The Reaction of Yeast Cystathionine β-Synthase Is Rate-Limited by the Conversion of Aminoacrylate to Cystathionine†

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ABSTRACT: Our studies of the reaction mechanism of cystathionine β-synthase from *Saccharomyces cerevisiae* (yeast) are facilitated by the spectroscopic properties of the pyridoxal phosphate coenzyme that forms a series of intermediates in the reaction of L-serine and L-homocysteine to form L-cystathionine. To characterize these reaction intermediates, we have carried out rapid-scanning stopped-flow and single-wavelength stopped-flow kinetic measurements under pre-steady-state conditions, as well as circular dichroism and fluorescence spectroscopy under steady-state conditions. We find that the gem-diamine and external aldimine of aminoacrylate are the primary intermediates in the forward half-reaction with L-serine and that the external aldimine of aminoacrylate or its complex with L-homocysteine is the primary intermediate in the reverse half-reaction with L-cystathionine. The second forward half-reaction of aminoacrylate with L-homocysteine is rapid. No primary kinetic isotope effect was obtained in the forward half-reaction with L-serine. The results provide evidence (1) that the formation of the external aldimine of L-serine is faster than the formation of the aminoacrylate intermediate, (2) that aminoacrylate is formed by the concerted removal of the α-proton and the hydroxyl group of L-serine, and (3) that the rate of the overall reaction is rate-limited by the conversion of aminoacrylate to L-cystathionine. We compare our results with cystathionine β-synthase with those of related investigations of tryptophan synthase and *O*-acetylserine sulfhydrylase.

Our studies of cystathionine β-synthase (CBS)[1] from *Saccharomyces cerevisiae* (yeast) are aimed at elucidating the mechanism and kinetic parameters of the enzyme reaction. This work has potential fundamental importance and medical relevance. Cystathionine β-synthase catalyzes the pyridoxal phosphate (PLP)-dependent conversion of L-serine and L-homocysteine to L-cystathionine in yeast and humans (Scheme 1). Mutations in the human enzyme or vitamin B_6 deficiency can lead to the accumulation of L-homocysteine (*1*, *2*). Elevated total plasma homocysteine is an important risk factor in coronary heart disease and other vascular diseases (*3*).

Cystathionine β-synthase from humans, yeast, and *Trypanosoma cruzi* have closely related sequences (*4*, *5*). Whereas the enzymes from yeast (*6*) and humans (*7*, *8*) are both composed of two domains, an N-terminal catalytic domain and a C-terminal regulatory domain, the enzyme from *T. cruzi* lacks the C-terminal regulatory domain. Human CBS is unique in its dependence on both PLP and heme (*8*, *9*). In contrast, CBS from yeast (*10*) or *T. cruzi* (*5*) does not contain heme. The discovery that deletion of the C-terminal domain of human CBS eliminates *S*-adenosyl-L-methionine activation (*11*) or binding (*8*) suggests that *S*-adenosyl-L-methionine is an allosteric activator that binds to the C-terminal regulatory domain. In contrast, CBS from yeast (*10*) and CBS from *T. cruzi* (*5*) are not regulated by *S*-adenosyl-L-methionine (*5*).

Crystals of the N-terminal catalytic domain of the human enzyme have recently been used for preliminary X-ray diffraction analysis (*12*) and for single-crystal microspectrophotometry (*13*). Exposure of the reduced form of enzyme crystals to carbon monoxide led to the complete release of the heme moiety (*13*). Addition of L-serine to the heme free crystals led to the formation of the key aminoacrylate intermediate which reacted with L-homocysteine to form L-cystathionine. These findings indicate that the heme moiety does not serve a direct catalytic role in the human enzyme (*13*). Recent studies of the PLP binding sites of the human and yeast enzymes using ^{31}P NMR and pulsed EPR spectroscopy provide evidence that the heme and PLP cofactors are not proximal in the human enzyme (*14*). These results are consistent with a regulatory role for heme in the human enzyme.

We have reported methods that yield high levels of homogeneous full-length yeast CBS (F-CBS) and of the truncated catalytic domain (T-CBS) (residues 1−353) (*6*, *10*). Our initial studies using absorbance and circular dichroism (CD) spectroscopy (*10*) demonstrated the formation and accumulation of an aminoacrylate intermediate (E-AA) from L-serine in the first half-reaction (stage I) shown in Scheme

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[1] Abbreviations: CBS, cystathionine β-synthase; F-CBS, full-length CBS; T-CBS, truncated CBS; PLP, pyridoxal phosphate; RSSF, rapid-scanning stopped-flow; OASS, *O*-acetylserine sulfhydrylase; CD, circular dichroism.

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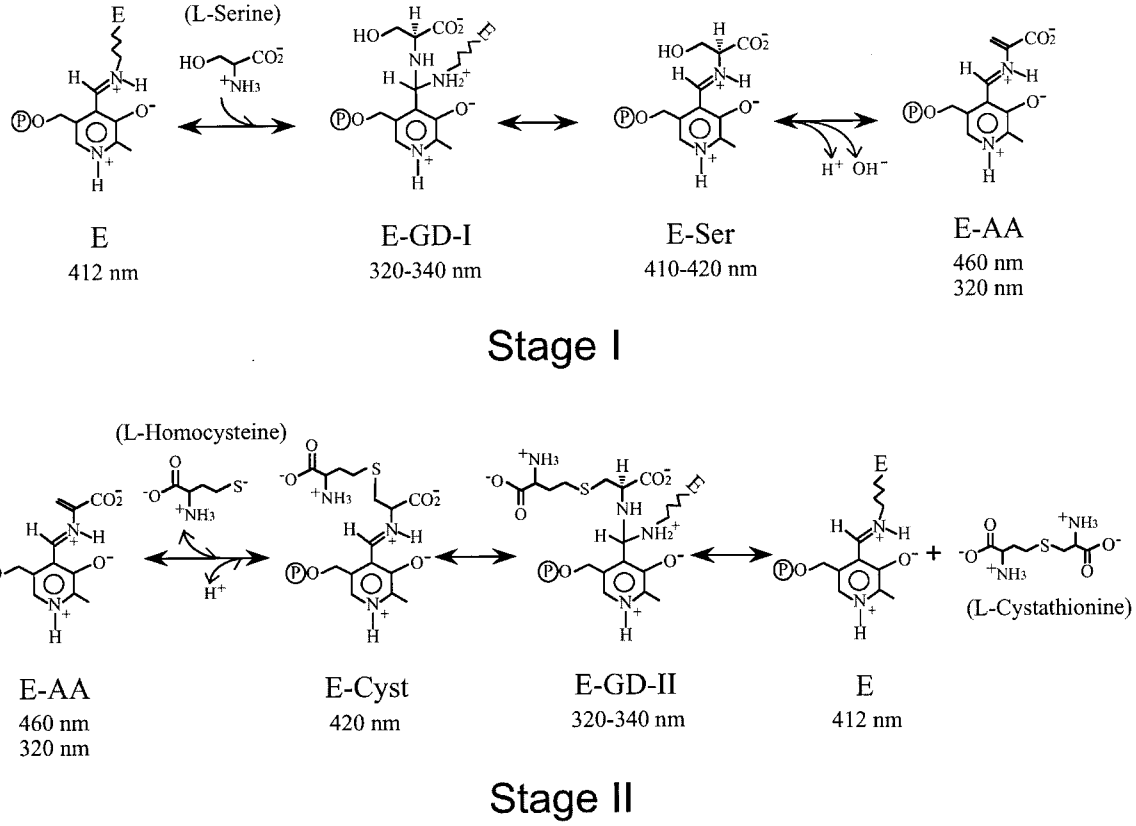
Scheme 1: Intermediates in the CBS Reaction, Where E Is the Internal Aldimine, E-Ser, E-AA, and E-Cyst Are the External Aldimines of PLP with L-Serine, Aminoacrylate, and L-Cystathionine, Respectively, and E-GD-I and E-GD-II Are the Gem-Diamines of L-Serine and L-Cystathionine, Respectively[a]



[a] Stage I is the first half-reaction with L-serine in the absence of a cosubstrate. Stage II is the second half-reaction obtained upon addition of L-homocysteine to E-AA. Quinonoid intermediates between E-Ser and E-AA or between E-Cyst and E-AA are possible intermediates that are not shown.

1. Addition of L-homocysteine to the preformed E-AA intermediate leads to a decrease in absorbance at 460 nm, providing evidence for the occurrence of the second half-reaction (stage II) shown in Scheme 1. The reaction of CBS with the product, L-cystathionine, also leads to formation of the E-AA intermediate. This result shows that the reaction is partially reversible. Steady-state kinetic studies of the CBS reaction provide additional evidence that the reaction of L-serine and L-homocysteine proceeds by the double displacement or ping-pong mechanism shown in Scheme 1 (*6*). Several other PLP enzymes, including tryptophan synthase (*15*) and *O*-acetylserine sulfhydrylase (OASS) (*16*, *17*), catalyze β-replacement reactions via mechanisms analogous to that shown in Scheme 1. For a review of PLP-dependent β-replacement and β-elimination reactions, see ref *18*.

In the work presented here, we have carried out RSSF and single-wavelength kinetic studies with the goals of detecting intermediates in the CBS reaction (Scheme 1) and of determining the rates of appearance and decomposition of these intermediates. We have used yeast T-CBS for this work because it is more stable and more soluble than F-CBS (*6*). Our results show that binding of L-serine as the external aldimine (E-Ser) is faster than formation of the aminoacrylate (E-AA) and that the rate-limiting step is the conversion of E-AA or an E-AA complex with L-homocysteine to L-cystathionine. We compare our results with CBS with those of related investigations of tryptophan synthase and OASS.

EXPERIMENTAL PROCEDURES

Materials. L-Cystathionine and L-serine were obtained from Fluka. L-Homocysteine was prepared from L-homocysteine thiolactone (Sigma) as described previously (*19*), diluted with 50 mM *N*,*N*-bis(2-hydroxyethyl)glycine buffer adjusted to pH 7.8 with NaOH. [α-^{2}H]-L-Serine was prepared as described previously (*20*).

Enzymes and Buffers. Truncated CBS (T-CBS) from *S. cerevisiae* was purified as described using the plasmid pT-SEC (*6*). Protein concentrations were determined as described previously (*6*). All experiments were carried out at 25 °C in 50 mM *N*,*N*-bis(2-hydroxyethyl)glycine buffer adjusted to pH 7.8 with NaOH.

Steady-State Spectroscopic Methods. Absorption spectra of T-CBS were recorded using a Hewlett-Packard 8452 diode array spectrophotometer thermostated at 25 °C by a Peltier junction temperature-controlled cuvette holder. CD measurements (mean residue ellipticity in degrees per square centimeter per decimole) were taken at 25 °C in a Jasco J-715 spectropolarimeter interfaced with a personal computer (Japan Spectroscopic Co., Easton, MD). A baseline was measured for each sample using buffer. The data for both sample and buffer were converted to absorbance, using functions built into the Jasco software, saved as text files, and imported into a spreadsheet program. The CD and absorbance spectra of the enzyme were calculated as the difference between the two files. Mean residue ellipticities were

converted to molar ellipticities (θ) by multiplication by the number of amino acids, 353. Fluorescence measurements were taken using a Photon Technology International (PTI) dual-excitation spectrofluorimeter thermostated at 25 °C.

Rapid-Scanning and Single-Wavelength Stopped-Flow Measurements. Enzyme solutions were 1 mg/mL (25.8 μM) in 50 mM *N*,*N*-bis(2-hydroxyethyl)glycine buffer adjusted to pH 7.8 with NaOH. RSSF measurements were taken with new instrumentation, which will be described in more detail elsewhere. The system consists of an Applied PhotoPhysics SF.17MV microvolume mixing module with custom-built optical and data acquisition systems comprised of a 75 W xenon lamp and two quartz first-surface mirrors, one flat and one parabolic, that focus and collimate the light from the lamp housing as a fairly tight beam with a diameter of ~2−3 mm onto the end of a quartz fiber optic cable for presentation of white light through the stopped-flow observation cuvette. The transmitted light is then refocused onto the end of a second quartz fiber optic bundle and presented to a grating spectrograph (Oriel model 77400, Multispec MS-125 spectrograph, 0.125 m) consisting of a Oriel model 77413 grating (600 l/mm, blazed at 200 nm to give high light throughput in the wavelength range from 250 to ~500 nm). The rainbow of light from the spectrograph is then imaged onto a 512-element diode array (Oriel model 78201-1236 Instaspec II PDA, with UV sensitive diodes, a 1 MHz PC-based controller card, and a 16 bit A/D converter). Data from the A/D converter are stored in the memory of a 333 MHz Pentium II Celeron personal computer as files in the form of time-resolved absorbance versus wavelength. Data were collected with a repetitive scan rate of 1.2 ms/scan. In each RSSF experiment, a set of 1000 spectra was collected at 25 °C with total scan acquisition times of either 1.2 or 3.6 s. The raw data were analyzed with the Instaspec software, and 25 representative spectra were selected from the raw data set for presentation in each RSSF figure.

Single-wavelength stopped-flow absorbance and fluorescence studies were carried out using an Applied PhotoPhysics mixing unit (mixing dead time = 1 ms) combined with custom-built optical and data acquisition systems involving quartz fiber optics, phototube detection, and data acquisition and data storage via a high-speed A/D converter and PC computer system operated under the control of software written by S. C. Koerber. Single-wavelength time courses were fitted using the Marquardt−Levenberg algorithm to an equation of the following general form (eq 1):

$$A_t = A_\infty \pm \sum A_i \exp(-t/\tau_i) \qquad (1)$$

where A_t and A_∞ are the absorbance values at time t and infinite time, respectively, A_i and τ_i represent the ith amplitude and relaxation time, respectively. All time courses were collected under pseudo-first-order conditions.

Peak-Fitting Analysis of RSSF Spectra. Single spectra for specific time points of the RSSF spectral set were extracted into separate files and analyzed with the help of commercial peak-fitting software (Peakfit, version 3.1, Jandel Scientific). The software applies the Marquardt−Levenberg algorithm in fitting a spectrum to a sum of peaks. The shape of each of the individual peak areas was described using a log-normal equation with four independent parameters for each peak as described previously (*21*, *22*). To correct for light scattering and baseline shifts, a curve of the general form $y = ax^{-4} + c$ was used. The parameters for the latter curve were estimated from absorbance measurements in the 600−800 nm region, where PLP-bound intermediates do not contribute to the absorbance.

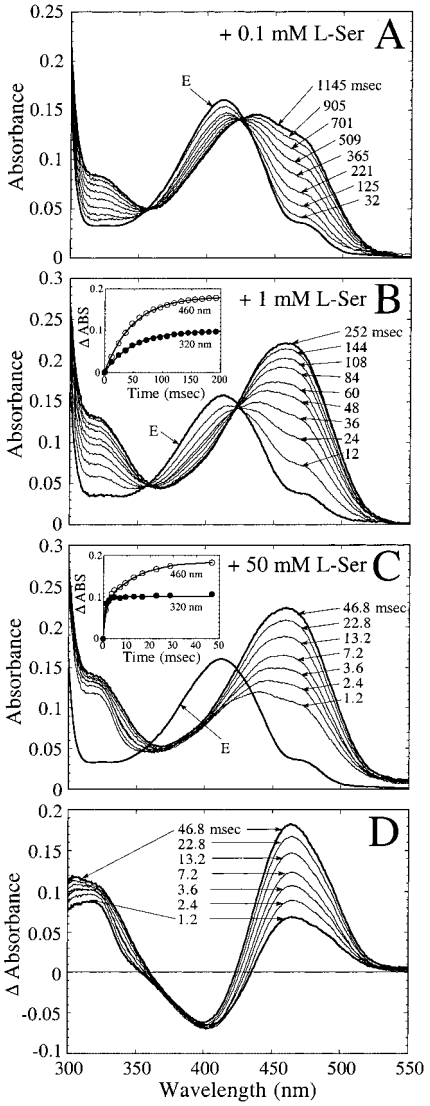


FIGURE 1: RSSF spectra obtained upon reaction of T-CBS with L-serine. Spectra were recorded at the indicated times after mixing with 0.1 (A), 1 (B), and 50 mM L-serine (C). Panel D shows the spectra from panel C after subtraction of the spectrum of the enzyme alone. The insets in panels B and C show the rates of change of absorbance at 320 and 460 nm.

RESULTS

To obtain information about the identity of intermediates in the reactions of yeast CBS (Scheme 1) and the rates of appearance and decomposition of these intermediates, we have carried out rapid-kinetic and steady-state spectroscopic studies.

Rapid-Scanning Stopped-Flow Experiments. The reaction of T-CBS with three different concentrations of L-serine (0.1, 1, and 50 mM) gives the RSSF spectra shown in Figure 1A−C. The spectra in the presence of a low concentration of L-serine (0.1 or 1 mM) exhibit two clear apparent isosbestic points (Figure 1A,B). The results suggest that the internal aldimine (E) is converted to a mixture of the two tautomeric

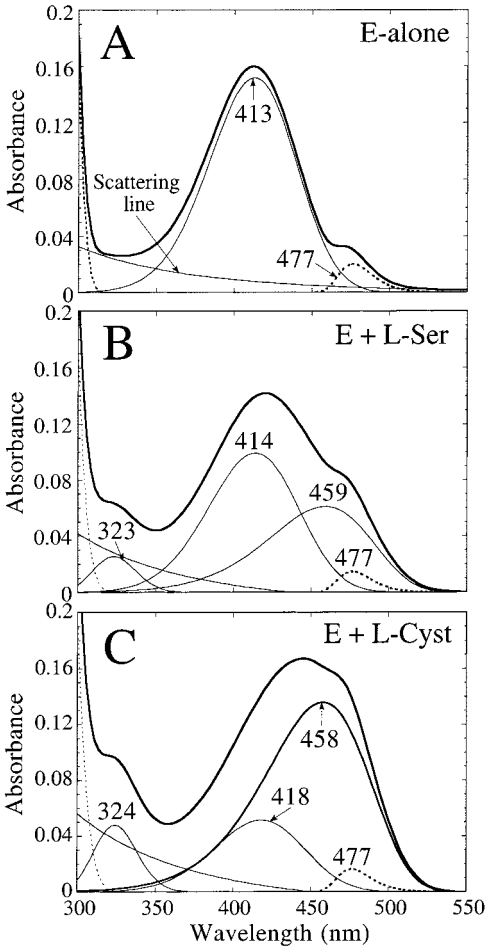


FIGURE 2: Theoretical log-normal fits for three spectra from Figures 1 and 4. Panel A is for the enzyme alone (T-CBS), panel B for the enzyme with 1 mM L-serine at 24 ms, and panel C for the enzyme with 2.5 mM L-cystathionine at 288 ms.

forms of the aminoacrylate intermediate (E-AA) with λ_{max} values of ~460 and ~320 nm, with no detectable accumulation of the gem-diamine (E-GD-I) and the external aldimine with L-serine (E-Ser) (Scheme 1). The rates of the increase in absorbance at ~460 and ~320 nm are identical (inset of Figure 1B), and the ratio of absorbancies at 460 and 320 nm is constant during the reaction with 1 mM L-serine. In contrast, the spectra in the presence of a high concentration of L-serine (50 mM) lack clear isosbestic points (Figure 1C), suggesting that an additional intermediate accumulates to detectable levels under these conditions. The initial rate of the increase at 320 nm is much faster than that at 460 nm (inset of Figure 1C). Further analysis of the data in Figure 1C by subtracting the spectrum of the enzyme alone from each of the spectra obtained after addition of 50 mM L-serine (Figure 1D) shows that a new species absorbing at ~320 nm is formed essentially completely within the dead time of the experiment (1.2 ms). This species is likely a gem-diamine (E-GD-I in Scheme 1) that builds up to detectable levels with 50 mM L-serine.

Figure 2 shows the results of peak-fitting analyses of selected spectra from Figure 1 and from the reaction of T-CBS with L-cystathionine (see below and Figure 4). Analysis of the spectrum of T-CBS alone (Figure 1A) identifies two peaks with λ_{max} values of 413 nm (internal aldimine, E) and 477 nm. The peak at 477 nm, which is not

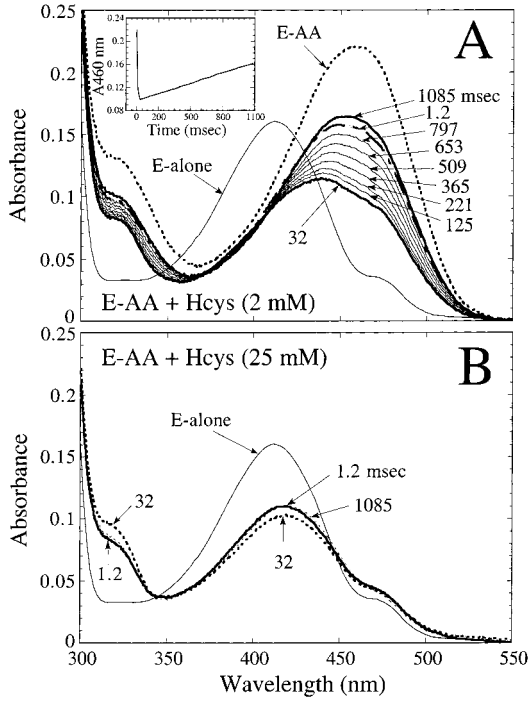


FIGURE 3: RSSF spectra obtained upon reaction of E-AA (premixed with 25 mM L-serine) with 2 (A) and 25 mM L-homocysteine (B) at the indicated times after mixing T-CBS with L-homocysteine. The time course of absorbance at 460 nm is shown in the inset of panel A.

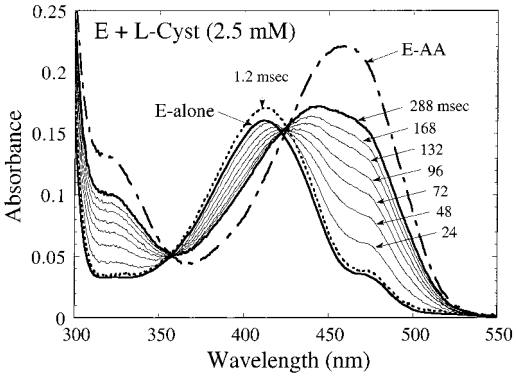


FIGURE 4: RSSF spectra obtained upon reaction of T-CBS with 2.5 mM L-cystathionine at the indicated times after mixing. The E-AA represents the aminoacrylate intermediate absorbance with L-serine. The curve labeled E-AA was obtained by the reaction of T-CBS with 50 mM L-serine after 46.8 ms (see Figure 1C).

observed in F-CBS (*10*), is a tightly bound chromophore, likely a PLP derivative. Attempts to remove this material by treatment with guanidine hydrochloride or potassium thiocyanate have been unsuccessful. The presence and invariance of this peak in all of the spectra of T-CBS collected in the presence of different substrates indicate that the unknown chromophore is catalytically inert. Analysis of the RSSF spectrum after reaction of T-CBS with 1 mM L-serine at 24 ms (Figure 2B) identifies four peaks with λ_{max} values of 414 nm (E), 459 and 323 nm (E-AA), and 477 nm (unknown). The fitting parameters for panels A−C of Figure 2 are shown in Table 1.

When the aminoacrylate intermediate (E-AA) is preformed by mixing T-CBS with L-serine and then reacted with 2 or 25 mM L-homocysteine, the spectra shown in Figure 3 are obtained. As shown in Figure 3A and in the inset, the

Table 1: Fitting Parameters for Spectra in Figure 2

panel	λ_{max}	amplitude	skewness	bandwidth	statistics
A	280.4	0.81	0.78	31.3	$r^2 = 0.999$
	413.0	0.17	0.91	65.9	$x^2 = 0.00662$
	476.7	0.02	1.36	25.9	
B	281.3	0.74	0.94	26.8	$r^2 = 0.998$
	322.9	0.03	1.07	32.3	$x^2 = 0.00873$
	414.0	0.10	0.91	68.0	
	459.0	0.61	0.77	79.2	
	476.7	0.01	1.36	25.9	
C	281.2	0.76	1.00	26.7	$r^2 = 0.998$
	324.1	0.05	1.07	32.3	$x^2 = 0.0113$
	418.3	0.05	0.91	68.0	
	457.9	0.14	0.77	79.2	
	476.7	0.02	1.36	25.9	

addition of a low concentration of L-homocysteine (2 mM) to E-AA results in a rapid decrease in absorbance at 460 nm followed by an increase as L-homocysteine is exhausted and the enzyme reacts with excess L-serine to form E-AA. Addition of a high concentration of L-homocysteine (25 mM) to E-AA (Figure 3B) results in the rapid formation of two bands (λ_{max} = 420 and 320 nm). The 420 nm band is likely due to the internal aldimine (E). The 320 nm band may be the gem-diamine of the L-cystathionine product (E-GD-II in Scheme 1).

Addition of L-cystathionine to F-CBS results in the accumulation of an aminoacrylate intermediate (E-AA) under steady-state conditions (*10*). As shown in Figure 4, addition of L-cystathionine (2.5 mM) to T-CBS yields RSSF spectra that exhibit two almost perfect apparent isosbestic points and are very similar to those observed with 1 mM L-serine in Figure 1B. Peak-fitting analysis of the RSSF spectrum of Figure 4 at 288 ms identifies four peaks with λ_{max} values of 418 nm (E), 458 and 324 nm (E-AA), and 477 nm (unknown) (Figure 2C). The rates of formation of the two bands at 460 and 320 nm are identical, and the ratio of their absorbancies at 460 and 320 nm is constant during the reaction (analysis of data from Figure 4). Thus, there is no evidence of a transitory gem-diamine intermediate in the reverse half-reaction (stage II). Addition of 5 mM L-homocysteine to the enzyme after reaction for 10 min with 2.5 mM L-cystathionine, as described for Figure 4, results in a rapid decrease in the absorbance at 460 nm, consistent with the reaction of L-homocysteine with E-AA to form L-cystathionine (data not shown).

Single-Wavelength Stopped-Flow Experiments. To better assess the formation and decay of individual intermediates with higher precision, we carried out single-wavelength stopped-flow experiments (Figure 5). The appearance of the aminoacrylate intermediate, formed upon mixing T-CBS with L-serine, was monitored at 460 nm as a function of the concentration of L-serine. Time courses were analyzed with curve-fitting software, and the relaxation rate parameters were determined. All curves were best fitted using a single exponential. A plot of the dependence of the relaxation rate ($1/\tau$) on the concentration of L-serine is shown in Figure 5A. Single-wavelength stopped-flow absorbance measurements at 460 nm were also used to determine the relaxation rates in the presence of 1 mM [α-^{2}H]-L-serine ($1/\tau = 30.8 \pm 1.5$ s^{-1}) or [α-^{1}H]-L-serine ($1/\tau = 31.9 \pm 1.5$ s^{-1}). The results show that there is no primary kinetic isotope effect in stage I. Single-wavelength stopped-flow kinetic studies show that

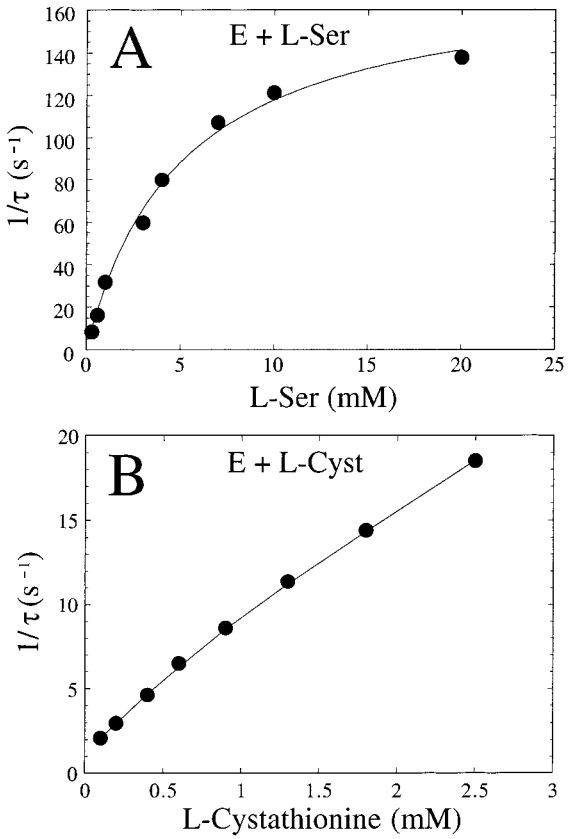


FIGURE 5: Dependence of the relaxation rates for the formation of the band at 460 nm on the concentration of L-serine (A) and L-cystathionine (B). L-Serine concentrations were 0.3, 0.6, 1.0, 3.0, 4.0, 7, 10, and 20 mM, and L-cystathionine concentrations were 0.1, 0.2, 0.4, 0.6, 0.9, 1.3, 1.8, and 2.5 mM. Data were obtained from single-wavelength stopped-flow absorbance time courses for the appearance of the aminoacrylate intermediate monitoring the absorbance at 460 nm. The data were fitted using the Marquardt−Levenberg algorithm (see the text) and were plotted with the average values of five time scans.

the reaction of L-cystathionine with T-CBS consists of two kinetic phases at low L-cystathionine concentrations. The amplitude of the faster phase (of increasing absorbance) corresponds to the formation of E-AA, and this phase dominates the time course. The slower phase (of decreasing absorbance and small amplitude) appears to correspond to the formation of E and E-Ser from E-AA as the steady state is achieved. The rate of the slow phase ($\sim$0.3 s^{-1}) shows little or no L-cystathionine concentration dependence. Figure 5B presents the dependence of the faster relaxation rate ($1/\tau$) on the concentration of L-cystathionine for the formation of E-AA (see Figure 4). Because of the low solubility of L-cystathionine, saturation of the rate is not reached over the range of L-cystathionine concentrations that were used. Reaction of the preformed aminoacrylate intermediate (E-AA) with the second substrate, L-homocysteine, was monitored at 460 nm in a single-wavelength stopped-flow experiment. The decay of the 460 nm band is too rapid to be measured even at low concentrations of L-homocysteine, and the reaction is complete within the mixing dead time (1 ms).

Analysis of the CBS Reaction Kinetics. The CBS reaction proceeds through a series of intermediates shown in Scheme 1. Because the gem-diamine intermediates (E-GD-I and E-GD-II) are usually not detected, this complex reaction can

be interpreted in terms of two simpler reactions involving external aldimine intermediates (E-Ser and E-Cyst) and E-AA. The reaction of the enzyme (E) with L-serine to form E-AA (stage I in Scheme 1 in the forward direction) can be simplified to eq 2:

$$E + Ser \underset{k_2}{\overset{k_1}{\leftrightarrow}} E\text{-}Ser \underset{k_4}{\overset{k_3}{\leftrightarrow}} E\text{-}AA + H_2O \quad (2)$$

The reaction of E with L-cystathionine to form E-AA (stage II in Scheme 1 in the reverse direction) can be simplified to eq 3:

$$E + Cyst \underset{k_7}{\overset{k_8}{\leftrightarrow}} E\text{-}Cyst \underset{k_5}{\overset{k_6}{\leftrightarrow}} E\text{-}AA + Hcys \quad (3)$$

Following the nomenclature of Czerlinski (*23*), these reactions are both five-component systems. Because the concentration of water is high (55 M) and constant, eq 2 can be simplified to a four-component system (eq 4):

$$E + Ser \underset{k_2}{\overset{k_1}{\leftrightarrow}} E\text{-}Ser \underset{k_4^*}{\overset{k_3}{\leftrightarrow}} E\text{-}AA \quad (4)$$

where $k_4^* = k_4[H_2O]$. The rate of formation of E-AA from L-serine shows a hyperbolic dependence on L-serine concentration (Figure 5A). This dependence indicates that the slow step in the reaction is the conversion of E-Ser to E-AA. The rate becomes independent of L-serine concentration when the rapid first step is saturated. When the low enzyme (E) concentration is neglected, the relaxation ($1/\tau$) is given by eq 5:

$$\frac{1}{\tau} = \frac{k_3[Ser]}{K_{21} + [Ser]} + k_4^* \quad (5)$$

where $K_{21} = k_2/k_1$ (*23*). Fitting the data of Figure 5A to eq 5 gave a k_3 of 177 ± 8 s^{-1} and a K_{21} of 4.6 ± 0.8 mM. The intercept of the graph was too small to yield an accurate value for k_4^*. Using fluorescence titration and neglecting the presence of E-Ser, we previously determined the apparent dissociation constant,[2] K_d', for formation of E-AA to be 14 μM (*6*). Because E-Ser is present in very small amounts, we can approximate that $K_d = (K_{21}k_4^*)/k_3$ and calculate that $k_4^* = (177 \times 14 \times 10^{-6})/(4.6 \times 10^{-3})$ (=0.54 s^{-1}), a value consistent with Figure 5A, and with the rate of the slow phase detected in the reverse reaction (0.3 s^{-1}).

Equation 3 cannot be simplified in a similar way because the concentration of L-homocysteine is low and changing. The time course of such a five-component system will show two concentration-dependent rate constants. Extrapolation of the rate data for the five-component system shown by eq 3 to zero L-cystathionine concentration where L-homocysteine is absent should give a value of zero, whereas extrapolation of the rate data for a four-component system will give a positive intercept (*23*). Figure 5B shows that the extrapolation of the relaxation rate for this reaction gives a positive intercept. This result suggests that L-homocysteine forms a stable complex with E-AA (E-AA-Hcys) to give a four-component system as shown by eq 6:

$$E + Cyst \underset{k_7}{\overset{k_8}{\leftrightarrow}} E\text{-}Cyst \underset{k_5}{\overset{k_6}{\leftrightarrow}} E\text{-}AA\text{-}Hcys \rightarrow \text{products (?)} \quad (6)$$

Previous steady-state kinetic studies provided evidence that L-homocysteine forms dead-end complexes with CBS by binding to the site from which it can carry out nucleophilic attack on E-AA (*6*). The system represented by eq 6 will show two relaxation rates given by eqs 7 and 8:

$$\frac{1}{\tau_1} = k_8[Cyst] + k_7 \quad (7)$$

$$\frac{1}{\tau_2} = \frac{k_6[Cyst]}{K_{78} + [Cyst]} + k_5 \quad (8)$$

where $K_{78} = k_7/k_8$. The linear dependence of the measured relaxation rates on L-cystathionine concentration might be taken to indicate that the slow step in this reaction is the second-order formation of E-Cyst. However, RSSF spectra recorded after addition of L-cystathionine to CBS (Figure 4) show a small increase in absorbance centered at 420 nm within the mixing time of the instrument (1.2 ms) followed by a slow increase in absorbance at 460 nm (E-AA or E-AA-Hcys). These results indicate that the external aldimine of L-cystathionine (E-Cyst) is formed very rapidly, but to an unknown extent, and is then slowly converted to E-AA or E-AA-Hcys. The linear concentration dependence of the relaxation rate in Figure 5B likely results from the use of low concentrations of L-cystathionine ([Cyst] $< K_{78}$) because of the low solubility, masking the expected hyperbolic behavior. Fitting the available values to eq 8 gives a k_5 of 1.2 ± 0.1 s^{-1}, a k_6 of 68.7 ± 5.6 s^{-1}, and a K_{78} of 7.45 ± 0.8 mM.

Figure 3 shows that addition of L-homocysteine to preformed E-AA produces very rapid changes in absorbance, indicating rapid binding. However, it should not be construed that this reaction is simply the reversal of eq 3 because earlier steady-state studies of the mechanism of L-cystathionine synthesis demonstrated severe substrate inhibition of the forward reaction by L-homocysteine (*6*). Analysis of the system showed that the major component present under steady-state conditions is a dead-end complex between E-Ser and L-homocysteine (E-Ser-Hcys) (*6*). Thus, the spectra in Figure 3 may represent a complex mixture of reaction intermediates and dead-end complexes.

Steady-State Spectroscopic Studies. Addition of 1 mM L-serine to T-CBS results in formation of the aminoacrylate intermediate (E-AA), which has a negative CD band centered at 460 nm (Figure 6A) and an absorption maximum at 460 nm (Figure 6B) (*10*). The aminoacrylate intermediate also exhibits a fluorescence emission maximum centered at 540 nm ($\lambda_{ex} = 460$ nm) (*6*). The CD and absorption spectra of T-CBS in the presence of 1 mM L-serine and 5 mM L-homocysteine are essentially identical to the spectra of T-CBS alone. In contrast, the CD spectrum for the external aldimine formed with L-alanine exhibits a negative band at 420 nm (*10*).

DISCUSSION

Our results provide new insights into the CBS reaction mechanism. Previous studies demonstrated that the kinetic mechanism of yeast CBS is ping-pong or double displace-

[2] K_d' is an apparent dissociation constant because it was measured by fluorescence and not by direct binding.

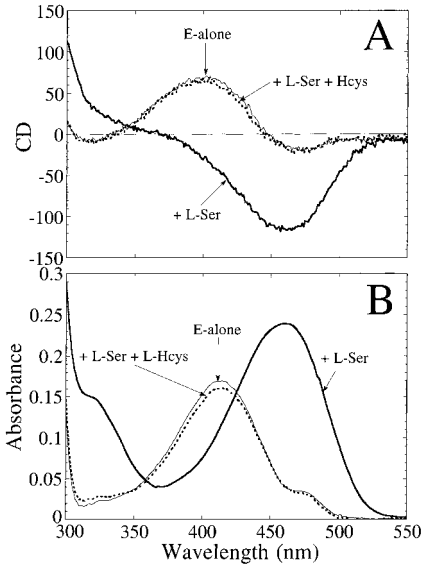


FIGURE 6: CD (A) and absorbance (B) spectra of T-CBS alone, T-CBS in the presence of 1 mM L-serine, and T-CBS in the presence of 1 and 5 mM L-homocysteine (~15 min after mixing). The absorbance and CD spectra were both calculated from the data obtained from the Jasco J-715 spectropolarimeter as described in Experimental Procedures.

ment as shown in Scheme 1 (*6*). Stage I results in the removal of the α-proton of L-serine and the β-elimination of the hydroxyl group of L-serine with formation of the external aldimine of aminoacrylate (E-AA). Stage II involves the reaction of E-AA with L-homocysteine to form L-cystathionine. Similar reaction mechanisms have been established for other PLP-dependent enzymes, including *O*-acetylserine sulfhydrylase (*16*, *17*) and tryptophan synthase (*15*). This discussion focuses on similarities and differences between the kinetic mechanisms of CBS and those of OASS and tryptophan synthase. The three enzymes belong to the same family of evolutionarily related PLP enzymes, termed the β-family (*24*) or fold type II (*25*). The sequence of CBS is more closely related to that of OASS than to that of any other enzyme in the β-family (*24*). All three enzymes catalyze β-replacement reactions with retention of configuration (reviewed in ref *17*).

As shown in Figure 1, the gem-diamine (E-GD-I) and the aldimine of aminoacrylate (E-AA) are the only clear intermediates detected by RSSF in stage I (Figure 1). In contrast, RSSF studies of the reactions of OASS (*21*) and tryptophan synthase (*26*, *27*) provide clear evidence that formation of the aldimine of aminoacrylate (E-AA) is preceded by the appearance and decay of the external aldimine of the substrate. The absence of a primary kinetic isotope effect on E-AA formation argues that removal of the α-proton of L-serine is not rate-limiting. Nevertheless, no quinonoid intermediate between E-Ser and E-AA is detected. Thus, either removal of the α-proton and β-elimination of the hydroxyl group of L-serine are concerted reactions or the quinonoid does not accumulate to detectable levels. Consequently, there is either a slow conformational change or a slow formation of the external aldimine that is followed by rapid removal of the α-proton and elimination of OH^-. In contrast, tryptophan synthase (*26*, *28*−*31*) and OASS (*20*, *21*) both exhibit primary kinetic isotope effects.

Figure 3 shows the results of addition of L-homocysteine to the preformed E-AA (stage II). When the concentration of L-homocysteine is lower than that of L-serine (Figure 3A), the E-AA intermediate reappears after L-homocysteine has been exhausted. When equal concentrations of L-serine and L-homocysteine are used (Figure 3B), E-AA disappears within the dead time of the RSSF instrument (1.2 ms). The spectra at 1.2 and 1085 ms exhibit a shoulder with a λ_{max} of 320 nm and a peak with a λ_{max} of 420 nm. The 320 nm species is likely the gem-diamine of the product, L-cystathionine (E-GD-II in Scheme 1). A possible quinonoid intermediate between E-Cyst and E-AA is not detected. The 420 nm species may be assigned to the free enzyme, to the external aldimine of L-cystathionine, or to one or more of the dead-end complexes (e.g., E-Hcys or E-Ser-Hcys) identified in previous steady-state studies (*6*). In an attempt to distinguish between these possibilities, we recorded the CD and absorbance spectra of the enzyme in the presence of L-serine alone or in the presence of L-serine and excess L-homocysteine (Figure 6). Our findings that the CD and absorbance spectra of the enzyme in the presence of L-serine and excess L-homocysteine are essentially identical to those of the enzyme alone suggest that the spectrum at the end of the reaction is due to the enzyme alone after dissociation of the product, L-cystathionine. The CD spectrum of the external aldimine of L-cystathionine would likely exhibit a negative CD band centered at ~420−430 nm as observed for the CD band of the external aldimine of L-alanine (*10*).

Formation of a quinonoid intermediate requires N-1 of PLP to be protonated or to become protonated as the intermediate forms (*17*). The quinonoid intermediate has been observed for a number of PLP-dependent enzymes that catalyze β-elimination reactions (*17*). For example, quinonoid intermediates are detected in both stage I and stage II of the reactions of tryptophan synthase with L-serine and with the cosubstrates indole (*26*, *27*, *29*, *32*), β-mercaptoethanol (*32*, *33*), or other nucleophiles (*34*, *35*). In contrast, no quinonoid intermediate is detected in RSSF studies of the OASS reaction (*17*), or of the CBS reaction (this work). The crystal structure of OASS shows that Ser272 is adjacent to N-1 of PLP. Tai and Cook have reasoned that formation of a quinonoid intermediate is disfavored in reactions of OASS because the p*K*'s of N-1 of PLP and the adjacent Ser272 are unmatched, making Ser272 unlikely to donate a proton to N-1 (*17*). Sequence alignments show that yeast CBS has Ser289 at the position equivalent to Ser272 in OASS. In contrast, the residue in tryptophan synthase (Ser377), which is adjacent to N-1 of PLP, is not homologous to OASS Ser272 or to the equivalent Ser289 in yeast CBS. Thus, formation of the quinonoid intermediate may be favored by the structure of tryptophan synthase but not by the structures of CBS and OASS. Replacement of tryptophan synthase Ser377 with Asp or Glu leads to the marked accumulation of quinonoid intermediates. The engineered Asp or Glu residue likely promotes formation of quinonoid intermediates by stabilizing the protonated N-1 of PLP and reducing the pK_a of the internal aldimine nitrogen (*36*).

As observed previously for F-CBS in steady-state measurements of CD and absorbance spectra (*10*), an intermediate with maximum absorbance at 460 nm is the only species detected in RSSF experiments in the reverse reaction of T-CBS with the product L-cystathionine (Figure 4). Although

we previously attributed this species to the external aldimine of aminoacrylate (E-AA) (*6*), our new kinetic analysis suggests that an E-AA-Hcys intermediate is also formed in this reaction. The RSSF spectra in Figure 4 resemble closely the spectra obtained in the reaction with 1 mM L-serine (Figure 1B). The finding that the absorbance at 460 nm disappears upon addition of excess L-homocysteine supports the assignment of the 460 nm intermediate to E-AA.

An important finding in this work is that the rate constant for E-AA formation in stage I (176 s^{-1}) at 25 °C is ~20-fold greater than the value of *V*/*Et* (8 s^{-1}) at 25 °C. [The value of *V*/*Et* is estimated from the value at 37 °C (*6*) using the empirical rule of a 2-fold reduction in rate for a 10 °C reduction in temperature.] Thus, formation of E-AA is not the rate-limiting step in the overall reaction. The present experiments indicate, for the first time, the presence of an E-AA-Hcys complex in the reverse reaction with L-cystathionine (see eq 6). Although such complexes are not usually considered in the mechanisms of other PLP enzymes, the E-AA-Hcys complex is consistent with our evidence for similar complexes (E-Hcys and E-Ser-Hcys) with other intermediates (*6*). Our kinetic analysis indicates that the binding reaction of L-homocysteine with E-AA to form E-AA-Hcys is very fast and that the conversion of E-AA-Hcys to E-Cyst is slow. Our finding that a quinonoid intermediate is not observed in stage II suggests that the conversion of E-AA-Hcys to E-Cyst is the rate-limiting step in the overall reaction. This suggestion is supported by the reasonable agreement between the forward rate constant for this reaction (1.2 s^{-1}) and the value estimated above for k_{cat} (8 s^{-1}). In contrast, formation of E-AA is rate-limiting in the OASS reaction (*21*), whereas product release is thought to be the rate-limiting step with tryptophan synthase (*37*).

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