Kinetics of the Yeast Cystathionine β-Synthase Forward and Reverse Reactions: Continuous Assays and the Equilibrium Constant for the Reaction†

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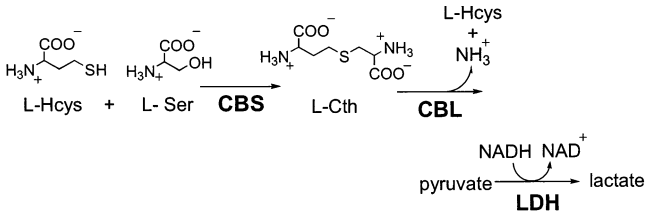
ABSTRACT: Cystathionine β-synthase (CBS) is a pyridoxal-phosphate-dependent enzyme that catalyzes a β-replacement reaction in which the hydroxyl group of serine (L-Ser) is displaced by the thiol of homocysteine (L-Hcys) to form cystathionine (L-Cth) in the first step of the trans-sulfuration pathway. A new continuous assay for the forward reaction, employing cystathionine β-lyase and L-lactate dehydrogenase as coupling enzymes, is described. It alleviates product inhibition by L-Cth and revealed that the values for K_m^{L-Ser} (1.2 mM) and for substrate inhibition by L-Hcys ($K_{iF1}^{L-Hcys} = 2.0$ mM) are lower than those previously reported. A continuous, 5,5′-dithio-bis-(2-nitrobenzoic acid) (DTNB)-based assay for the CBS-catalyzed hydrolysis of L-Cth to L-Ser and L-Hcys provides a tool for investigation of the reverse reaction ($k_{catR} = 0.56$ s^{-1}, $K_m^{L-Cth} = 0.083$ mM). The k_{catR}/K_m^{L-Cth} versus pH profile of ytCBS is bell-shaped with a pH optimum of 8.3, and the pK_a values for the acidic and basic limbs are 8.05 and 8.63, respectively. The latter is assigned to the α-amino group of L-Cth (p$K_a = 8.54$). The internal aldimine of ytCBS remains protonated at pH < 11; therefore, the acidic pK_a is assigned to an enzyme functionality that is not associated with the internal aldimine. K_{eq} was determined directly and from the kinetic parameters, and the values are 0.61 and 1.2 μM, respectively.

Cystathionine β-synthase (CBS)[1] catalyzes a β-replacement (Scheme 1) in which the hydroxyl group of serine (L-Ser) is exchanged for the thiol of homocysteine (L-Hcys, ref *1*) to form cystathionine (L-Cth); a reaction that is similar to those catalyzed by the evolutionarily related enzymes tryptophan synthase (TrpS, ref *2*) and *O*-acetylserine sulfhydrylase (OASS, ref *3*). CBS functions in the trans-sulfuration pathway that converts Hcys to cysteine (*4*). In humans, CBS mutants with decreased activity result in elevated plasma levels of Hcys, which is a major cause of homocystinuria (*5*, *6*), an autosomal recessive disease with clinical manifestations including arteriosclerosislike vascular damage and skeletal abnormalities (*7*). Nearly 100 homocystinuria-associated CBS alleles have been identified (*8*). Elevated plasma concentration of Hcys has been recognized as an independent risk factor for heart disease (*9*).

Scheme 1. Coupled-Coupled Enzyme Assay for CBS[a]



[a] CBS catalyzes replacement of the β-hydroxyl group of L-Ser with L-Hcys to form L-Cth. The latter is converted to L-Hcys, pyruvate, and NH_3 by CBL. Pyruvate reduction by LDH is accompanied by NADH oxidation, which is followed spectrophotometrically.

CBS exists as a mixture of homotetramers and octamers in which the individual subunits contain both a catalytic and a regulatory domain (*10*, *11*). Full-length CBS is prone to aggregation, which has hindered attempts at crystallization (*11*−*13*). Removal of the regulatory domain alleviates this problem and results in subunits of ∼40−45 kDa that form active homodimers (*11*, *12*). The catalytic domain of CBS is the most conserved portion with respect to CBS orthologs and to evolutionarily related enzymes from bacteria, yeast, and plants. The catalytic domain of human CBS (hCBS) is 47% identical to yeast CBS (yCBS) and shares 30−34% identity with plant and bacterial OASS. The crystal structure for the catalytic domain of hCBS shows that the catalytic core (*14*, *15*) is similar to that of OASS from *Salmonella typhimurium* (*16*).

Mammalian CBS is unique in that it is the only enzyme known to contain both pyridoxal phosphate (PLP) and heme

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[1] Abbreviations: AA, aminoacrylate; AMPSO, *N*-(1,1-dimethyl-2-hydroxyethyl)-3-amino-2-hydroxypropanesulfonic acid; CAPS, 3-(cyclohexylamino)-1-propanesulfonic acid; CBL, cystathionine β-lyase; CBS, cystathionine β-synthase; hCBS, human CBS; yCBS, yeast CBS; ytCBS, truncated yCBS (catalytic domain); CGL, cystathionine γ-synthase; Cth, cystathionine; DMSO, dimethyl sulfoxide; DTNB, 5, 5′-dithio-bis-(2-nitrobenzoic acid); EDTA, ethylenediaminetetraacetic acid; Hcys, homocysteine; HPLC, high-pressure liquid chromatography; LDH, L-lactate dehydrogenase; NADH, β-nicotinamide adenine dinucleotide (reduced form); Ni-NTA, nickel nitrilotriacetic acid; OASS, *O*-acetylserine sulfhydrylase; PCR, polymerase chain reaction; PLP, pyridoxal 5′-phosphate; TAPS, *N*-[tris(hydroxymethyl)methyl]-3-aminopropane sulfonic acid; TLC, thin-layer chromatography; TNBSA, 2,4,6-trinitrobenzenesulfonic acid; TrpS, tryptophan synthase.

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(*17*). Mechanistic studies of PLP enzymes are frequently facilitated by presteady-state kinetic investigations that monitor the coenzyme-associated intermediates. Such studies are precluded in hCBS because of the overlapping absorbance spectra of the PLP and heme cofactors. yCBS catalyzes the same reaction as hCBS but does not contain heme; therefore, it provides a useful surrogate for the human enzyme (*18*, *19*).

Here we report a new continuous assay for the investigation of the CBS-catalyzed condensation of L-Ser and L-Hcys to L-Cth. The steady-state characterization of the truncated form of yCBS (ytCBS) is reinvestigated with this assay. Conditions are also described for a continuous assay to monitor the reverse CBS reaction (L-Cth → L-Ser + L-Hcys). The pH dependence of this reaction is described. The value of the equilibrium constant was determined directly and from the Haldane relationship derived for the reaction.

EXPERIMENTAL PROCEDURES

Reagents. L-Cth [(*R*)-*S*-(2-amino-2-carboxyethyl)-L-homocysteine] and L-Ser were purchased from Fluka. L-Lactate dehydrogenase (LDH), L-Hcys thiolactone, D,L-Hcys thiolactones, and Cth (mixture of four stereoisomers: L-, D-, L-allo, and D-allo) were obtained from Sigma. Hcys was prepared from the thiolactone (*20*). Protease inhibitor (Complete EDTA-free) tablets were a Roche product. DEAE sepharose fast-flow resin was purchased from Amersham Pharmacia Biotech. Macro-Prep ceramic hydroxyapatite Type I resin was obtained from BioRad. Toyopearl butyl-650M resin was from TosoHaas. Ni-NTA resin was a Qiagen product. DTNB and TNBSA were from Pierce.

Cloning and His-Tagging of Cystathionine β-lyase (CBL). The *metB* gene encoding CBL (*21*) was PCR amplified from the genomic DNA of *Escherichia coli* MG1655 with the CBL-specific primers 5′-GCT ACC AAA ATC AGC GGC GAT ATC GTT GG-3′ and 5′-CCT GTG GAA CAG CGT TTA AGC CTT TAT CTG CC-3′. Restriction sites and a C-terminal, six-histidine tag were subsequently introduced by PCR with the primers 5′-GG AAT CCC GCC *ATG* GCG GAC AAA AAG C-3′ and 5′-T CCC CCC GGG TTA ATG GTG GTG ATG GTG GTG TAC AAT TCG CGC AAA ACC GGC GTC C-3′. (Restriction sites are underlined, and the ATG start codon is in italics.) The CBL gene was introduced into the pTrc-99a vector (Amersham Pharmacia Biotech) and transformed into the *E. coli* strain DH10B (Gibco BRL) via electroporation (Gene Pulser, BioRad).

Overexpression and Purification of ytCBS. The *E. coli* expression system for ytCBS was the generous gift of Dr. Edith Miles (National Institutes of Health). The procedure of Jhee et al. (*11*, *19*) was employed for the purification of ytCBS, with the following modifications. A 3-L (4 × 750 mL in 2.8-L baffled Fernbach flasks) culture was grown and induced as described (*11*, *19*), but δ-aminolevulinic acid was omitted because yCBS does not contain heme (*18*, *19*). The cells were disrupted by incubation with 1 mg/mL lysozyme on ice for 20 min followed by repeated (8 × 30 s) cycles in a Bead Beater (Biospec Products). The resulting ∼120 mg (40 mg/L yield) of ytCBS was ≥95% pure, as determined by SDS−PAGE, and had the same specific activity as that reported by Jhee et al. (*11*).

Overexpression and Purification of CBL. Purification of CBL was facilitated by the introduced C-terminal histidine tag. One liter of LB media in a 2.8-L Fernbach flask was warmed at 37 °C for 3 h followed by addition of ampicillin to 100 μg/mL and inoculation with 20 mL of an overnight culture of pTrc-99a/CBL in *E. coli* DH10B. IPTG was added to 0.1 mM when the OD_{600} reached 0.5, and the cells were incubated at 30 °C for a further 6 h. The harvested cell pellet was suspended in 30 mL of buffer A (50 mM potassium phosphate (pH 7.8), 10 mM β-mercaptoethanol, 0.1 mM PLP, and 10 mM imidazole) containing one Complete EDTA-free tablet (Roche) and 20 μg/mL DNAse I. Cells were disrupted by incubation with 1 mg/mL lysozyme and repeated cycles in a Bead Beater (Biospec Products) as described above for ytCBS. The lysate was centrifuged, the supernatant was loaded onto a 1.5 × 10-cm column of Ni-NTA resin (Qiagen), and the column was washed with 200 mL of buffer A. The enzyme was eluted with a 400-mL linear gradient of 10−400 mM imidazole in buffer A. The CBL-containing fractions were pooled, concentrated, and dialyzed against buffer C (50 mM potassium phosphate (pH 7.8), 1 mM EDTA, 1 mM DTT, and 20 μM PLP). Approximately 150 mg of ≥95% pure CBL was obtained from the 1-L culture. The specific activity was 1.23×10^4 μmol pyruvate h^{-1} mg^{-1}, which is comparable to the literature value of 1.29×10^4 μmol pyruvate h^{-1} mg^{-1} for untagged CBL (*22*, *23*).

Enzyme Assays. Activity was measured in a total volume of 1 mL at 37 °C on a HP 8453 or a Perkin-Elmer Lambda 6 UV−vis spectrophotometer. The assay buffer was comprised of 50 mM Tris (pH 8.6) and 20 μM PLP. Samples were equilibrated at 37 °C, and a background rate was recorded prior to initiation of the reaction. Data were fit by nonlinear regression with the program SAS (SAS Institute, Cary, NC).

CBS DTNB Assay. Reactions were carried out in assay buffer containing 2 mM DTNB (20 μL of a 100 mM stock of DTNB in DMSO) and 0.015−5.0 mM L-Cth or 0.05−25 mM of the mixed isomers of Cth. The reactions were initiated by the addition of ytCBS to 0.64 μM. Absorbance changes were monitored at 412 nm ($\Delta\epsilon_{412} = 13\,600$ M^{-1} cm^{-1}). The velocity of the reaction is independent of added [PLP] (0−100 μM) and of the presence of 0−2 mg/mL BSA or DMSO (≤10%) and is linearly dependent on [ytCBS] between 0.026 and 1.03 μM.

k_{catR} and K_m^{L-Cth} values were obtained by fitting the data to the Michaelis−Menten equation, and k_{catR}/K_m^{L-Cth} was obtained independently from

$$\frac{v}{[E]} = \frac{k_{catR}[\text{L-Cth}]/K_m^{L-Cth}}{1 + [\text{L-Cth}]/K_m^{L-Cth}} \quad (1)$$

CBS Coupled-Coupled Assay. CBL and LDH concentrations of 0.4 μM and 1.3 μM, respectively, were selected for the ytCBS assay because they are each well into the plateau region of the coupling enzymes (Figure 1, insets A and B) for the range of ytCBS concentrations assayed. Reactions were carried out in assay buffer containing 150 μM NADH, 1.3 μM LDH, 0.4 μM CBL, 0.025−15 mM L-Hcys, and 0.05−20 mM L-Ser. They were initiated by the addition of

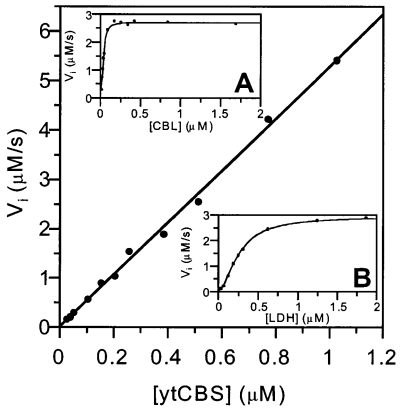


FIGURE 1: Dependence of the rate of NADH oxidation in the coupled-coupled CBL/LDH assay upon [ytCBS], [CBL] (inset A), and [LDH] (inset B). Reactions were in 1-mL volumes at 37 °C, and the rates were monitored at 340 nm. Conditions: 50 mM Tris (pH 8.6), 150 μM NADH, 20 μM PLP, 1 mM L-Ser, 1 mM L-Hcys and (main panel) 0.026−1.03 μM ytCBS, 0.4 μM CBL, and 1.3 μM LDH; (inset A) 0.52 μM ytCBS, 0.084−4.2 μM CBL, and 1.3 μM LDH; (inset B) 0.52 μM ytCBS, 0.4 μM CBL, and 0.031−3.1 μM LDH.

0.026−1.03 μM ytCBS, and the conversion of NADH to NAD^+ was monitored at 340 nm ($\Delta\epsilon_{340} = 6200\ M^{-1}\ cm^{-1}$). The rate of NADH oxidation in the CBL/LDH continuous assay is linearly dependent on ytCBS concentration over the specified range (Figure 1).

Evaluation of the pH Dependence of the Kinetic Parameters of ytCBS. The pH dependence of the kinetic parameters for the ytCBS-catalyzed conversion of L-Cth to L-Hcys and L-Ser was determined in the DTNB assay. A three-component buffer, comprised of 50 mM MOPS (pK_a = 7.2), 50 mM Bicine (pK_a = 8.3), and 50 mM proline (pK_a = 10.7) was employed to maintain constant ionic strength ($I_c = 0.28$) (*11*, *24*). The pH was adjusted to 11.2 with sodium hydroxide prior to back-titration with HCl to the desired pH (*11*, *24*). The kinetic measurements were carried out from pH 6.3−9.7 in the presence of 0.05−25 mM Cth, 20 μM PLP, and 2 mM DTNB at 37 °C. The pH dependence of k_{catR} was fitted to eq 2 and that of k_{catR}/K_m^{L-Cth} was fitted to the bell-shaped curve described by eq 3:

$$k_{catR} = \frac{(k_{catR})_{max}}{1 + 10^{(pK_{a1} - pH)}} \quad (2)$$

$$k_{catR}/K_m^{L-Cth} = \frac{(k_{catR}/K_m^{L-Cth})_{max}}{1 + 10^{(pK_{a1} - pH)} + 10^{(pH - pK_{a2})}} \quad (3)$$

where $(k_{catR})_{max}$ and $(k_{catR}/K_m^{L-Cth})_{max}$ are the upper limit values for the kinetic parameters.

Spectrophotometric Titration of the ytCBS Internal Aldimine. The internal aldimine of ytCBS was titrated versus pH with 20 μM enzyme in 5 mM TAPS (pK_a 8.4, pH 7.24) containing 0.5 M KCl. The pH was varied by successive additions of 0.5 M AMPSO (pK_a 9.0, pH 10.6) below pH 9.0; 0.5 M CAPS (pK_a 10.4, pH 11.5) between pH 9.0−10.5; and 1.0 M NaOH above pH 10.5. The enzyme solution was drawn through a 0.2-μm filter to reduce light scattering from precipitate, and the pH of the solution was determined prior to each absorbance measurement. Spectra were recorded from 250 to 500 nm on a UVIKON 360 double-beam spectrophotometer (KONTRON Instruments). The data recorded at 425 and 360 nm were fitted to eqs 4 and 5, respectively:

$$A = \frac{A_1 - A_2}{1 + 10^{(pH - pK_{spec})}} + A_2 \quad (4)$$

$$A = \frac{A_1 - A_2}{1 + 10^{(pK_{spec} - pH)}} + A_2 \quad (5)$$

where A_1 and A_2 are the high or low absorbance limits at a particular wavelength, respectively.

Derivatization with 2,4,6-Trinitrobenzenesulfonic acid (TNBSA). Reactions were carried out in 50 mM potassium phosphate (pH 8.6) containing 5 mM L-Cth and were initiated by the addition of ytCBS to 0.64 μM. Aliquots (10 μL) were quenched by transfer to 600 μL of 50 mM potassium phosphate, pH 11. TNBSA was added to 500 μM, and the samples were incubated for 2 h in the dark at 25 °C prior to quenching with 30 μL of glacial acetic acid (*25*). Quenched reactions were separated by HPLC (Varian ProStar) on an Alltech Nucleosil C18 column and 76.5:12.5:1 H_2O/CH_3-CN/acetic acid at a flow rate of 1 mL/min.

RESULTS

Continuous Assay for the CBS-Catalyzed Production of L-Cth from L-Ser and L-Hcys. CBL and LDH provide a coupled-coupled assay for CBS (Scheme 1). The kinetic parameters for the CBL-catalyzed conversion of L-Cth to L-Hcys, pyruvate, and NH_3 ($k_{cat} = 150\ s^{-1}$, $K_m^{L-Cth} = 340$ μM, and $k_{cat}/K_m^{L-Cth} = 4.3 \times 10^5\ M^{-1}\ s^{-1}$) and of L-Ser to pyruvate and NH_3 ($k_{cat} = 0.75\ s^{-1}$, $K_m^{L-Ser} = 3.6$ mM, and $k_{cat}/K_m^{L-Ser} = 210\ M^{-1}\ s^{-1}$), as well as those for the LDH-catalyzed reduction of pyruvate to lactate ($k_{cat} = 320\ s^{-1}$, $K_m^{Pyr} = 1.4$ mM, and $k_{cat}/K_m^{Pyr} = 2.3 \times 10^5\ M^{-1}\ s^{-1}$) were determined under the CBS assay conditions. The k_{cat} of CBL for L-Cth is comparable to the literature value ($k_{cat} = 187\ s^{-1}$, ref *23*); however, the reported K_m^{L-Cth} (42 μM, ref *23*) is 8-fold less than that determined in this study. The difference in K_m^{L-Cth} values may be due in part to the lower pH (8.6) in the present study versus pH 9.0 in ref *23* or to the added histidine tag. Although CBL is able to use L-Ser as a substrate, the k_{cat}/K_m^{L-Ser} is 0.5% that of L-Cth, and this activity does not interfere in the assay.

Substrate and Product Inhibition of ytCBS. The continuous nature of the CBL/LDH assay eliminates L-Cth accumulation and thus precludes the product inhibition that complicates parameter evaluation in the discontinuous assay. Sample data are shown in Figure 2. The decrease in v_i/[E] at elevated substrate concentrations is indicative of substrate inhibition, which is negligible for L-Ser (Figure 2A) and pronounced for L-Hcys (Figure 2B). Substrate inhibition of CBS by L-Hcys has been described for both hCBS (*26*) and yCBS (*11*). The model proposed by Jhee et al. (*11*), which incorporates two forms of substrate inhibition by L-Hcys into the ping-pong mechanism of yCBS, is the same as that shown in Scheme 2, except for the K_i^{L-Cth} term. The data obtained

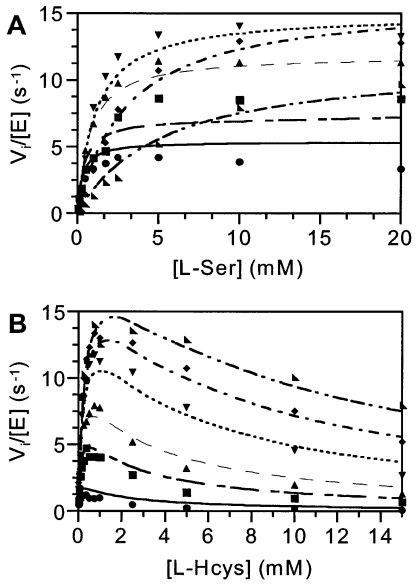
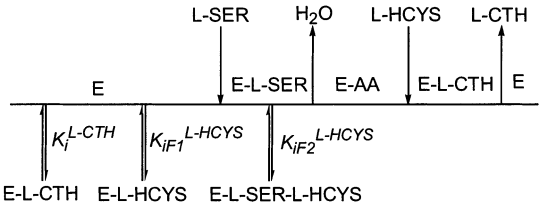


FIGURE 2: Steady-state initial velocity kinetics for ytCBS showing the dependence of the reaction on [L-Ser] and on [L-Hcys]. (A) Effect of L-Ser concentration at fixed concentrations of L-Hcys (0.10 mM, ●; 0.15 mM, ■; 0.375 mM, ▲; 0.75 mM, ▼; 5 mM, ♦; 15 mM, right triangle). (B) Effect of L-Hcys concentration at fixed concentrations of L-Ser (0.125 mM, ●; 0.5 mM, ■; 1.0 mM, ▲; 2.5 mM, ▼; 5.0 mM, ♦; 10 mM, right triangle). The fit of the entire data set to eq 6 is represented by the lines. The fitted parameters are given in Table 1. The reactions were initiated by addition of ytCBS (0.026−1.03 μM). Other conditions were as described in the legend to Figure 1.

Scheme 2. Kinetic Mechanism Describing the CBS-Catalyzed Condensation of L-Ser with L-Hcys to Form L-Cth[a]



[a] L-Ser binds to the enzyme to form the external aldimine (E−L-Ser). H_2O is eliminated to form the aminoacrylate complex (E−AA). L-Hcys reacts with E−AA to form the external aldimine of L-Cth (E−L-Cth) from which L-Cth is released. Product inhibition (K_i^{L-CTH}) results when L-Cth accumulates since L-Ser and L-Cth compete for the unliganded form of the enzyme (E). L-Hcys can bind to either E (K_{iF1}^{L-HCYS}) or E−Ser (K_{iF2}^{L-HCYS}).

for ytCBS in the CBL/LDH coupled assay were fit to this model with

$$\frac{v}{[E]} = [k_{catF}[\text{L-Ser}][\text{L-Hcys}]]/ \left[K_m^{L-Hcys}[\text{l-Ser}]\left(1+\frac{[\text{L-Hcys}]}{K_{iF1}^{L-Hcys}}\right) + K_m^{L-Ser}[\text{L-Hcys}] + [\text{L-Ser}][\text{L-Hcys}]\left(1+\frac{[\text{L-Hcys}]}{K_{iF2}^{L-Hcys}}\right)\right] \quad (6)$$

K_{iF1}^{L-Hcys} is the E−Hcys dissociation constant, and K_{iF2}^{L-Hcys} is the dissociation constant for the E−Ser−Hcys complex because of L-Hcys association with the E−Ser form of the enzyme before the aminoacrylate (AA) can be formed. The fitted parameters are presented in Table 1. The value obtained

Table 1: Kinetic Parameters for the Forward and Reverse ytCBS-catalyzed Reactions[a]

	this study	ref 11
L-serine + L-homocysteine → L-cystathionine		
k_{catF} (s^{-1})	21.5 ± 0.9[b]	16.0 ± 0.1[c]
K_m^{L-SER} (mM)	1.2 ± 0.1[b]	5.34 ± 0.64[c]
K_m^{L-HCYS} (mM)	0.30 ± 0.03[b]	0.445 ± 0.059[c]
K_{iF1}^{L-HCYS} (mM)	2.0 ± 0.4[b]	23.1 ± 7.9[c]
K_{iF2}^{L-HCYS} (mM)	18 ± 4[b]	22.3 ± 4.1[c]
L-cystathionine → L-homocysteine + L-serine		
k_{catR} (s^{-1})[d]	0.56 ± 0.01	
K_m^{L-CTH} (μM)[d]	83 ± 3	
K_{iR}^{L-SER} (μM)	15 ± 1[e]	14 ± 0.25[f]

[a] Subscripts are defined in Scheme 2 and eq 6. [b] Kinetic measurements were carried out in 50 mM Tris (pH 8.6) containing 20 μM PLP, 0.4 μM CBL, 1.3 μM LDH, 150 μM NADH, 0.025−15 mM L-Hcys, 0.05−20 mM L-Ser, and 0.026−1.03 μM ytCBS at 37 °C. The data were fit to eq 6. [c] Data were fit to eq 6 by Jhee et al. (*11*). [d] Kinetic measurements were carried out in 50 mM Tris (pH 8.6), 20 μM PLP, 2 mM DTNB, 0.015−5 mM L-Cth, and 0.64 μM ytCBS at 37 °C. Data were fit to the Michaelis−Menten equation. [e] L-Ser was varied between 0 and 5.0 mM at fixed concentrations of L-Cth (0.01−2.0 mM). Data were fit to eq 8. [f] Jhee et al. (*11*) determined K_d^{L-SER} as aminoacrylate formation, monitored by fluorescence.

for k_{catF} (21.5 ± 0.9 s^{-1}) is 1.3-fold greater than that reported previously (Table 1, ref *11*), while those of K_m^{L-Ser} (1.2 ± 0.1 mM) and K_{iF1}^{L-Hcys} (2.0 ± 0.4 mM) are 4.5 and 12-fold less, respectively, than those reported by Jhee et al. (Table 1, ref *11*). The K_m^{Hcys} is 2-fold greater for D,L-Hcys than for L-Hcys; however, the k_{catF} and K_m^{L-Ser} of ytCBS are unaltered by the presence of D-Hcys (D,L-Hcys vs L-Hcys), indicating that it is neither a substrate nor an inhibitor for the ytCBS-catalyzed condensation of L-Ser and L-Hcys.

Continuous Assay for the Reverse Reaction: CBS-Catalyzed Conversion of L-Cth to L-Ser and L-Hcys. Brown and Gordon (*27*) observed that L-Hcys formation could be monitored continuously in the CBS-catalyzed hydrolysis of L-Cth to L-Ser and L-Hcys, which is the reverse of the reaction effected under physiological conditions. However, the method was employed only for the quantitation of CBS and was not developed as an assay for kinetic investigations. Its use has not been reported since the initial description in 1971. Optimization of the conditions of the DTNB assay for the determination of the steady-state kinetic parameters of ytCBS is described here. The presence of 2 mM DTNB in the reaction mixture does not affect the activity of ytCBS. L-Hcys formation is monitored continuously with DTNB in the ytCBS-catalyzed hydrolysis of L-Cth to L-Ser and L-Hcys. The rate was determined as a function of [L-Cth], and the Michaelis−Menten parameters are included in Table 1. The k_{catR} for L-Cth and Cth are identical, and the K_m^{Cth} is 4-fold higher than the K_m^{L-Cth}, indicating that the other three stereoisomers of Cth (D, L-allo, and D-allo) are neither substrates of ytCBS nor inhibitors of the ytCBS-catalyzed hydrolysis of L-Cth to L-Ser and L-Hcys.

pH Dependence of ytCBS Activity. Figure 3 shows the pH dependence of k_{catR} and k_{catR}/K_m^{L-Cth} for the ytCBS-catalyzed hydrolysis of L-Cth to L-Hcys and L-Ser. The fit of the k_{catR} data to eq 2 yielded a single pK_a value of 7.27 ± 0.06, while the k_{catR}/K_m^{L-Cth} data fit to eq 3 gave values of pK_{a1} = 8.05 ± 0.09 and pK_{a2} = 8.63 ± 0.09 (Table 2).

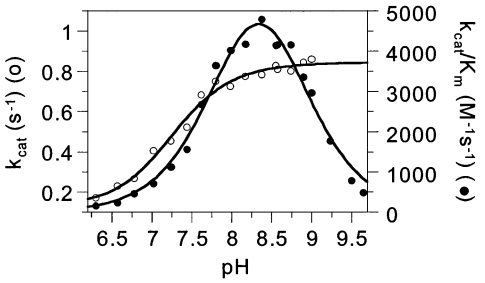


FIGURE 3: pH dependence of k_{catR} and k_{catR}/K_m^{L-Cth} for the ytCBS-catalyzed hydrolysis of L-Cth to L-Ser and L-Hcys monitored with the DTNB assay. The lines represent the nonlinear regression fits of k_{catR} vs pH to eq 2 and of k_{catR}/K_m^{L-Cth} vs pH to eq 3. Reactions were in 1-mL volumes at 37 °C, and the rates were monitored at 412 nm. Conditions: 50 mM MBP (50 mM MOPS, 50 mM bicine, and 50 mM proline), 20 μM PLP, 2 mM DTNB, 0.015−5 mM L-Cth, and 0.64 μM ytCBS.

Table 2: Parameters Determined from the pH Dependence of the Reaction of ytCBS with L-Cth

	limiting value	pK_{a1}	pK_{a2}
k_{catR}[a]	0.84 ± 0.02 (s^{-1})	7.27 ± 0.06	
k_{catR}/K_m^{L-CTH}[a]	9000 ± 1200 ($M^{-1}s^{-1}$)	8.05 ± 0.09	8.63 ± 0.10
L-cystathionine		8.54 ± 0.01	9.63 ± 0.01

[a] Kinetic measurements were carried out from pH 6.3−9.7 in MBP buffer containing 0.05−25 mM Cth, 20 μM PLP, and 2 mM DTNB at 37 °C. Data for k_{catR} and k_{catR}/K_m^{L-CTH} vs pH were fit to eqs 2 and 3, respectively.

The pK_a values of the α-NH_2 groups of L-Cth were determined by direct titration with NaOH as 8.54 ± 0.01 and 9.63 ± 0.01 at 25 °C. The former value is within experimental error of that determined for the basic limb of the k_{catR}/K_m^{L-Cth} versus pH plot (Table 2, Figure 3).

Spectrophotometric Titration of the ytCBS Internal Aldimine. There is a shift in ytCBS absorbance from 277 to 289 nm at pH > 11 (Figure 4A) that likely reflects tyrosine ionization (pK_a = 10.13, ref *28*, *29*). The 412 nm absorbance of ytCBS is lost at pH >11 (Figure 4B) concurrently with the tyrosine 277−289 nm shift. It is possible that PLP dissociates from the enzyme at high pH because the absorbance shifts to a maximum of 390 nm (Figure 4B), which is equal to that of free PLP (*30*).

Reversibility of the ytCBS-Catalyzed Reaction. While earlier characterizations of the CBS reaction focused on the thermodynamically favorable and physiologically relevant formation of L-Cth, the data presented in Table 1 show that the reaction can be followed in the reverse direction under steady-state conditions. This has also been demonstrated in presteady-state studies (*31*, *32*). Derivatization of the reaction products with TNBSA, subsequent separation by reversed-phase HPLC, and comparison with TNBSA-derivatized standards (data not shown) demonstrated that ytCBS catalyzes the conversion of L-Cth to L-Hcys and L-Ser. In addition, no conversion of NADH to NAD^+ was observed when 0.64 μM ytCBS was incubated with 5 mM L-Cth in the presence of 3.1 μM LDH and 150 μM NADH, indicating that no pyruvate or α-ketobutyrate were produced. These two observations, in combination with the DTNB assay for CBS described above, demonstrate that the CBS-catalyzed reaction can be monitored in both directions.

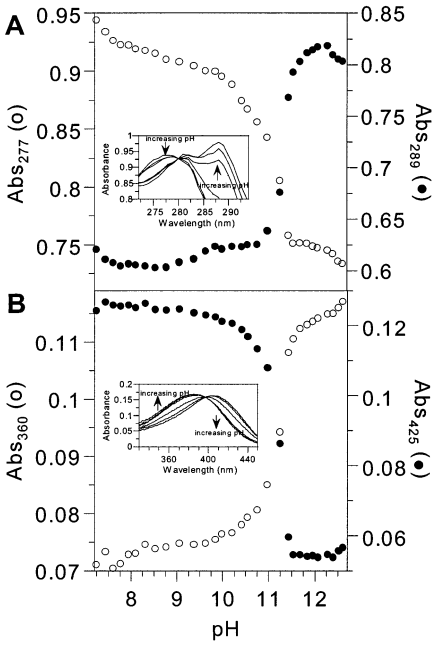


FIGURE 4: Spectrophotometric pH titration of 20 μM ytCBS. (A) Absorbance at 277 (O) and 289 nm (●) vs pH. (B) Absorbance at 360 (O) and 425 nm (●) vs pH. (Insets A and B) pH dependence of the UV−vis spectra at pH 7.24, 10.54, 10.97, 11.24, 11.53, and 12.06.

The equilibrium constant (K_{eq}) for the ytCBS reaction was determined directly by measurement of the L-Hcys concentration at equilibrium. ytCBS (2.6 μM) was incubated with 5 mM L-Cth, and aliquots were removed at time-points from 0 to 190 min. The observed equilibrium concentration of L-Hcys from the DTNB assay was 55 μM. This concentration was attained after 30 min and did not vary over at least the next 160 min. Additional aliquots of ytCBS did not result in a change in the L-Hcys concentration, confirming that the reaction had attained equilibrium. The calculated L-Ser and L-Cth concentrations at equilibrium were 55 μM and 4.95 mM, respectively, and K_{eq} was calculated as 0.61 μM from

$$K_{eq} = \frac{[\text{L-Ser}][\text{L-Hcys}]}{[\text{L-Cth}]} \tag{7}$$

The K_{eq} value was also calculated from the rates of the forward and reverse reactions. Ping-pong bi bi hydrolytic enzymes such as CBS are treated as ordered bi uni because the water concentration is invariant (*33*). The rates of the ytCBS-catalyzed conversion of 0.01−2.0 mM L-Cth to L-Ser and L-Hcys were measured in the presence of 0−5.0 mM L-Ser (Figure 5), and the data were fit by nonlinear regression to the following equation (*33*):

$$\frac{v}{[E]} = \frac{k_{cat}[\text{L-Cth}]}{K_m^{L-Cth}\left(1 + \frac{[\text{L-Ser}]}{K_{iR}^{L-Ser}}\right) + [\text{L- Cth}]} \tag{8}$$

The value of K_{iR}^{L-Ser} (15 ± 1 μM) obtained is a measure of product inhibition by L-Ser of the ytCBS-catalyzed hydrolysis of L-Cth. This value is within experimental error of the K_d^{L-Ser} value for ytCBS (14 μM) determined from measurement of AA formation (*11*).

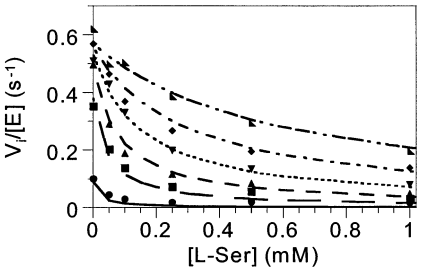


FIGURE 5: L-Ser product inhibition of the ytCBS-catalyzed hydrolysis of L-Cth to L-Ser and L-Hcys. L-Ser was varied between 0 and 5.0 mM at fixed concentrations of L-Cth (0.01 mM, ●; 0.1 mM, ■; 0.25 mM, ▲; 0.5 mM, ▼; 1.0 mM, ♦; 2.0 mM, right triangle). The fit of the entire data set to eq 8 is represented by the lines. The fitted parameter K_{iR}^{L-Ser} is given in Table 1. The reactions were initiated by addition of 0.64 μM CBS. The buffer was 50 mM Tris (pH 8.6), and other conditions were as described in the legend to Figure 3.

The Haldane equation (eq 9, ref *33*) gives the kinetically determined value of K_{eq}.

$$K_{eq} = \frac{k_{catF} K_m^{L-Cth}}{k_{catR} K_m^{L-Hcys} K_{iR}^{L-Ser}} \quad (9)$$

The calculated value ($K_{eq} = 1.4 \pm 0.3\ \mu$M) is ~2-fold greater than that determined by direct measurement of L-Hcys concentration at equilibrium (0.61 μM).

DISCUSSION

Continuous Assay for the CBS-Catalyzed Production of L-Cth from L-Ser and L-Hcy Eliminates Product Inhibition by L-Cth. Prior kinetic investigations of CBS activity relied predominantly on the ^{14}C-L-Ser endpoint assay in which ^{14}C-labeled L-Cth resulting from the reaction of ^{14}C-L-Ser with L-Hcys is separated from the substrates by thin-layer chromatography or HPLC and quantitated by scintillation counting (*34*, *35*). This assay is sensitive, but laborious, and ^{14}C-L-Ser is expensive. In addition, the accumulated L-Cth product competes with L-Ser for free enzyme (E, Scheme 2). These limitations motivated the search for a continuous assay. Commonly available enzymes catalyzing reactions that capture nascent L-Cth that could be employed as coupling enzymes are limited to those of the trans-sulfuration pathway, CBL and cystathionine γ-lyase (CGL). CBL converts L-Cth to L-Hcys, NH_3, and pyruvate. The latter is conveniently monitored by the standard LDH assay. Under ytCBS assay conditions, both k_{cat}/K_m^{L-Cth} of CBL ($4.3 \times 10^5\ M^{-1}\ s^{-1}$) and k_{cat}/K_m^{Pyr} of LDH ($2.3 \times 10^5\ M^{-1}\ s^{-1}$) are at least an order of magnitude greater than the k_{catF}/K_m^{L-Ser} ($1.8 \times 10^4\ M^{-1}\ s^{-1}$) for ytCBS. Thus, a continuous assay is practical for ytCBS. The concentration of LDH required, assuming a short lag period ($\tau = 4$ s), was estimated as 1.1 μM (eq 10) (*36*, *37*).

$$[LDH] = \frac{K_m^{Pyr}}{k_{cat}^{LDH} \times \tau} \quad (10)$$

This value was employed to calculate a CBL concentration of 0.4 μM, as that required to give an overall lag period of 10 s, via eq 11 (*36*, *37*).

$$[CBL] = \frac{K_m^{L-CTH}/k_{cat}^{CBL}}{\tau - K_m^{Pyr}/k_{cat}^{LDH} \times [LDH]} \quad (11)$$

The calculated LDH and CBL concentrations were subsequently optimized by independently varying the concentration of each (Figure 1). The results demonstrate the effectiveness of these enzymes for the continuous quantitation of L-Cth production by CBS (Scheme 1). Additional advantages of this assay are that it is nonradioactive and less costly and time-consuming than the ^{14}C-L-Ser assay, and it eliminates product inhibition by L-Cth.

There is significant variation in the reported values of kinetic constants for both yCBS and hCBS. For example, k_{catF} values of 8.0 and 16 s^{-1}, K_m^{L-Ser} values of 2.2 and 5.34 mM, and K_m^{L-Hcys} values of 2.3 and 0.445 mM have been reported for the full-length form of yCBS under similar experimental conditions of 100 mM Tris, pH 8.3 (*38*), and 200 mM Tris, pH 8.6 (*11*). Therefore, this variation is likely due to substrate and product inhibition rather than a difference in experimental conditions. The 4.5- and 12-fold reductions in the values of K_m^{L-Ser} (1.2 ± 0.1 mM) and K_{iF1}^{L-Hcys} (2.0 ± 0.4 mM) determined here (Table 1) as compared to reported values (*11*) are due to relief of product inhibition by the continuous CBL/LDH assay.

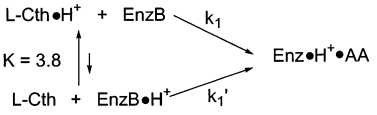
The TNBSA derivatization assay provides a novel discontinuous alternative to the ^{14}C-L-Ser method. It does require separation of modified products by TLC or HPLC and therefore offers no advantage other than cost and avoidance of radioactivity over the ^{14}C-L-Ser assay. It may be useful in specific applications where the continuous CBL/LDH assay is not practical.

CBS-Catalyzed Hydrolysis of L-Cth to L-Ser and L-Hcys Can Be Monitored Continuously. DTNB is a convenient reagent to monitor the rates of release of nascent thiols continuously (*39*). It is used in CBL and CGL assays (*22*, *40*). The k_{cat} value of the ytCBS-catalyzed condensation of L-Hcys and L-Ser to L-Cth is 38-fold greater ($k_{catF} = 21.5\ s^{-1}$) than that of the reverse reaction, determined by the DTNB assay ($k_{catR} = 0.56\ s^{-1}$, Table 1).

pH Dependence of ytCBS Activity and Spectral Properties of the Enzyme. The absorption spectra of yCBS and OASS, at physiological pH, exhibit λ_{max} values near 412 nm, which are diagnostic for the protonated form of the internal aldimine (*19*, *41*). Loss of this proton is accompanied by a shift in the spectrum of PLP to λ_{max} ~360 nm (*42*, *43*). The aldimines do titrate near neutral pH in some aminotransferases (*44*). However, the absorption spectra of TrpS and OASS, enzymes more closely related to CBS, are independent of pH from 6.9 to 10.4 and 5.5−10.85, respectively (*24*, *41*). The data of Figure 4 show that the spectrum of the internal aldimine of ytCBS is also independent of pH from 7.24 to 11. The decrease in absorbance at 412 nm and increase at 390 nm at pH > 11 are likely due to dissociation of PLP from ytCBS (Figure 4B, ref *30*). This occurs concomitantly with the 277−289 nm shift (Figure 4A), which is indicative of tyrosine ionization (*28*, *29*). Therefore, it is likely that a conformational change of the protein is required to allow access of base to the aldimine.

The pH optimum of the ytCBS k_{catR}/K_m^{L-Cth} versus pH profile for the hydrolysis of L-Cth to L-Ser and L-Hcys is

Scheme 3. Kinetically Indistinguishable Pathways Leading to the First Irreversible Step (Release of L-Hcys) in the Reaction of L-Cth with ytCBS



8.3 (Figure 3). Values of 8.5 for hCBS (*26*) and yCBS (*11*) and the range of 8.3−8.6 for rat CBS (*20*) have been reported for the specific activity at a single substrate concentration for the forward reaction. The k_{catR} versus pH profile of ytCBS (Figure 3) exhibits only an acidic limb with a pK_a of 7.27 ± 0.06. The k_{catR}/K_m^{L-Cth} versus pH profile of ytCBS is bell-shaped (Figure 3) with pK_a values of 8.05 ± 0.09 (pK_{a1}, acidic limb) and 8.63 ± 0.10 (pK_{a2}, basic limb). The pH independence of the internal aldimine of ytCBS indicates that this group cannot be assigned to one of those that titrate in the pH-activity profiles. The acidic limb (pK_{a1}) is thus assigned to an enzyme group other than the imino moiety of the internal aldimine. It is possible that the pK_a of this group is lowered from 8.05 (k_{catR}/K_m^{L-Cth} vs pH) to 7.27 (k_{catR} vs pH) in the ytCBS−L-Cth complex.

The basic limb (pK_{a2}) of the k_{catR}/K_m^{L-Cth} versus pH profile is assigned to the pK_a of the α-amino group of L-Cth (pK_a = 8.54 ± 0.01), indicating that the substrate formally must bind in the ammonium state. The pH versus k_{catR}/K_m^{L-Cth} profile presents an enzyme functionality with pK_a = 8.05; that of L-Cth is 8.54 ± 0.01; thus, the association reaction can be written in either of the two kinetically indistinguishable forms shown in Scheme 3. The limiting values of k_1 (1.5×10^4 M^{-1} s^{-1}) and k_1' (5.8×10^4 M^{-1} s^{-1}) were calculated from eqs 12 and 13, respectively.

$$\frac{k_{cat}}{K_m^{L-CTH}} = k_1 \frac{[H^+]}{[H^+] + K_a^{L-CTH}} \frac{K_a^E}{[H^+] + K_a^E} \quad (12)$$

$$\frac{k_{cat}}{K_m^{L-CTH}} = k_1' \frac{K_a^{L-CTH}}{[H^+] + K_a^{L-CTH}} \frac{[H^+]}{[H^+] + K_a^E} \quad (13)$$

The value of neither k_1 nor k_1' exceeds the diffusion controlled limit; therefore, the prototropy of the associating forms cannot be assigned.

Equilibrium Constant. The value of 0.61 μM calculated for K_{eq} by direct measurement of the L-Hcys concentration at equilibrium is about half of that determined from the kinetic parameters (K_{eq} = 1.4 ± 0.3 μM). The physiological [L-Ser] in yeast ranges between 1 and 20 mM depending on the growth phase of the cells (*45*); thus, [L-Hcys]/[L-Cth] ≤ 1×10^{-3}, indicating that the CBS-catalyzed reaction strongly favors L-Cth in vivo.

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