Yeast Cystathionine β-Synthase Reacts with L-Allothreonine, a Non-Natural Substrate, and L-Homocysteine To Form a New Amino Acid, 3-Methyl-L-cystathionine†

Kwang-Hwan Jhee,‡ Dimitri Niks,§ Peter McPhie,‡ Michael F. Dunn,§ and Edith Wilson Miles*,‡

Laboratory of Biochemistry and Genetics, National Institute of Diabetes and Digestive and Kidney Diseases, National Institutes of Health, Bethesda, Maryland 20892-0830, and Department of Biochemistry, University of California, Riverside, California 92521

Received September 4, 2001; Revised Manuscript Received December 13, 2001

ABSTRACT: Our studies of the reaction mechanism of cystathionine β-synthase from yeast (*Saccharomyces cerevisiae*) are facilitated by the spectroscopic properties of the pyridoxal phosphate coenzyme. The enzyme catalyzes the reaction of L-serine with L-homocysteine to form L-cystathionine through a series of pyridoxal phosphate intermediates. In this work, we explore the substrate specificity of the enzyme by use of substrate analogues combined with kinetic measurements under pre-steady-state conditions and with circular dichroism and fluorescence spectroscopy under steady-state conditions. Our results show that L-allothreonine, but not L-threonine, serves as an effective substrate. L-Allothreonine reacts with the pyridoxal phosphate cofactor to form a stable 3-methyl aminoacrylate intermediate that absorbs maximally at 446 nm. The rapid-scanning stopped-flow results show that the binding of L-allothreonine as the external aldimine is faster than formation of the 3-methyl aminoacrylate intermediate. The 3-methyl aminoacrylate intermediate reacts with L-homocysteine to form a new amino acid, 3-methyl-L-cystathionine, which was characterized by nuclear magnetic resonance spectroscopy. This new amino acid may be a useful analogue of L-cystathionine.

This work is aimed at an improved understanding of the mechanism and substrate specificity of cystathionine β-synthase (EC 4.2.1.22), which catalyzes the reaction of L-serine and L-homocysteine to form L-cystathionine. The human enzyme plays an important role in detoxifying plasma homocysteine, which is an important risk factor in coronary heart disease and other human diseases (*1*, *2*). Pyridoxal phosphate (PLP)[1] is the essential cofactor for both the yeast heme-independent cystathionine β-synthase (*3*, *4*) and the human heme-dependent cystathionine β-synthase (*5*). The absence of heme in the yeast enzyme facilitates the detection of PLP intermediates in the cystathionine β-synthase reaction (Scheme 1). The mechanism in Scheme 1, which is based on that of other enzymes that catalyze PLP-dependent β-replacement reactions (*6*, *7*), shows that the reaction occurs in two stages. In stage I, the external aldimine of the substrate (ES1) is converted by loss of the α-proton and elimination of the β-hydroxyl group to an unsaturated imine (ES2). In stage II, ES2 adds a nucleophile, L-homocysteine, to form the external aldimine of the product (ES3). Our initial studies of yeast cystathionine β-synthase using absorbance and circular dichroism spectroscopy (*3*) demonstrated the formation of ES2 from L-serine in stage I and the disappearance of ES2 upon addition of L-homocysteine in stage II. Steady-state kinetic studies provide evidence that the cystathionine β-synthase reaction proceeds by the ping-pong mechanism shown in Scheme 1 (*8*). Rapid-scanning stopped-flow and single-wavelength kinetic studies demonstrate that binding of L-serine as the external aldimine (ES1) is faster than formation of the aminoacrylate intermediate (ES2) and that the rate-limiting step is the reaction of ES2 with L-homocysteine to form L-cystathionine (*9*).

Investigations of the domain architecture of cystathionine β-synthase from yeast (*8*) and humans (*10*, *11*) reveal that each enzyme is composed of an N-terminal catalytic domain and a C-terminal regulatory domain. In contrast, the enzyme from *Trypanosoma cruzi* has only a catalytic domain (*12*). The deduced amino acid sequences of the catalytic domain of cystathionine β-synthase from several sources are significantly homologous to each other (*12*) and to the sequences of several other PLP-dependent enzymes in the β-family (*13*) or fold type II (*14*). These enzymes include *O*-acetylserine sulfhydrylase, threonine deaminase, and tryptophan synthase (*8*). Cystathionine β-synthase from rats and humans is novel both in its dependence on heme (*5*) and in its regulation by *S*-adenosyl-L-methionine (*15*). The observation that the yeast enzyme lacks heme as well as a 66-residue N-terminal extension suggests that the N-terminal extension of the human enzyme is involved in heme binding (*16*). The crystal structure of a truncated form of human cystathionine β-synthase (*17*) containing residues 1−413 confirms that heme binds to the N-terminal domain. The heme iron coordinates

† Supported by NIGMS Grant GM55749 (M.F.D.).

* To whom correspondence should be addressed. Telephone: (301) 496-2763. Fax: (301) 402-0240. E-mail: EdithM@intra.niddk.nih.gov.

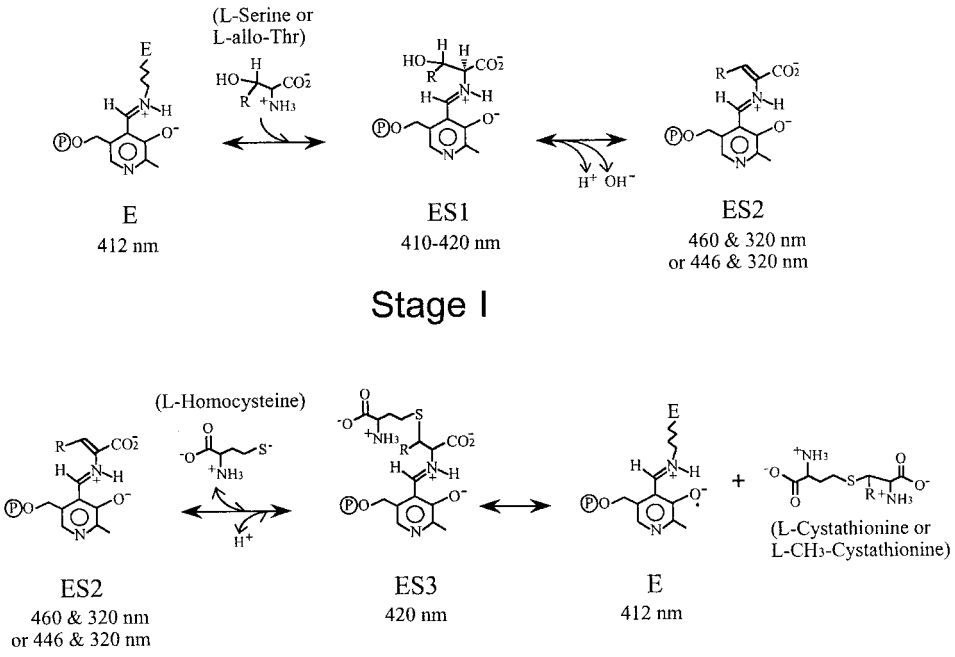
‡ National Institutes of Health.

§ University of California.

[1] Abbreviations: T-CBS, truncated cystathionine β-synthase; PLP, pyridoxal phosphate.

10.1021/bi011756t CCC: $22.00
Published on Web 01/18/2002

Scheme 1: Intermediates in the Reactions of Cystathionine β-Synthase[a]



[a] Stage I is the first half-reaction with L-serine (R = H) or L-allothreonine (R = CH_3) in the absence of a cosubstrate. Stage II is the second half-reaction obtained upon addition of L-homocysteine to the E−AA or E−CH_3-AA species.

Cys52 and His65 and is located ~20 Å from PLP. However, the role of heme in human cystathionine β-synthase is still not clear. Evidence that heme does not serve a catalytic role is provided by the finding that heme-free crystals of the catalytic domain of human cystathionine β-synthase exhibit catalytic activity (*18*) and that human cystathionine β-synthase having residues 1−70 deleted retains ~25% of the wild-type activity (J. P. Kraus, unpublished results cited in ref *17*). Heme may serve a regulatory role. The finding that the heme binding site is spatially adjacent to an oxidoreductase active site motif in the structure of the human enzyme could explain the regulation of cystathionine β-synthase activity by redox changes (*17*). Recent ^{31}P NMR studies of PLP in human cystathionine β-synthase provide structural evidence for transmission to the active site of an oxidation state change in the heme pocket consistent with a regulatory role for heme (*16*). The discovery that deletion of the C-terminal domain of the human enzyme eliminates *S*-adenosyl-L-methionine activation (*11*) suggests that *S*-adenosyl-L-methionine is an allosteric activator that binds to the C-terminal regulatory domain.

In the work presented here, we have explored the mechanism and substrate specificity of yeast cystathionine β-synthase by using analogues of the natural substrate L-serine. To detect intermediates shown in Scheme 1 and to determine the rates of the appearance and decomposition of these intermediates, we have used absorbance, circular dichroism, and fluorescence spectroscopies under steady-state conditions and rapid-scanning stopped-flow kinetic measurements under pre-steady-state conditions. The N-terminal catalytic domain of yeast cystathionine β-synthase (T-CBS) was used because it is more stable and soluble than the full-length yeast enzyme (*8*). Our results show that L-allothreonine, but not L-threonine, serves as an effective substrate. L-Allothreonine reacts with the PLP cofactor to form a stable PLP−3-methyl aminoacrylate intermediate (ES2 in Scheme 1 where R is CH_3) with a λ_{max} of 446 nm. The 3-methyl aminoacrylate intermediate reacts with L-homocysteine in stage II to form a product. The rapid-scanning stopped-flow results show that the binding of L-allothreonine as the external aldimine (ES1) is faster than formation of the 3-methyl aminoacrylate (ES2), as found previously for the natural substrate L-serine (*9*). The reaction product was identified as 3-methyl-L-cystathionine by nuclear magnetic resonance spectroscopy. This new amino acid is a potentially useful analogue of L-cystathionine. Thus, cystathionine β-synthase may prove to be beneficial for the synthesis of new amino acids.

EXPERIMENTAL PROCEDURES

Materials. L-Serine was obtained from Fluka. L-Allothreonine, L-threonine (98% pure), and D,L-homocysteine were purchased from Sigma. L-Homocysteine was prepared from L-homocysteine thiolactone (Sigma) as described previously (*19*) and diluted with 50 mM *N*,*N*-bis(2-hydroxyethyl)glycine buffer adjusted to pH 7.8 with NaOH.

Enzymes and Buffers. Truncated cystathionine β-synthase (T-CBS) from yeast (*Saccharomyces cerevisiae)* was purified as described from *Escherichia coli* XL1-Blue cells transformed with the plasmid pT-SEC (*8*).[2] Protein concentrations were determined as described previously (*8*). 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

[2] This expression vector is available from the American Type Culture Collection, 10801 University Blvd., Manassas, VA 20110-2209, as ATCC Number 87792.

junction temperature-controlled cuvette holder. Circular dichroism 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. 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. The rapid-scanning stopped-flow system has been described previously (*9*). A more detailed description of this instrumentation will be presented elsewhere. Data were collected with a repetitive scan rate of 1.2 ms/scan. In each rapid-scanning stopped-flow experiment, a set of 1000 spectra was collected at 25 °C with total scan acquisition times of 1.2 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 rapid-scanning stopped-flow figure.

Single-wavelength stopped-flow absorbance and fluorescence studies were carried out using an Applied PhotoPhysics mixing unit (mixing dead time of 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 personal 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):

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

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

^{1}H NMR Spectroscopy. All ^{1}H NMR experiments were performed using either a Varian Inova 500 MHz or a Varian Inova 300 MHz spectrometer. All spectra were carried out in 10% D_2O using a presaturation pulse for water suppression. Spectral analysis was performed using VNMR 6.1c and MestRe-C 2.3a.

Product Synthesis, Isolation, and Identification. L-Allothreonine (80.0 mM), D,L-homocysteine (9.0 mM), and T-CBS (0.15 mg/mL) were allowed to react for 1−2 h. The enzyme was separated using a Microcon-10 microconcentrator (the microconcentrator membrane was prewashed with ddH_2O to remove glycerol). An L-allothreonine stock solution was brought to pH 7.8 before dilution to the final concentration given above, and used as the only buffering agent in the reaction mixture. The product was subjected to silica plate TLC analysis and developed using a 60:15:25 butanol/acetic acid/water solvent. Part of the plate was sprayed with Ninhydrin reagent for free amine analysis to locate the putative product (a companion plate was used to determine the location of the substrates). The rest of the plate was scraped at the location of the putative product. The product was extracted using ddH_2O, and traces of silica were removed using another prewashed microconcentrator. The product was then lyophilized and subjected to ^{1}H NMR analysis. Both one-dimensional (1D) proton and two-dimensional (2D) TOCSY (total correlation spectroscopy) spectra of the product were obtained. 1D proton spectra were also obtained for L-allothreonine and D,L-homocysteine to facilitate assignments and to compare with previously published spectra (spectrum 11978M) (*20*) and (spectrum 30761M) (*21*).

For product identification via difference spectra measurements, the reaction of 10.0 mM L-allothreonine, 10.0 mM D,L-homocysteine, and T-CBS (0.03 mg/mL) was monitored by ^{1}H NMR over the course of 24 h. A spectrum of the two substrates together also was taken before addition of the enzyme. Then a difference spectrum was constructed (product formation after 24 h minus substrates). Assignments were made with the help of the data from the 1D and 2D product spectra.

RESULTS

The spectroscopic properties of PLP provide a sensitive probe for detecting intermediates in β-replacement reactions (Scheme 1). To further characterize the mechanism and substrate specificity of yeast cystathionine β-synthase, we have carried out rapid-kinetic and steady-state spectroscopic studies with analogues of the natural substrate L-serine (Scheme 1).

Steady-State Spectroscopic Studies. Figure 1A shows that addition of 50 mM L-allothreonine to T-CBS resulted in the disappearance of the 412 nm absorbance band attributed to the internal aldimine (E in Scheme 1) and the appearance of a new spectroscopic species with a major band centered at 446 nm. This species is likely the 3-methyl aminoacrylate Schiff base (E−CH_3−AA; ES2 in Scheme 1 where R is CH_3). Circular dichroism spectra recorded under the same conditions (Figure 1C) show the conversion of the positive 412 nm band to a negative band centered at 446 nm. These results are very similar to those obtained with the natural substrate L-serine, which yields an absorption band at 460 nm and a negative circular dichroism band centered at 460 nm (Figure 1A,C) that were previously ascribed to the external aldimine of aminoacrylate (E-AA; ES2 in Scheme 1 where R is H) (*3*). The E-AA and E−CH_3-AA species have similar absorbancies at 460 and 446 nm, respectively, and at 320 nm (Figure 1A). Addition of 50 mM L-threonine to T-CBS resulted in a much slower conversion of E to ES2.

When the 3-methyl aminoacrylate intermediate (3-CH_3-AA) was preformed by mixing T-CBS with 1 mM L-allothreonine and then mixed with 5 mM L-homocysteine, the absorbance and circular dichroism spectra shown in panels B and C of Figure 1 were obtained. These spectra are essentially identical to the spectra of T-CBS alone, demonstrating the conversion of E-3-CH_3-AA to E plus product in stage II of the reaction shown in Scheme 1. Similar results were reported previously for the reaction of the E−AA species and L-homocysteine (*9*).

Rapid-Scanning Stopped-Flow Spectroscopy. The reaction of T-CBS with 50 mM L-allothreonine gave the rapid-scanning stopped-flow spectra shown in Figure 2A. The spectra exhibit two clear apparent isosbestic points. The

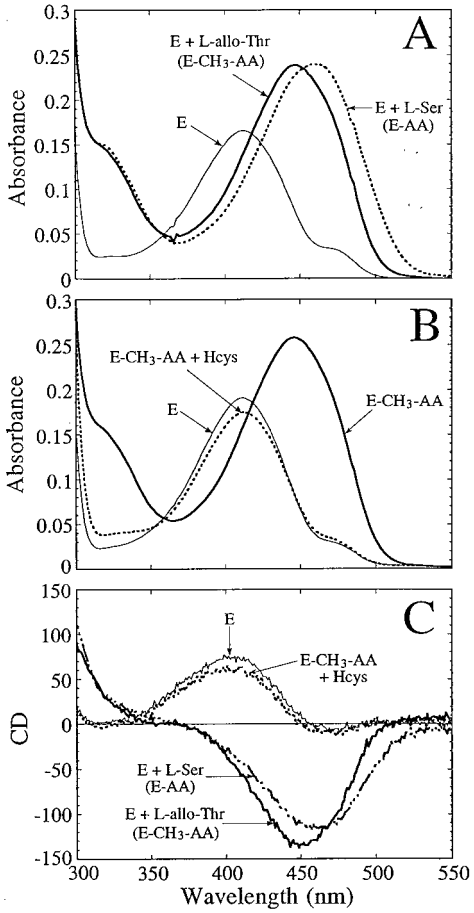


FIGURE 1: Absorbance and circular dichroism spectra of T-CBS. (A) Absorbance spectra of T-CBS alone (E) and in the presence of 50 mM L-serine or 50 mM L-allothreonine. (B) Absorbance spectrum of T-CBS in the presence of 1 mM L-allothreonine before and 15 min after mixing with 5 mM L-homocysteine. (C) Circular dichroism spectra of T-CBS alone, in the presence of 1 mM L-serine, and in the presence of 1 mM L-allothreonine before and 15 min after mixing with 5 mM L-homocysteine. The absorption spectrum of T-CBS exhibits a shoulder at 477 nm, which is catalytically inert (*9*).

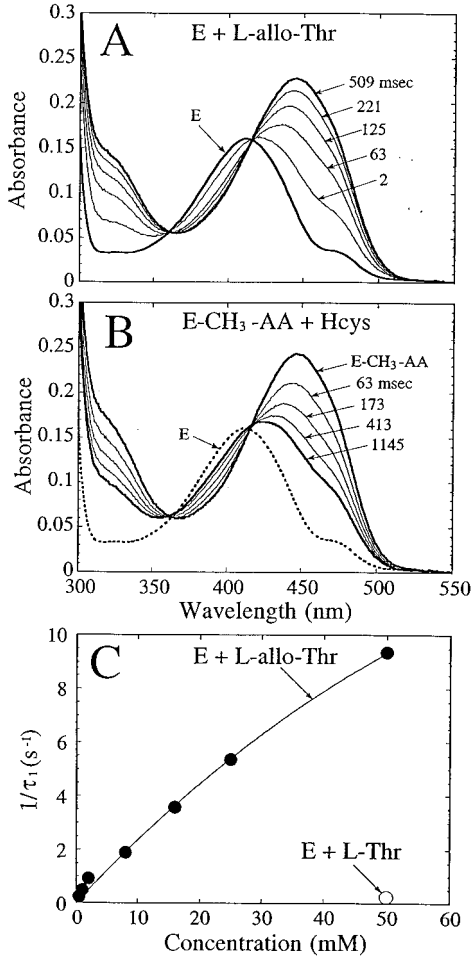


FIGURE 2: Rapid-scanning stopped-flow spectra of the reactions of T-CBS with L-allothreonine and L-homocysteine. (A) Spectra at the indicated times after mixing T-CBS with 50 mM L-allothreonine. (B) Spectra at the indicated times after mixing CH_3-AA (T-CBS premixed with 25 mM L-allothreonine) with 25 mM L-homocysteine. (C) Dependence of the relaxation rates for the formation of the band at 446 nm on the concentration of L-allothreonine [(●) 0.5, 1.0, 2.0, 8.0, 16.0, 25, and 50 mM] and L-threonine [(○) 50 mM]. Data were obtained from single-wavelength stopped-flow absorbance time courses for the appearance of the aminoacrylate intermediate monitoring the absorbance at 446 nm. The data were fitted using the Marquardt−Levenberg algorithm (see the text) and were plotted with the average values of five time scans.

results suggest that the internal aldimine (E) is converted to a mixture of the two forms of the 3-methyl aminoacrylate intermediate (ES2) (λ_{max} = 446 and 320 nm) with no detectable accumulation of external aldimine with L-allothreonine (ES1) (Scheme 1). The rates of increase in absorbance at 446 nm (9.4 ± 0.14 s^{-1}) and 320 nm (9.5 ± 0.14 s^{-1}) are identical, suggesting that there is no additional detectable intermediate between E and ES2 under these conditions. The first half-reaction of *O*-acetylserine catalyzed by *O*-acetylserine sulfhydrylase also yields two absorbance bands at 470 and 330 nm that have been attributed to two forms of aminoacrylate (*22*). The ratio of absorbancies at 470 and 330 nm is constant during the course of the reaction. The authors suggested that the 330 nm band is due to an electronic transition of the aminoacrylate and likely represents a second tautomeric form of the intermediate, or a form in which the intramolecular hydrogen bond between the imine nitrogen and the 3-hydroxyl of PLP is for some reason not allowed. In contrast, addition of a high concentration of L-serine (50 mM) to T-CBS resulted in the detection of a transitory gem-diamine intermediate (*9*). Table 1 compares the relaxation rates for the appearance of ES2 in the reaction with 50 mM L-serine (176 ± 7.56 s^{-1}) (*9*) and with L-allothreonine (9.4 ± 0.14 s^{-1}). However, the K_d for L-allothreonine is ~7 times higher than for L-serine, and thus,

Table 1: Comparison of the Kinetic Parameters for Reactions of L-Serine and L-Allothreonine with T-CBS in the Presence or Absence of L-Homocysteine

substrate	$1/\tau_1$ (s^{-1})[a] for ES2 formation	$1/\tau_2$ (s^{-1})[b] for ES2 reaction with L-homocysteine	K_d' (μM)[c]	ref
L-serine	176 ± 7.56	>400	14 ± 0.25	*8, 9*
L-allothreonine	9.4 ± 0.14	1.18 ± 0.28	106 ± 1.96	this work

[a] The relaxation rates ($1/\tau_1$) for ES2 formation (Scheme 1, stage I) were calculated from the change in absorbance at 460 nm with 50 mM L-serine or at 446 nm with 50 mM L-allothreonine. [b] The relaxation rates ($1/\tau_2$) for ES2 decay (Scheme 1, stage II) were calculated from the change in absorbance at 460 or 446 nm after addition of 2 mM L-homocysteine to T-CBS premixed with L-serine (25 mM final concentration) or L-allothreonine (25 mM final concentration), respectively. [c] The apparent dissociation constants (K_d') were measured by fluorescence titrations (see Figure 3).

50 mM L-allothreonine may be insufficient to give detectable amounts of the gem-diamine.

When the 3-methyl aminoacrylate intermediate (3-CH_3-AA) was preformed by mixing T-CBS with 25 mM L-allothreonine and then mixed with 25 mM L-homocysteine, the spectra shown in Figure 2B were obtained. The rapid-scanning stopped-flow spectra exhibit two clear apparent isosbestic points (Figure 2B). The rates of decrease in absorbance at 446 nm ($4.77 \pm 0.26\ s^{-1}$) and 320 nm ($4.65 \pm 0.25\ s^{-1}$) are essentially identical. The results suggest that the 3-methyl aminoacrylate intermediate (3-CH_3-AA) is converted to internal aldimine (E) with no detectable accumulation of the external aldimine of 3-methyl-L-cystathionine (ES3) (Scheme 1). In contrast, the rate of reaction of L-homocysteine with the E−AA species is too fast to measure even at a low concentration (2 mM) of L-homocysteine (*9*). Table 1 compares the relaxation rates for the disappearance of ES2 upon addition of 2 mM L-homocysteine in the presence of L-serine ($>400\ s^{-1}$) or L-allothreonine ($1.18 \pm 0.28\ s^{-1}$).

Single-Wavelength Stopped-Flow Spectroscopy. To better assess the formation and decay of individual intermediates with higher precision, we carried out single-wavelength stopped-flow experiments (Figure 2C). The appearance of the 3-methyl aminoacrylate intermediate, formed upon mixing T-CBS with L-allothreonine, was monitored at 446 nm as a function of the concentration of L-allothreonine. 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 ($1/\tau_1$) on the concentration of L-allothreonine is shown in Figure 2C. As a result of the low solubility of L-allothreonine (~100 mM) and high dissociation constant (Table 1), saturation of the rate was not reached over the concentration range of L-allothreonine that was used. Addition of L-threonine to T-CBS also resulted in ES2 formation. The relaxation rate for the formation of ES2 with 50 mM L-threonine ($0.14 \pm 0.003\ s^{-1}$) is 67 times slower than with 50 mM L-allothreonine (Figure 2C), and the apparent dissociation constant of L-allothreonine (0.106 mM) is 60 times smaller than that of L-threonine (6.35 mM) (see below and the inset of Figure 3B). It appears likely that the L-threonine that was used contained ~1.6% L-allothreonine.

Fluorescence Spectroscopy. The aminoacrylate intermediate formed from L-serine (ES2) exhibits a fluorescence emission maximum centered at 540 nm when excited at 460 nm (Figure 3A) (*9*). The 3-methyl aminoacrylate intermediate formed from L-allothreonine also exhibits a fluorescence emission maximum centered at 525 nm when excited at 446 nm (Figure 3A). Measurement of fluorescence emission as a function of substrate concentration yielded apparent K_d' values[3] of 106 ± 1.96 μM for L-allothreonine and 6.35 ± 0.24 mM for L-threonine (Figure 3B). The apparent K_d' value for L-serine is 14 ± 0.25 μM (*9*) (Table 1).

Analysis of the Reaction Kinetics. The cystathionine β-synthase reaction with L-allothreonine proceeds through a series of intermediates shown in Scheme 1. Stage I involves two intermediates, the external aldimine of L-allothreonine

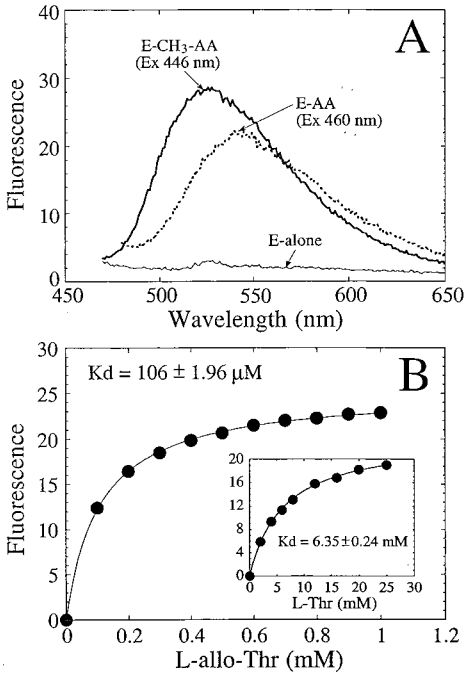


FIGURE 3: Effects of substrates on the fluorescence properties of T-CBS. (A) Fluorescence emission spectra for T-CBS alone (excitation at 446 nm) and in the presence of 10 mM L-allothreonine (excitation at 446 nm) or of 10 mM L-serine (excitation at 460 nm). (B) Plots of the fluorescence emission of T-CBS (1 μM) vs L-allothreonine concentration, or L-threonine concentration (inset). The increase in fluorescence emission at 525 nm (excitation at 446 nm) was determined at 5 min after each addition of L-allothreonine or L-threonine. The apparent K_d' values (106 ± 1.96 μM) for L-allothreonine and L-threonine (6.35 ± 0.24 mM) were calculated from the relation $\Delta F = \Delta F_{max}$[L-allo-Thr]/$K_d$ + [L-allo-Thr]. The species with maximum emission at 525 nm is attributed to the external aldimine of the 3-methyl aminoacrylate intermediate (see the Results). The inset shows an analogous fluorescence titration with L-threonine.

(E−L-allo-Thr) and the 3-methyl aminoacrylate intermediate (E−CH_3-AA) as shown by eq 2, as described previously for the reaction with L-serine (*9*).

$$\text{E} + \text{L-allo-Thr} \underset{k_2}{\overset{k_1}{\rightleftarrows}} \text{E}-\text{allo-Thr} \underset{k_4}{\overset{k_3}{\rightleftarrows}} \text{E}-\text{CH}_3\text{-AA} \quad (2)$$

The rate of formation of the E−CH_3-AA species from L-allothreonine shows a hyperbolic dependence on substrate concentration (Figure 2C). This dependence indicates that the slow step in the reaction is the conversion of the E−L-allo-Thr species to the E−CH_3-AA species. When the low concentration of enzyme (E) is neglected, the relaxation ($1/\tau$) is given by eq 3:

$$\frac{1}{\tau} = \frac{k_3[\text{allo-Thr}]}{K_{21} + [\text{allo-Thr}]} + k_4 \quad (3)$$

where $K_{21} = k_2/k_1$. Fitting the data of Figure 2C to eq 3 gave a k_3 of 47 ± 12.1 s^{-1}, a k_4 of 0.266 ± 0.088 s^{-1}, and a K_{21} of 212 ± 66 mM. These values may be compared with those derived in earlier work using L-serine, the natural substrate (*9*). The dissociation constant for formation of the external aldimine (K_{21}) is increased almost 50-fold from 4.6 mM for L-serine to 212 mM for L-allothreonine, indicating that it is more difficult to form the external aldimine of L-allothreonine. However, the rate constants for the transformation

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

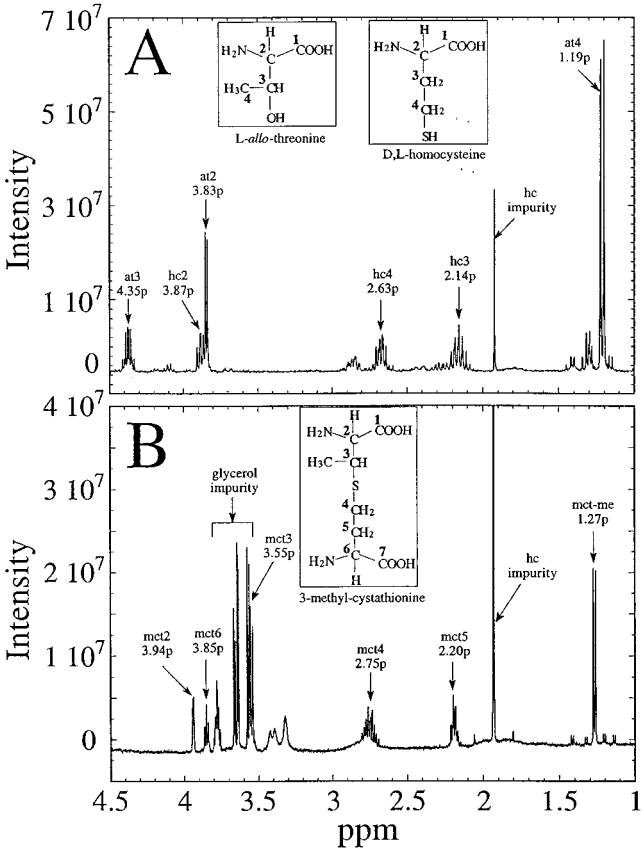


FIGURE 4: ^{1}H NMR spectra of substrates L-allothreonine and D,L-homocysteine (A) and the ^{1}H NMR spectrum of the product 3-methylcystathionine (B). Abbreviations are used at each analyzed peak: at, L-allothreonine; hc, D,L-homocysteine; mct, 3-methylcystathionine; and me, the C-3 methyl group. The numbers after each abbreviation represent the carbon numbers of each compound.

between the external aldimine and the 3-methyl aminoacrylate are reduced only 3-fold compared to those for L-serine ($k_3 = 177$ s^{-1} and $k_4 = 0.54$ s^{-1}). Once formed, the external aldimine of L-allothreonine is almost as reactive as that formed from the natural substrate L-serine.

NMR Spectroscopy. Isolation of the product from reaction of L-allothreonine with D,L-homocysteine (see Experimental Procedures) revealed the formation of a new amino acid. Structural determination by ^{1}H NMR establishes that the reaction product is the cystathionine analogue 3-methyl-L-cystathionine. Figure 4A shows there are two sets of signals each with three resonances. One set consists of resonances centered around 4.35 (multiplet), 3.83 (doublet), and 1.19 ppm (doublet) belonging to L-allothreonine (at3, at2, and at4, respectively). The other set consists of resonances centered around 3.87 (triplet), 2.63 (multiplet), and 2.14 ppm (multiplet) belonging to D,L-homocysteine (hc2, hc4, and hc3, respectively). Integration of these sets gives relative ratios for the areas under each envelope of 0.8, 1.0, and 3.0, respectively, for L-allothreonine and 1.2, 2.0, and 2.0, respectively, for D,L-homocysteine. Figure 4B shows the six separate envelopes of resonances for the product 3-methyl-L-cystathionine centered at 3.94 (doublet, mct2), 3.85 (triplet, mct6), 3.55 (multiplet, mct3), 2.75 (multiplet, mct4), 2.20 (multiplet, mct5), and 1.27 ppm (doublet, mct-me). Integration gives relative ratios for the areas under each envelope of 0.9, 1.0, 0.8, 2.3, 1.9, and 3.0, respectively. The integration ratio of the 3.55 ppm resonance was obtained by subtracting

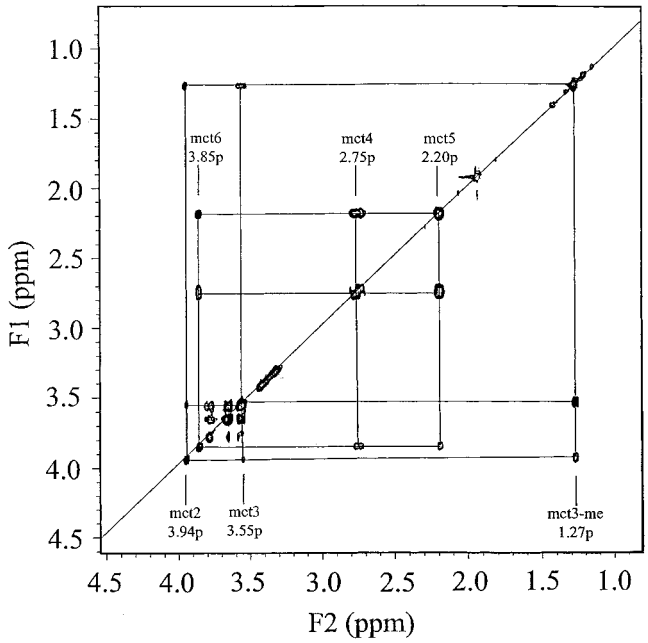


FIGURE 5: 2D TOCSY spectrum of the product 3-methylcystathionine. Peak abbreviations: mct, 3-methylcystathionine; and me, the C-3 methyl group. The numbers after each abbreviation represent the carbon numbers of each compound.

the ratio of the resonances centered at 3.65 ppm from that of the resonance centered at 3.56 ppm (the 3.55 ppm resonance is obscured by those at 3.56 ppm; see Figure 5).

Figure 5 shows the 2D TOCSY spectrum of the product 3-methyl-L-cystathionine, obtained as described in Experimental Procedures. The key structural features of the spectrum are the two sets of correlated resonances (there is no coupling across the sulfur ether bond). One set results from the contribution of the L-allothreonine moiety (resonances centered at 3.94, 3.55, and 1.27 ppm) and the other from the D,L-homocysteine moiety (resonances centered at 3.85, 2.75, and 2.20 ppm). The spectrum also shows the correlation of the protons in the glycerol impurity (spectral region of 3.5−3.8 ppm). The 3.55 ppm resonance arising from the C-3 proton of the product is buried in the glycerol spectral envelope but can be seen correlated to two other protons (mct2 and mct-me).

The difference spectrum in Figure 6 was constructed as described in Experimental Procedures. It shows six negative resonances arising from the disappearance of the substrates L-allothreonine (resonances centered at 4.35, 3.83, and 1.19 ppm; at3, at2, and at4, respectively) and D,L-homocysteine (resonances centered at 3.87, 2.63, and 2.14 ppm; hc2, hc4, and hc3, respectively), as well as six positive signals from the formation of the product, 3-methyl-L-cystathionine (resonances centered at 3.94, 3.85, 3.55, 2.75, 2.20, and 1.27 ppm; mct2, mct6, mct3, mct4, mct5, and mct3-me, respectively). The negative signals are in excellent agreement with the spectrum of the substrates in Figure 4A, while the positive signals are in excellent agreement with the spectrum of the product in Figure 4B. The multiplet arising from the splitting of the C-3 proton (3.55 ppm) by adjacent α-proton and methyl resonances buried in Figure 4B is readily apparent here.

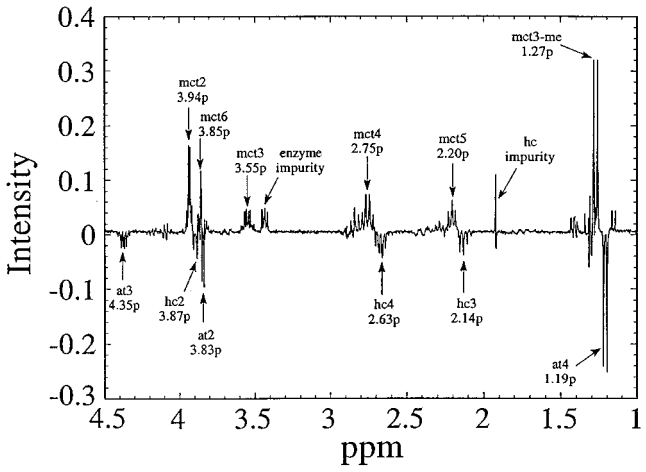


FIGURE 6: ^{1}H NMR difference spectrum of substrates L-allothreonine and D,L-homocysteine and the product 3-methylcystathionine. Peak abbreviations: at, L-allothreonine; hc, L-homocysteine; mct, 3-methylcystathionine; and me, the C-3 methyl group. The numbers after each abbreviation represent the carbon numbers of each compound.

DISCUSSION

A large number of PLP-dependent enzymes catalyze β-elimination and β-replacement reactions with amino acids that have an electronegative substituent in the β-position (*6*, *7*). The β-replacement reactions are thought to proceed through two stages, as shown in Scheme 1 for the reactions of cystathionine β-synthase. Stage I results in formation of an unsaturated imine (ES2 in Scheme 1) that can either add a different nucleophile than the one eliminated (β-replacement) or be converted to an α-keto acid and ammonia (β-elimination). Spectroscopic evidence for the transient appearance of the PLP aldimine of an unsaturated imine (λ_{max} = 455 nm), termed aminocrotonate or 3-methyl aminoacrylate, was first reported in the reaction of L-threonine deaminase with L-threonine (*23*). In these cases, the 455 nm band was not detected upon addition of L-allothreonine (*24*). Spectroscopic evidence for an unsaturated imine intermediate has also been obtained in the reactions of D-serine dehydratase (*25*), *O*-acetylserine sulfhydrylase (*26*), tryptophan synthase (*27*), and cystathionine β-synthase (*3*, *18*).

Our results show that a 3-methyl aminoacrylate intermediate is formed in the reaction of cystathionine β-synthase with L-allothreonine, but not with L-threonine, and that the 3-methyl aminoacrylate intermediate reacts with L-homocysteine to form 3-methyl-L-cystathionine (Figures 1−3). The absorbance, circular dichroism, and fluorescence maxima of the 3-methyl aminoacrylate intermediate are displaced 14 nm (from 460 to 446 nm) as compared to the corresponding values for the aminoacrylate intermediate formed with L-serine. The spectroscopic properties of the 3-methyl aminoacrylate intermediate provide a convenient probe for measuring the rates of formation and disappearance of this intermediate (Figure 2 and Table 1) and for measuring the apparent binding constant for L-allothreonine (Figure 3B and Table 1). The rapid-scanning stopped-flow results show that the binding of L-allothreonine as the external aldimine is faster than formation of the 3-methyl aminoacrylate intermediate.

The ^{1}H NMR spectrum in Figure 4B establishes that the product of the reaction of L-allothreonine and L-homocysteine is 3-methyl-L-cystathionine, a new amino acid. Since the stereochemistry of the β-replacement reaction of cystathionine β-synthase with L-serine and L-homocysteine proceeds with retention of configuration at C-3 (*28*), according to the arguments put forward by Tai and Cook (*29*), the mechanism of the reaction should occur via an E_1 syn pathway. The β-replacement reactions catalyzed by *O*-acetylserine sulfhydrylase (*30*) and tryptophan synthase (*31*) also proceed stereospecifically with retention of configuration at C-3. A closely related amino acid, γ-methyl-L-cystathionine, has been identified by ^{1}H NMR to be the product of the reaction of cystathionine γ-synthase with L-allylglycine and L-cysteine (*32*).

The specificity of the reaction of cystathionine β-synthase for L-allothreonine rather than L-threonine likely has steric origins. Although an X-ray structure of human cystathionine β-synthase has recently been published (*17*), a structure of the enzyme with bound ligands is not yet available. Therefore, we have attempted to explore specificity by modeling the L-threonine external aldimine structure using the known structures of external aldimines reported for *O*-acetylserine sulfhydrylase, which is most closely related to cystathionine β-synthase, and for tryptophan synthase. When the L-threonine external aldimine is modeled into the *O*-acetylserine sulfhydrylase active site using the coordinates for the external aldimine of L-methionine (*33*), the L-threonine methyl group of the external aldimine structure occupies a position that is severely sterically hindered by clashes with the PLP phosphoryl group, PLP C-5′, and PLP C-4′. These steric clashes would not occur in the corresponding L-allothreonine structure. A similar modeling result was found using the structures of the external aldimines of tryptophan synthase (*34*). The specificities of *O*-acetylserine sulfhydrylase[4] and tryptophan synthase[5] for L-allothreonine and L-threonine are currently under investigation. L-Threonine, but not L-allothreonine, serves as an S-substituent acceptor in a β-replacement reaction of tryptophan synthase with thiobenzyl alcohol (*35*). However, the rate of this reaction is only ∼1% of the rate of the analogous reaction with L-serine. Consequently, a detailed explanation of the yeast T-CBS specificity must await further structure determinations.

3-Methyl-L-lanthionine, which has one fewer carbon atom than 3-methyl-L-cystathionine, has been identified as an amino acid component in the polypeptide chain of lantibiotics (*36*). Lantibiotics are a group of antibiotics that are produced by bacteria and that have high antimicrobial activity (*36*, *37*). The lantibiotics are formed from a ribosomally synthesized prepeptide by posttranslational modification reactions that generate the unusual thioether amino acids lanthionine and 3-methyllanthionine. In these reactions, the hydroxyl groups of L-serine and L-threonine are first dehydrated to yield the αβ-unsaturated amino acids 2,3-didehydroalanine and 2,3-didehydrobutyrine, respectively, which undergo an intramolecular Michael addition with neighboring Cys residues to form thioether-bridged dicarboxy di-amino acids (*36*). These reactions are similar to the β-elimination and

[4] Unpublished results of Paul F. Cook, University of Oklahoma, Norman, OK.

[5] Unpublished results of Dimitri Niks, University of California, Riverside, CA.

β-replacement reactions shown in Scheme 1. Since the deduced amino acid sequences of the enzymes involved in the lantibiotic Pep5 biosynthetic gene cluster are not at all similar to known sequences (*37*), the enzymes are not homologous to known PLP-dependent enzymes.

Our results may provide a practical method for the synthesis of 3-methyl-L-cystathionine, which is a potentially useful analogue of L-cystathionine. The closely related enzyme, tryptophan synthase, has proven to be useful for the synthesis of a wide variety of amino acids, including *S*-(β-hydroxyethyl)-L-cysteine (*38*, *39*), *S*-benzyl-L-cysteine (*35*), aza-L-tryptophans (*40*), chloro-L-tryptophans (*41*), selenoloalanines (*42*), and dihydroiso-L-tryptophan (*43*, *44*).

ACKNOWLEDGMENT

We thank Dr. Dan Borchardt for his assistance in collection and analysis of the ^{1}H NMR data and Donald Keidel for his modeling work with Insight II.

REFERENCES

1. Mudd, S. H., Levy, H. L., and Skovby, F. (1995) in *The Metabolic Basis of Inherited Disease* (Scriver, C. R., Beaudet, A. L., Sly, W. S., and Valle, D., Eds.) 6th ed., pp 1272−1327, McGraw-Hill, New York.
2. Refsum, H., Ueland, P. M., Nygard, O., and Vollset, S. E. (1998) *Annu. Rev. Med. 49*, 31−62.
3. Jhee, K.-H., McPhie, P., and Miles, E. W. (2000) *J. Biol. Chem. 75*, 11541−11544.
4. Maclean, K. N., Janosik, M., Oliveriusova, J., Kery, V., and Kraus, J. P. (2000) *J. Inorg. Biochem. 81* (3), 161−171.
5. Kery, V., Bukovska, G., and Kraus, J. P. (1994) *J. Biol. Chem. 269* (41), 25283−25288.
6. Davis, L., and Metzler, D. E. (1972) in *The Enzymes* (Boyer, P. D., Ed.) Vol. 7, pp 33−74, Academic Press, New York.
7. Miles, E. W. (1986) in *Pyridoxal Phosphate: Chemical, Biochemical and Medical Aspects, Part B* (Dolphin, D., Poulson, D., and Avramovic, O., Eds.) Vol. 1B, pp 253−310, John Wiley and Sons, New York.
8. Jhee, K.-H., McPhie, P., and Miles, E. W. (2000) *Biochemistry 39* (34), 10548−10556.
9. Jhee, K.-H., Niks, D., McPhie, P., Dunn, M. F., and Miles, E. W. (2001) *Biochemistry 40* (36), 10873−10880.
10. Kery, V., Poneleit, L., and Kraus, J. P. (1998) *Arch. Biochem. Biophys. 355* (2), 222−232.
11. Shan, X., and Kruger, W. D. (1998) *Nat. Genet. 19* (1), 91−93.
12. Nozaki, T., Shigeta, Y., Saito-Nakano, Y., Imada, M., and Kruger, W. D. (2001) *J. Biol. Chem. 276* (9), 6516−6523.
13. Alexander, F. W., Sandmeier, E., Mehta, P. K., and Christen, P. (1994) *Eur. J. Biochem. 219*, 953−960.
14. Grishin, N. V., Phillips, M. A., and Goldsmith, E. J. (1995) *Protein Sci. 4* (7), 1291−1304.
15. Finkelstein, J. D., and Harris, B. (1975) *Arch. Biochem. Biophys. 171* (1), 282−286.
16. Kabil, O., Taoka, S., LoBrutto, R., Shoemaker, R., and Banerjee, R. (2001) *J. Biol. Chem. 276*, 19350−19355.
17. Meier, M., Janosik, M., Kery, V., Kraus, J. P., and Burkhard, P. (2001) *EMBO J. 20* (15), 3910−3916.
18. Bruno, S., Schiarettti, F., Burkhard, P., Kraus, J. P., and Mozzarelli, A. (2001) *J. Biol. Chem. 276* (1), 16−19.
19. Drummond, J. T., Jarrett, J., Gonzalez, J. C., Huang, S., and Matthews, R. G. (1995) *Anal. Biochem. 228* (2), 323−329.
20. Spectrum 11978M (1971) Sadtler Research Laboratories, Philadelphia.
21. Spectrum 30761M (1980) Sadtler Research Laboratories, Philadelphia.
22. Woehl, E. U., Tai, C. H., Dunn, M. F., and Cook, P. F. (1996) *Biochemistry 35* (15), 4776−4783.
23. Tokushige, M., Nakazawa, A., Shizuta, Y., Okada, Y., and Hayaishi, O. (1968) in *Symposium on Pyridoxal Enzymes* (Yamada, K., Katunuma, N., and Wada, H., Eds.) pp 105−108, Maruzen, Tokyo.
24. Rabinowitz, K. W., Niederman, R. A., and Wood, W. A. (1973) *J. Biol. Chem. 248*, 8207−8215.
25. Schnackerz, K. D., Ehrlich, J. H., Giesemann, W., and Reed, T. A. (1979) *Biochemistry 18*, 3557−3563.
26. Cook, P. F., and Wedding, R. T. (1976) *J. Biol. Chem. 251*, 2023−2029.
27. York, S. S. (1972) *Biochemistry 11* (14), 2733−2740.
28. Borcsok, E., and Abeles, R. H. (1982) *Arch. Biochem. Biophys. 213*, 695−707.
29. Tai, C.-H., and Cook, P. F. (2001) *Acc. Chem. Res. 34*, 49−59.
30. Floss, H. G., Schleicher, E., and Potts, R. (1976) *J. Biol. Chem. 251* (18), 5478−5482.
31. Tsai, M. D., Schleicher, E., Potts, R., Skye, G. E., and Floss, H. G. (1978) *J. Biol. Chem. 253* (15), 5344−5349.
32. Brzovic′, P., Holbrook, E. L., Greene, R. C., and Dunn, M. F. (1990) *Biochemistry 29*, 442−451.
33. Burkhard, P., Tai, C. H., Ristroph, C. M., Cook, P. F., and Jansonius, J. N. (1999) *J. Mol. Biol. 291* (4), 941−953.
34. Rhee, S., Parris, K. D., Hyde, C. C., Ahmed, S. A., Miles, E. W., and Davies, D. R. (1997) *Biochemistry 36* (25), 7664−7680.
35. Esaki, N., Tanaka, H., Miles, E. W., and Soda, K. (1983) *Agric. Biol. Chem. 47*, 2861−2864.
36. Guder, A., Wiedemann, I., and Sahl, H. G. (2000) *Biopolymers 55* (1), 62−73.
37. Meyer, C., Bierbaum, G., Heidrich, C., Reis, M., Suling, J., Iglesias-Wind, M. I., Kempter, C., Molitor, E., and Sahl, H. G. (1995) *Eur. J. Biochem. 232* (2), 478−489.
38. Goldberg, M. E., and Baldwin, R. L. (1967) *Biochemistry 6* (7), 2113−2119.
39. Miles, E. W., Hatanaka, M., and Crawford, I. P. (1968) *Biochemistry 7* (8), 2742−2753.
40. Sloan, M. J., and Phillips, R. S. (1992) *Bioorg. Med. Chem. Lett. 2* (9), 1053−1056.
41. Lee, M., and Phillips, R. S. (1992) *Bioorg. Med. Chem. Lett. 2* (12), 1563−1564.
42. Welch, M., and Phillips, R. S. (1999) *Bioorg. Med. Chem. Lett. 9* (5), 637−640.
43. Roy, M., Keblawi, S., and Dunn, M. F. (1988) *Biochemistry 27* (18), 6698−6704.
44. Ferrari, D., Yang, L.-H., Miles, E. W., and Dunn, M. F. (2001) *Biochemistry 40*, 7421−7432.

BI011756T