Stopped-flow Kinetic Analysis of the Reaction Catalyzed by the Full-length Yeast Cystathionine β-Synthase*

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Cystathionine β-synthase found in yeast catalyzes a pyridoxal phosphate-dependent condensation of homocysteine and serine to form cystathionine. Unlike the homologous mammalian enzymes, yeast cystathionine β-synthase lacks a second cofactor, heme, which facilitates detailed kinetic studies of the enzyme because the different pyridoxal phosphate-bound intermediates can be followed by their characteristic absorption spectra. We conducted a rapid reaction kinetic analysis of the full-length yeast enzyme in the forward and reverse directions. In the forward direction, we observed formation of the external aldime of serine (14 mM s⁻¹) and the aminoacrylate intermediate (15 s⁻¹). Homocysteine binds to the aminoacrylate with a bimolecular rate constant of 35 mM⁻¹ s⁻¹ and rapidly converts to cystathionine (180 s⁻¹), leading to the accumulation of a 420 nm absorbing species, which has been assigned as the external aldime of cystathionine. Release of cystathionine is slow (k = 2.3 s⁻¹), which is similar to kₐₚ (1.7 s⁻¹) at 15 °C, consistent with this being a rate-determining step. In the reverse direction, cystathionine binds to the enzyme with a bimolecular rate constant of 1.5 mM⁻¹ s⁻¹ and is rapidly converted to the aminoacrylate without accumulation of the external aldime. The kinetic behavior of the full-length enzyme shows notable differences from that reported for a truncated form of the enzyme lacking the C-terminal third of the protein (Jhee, K. H., Niks, D., McPhie, P., Dunn, M. F., and Miles, E. W. (2001) Biochemistry 40, 10873–10880).

In mammals, cystathionine β-synthase catalyzes the first step in the “reverse” trans-sulfuration pathway that converts the essential amino acid, methionine, to cysteine. In addition, it plays a key role in regulating the intracellular concentrations of homocysteine, a sulfur-containing amino acid that is correlated with a number of diseases at elevated levels including neural tube defects, cardiovascular diseases, and Alzheimer’s disease (1–3). Mutations in cystathionine β-synthase are the single most common cause of severe hyperhomocysteinemia, and approximately half of the patients are pyridoxine-responsive, i.e. they benefit from treatment with high doses of the vitamin B₆ precursor, pyridoxine (4). Thus far, >90 different mutations have been described in the cystathionine β-synthase gene in homocystinuric patients, with the vast majority being missense and private mutations (5). Cystathionine β-synthase catalyzes the condensation of serine and homocysteine to give cystathionine in a pyridoxal phosphate (PLP)¹-dependent reaction. Cystathionine is subsequently cleaved in the trans-sulfuration pathway by another PLP-dependent enzyme, γ-cystathionase, to give cysteine and α-ketoglutarate.

Cystathionine β-synthases from Trypanosoma cruzi, Saccharomyces cerevisiae, and humans have highly homologous sequences. All are predicted to belong to the β or Fold II family of PLP-dependent enzymes (6, 7). This similarity is borne out in the three-dimensional structure of the catalytic PLP-containing core of the enzyme, which has been reported recently (8), and resembles those of related PLP enzymes, viz. O-acetyl serine sulfhydrylase (9) and threonine deaminase (10). A major difference between the lower and higher eukaryotic cystathionine β-synthases that have been characterized so far is the presence of a second cofactor, iron protoporphyrin IX (11), in the mammalian enzymes, which has unusual spectroscopic properties (12–15). The binding site for the heme is located in a 66-amino acid-long N-terminal extension that is missing in the yeast enzyme (8, 16). The heme is distant from the PLP, which is bound in the active site, and appears to play a regulatory role. In addition, S-adenosylmethionine serves as an allosteric effector of the mammalian but not the yeast enzyme, and binds to the C-terminal region of the protein (17).

The full-length yeast and human enzymes appear to exist in multiple oligomeric states as suggested by their broad elution profiles on gel filtration columns and their range from tetramer to octamer (13, 18). A hypersensitive proteolysis site results in the facile generation of a truncated species, the catalytic core, which exists as a dimer (18, 19). The dimeric human and yeast forms display high levels of enzyme activity and differences in their steady-state kinetic properties versus the corresponding full-length enzymes. A pre-steady-state kinetic characterization of the truncated form of the yeast enzyme has been reported recently (20). The truncated human enzyme retains its heme but loses sensitivity to regulation by S-adenosylmethionine (19). Although the reaction catalyzed by cystathionine β-synthase superficially resembles those catalyzed by other PLP-dependent β-replacement enzymes such as tryptophan synthase and O-acetyl serine sulfhydrylase, mechanistic and kinetic studies with potential suicide inactivators on the rat enzyme, reported by Borcsok and Abeles (21), have suggested some notable differences (21).

In general, rapid reaction kinetic analyses of PLP-dependent enzymes provide a rich source of information on the identity of catalytic intermediates. However, the presence of heme in the human enzyme dwarfs the spectroscopic signatures associated with the PLP species and renders these measurements difficult. As a first step toward identifying the intermediates in the reaction catalyzed by cystathionine β-synthase, we used rapid

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¹ The abbreviation used is: PLP, pyridoxal phosphate.
reaction kinetics to characterize the full-length tetrameric form of the yeast enzyme as both a model and a guide for future studies on the human enzyme. We report a number of significant differences in the kinetics of the full-length yeast enzyme compared with that of the truncated form, engineered by deletion of C-terminal residues 354–507, which was reported recently (20).

**EXPERIMENTAL PROCEDURES**

**Materials**—Serine, D,L-homocysteine, and l-cystathionine were purchased from Sigma. The concentration of homocysteine was determined spectrophotometrically using Ellman’s reagent (22).

**Purification and Steady-state Kinetic Analysis of Yeast Cystathionine β-Synthase**—Full-length yeast cystathionine β-synthase was purified as described previously using a recombinant expression vector, pSEC (23), provided by Dr. Edith Miles (National Institutes of Health, Bethesda, MD). The activity of the enzyme and the steady-state kinetic parameters were measured in the radioactive assay as described previously (13). To determine the effects of preincubation with either serine or homocysteine, the enzyme was mixed with the first substrate for 5 min at 37 °C before being exposed to the second substrate, and the reaction was allowed to proceed for 30 min. For determination of substrate Km values, the concentration of the second substrate was fixed at 30 mM, whereas that of the first was varied.

**Rapid Reaction Kinetics**—Stopped flow experiments were performed on an Applied Photophysics spectrophotometer (SX.1MV8) equipped with a photodiode array detector. The temperature of the mixing chamber was maintained at 15 ± 1 °C and was controlled by a circulating water bath. A solution containing yeast cystathionine β-synthase (18 μM) in 0.2 mM Tris buffer, pH 8, was mixed with varying concentrations of substrate in the same buffer, as indicated in the figure legends. When the reaction of preformed aminoacrylate with homocysteine was monitored, 18 μM enzyme was premixed with 30 mM serine in 0.2 M Tris, pH 8.0, and mixed rapidly with varying concentrations of homocysteine. Because of the limited solubility of cystathionine, a stock solution (0.5 M) was made in 0.8 N NaOH, and aliquots of this solution were diluted to give the desired concentration in 0.2 M Tris buffer, pH 8. The concentrations of enzyme and substrate refer to those before mixing; both solutions were diluted 2-fold after mixing. The reactions were followed with a photo-diode array detector.

Typically, time-dependent spectra were analyzed by the single value decomposition algorithm which is an integral feature of Pro-K program (Global Analysis for Spectra, Kinetic Data, version 4.20) supplied by Applied Photophysics. A set of output matrices comprising ordered sets of basis spectra and time-dependent amplitudes was obtained together with a photo-diode array detector. The number of significant singular values is a model-free indication of the number of independent components present in the original data set. A kinetic model was built based on the number of independent spectra predicted by this method. The reported rate constants are the average of at least two different rapid mixing experiments. The spectra (recorded between 300 and 600 nm and between 1.3 and 500 ms), each containing 57,200 data points, were fit to Equation 1,

\[
\text{OD} = C \cdot e^{-kt} \quad (\text{Eq. 1})
\]

where C is the amplitude, k is the rate constant, and OD, the optical density, is the sum of the absorbance of the individual species (λi) contributing to a given spectrum, each of which is described by Equation 2.

\[
\text{OD(λi)} = C(λi) \cdot e^{-kt} \quad (\text{Eq. 2})
\]

**Table I**

Summary of steady-state kinetic parameters for full-length yeast cystathionine β-synthase determined under serine or homocysteine preincubation conditions

| Parameter | Preincubation condition | Serine | Homocysteine |
|-----------|------------------------|-------|--------------|
| Vmax (μmol mg⁻¹ h⁻¹) | 1080 ± 40 | 948 ± 9 |
| Kcat (s⁻¹) at 37 °C | 16.8 ± 0.6 | 14.7 ± 0.1 |
| Km, Ser (mM) | 2.3 ± 1.1 | 1.2 ± 0.2 |
| Km, Hcy (mM) | 3.5 ± 0.6 | 4.9 ± 2.2 |

*This was calculated per mole of active site with a molecular mass of 56 kDa.

**FIG. 1.** Spectral changes resulting from the addition of serine to the cystathionine β-synthase. Enzyme (18 μM) was mixed with varying concentrations of serine in 0.2 M Tris, pH 8.0. A, absorption changes seen between 1.3 and 500 ms after mixing 1.5 mM serine with enzyme are shown. The resting enzyme has an absorption maximum at 412 nm (internal aldimine) that is converted to a species with an absorption maximum at 460 nm, assigned as the aminoacylate. B, global fitting of the time-dependent spectra in A reveals the presence of three species with absorption maxima at 412 (a), 416 (b), and 460 nm (c), respectively. C, dependence of the apparent rates of formation of the external aldimine (v) and the aminoacylate (c) on the concentration of serine.
ity of the yeast enzyme is unaffected by whether it is preincubated with serine or homocysteine. The yeast cystathionine \(\beta\)-synthase displays a bell-shaped dependence on pH with an optimum at 8.1 and inflection points corresponding to pKa values of 7.7 ± 0.1 and 8.8 ± 0.1, respectively (data not shown).

**Reaction of Cystathionine \(\beta\)-Synthase with Serine—Absorbance changes resulting from rapid mixing of cystathionine \(\beta\)-synthase with serine are shown in Fig. 1A. Global analysis of the spectrum (Fig. 1B) indicated the presence of three species assigned as the internal aldime (\(\lambda_{\text{max}} = 412\) nm), the external aldime (\(\lambda_{\text{max}} = 416\) nm), and the aminoacrylate (\(\lambda_{\text{max}} = 460\) nm). There was no evidence for a gem-diamine (with a \(\lambda_{\text{max}} \approx 320\) nm) at high serine concentrations or for a tautomeric form of the aminoacrylate (with a \(\lambda_{\text{max}} \sim 320\) nm) at low serine concentration as reported for the truncated yeast cystathionine \(\beta\)-synthase (20). Formation of the external aldime was linearly dependent on the concentration of serine (Fig. 1C) and yielded a bimolecular rate constant of 14 mM\(^{-1}\) s\(^{-1}\) and a \(K_d\) for serine of 1.5 ± 0.5 mM. Formation of the aminoacrylate showed saturation dependence on the concentration of serine and a maximal rate constant of 15 ± 0.4 s\(^{-1}\). For comparison, the \(k_{\text{cat}}\) for yeast cystathionine \(\beta\)-synthase at 15 °C is 1.7 s\(^{-1}\) calculated per mole of monomer of 56 kDa molecular mass.

**Reaction of E-Aminoacrylate with Homocysteine—Kinetics of the second half reaction, i.e. addition of homocysteine to the aminoacrylate intermediate to form cystathionine, was followed by preincubating 18 \(\mu\)M enzyme with 30 mM serine to preform the aminoacrylate, which was then rapidly mixed with varying concentrations (0–60 \(\mu\)M) of \(\Delta\)-l-homocysteine. At low concentrations of homocysteine, a decrease in the absorbance of the aminoacrylate, a decrease in the absorbance of the aminoacrylate intermediate is observed at 460 nm with a concomitant blue shift in the spectrum (Fig. 2A). In contrast, the disappearance of the preformed aminoacrylate with the truncated yeast cystathionine \(\beta\)-synthase was reported to be too fast to measure even at low homocysteine concentration (20). However, at high concentrations of homocysteine, the decay of the aminoacrylate is completely missed (Fig. 2B), indicating that the reaction is rapid and largely over within the dead time of the instrument (2.2 ms). Instead, a rapid decrease at 420 nm, with a \(k_{\text{on,app}}\) of 180 ± 5 s\(^{-1}\), is observed followed by a slow increase in absorption at the same wavelength with a rate constant of 0.7 s\(^{-1}\) (Fig. 2C). The species at 420 nm is tentatively assigned as the external aldime of cystathionine, and its rate of formation is identical to the rate of disappearance of the 460 nm aminoacrylate at low concentrations of homocysteine (Fig. 2D). The nature of the change leading to an increase in the extinction coefficient of the 420 nm-absorbing species at a rate of 0.7 s\(^{-1}\) is not understood, and it is too slow to be catalytically relevant.

The dependence of the rate of disappearance of the aminoacrylate on the concentration of homocysteine yields a bimolecular rate constant of 35 ± 0.6 mM\(^{-1}\) s\(^{-1}\) and a \(K_d\) for homocysteine of 0.68 ± 0.03 mM (Fig. 2D).

**Reaction of Enzyme with Cystathionine—Rapid mixing of...
enzyme with cystathionine resulted in conversion of the external aldimine (λ_max = 412 nm) to the aminoacrylate with a λ_max of 460 nm (Fig. 3A). A clean isosbestic point is observed at 426 nm, indicating that an intermediate such as the external aldimine of cystathionine does not accumulate to detectable levels. Hence, the complexes between enzyme and cystathionine formed in the forward and reverse directions appear to be different. When equimolar serine and homocysteine are mixed with enzyme, the spectrum of the enzyme (λ_max = 420 nm) is consistent with the presence of the external aldimine of cystathionine (Fig. 2B), for which release is slow and presumably limits the overall reaction. In contrast, when the enzyme is mixed with cystathionine, the external aldimine is not detected, and the aminoacrylate form accumulates (Fig. 3A). The dependence of the rate of aminoacrylate formation on the concentration of cystathionine yields a bimolecular rate constant of 1.5 ± 0.3 mM⁻¹ s⁻¹ and a K_d for cystathionine of 1.6 ± 0.3 mM (Fig. 3B). The limited solubility of cystathionine precluded measurements at concentrations above 15 mM.

**DISCUSSION**

Differences in the spectroscopic signatures associated with the bound intermediates in PLP-dependent enzymes provide a convenient means for analyzing the kinetics of their formation and decay. Steady-state kinetic analysis of the yeast cystathionin-β-synthase indicates that it catalyzes a ping-pong reaction in which serine binds to generate an enzyme-bound aminoacrylate intermediate that reacts with homocysteine to give cystathionine (18). In this study, we report a pre-steady-state kinetic analysis of the reaction catalyzed by the full-length yeast enzyme. AA and Cyst denote aminoacrylate and cystathionine, respectively.

**FIG. 3.** Spectral changes resulting from the addition of cystathionine to cystathioninase synthase. Enzyme (18 μM) in 0.2 M Tris, pH 8.0, was mixed with varying concentrations of L-cystathionine in the same buffer. A, spectral changes observed between 2.5 and 1000 ms after addition of 30 mM cystathionine. Conversion of the 412 nm resting enzyme spectrum to a 460-nm aminoacrylate species is observed with an isosbestic point at 426 nm. B, dependence of the apparent rate for formation of the aminoacrylate species on the concentration of cystathionine.

| [L-cystathionine], mM | k_{cat} for Aminoacrylate (s⁻¹) |
|-----------------------|---------------------------------|
| 0                     | 0                               |
| 1                     | 5                               |
| 2                     | 10                              |
| 5                     | 15                              |
| 10                    | 20                              |

**SCHEME I.** Intermediates in the reaction catalyzed by cystathioninase synthase and their absorption maxima as determined in this study. The two gem-diamine intermediates (GD-I and GD-II), shown in square brackets, were not observed in the pre-steady-state kinetic analysis of the full-length yeast enzyme. AA and Cyst denote aminoacrylate and cystathionine, respectively.

**SCHEME II.** Minimal kinetic scheme for reaction catalyzed by yeast cystathioninase synthase in the forward and reverse directions. The values shown in parentheses in A are taken from B, where the reaction was followed in the reverse direction.
a poor leaving group (OH\textsuperscript{−}) from the substrate, and would require enzyme assisted protonation (Scheme I). In contrast, formation of the aminoacrylate from cystathionine in the reverse reaction involves elimination of a better leaving group (thiolate) and may explain the difference in the rates (Scheme II).

In the truncated enzyme, the external aldimine of serine was not observed, and the $k_{\text{obsd}}$ reported for aminoacrylate formation (177 s\textsuperscript{−1}) is comparable with the rate we observe for the disappearance of the internal aldimine at high serine concentration (−200 s\textsuperscript{−1}, Fig. 1C). The observation of the external aldimine intermediate with the full-length enzyme allows deconvolution of the $k_{\text{obsd}}$ into bi- and unimolecular rate constants as shown in Fig. 1C.

The second-half reaction, i.e. conversion of the aminoacrylate to cystathionine, can be monitored by mixing preformed aminoacrylate with homocysteine. The disappearance of the aminoacrylate with homocysteine, where the reaction is over in the dead time of the instrument. The product of this reaction has an absorption maximum at 420 nm and is assigned as the external aldimine of cystathionine. The rate constant for the decrease in absorbance at 420 nm (180 ± 5 s\textsuperscript{−1}) parallels the rate constant for the disappearance of the aminoacrylate at low homocysteine concentrations where the rate can be measured (Fig. 2D).

The reaction of cystathionine and enzyme to form an aminoacrylate species shows monophasic kinetics, and formation of an external aldimine intermediate is not observed (Fig. 3A).

The accumulation of the 420 nm species in the forward direction assigned as the external aldimine of cystathionine (Fig. 2B) indicates that product release is rate-limiting, a conclusion that was also reached with the truncated enzyme (20).

In summary, notable differences are observed in the pre-steady-state kinetic analysis of intermediates in the reactions catalyzed by full-length and truncated yeast cystathionine $\beta$- synthase. A gem-diamine intermediate is seen in the presence of serine with the truncated but not full-length enzyme, whereas the external aldimine of serine is observed with full-length but not truncated enzyme. Similarly, a 320 nm absorbance assigned as the gem-diamine of cystathionine is observed when the aminoacrylate is mixed with homocysteine in the truncated but not the full-length enzyme. Quinonoid intermediates are not seen with either enzyme form, which is similar to the reaction catalyzed by the closely related PLP-dependent enzyme, O-acetyl serine sulfhydrylase (24). This has been rationalized by Cook and co-workers (9) to result from the mismatch in the $pK_a$ values of the N1 of PLP and Ser-272, making protonation of N1 unlikely and thereby disfavoring quinonoid formation. A homologous serine is conserved in both yeast and human cystathionine $\beta$- synthase (25). The studies with the full-length yeast enzyme should be useful in elucidating the kinetic mechanism of the human enzyme.

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