of the 10 full data sets by eq 6 (model 3) are as follows: F-CBS, $V_{max} = 1115 \pm 51$ μmol mg^{-1} h^{-1}, K_m(Ser) = 11.23 ± 0.92 mM, K_m(Hcys) = 0.615 ± 0.051 mM, K_i(Hcys) = 46.5 ± 13.4 mM, K_j(Hcys) = 18.7 ± 2.3 mM (sum of squares = 3.46E04); T-CBS, $V_{max} = 1492 \pm 86$ μmol mg^{-1} h^{-1}, K_m(Ser) = 5.34 ± 0.64 mM, K_m(Hcys) = 0.445 ± 0.059 mM, K_i(Hcys) = 23.1 ± 7.9 mM, K_j(Hcys) = 22.3 ± 4.1 mM (sum of squares = 3.22E05).

CBS (Table 2). The most important difference between the two is that the human enzyme binds heme whereas the yeast enzyme does not (*29*, *30*). Thus, heme does not appear to play an essential catalytic role, although it may be involved in L-homocysteine binding or activation as proposed (*26*) (see below). Although yeast CBS (*6*, *16*) is slightly shorter than human CBS (*5*, *6*) (507 vs 551 amino acids), the predicted sequences of the two enzymes share 38% identity and 72% similarity (*6*) (see Scheme 1). Our findings that a truncated form of yeast CBS (T-CBS, residues 1−353) bound PLP stoichiometrically (Figure 4) and exhibited catalytic activity (Figures 1 and 2 and Table 2) establish that the N-terminal residues 1−353 function as a catalytic domain (Scheme 1). Similar findings have been reported for truncated forms of human CBS obtained by proteolysis (*12*) or by mutation (*13*, *23*).

The human enzyme is activated either by SAM (*12*, *13*, *20*, *26*) or by C-terminal truncation (*12*, *13*) (Table 2). Once truncated, however, human CBS (T-CBS) is no longer activated by SAM (*12*, *13*) and no longer binds SAM (*23*), supporting the hypothesis that the C-terminal domain of human CBS is a regulatory domain and is required for SAM regulation. In contrast, the yeast enzyme was activated by C-terminal truncation (Figures 1 and 2) but not by SAM (Table 2), suggesting that the C-terminal domain of yeast CBS (residues 354−507) serves a regulatory role that is independent of SAM. A previous report that yeast CBS is activated by SAM (*21*) is not supported by our findings or by work in press from the Kraus group (*30*).

Human CBS (F-CBS) forms tetramers and higher multimers, whereas the N-terminal domain (T-CBS) forms dimers (Table 2) (*12*). Yeast F-CBS also formed a mixture of tetramers and octamers, whereas T-CBS formed a dimer (Figure 3 and Table 2). Our results support the hypothesis that the C-terminal domain plays a role in the interaction of the subunits to form higher oligomeric structures.

Human and yeast CBS exhibit similar pH optima (Table 2). Steady-state kinetic data for yeast F-CBS and T-CBS (Figure 5A−D) were hyperbolic at low substrate concentrations but exhibited substrate inhibition at high concentrations of L-homocysteine. Data obtained at low substrate concentrations were also plotted by double reciprocal plots of initial rate against the concentration of one substrate at fixed concentrations of the other substrate (Figure 5E,F). The two partial data sets for T-CBS at low substrate concentration fit well to eq 3 using the PC-MLAB program and yielded parallel lines, diagnostic of a ping-pong mechanism (*39*). Fits of corresponding data for F-CBS were less satisfactory (data not shown).

$$v_0 = \frac{V_{max}[Ser][Hcys]}{K_m(Hcys)[Ser] + K_m(Ser)[Hcys] + [Ser][Hcys]} \quad (3)$$

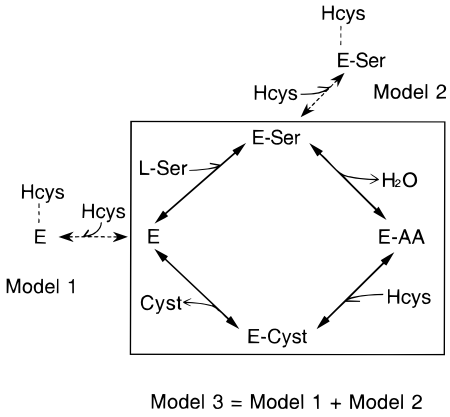
where V_{max} is the maximum velocity and K_m(Ser) and K_m(Hcys) are the Michaelis constants for L-serine (Ser) and L-homocysteine (Hcys). This behavior is consistent with the ping-pong mechanism shown in Scheme 2 for the reactions and intermediates within the box.

This mechanism is consistent with our previous finding that the reaction of L-serine with yeast F-CBS in the absence of L-homocysteine produces the external aldimine of aminoacrylate (E-AA), which absorbs at 460 nm and has a strong negative circular dichroism band at 460 nm (*29*). Analogous results have been obtained with T-CBS (data not shown). Addition of L-homocysteine to F-CBS (*29*) or to T-CBS in the presence of L-serine resulted in a transient decrease in absorbance at 460 nm, which is attributed to E-AA, providing additional evidence that the 460 nm spectroscopic species is a catalytic intermediate. Although addition of the substrate analogue L-alanine to yeast CBS produced the external aldimine of L-alanine (E-Ala), which absorbed at 420 nm and had a negative circular dichroism band at 420 nm (*29*), addition of L-homocysteine to yeast T-CBS resulted in no change in the absorption or circular dichroism spectrum. These results provide evidence that L-homocysteine does not form an external aldimine (E-Hcys) with yeast CBS (see below).

Lower rates at high substrate concentrations are commonly seen with enzymes that catalyze reactions by a ping-pong mechanism (*39*). This substrate inhibition is usually ascribed to the binding of one or both of the substrates to the wrong form of the enzyme, thereby diverting the enzyme from the productive pathway and altering the distribution of enzyme−substrate intermediates (*39*). For example, the binding of the cosubstrate indole to tryptophanase in the presence of an amino acid alters the equilibrium distribution of enzyme−substrate intermediates (*40*). We propose that the inhibition of F-CBS and T-CBS by high concentrations of L-homocysteine (Figure 5A−D) could occur by one of three models, 1−3, described in Scheme 2. The kinetic data obtained for T-CBS and F-CBS gave the best fit to eq 6 for model 3, as judged by the sum of squares function using the PC-MLAB program (see Figure 5A−D and legend).

The k_{cat} value for yeast F-CBS (17.3 s^{-1}) was close to the values reported for the full-length human enzyme in the presence of SAM (*12*, *23*) (Table 2). Interestingly, the k_{cat} value for yeast T-CBS (16.0 s^{-1}) was similar to that for yeast F-CBS (17.3 s^{-1}), despite the higher specific activity and V_{max} of T-CBS. The differences between the relative k_{cat} and V_{max} values for F-CBS and T-CBS result from the lower molecular mass of T-CBS. The differences between the relative specific activities and V_{max} values for the two enzymes result from the lower saturation of F-CBS than of T-CBS by substrates under the conditions for assay of specific activity. The K_m values for L-serine and L-homocysteine were somewhat lower for T-CBS than for F-CBS. The K_m values for L-serine in our work are higher than that reported previously for the yeast CBS (*21*) and than most of the values reported for the human F-CBS and T-CBS (*12*, *23*) (Table 2). The K_d values for L-serine are also higher for yeast F-CBS and T-CBS than for human CBS (*23*). The K_m values for L-homocysteine in our work are lower than that reported previously for the yeast CBS (*21*) and than most of the values reported for the human F-CBS and T-CBS (*12*, *23*) (Table 2). Our finding that higher concentrations of

Scheme 2: Reaction Mechanism of CBS[a]



Model 3 = Model 1 + Model 2

[a] The reactions and intermediates inside the box are for a ping-pong mechanism: L-serine binds to the enzyme, E, to form the external aldimine, E-Ser. β-Elimination of water converts E-Ser to the external aldimine of aminoacrylate, E-AA, which undergoes nucleophilic attack (β-replacement) by L-homocysteine (Hcys) to form the external aldimine of L-cystathionine, E-Cyst, which breaks down to give the free enzyme, E, and the product L-cystathionine (Cyst). The reactions and intermediates outside the box represent models 1 and 2 for substrate inhibition by L-homocysteine. In model 1, L-homocysteine (Hcys) binds to E to form a noncovalent dead-end complex E·Hcys. In model 2, Hcys binds to E-Ser to produce a different dead-end complex. In model 3, Hcys binds to both E and E-Ser (i.e., model 3 = model 1 + model 2). Our finding that addition of L-homocysteine to the free enzyme produced no spectroscopic changes (see Discussion) implies that Hcys does not form an external aldimine with E but probably binds to the site to which Hcys binds prior to nucleophilic attack on E-AA. Binding of Hcys to a second site in model 1, 2, or 3 could divert the enzyme from the productive pathway and alter the equilibrium distribution of enzyme−substrate intermediates. Steady-state equations for models 1-3 are eq 4-6, respectively:

$$v_0 = V_{max}[Ser][Hcys]/\{K_m(Ser)[Hcys](1 + [Hcys]/K_i(Hcys)) + K_m(Hcys)[Ser] + ([Ser][Hcys])\} \quad (4)$$

$$v_0 = V_{max}[Ser][Hcys]/\{K_m(Hcys)[Ser] + K_m(Ser)[Hcys] + [Ser][Hcys](1 + [Hcys]/K_j(Hcys))\} \quad (5)$$

$$v_0 = V_{max}[Ser][Hcys]/\{K_m(Ser)[Hcys](1 + [Hcys]/K_i(Hcys)) + K_m(Hcys)[Ser] + [Ser][Hcys](1 + [Hcys]/K_j(Hcys))\} \quad (6)$$

In eq 4−6, K_i(Hcys) and K_j(Hcys) are the inhibition constants for Hcys, describing inhibition arising from formation of the two different dead-end complexes. The best fits of the data were to model 3 (Figure 5A−D).

L-homocysteine inhibited the yeast enzyme (Figure 5B−D and legend) has not been reported for the full-length wild-type human enzyme. Substrate inhibition by L-homocysteine has been described for the truncated human enzyme (*26*), and severe substrate inhibition by L-homocysteine has been described for a mutant form of the enzyme, V168M (*24*). Thus, yeast F-CBS and T-CBS exhibit small, but significant, differences in their steady-state parameters and also exhibit significant differences from the human enzyme in these parameters. The inhibition of the yeast enzyme by L-homocysteine and the low K_m for L-homocysteine may reflect a different mode of binding of L-homocysteine by the yeast and human enzymes. L-Homocysteine may bind to heme and be activated by heme in human CBS (*26*). In contrast, L-homocysteine must bind to a heme-independent binding site in yeast CBS. Thiols are known to be cosubstrates for other heme-independent PLP-dependent enzymes. For example, *O*-acetylserine sulfhydrylase reacts with *O*-acetyl-L-serine and hydrogen sulfide to form L-cysteine and acetate by a ping-pong mechanism (*41*). Tryptophan synthase reacts with L-serine and 2-mercaptoethanol to form *S*-hydroxyethyl-L-cysteine and water (*42*). Crystallographic studies demonstrate that L-cysteine binds to a second site in cystathionine γ-synthase in the equivalent position of the three-carbon moiety of the product L-cystathionine (*43*).

The yeast and human enzymes, then, have similar domain architectures consisting of an N-terminal catalytic domain and a C-terminal regulatory domain but exhibit differences in cofactor content and in steady-state kinetic parameters. Yeast CBS, unlike human CBS, is not a heme protein and is not activated by SAM. Because the yeast N-terminal catalytic domain, T-CBS, is more stable and less prone to aggregate than F-CBS, it is easier to handle and is potentially more suitable for structure determination by X-ray crystallography.

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