Bryant, D. A., Glazer, A. N., & Eiserling, F. A. (1976) *Arch. Microbiol. 110*, 61–75.
Csatorday, K., Guard-Friar, D., MacColl, R., & Berns, D. S. (1988) *Photochem. Photobiol. 47*, 285–291.
Gantt, E. (1980) *Int. Rev. Cytol. 66*, 45–80.
Gantt, E. (1981) *Annu. Rev. Plant Physiol. 32*, 327–347.
Guard-Friar, D., & MacColl, R. (1986) *Photochem. Photobiol. 43*, 81–85.
Guard-Friar, D., Hanzlik, C., & MacColl, R. (1989) *Biochim. Biophys. Acta 973*, 118–123.
Hiller, R. G., & Martin, C. D. (1987) *Biochim. Biophys. Acta 923*, 98–102.
MacColl, R., & Guard-Friar, D. (1983) *Biochemistry 22*, 5568–5572.
MacColl, R., & Guard-Friar, D. (1987) *Phycobiliproteins*, 218 pp, CRC Press, Boca Raton, FL.
MacColl, R., Guard-Friar, D., & Csatorday, K. (1983) *Arch. Microbiol. 135*, 194–198.
MacColl, R., Guard-Friar, D., Ryan, T. J., Csatorday, K., & Wu, P. (1988) *Biochim. Biophys. Acta 934*, 275–281.
Martin, C. D., & Hiller, R. G. (1987) *Biochim. Biophys. Acta 923*, 88–97.
O'Carra, P., Murphy, R. F., & Killilea, S. D. (1980) *Biochem. J. 187*, 303–309.
O'hEocha, C., O'Carra, P., & Mitchell, D. (1964) *Proc. R. Ir. Acad., Sect. B 63B*, 191–200.
Ong, L. J., Glazer, A. N., & Waterbury, J. B. (1984) *Science 224*, 80–83.
Saffo, M. B. (1987) *BioScience 37*, 654–664.
Scheer, H. (1981) *Angew. Chem., Int. Ed. Engl. 20*, 241–261.
Sidler, W., Kumpf, B., Suter, F., Morisset, W., Wehrmeyer, W., & Zuber, H. (1985) *Biol. Chem. Hoppe-Seyler 366*, 233–244.
Siebzehnrübl, S., Fischer, R., Kufer, W., & Scheer, H. (1989) *Photochem. Photobiol. 49*, 753–761.
Yu, M.-H., Glazer, A. N., Spencer, K. G., & West, J. A. (1981) *Plant Physiol. 68*, 482–488.
Zilinskas, B. A., & Greenwald, L. S. (1986) *Photosynth. Res. 10*, 7–35.
Zuber, H. (1985) *Photochem. Photobiol. 42*, 821–844.
Zuber, H. (1987) in *The Light Reactions* (Barber, J., Ed.) pp 197–259, Elsevier Science Publishers B. V. (Biomedical Division), Amsterdam.

Purification and Properties of Cystathionine γ-Synthase from Overproducing Strains of *Escherichia coli*†

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ABSTRACT: To characterize the methionine biosynthetic enzyme cystathionine γ-synthase from *Escherichia coli*, we have constructed high copy number plasmids containing the *metB* structural gene but lacking the closely linked *metJ* regulatory gene. When cloned into an appropriate strain, these plasmids can direct the overproduction of cystathionine γ-synthase such that about 10% of the soluble protein is this enzyme. An efficient purification scheme has been developed that has allowed us to obtain gram quantities of enzyme. The active form is a tetramer with subunits of about 40 000 daltons and one pyridoxal phosphate cofactor per monomer. The kinetic constants for several enzyme-catalyzed reactions were determined at 25 °C. The K_m value for the elimination reaction with *O*-succinyl-L-homoserine was calculated to be 0.33 mM with maximal velocity of 460 min^{-1}. The K_m for the elimination (deamination) reaction with vinylglycine was 5.6 mM with maximal velocity of 900 min^{-1}. The K_m values for the replacement reaction were calculated to be 1.0 mM for *O*-succinyl-L-homoserine and 0.05 mM for L-cysteine with maximal velocity of 700 min^{-1}. The enzyme shows an absorption band at 422 nm (ϵ = 8463 M^{-1} cm^{-1}) attributable to the Schiff base form of the pyridoxal phosphate cofactor. Steady-state spectra of reaction complexes show appearance of new longer wavelength absorbing materials during reaction with *O*-succinyl-L-homoserine, vinylglycine, or vinylglycine and L-cysteine. Reaction with *O*-succinyl-L-homoserine and L-cysteine produces only a red shift and slight reduction of the band at 422 nm.

Cystathione γ-synthase (EC 4.2.99.9), the product of the *metB* gene of *Escherichia coli*, catalyzes a γ-replacement reaction to form L-cystathionine and succinate from *O*-succinyl-L-homoserine and L-cysteine (eq 1), or in the absence of cysteine catalyzes a γ-elimination reaction to convert *O*-succinyl-L-homoserine to α-ketobutyrate, ammonia, and succinate (eq 2). The mechanism of the reactions catalyzed by

O-succinyl-L-homoserine + L-cysteine → L-cystathionine + succinate (1)

O-succinyl-L-homoserine + H_2O → α-ketobutyrate + ammonia + succinate (2)

the enzyme from *Salmonella typhimurium* has been the subject of several studies [for example, see Guggenheim and Flavin (1971) and Johnston et al. (1979)], but mechanistic

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questions remained unresolved [see Brzovič et al. (1990)]. Since we had cloned the *metB* gene from *E. coli* (Zakin et al., 1982), we decided to construct strains that overproduce its product to allow preparation of large amounts of enzyme for definitive mechanistic studies.

The *metB* gene, like other members of the methionine regulon, is subject to repression by the *metJ* gene product. Since *metJ* is adjacent to *metB* on the *E. coli* chromosome (Saint-Girons et al., 1984), the plasmids available to us had functional copies of both genes and the expression of *metB* was repressed. In this paper, we describe construction of plasmids in which deletion of the *metJ* regulatory region proximal to *metB* resulted in great overproduction of the *metB* gene product (~ 10% of the soluble cellular protein), purification of gram quantities of the enzyme from such strains, and properties of the pure enzyme.

Materials and Methods

Materials. D-Methionine sulfoxide was synthesized as described by Greene (1973). *Eco*RI was a gift of Dr. Ti-Sheng Young and Dr. Sung-Hou Kim (UC, Berkeley). Octyl-Sepharose CL-4B and Sephacryl S-200 were from Pharmacia. Bio-Gel P6DG was from Bio-Rad. Ammonium sulfate solutions were prepared according to the tables of Wood (1976).

Microbiological Procedures. Growth of cells, preparation of plasmid DNA, transformation, and other related procedures were done by standard methods as described in Miller (1972) or Maniatis et al. (1982), sometimes with minor modifications. Digestion of pRCG134 (10 μg) with *Bst*EII was at 60 °C using 3 units of enzyme in 0.1 mL for 16 h followed by phenol extraction and ethanol precipitation. The BAL-31 reaction was done in 60 μL using 2 μg of DNA and 0.8 unit of enzyme incubated at 30 °C for 3, 6, or 9 min, followed by addition of 0.5 M EDTA[1] to a final concentration of 0.12 M, phenol extraction, and precipitation with ethanol. The DNA (0.4 μg) was ligated with 3 units of BRL T4 DNA ligase in 20 μL for 21 h at room temperature.

Assay of Cystathionine γ-Synthase. The elimination reaction to form α-ketobutyrate, succinate, and ammonia from *O*-succinyl-L-homoserine (OSHS) was used for routine enzyme assay. The formation of α-ketobutyrate was monitored by measurement of the lactate dehydrogenase catalyzed oxidation of reduced nicotinamide adenine dinucleotide (NADH). One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of α-ketobutyrate in 1 min.

To measure the enzyme activities in toluenized cells or crude cell extracts, the two-step assay of Kaplan and Flavin (1966a) was used. Cells or cell extracts were incubated with 5 mM OSHS in 0.2 M Tris-HCl, pH 8.1, and 0.25 mM pyridoxal 5′-phosphate (PLP) at 37 °C. The reaction was stopped with perchloric acid, cellular debris and protein were pelleted, and the supernate was neutralized with potassium carbonate. The α-ketobutyrate present in aliquots (25–200 μL) of the supernate was assayed by using lactate dehydrogenase in 50 mM potassium phosphate, pH 7.5, with 0.25 mg/mL NADH (Meister, 1950). This procedure was also used with 50 mM potassium pyrophosphate buffers at 25 °C to determine the optimum pH for α-ketobutyrate formation.

For enzyme samples that had been purified beyond the ammonium sulfate precipitation step, a continuous assay of elimination was used at room temperature (Kaplan & Flavin, 1966a), where the oxidation of NADH was measured spectrophotometrically at 340 nm in 1.0 mL containing 50 mM potassium pyrophosphate, pH 8.2, 12.5 μM PLP, 0.3 mM NADH, 1.25 mM L-OSHS (or 2.5 mM *O*-succinyl-DL-homoserine), and 0.2 mg of beef heart lactate dehydrogenase.

Cystathionine formation catalyzed by cystathionine γ-synthase was assayed by using a modification of the procedure previously described (Krueger et al., 1978). Enzyme was preincubated in a solution of 50 mM potassium pyrophosphate, pH 7.8, 1 mM L-cysteine, and 10 mM DTT in a total volume of 0.1 mL. The reaction was initiated by the addition of 1 mM *O*-succinyl-L-[^{14}C]homoserine (0.5 mCi/mmol) (Greene, 1977) and then incubated at 25 °C for 10–30 min. The reaction was stopped by the addition of 0.9 mL of 3% perchloric acid containing 1 mM carrier L-cystathionine; 0.8 mL was applied to a column (4 × 40 mm) of Dowex 50-X8(H) and washed with 0.3% Brij 35. After substrate was removed by washing the column with 1 N HCl (4 × 2.5 mL), product was eluted with 2.5 mL of 2 N ammonia. Two milliliters of product eluate was acidified with 1 mL of 4.8 N HCl and counted in 5 mL of RPI 3a70B complete counting cocktail with a Packard Tri-Carb liquid scintillation spectrometer. One unit is defined as the amount of enzyme that catalyzes the formation of 1 μmol of cystathionine in 1 min.

Protein concentration was determined by the microbiuret method using bovine serum albumin as a standard (Greene et al., 1973).

Growth of Plasmid-Bearing Strains for Enzyme Purification. Strain RG326(pLLH346) was grown at 37 °C in Davis–Mingioli (Davis & Mingioli, 1950) minimal glucose medium without citrate, supplemented with 0.4 mM D-methionine sulfoxide, 2 μg/mL pyridoxine, 5 μg/mL thiamin, and 15 μg/mL tetracycline with vigorous shaking (150 rpm) until an absorbance of 3.0 at 550 nm was reached (typically 18 h). Cells were harvested and washed with 20 mM Tris-HCl, pH 7.6. The cell pellets, which were noticeably yellow due to overproduction of the PLP-containing cystathionine γ-synthase, were stored at −70 °C until processed. The yield was 4.5–5 g of cells (wet weight) per liter.

Purification of Cystathionine γ-Synthase from E. coli. All steps were done at 4 °C unless specified otherwise.

(*a*) *Crude Extract.* Cells (50–75 g) were disrupted by sonication (30 min in 10-min bursts on an ice bath) in 2 volumes of phosphate buffer (50 mM potassium phosphate, pH 7.2, 1 mM EDTA) containing 0.05 mM PLP, 1 μg/mL DNAse I, and 50 μg/mL phenylmethanesulfonyl fluoride. Cell debris was pelleted and discarded.

(*b*) *Streptomycin Sulfate Precipitation.* A half-volume of 10% streptomycin sulfate was slowly added to crude extract with stirring. After an additional 30 min of stirring, precipitated nucleic acids were removed by centrifugation.

(*c*) *Heat Treatment.* PLP was added to a concentration of 0.2 mM. One volume of boiling phosphate buffer containing 0.2 mM PLP was then added with continuous stirring to rapidly raise the temperature to 60 °C. The solution was placed in a 60 °C water bath for 3 min and was then rapidly cooled to 4 °C by placing it in a dry ice/ethanol bath with stirring. Denatured proteins were pelleted and discarded.

(*d*) *Ammonium Sulfate Precipitation.* The proteins in the heat-treated supernate were precipitated by the slow addition of solid ultrapure ammonium sulfate to a concentration of 3 M. The solution was stirred for 1 to several hours (up to overnight). Proteins were pelleted, and the yellow pellet was stored at −70 °C.

[1] Abbreviations: EDTA, ethylenediaminetetraacetic acid; OSHS, *O*-succinyl-L-homoserine; NADH, nicotinamide adenine dinucleotide; PLP, pyridoxal 5′-phosphate; DTT, dithiothreitol; SDS, sodium dodecyl sulfate; PAGE, polyacrylamide gel electrophoresis; VG, D,L-vinylglycine, CS, cystathionine γ-synthase.

(*e*) *Octyl-Sepharose Chromatography.* The protein pellet from the previous step was resuspended in a volume of phosphate buffer sufficient to bring the ammonium sulfate concentration to 1.4 M. Half of the sample was pumped at 26 mL/h onto a 2.5 × 30 cm column of octyl-Sepharose preequilibrated with the same buffer. Absorbance of the column effluent at 280 nm was recorded. The column was washed with 1.4 M ammonium sulfate in phosphate buffer until a large protein peak had emerged. Cystathionine γ-synthase was then eluted with phosphate buffer containing 0.8 M ammonium sulfate. Active fractions from two columns were pooled, and the protein was precipitated by raising the ammonium sulfate concentration to 2.6 M.

(*f*) *Sephacryl S-200 Chromatography.* The protein pellet was resuspended in a small volume of phosphate buffer and centrifuged briefly to remove insoluble material. The sample was then subjected to slow ammonium sulfate fractionation by successively bringing the salt concentration to 1.4, 1.6, 1.8, and 2.0 M by addition of 3.8 M ammonium sulfate, pH 7. At this point, the protein sometimes formed crystals. The enzyme was recovered by centrifugation and resuspended in a small volume of phosphate buffer, and half of the sample was layered onto each of two 2.5 × 50 cm columns of Sephacryl S-200 preequilibrated in the same buffer. The flow rate was 4 mL/h, and elution of 280-nm-absorbing material was recorded. Active fractions were pooled, concentrated with ammonium sulfate, and then subjected to slow ammonium sulfate fractionation as described above. The enzyme was desalted by chromatography on Bio-Gel P6DG and stored at −70 °C in filtered phosphate buffer at 20 mg/mL. The total yield, from several of such preparations, was 900 mg of enzyme from 165 g (wet weight) of cells.

Sedimentation Equilibrium Centrifugation. The weight-average molecular weight of the native enzyme was measured by equilibrium sedimentation in a Beckman Model E ultracentrifuge equipped with optical scanner and monochromator. The sample was in phosphate buffer at a concentration of 0.2 mg/mL. Equilibrium was reached at rotor speeds of 12 000 and 15 000 rpm. Optical scans were recorded at 280 and 420 nm. Molecular weight was calculated from the sedimentation data by using the partial specific volume (0.7351) calculated from the amino acid composition of the protein (Cohn & Edsall, 1943) and a standard least-squares fitting procedure (Bevington, 1969).

Spectroscopic Studies. Absorption spectra were obtained by using a Hewlett-Packard 8452A diode array spectrophotometer at an enzyme concentration of 0.5 mg/mL (3.9 μM) in phosphate buffer. Steady-state spectra of enzyme plus substrates were recorded after addition of substrates at 20–25 mM to a concentration of 10 mM.

Results and Discussion

Construction of Strains Overproducing Cystathionine γ-Synthase. Previous attempts to construct strains that overproduce cystathionine γ-synthase failed because high copy number plasmids carrying *metB* (e.g., pRCG134; Zakin et al., 1982) also carried *metJ*, the gene for the aporepressor of the *met* regulon. Strains transformed with such plasmids require methionine for growth because they contain an elevated level of repressor that shuts down the *met* regulon (Smith & Greene, 1984). We therefore found it necessary to delete *metJ* coding sequences to allow full expression of the cloned *metB* gene. In addition to bacterial and plasmid DNA, pRCG134 carries a short segment of λ DNA adjacent to the *metJ* gene. This segment contains three *Bst*EII sites, which are convenient for production of a linear fragment to be digested with BAL-31.

Table I: Cystathionine γ-Synthase Activities of MS100 or RG326 Transformants

plasmid	deletion size[b] (kb)	enzyme activities of crude extracts[a] (milliunits/mg) MS100	RG326
pLLH304	0.1	50	895
pLLH305	0.1	37	1190
pLLH309	0.1	75	100
pLLH312	0.8	29	717
pLLH340	0.9	62	1135
pLLH346	0.9	36	1914
pLLH353	0.2	32	1150
pLLH354	0.2	32	1484
pLLH604	0.5	16	830
pLLH620	0.7	11	653
pLLH621	0.9	21	1290
pLLH631	0.5	32	608
pLLH653	0.5	12	741
pLLH924	0.9	nd	372
pLLH925	0.6	19	753
pLLH951	0.9	49	1456
pLLH955	0.9	61	1308
pRCG134	none	1	nd
pLLH1	none	nd	32
none		nd	86

[a] Measured by the two-step assay at 37 °C. [b] Determined from the size of *Mlu*I A and *Rsa*I B fragments by gel electrophoresis.

Digestion of plasmid pRCG134 with *Bst*EII produced three fragments, two small (1.25 and 1.35 kb) and one large (6.47 kb). The large fragment contains the *E. coli metB*, *metJ*, and *metL* genes along with plasmid maintenance and tetracycline resistance genes. The *Bst*EII sites at both ends of the large fragment have the same sequence, allowing it to be directly circularized by treatment with DNA ligase to produce pLLH1. The structure of pLLH1 deduced from the known sequences of its components (Sutcliffe, 1979; Duchange et al., 1983; Saint-Girons et al., 1984) is given in Figure 1. The *Bst*EII digest was treated with BAL-31 under conditions expected to remove less than 2 kb of DNA (Gray et al., 1975). At 3, 6, and 9 min, samples were removed, concentrated, and treated with T4 DNA ligase. The reaction mixtures were used to transform calcium chloride treated cells of strain MM294 [*endA*, *hsdR*, *thi*, *pro*, *r*(*k*)] (Roberts et al., 1979) which exhibits high transformation efficiency. MM294 transformants were selected for growth on methionine-free tetracycline plates. MM294(pLLH1) or MM294(pRCG134) does not grow on such plates without addition of methionine to overcome *metJ*-mediated repression of the chromosomal *met* regulon. Of 165 clones tested, 51 were found to harbor plasmids with deletions in the *metJ* gene (12, 16, and 23 clones recovered after BAL-31 treatment for 3, 6, and 9 min, respectively). Each of these was then used to transform strain MS100 (*metB1*, *relA1*, *recBC*) (Smith & Greene, 1984) to methionine prototrophy by selection on methionine-free tetracycline plates. From the 51 transformations, 17 yielded clones that grew on the selective medium, indicating that the plasmids contained functional copies of the *metB* gene (8, 5, and 4 clones recovered after treatment for 3, 6, and 9 min, respectively, with BAL-31). Plasmids purified from such strains were used to transform RG326 (*metA26*, *metJ97*, *thi*, *lacY1*, *lacZ4*, *galK2*, *tsx6*, *xyl*) (Greene et al., 1973) to tetracycline resistance. The resulting constructs were used as material from which to purify the *metB* gene product, cystathionine γ-synthase.

Table I gives the enzyme activities of MS100 and RG326 transformants of the deletion plasmids and of control strains transformed with pRCG134 or pLLH1. The approximate sizes of the deletions as measured by changes in the electro-

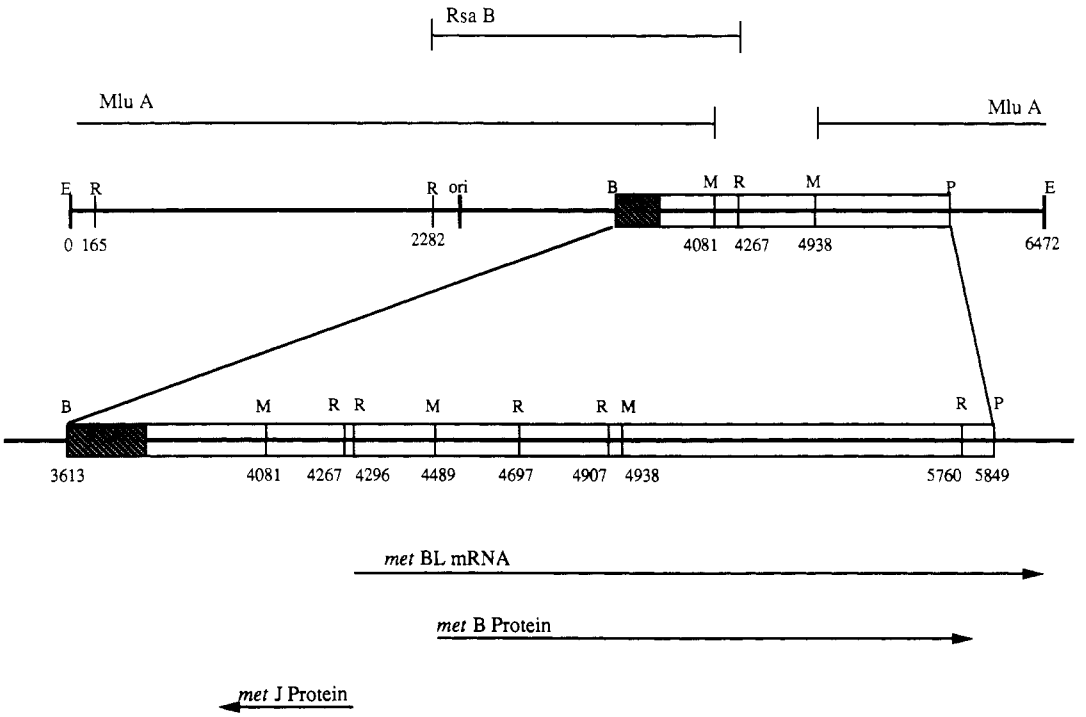

FIGURE 1: Restriction sites used in mapping deletions. Plasmid pLLH1 is shown with the relevant restriction sites indicated. Bacterial DNA is indicated by the triple line and phage DNA by the shaded area. The region between the *Bst*EII and *Pvu*I sites is enlarged to show restriction sites left out of the upper drawing for simplicity. The MluA (5615 bp) and RsaB (1985 bp) fragments are designated. The start site (4352; Duchange et al., 1983) and direction of the *metBL* transcript are indicated as well as the translational reading frames for the *metB* (4486–5646; Duchange et al., 1983) and *metJ* (4209–3892; Saint-Girons et al., 1984) products. B, *Bst*EII; E, *Eco*RI; M, *Mlu*I; P, *Pvu*I; R, *Rsa*I.

phoretic migration of the *Mlu*I A (5615 bp in pLLH1) or *Rsa*I B (1985 bp in pLLH1) restriction fragments are also listed. With the possible exception of pLLH309, all of the plasmid-borne *metB* genes are repressible. The cystathionine γ-synthase activities among the RG326 transformants range from 100 milliunits/mg of protein (pLLH309) to 1914 milliunits/mg of protein (pLLH346), with no close relationship between specific activity and extent of deletion. Since BAL-31 digests both ends of the linear DNA fragment, the extent of *metJ* deletion is unknown. With two exceptions, all of the plasmids retain the *Mlu*I site in the *metJ* coding sequence (position 4081 in Figure 1). pLLH312 lacks that *Mlu*I site, but retains the *Rsa*I site at position 4296, while pLLH621 lacks both of these restriction sites. Because of the selection procedure, it is also possible that some of the plasmids may have spontaneous mutations in *metJ*. Without being certain of the significance of the differences in specific activities of the strains, we chose RG326(pLLH346) to use for enzyme purification.

Purification of Cystathionine γ-Synthase. The enzyme was purified from RG326(pLLH346) cells that had been grown on medium supplemented with 0.4 mM D-methionine sulfoxide. Methionine-limited growth was used to allow amplification of the plasmid as well as to relieve any possible methionine-mediated inhibition of enzyme synthesis. A *metA* mutant was used since Savin et al. (1972) have reported that a *metA* strain of *S. typhimurium* makes higher amounts of cystathionine γ-synthase than the wild type or other types of methionine auxotrophs. The results of a typical fractionation from RG326(pLLH346) are shown in Table II. In the preparation described, steps 5–7 were done on half the material obtained from step 4. The octyl-Sepharose chromatography usually gave at least 4-fold purification when less-rich sources were used. When extracts from overproducing strains were used, the octyl-Sepharose eluates are about 80–90% pure. Cystathionine γ-synthase was purified about 9-fold over the starting material with a 60% yield. Several such preparations yielded 900 mg of enzyme from fractionation of extracts from 165 g of cells. This material had a specific activity of 9 units/mg. When subjected to ammonium sulfate back extraction, the specific activity was raised to 10 with little activity loss. Kaplan and Flavin (1966a) and Johnston et al. (1979) reported specific activities of 18.2 and 20, respectively, at 37 °C. This is comparable to our material, considering the difference in assay temperature. After the material was desalted on Bio-Gel P6DG, it was used for the spectral analyses described in Brzović et al. (1990). SDS–PAGE of purified enzyme is shown in Figure 2. The preparation shows one major band and a few impurities composing no more than 1–2% by comparison of intensities of impurity bands to those of smaller amounts of the major band (data not shown).

Table II: Purification of Cystathionine γ-Synthase from RG326(pLLH346)

step	mL	units[a]	mg	units/mg	% yield
(1) crude extract, 71.1 g of cells	180	9773	8568	1.14	100
(2) 3.3% streptomycin sulfate	265	11130	6612	1.68	114
(3) heat treatment 3 min, 60 °C	530	9184	5088	1.81	94
(4) 3 M ammonium sulfate pellet		8720	4188	2.08	89
(5) octyl-Sepharose (half of step 4)	127	4320	nd	nd	88
(6) ammonium sulfate fractionation	10	3400	nd	nd	70
(7) Sephacryl S-200	85	2870	319	9.0	60

[a] Activity was measured by the two-step assay at 37 °C for steps 1–4 and by the continuous assay at 25 °C for steps 5–7. For ease of comparison, the unit values in steps 1–4 have been corrected for the difference in assay procedures by dividing by a factor of 1.5.

Molecular Weight. The enzyme migrated as a single major band corresponding to a polypeptide of 39 000 when subjected

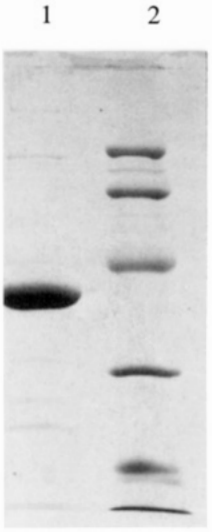

FIGURE 2: SDS–polyacrylamide (12%) gel electrophoresis of purified cystathionine γ-synthase. Denaturing gels were run according to the method of Laemmli (1970) without the addition of SDS to the gel. (Lane 1) 5 μg of enzyme. (Lane 2) Molecular weight standards (Bio-Rad): phosphorylase *b* (97 400), bovine serum albumin (66 200), ovalbumin (42 700), carbonic anhydrase (31 000), and soybean trypsin inhibitor (21 500).

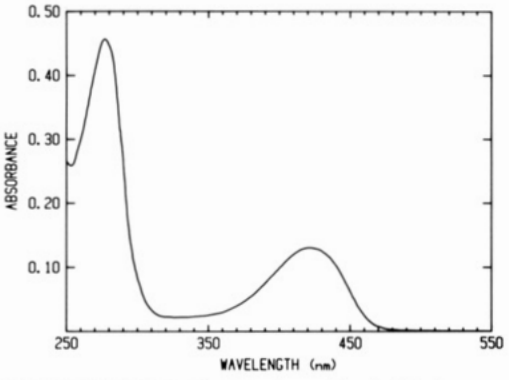

FIGURE 3: UV–visible absorption spectrum of cystathionine γ-synthase, 0.5 mg/mL, in 50 mM potassium phosphate, pH 7.2, and 1 mM EDTA.

to SDS–polyacrylamide gel electrophoresis (Figure 2). This is comparable to the value of 40 000 determined for the enzymes from *E. coli* (Tran et al., 1983) and *S. typhimurium* (Kaplan & Flavin, 1966b). From the DNA sequence of the *E. coli* gene, the subunit was calculated to be 41 503 (Duchange et al., 1983).

Sedimentation equilibrium analysis yielded an average value of 160 184 (standard deviation = 1169) for the tetramer. This agrees with the value of 160 000 determined by gel filtration for the enzyme from *E. coli* (Tran et al., 1983) and by sedimentation equilibrium for the enzyme from *S. typhimurium* (Kaplan & Flavin, 1966b). Therefore, the native enzymes of both bacterial species are tetramers of identically sized subunits. Cross-linking studies with dimethyl suberimidate confirmed the tetrameric structure of the native enzyme (Holbrook, 1984).

Spectra. The native enzyme displays absorption peaks at 278 and 422 nm as shown in Figure 3. The longer wavelength peak is typical of PLP enzymes and due to the cofactor in a Schiff base form with lysine 198 (Martel et al., 1987). An approximate extinction coefficient of the enzyme-bound Schiff base of PLP is 8463 M^{-1} cm^{-1} for the monomer or 33 850 M^{-1} cm^{-1} for the tetramer. The ratio of absorbances at 278 and 422 nm is approximately 3.6. The PLP content of the enzyme, determined spectrophotometrically after treatment of the enzyme with perchloric acid and phenylhydrazine by the method of Wada and Snell (1961), is 1 mol/40 kg of protein (1 mol of subunit), or 4 molecules of cofactor/tetramer. The *S. typhimurium* enzyme also contains one cofactor per subunit with molar absorbance at 422 nm of 39 000 M^{-1} cm^{-1} and ratio of absorbances at 280 and 422 nm of 3.85 (Kaplan & Flavin, 1966b).

Table III: Kinetic Parameters of Cystathionine γ-Synthase

	substrate	K_m (mM)	k_{cat},[a] (min^{-1})
elimination	OSHS	0.33	460
	VG	5.6	900
γ-replacement[b]	OSHS	1.0	700
	cysteine	0.05	

[a] Moles of product formed per mole of subunit at 25 °C. [b] Calculated from data using the Ping-Pong program of Cleland (1979) fit to the equation $v = V_m AB/(K_{mA}B + K_{mB}A + AB)$, where v is velocity, V_m is maximal velocity (k_{cat}), K_m is the Michaelis–Menten constant, A is OSHS, and B is cysteine.

The native enzyme exhibits positive circular dichroism with a maximum at 422 nm and $[\Theta]$ = 185 deg cm^2 $dmol^{-1}$ (Holbrook, 1984).

Steady-State Kinetics. The steady-state kinetics of the elimination reactions were measured at room temperature (~25 °C) and pH 8.2 by using either OSHS or DL-vinylglycine (VG) as substrates. As shown in Table III, the K_m for OSHS was calculated to be 0.33 mM, and maximum velocity (k_{cat} or turnover number) was 460 min^{-1} (moles of product formed per mole of subunit). These values are to be compared to those obtained for the enzyme isolated from *S. typhimurium* (Guggenheim & Flavin, 1971; Kaplan & Flavin, 1966a), which showed a K_m of 0.3 mM and a turnover number of 750 min^{-1} at 37 °C. The higher temperature accounts for the faster reaction rate. With the alternate substrate VG, the K_m is 5.6 mM, much higher than that for the normal substrate. The maximum velocity is 900 min^{-1}, about twice as fast as the elimination reaction from OSHS. Johnston et al. (1979), working with the enzyme from *S. typhimurium*, reported a K_m of 5.2 mM for VG and a k_{cat} of 1770 min^{-1} at 37 °C (about twice as fast as elimination from OSHS). Considering the differences in temperature, these values are very similar.

L-Cystathionine (the product of the replacement reaction) did not inhibit the elimination reaction at concentrations up to 1.5 mM (with OSHS concentrations from 0.2 to 2.5 mM). We did not test higher concentrations due to the limited solubility of cystathionine, but Guggenheim and Flavin (1969) report that cystathionine is a competitive inhibitor of the *S. typhimurium* enzyme, with a K_i of 5 mM.

The optimum pH for γ-elimination from OSHS (5 mM) was determined by measuring reaction rates using the two-step assay over the pH range 6.8–10.2. The velocity increased to about pH 8.8 and then remained constant. The optimum reported for the *S. typhimurium* enzyme of pH 8.2 (Kaplan & Flavin, 1966a) used a continuous coupled assay and therefore represented a compromise between the optima for the formation of α-ketobutyrate by cystathionine γ-synthase and for the oxidation of NADH by lactate dehydrogenase.

As was shown for the *S. typhimurium* cystathionine γ-synthase (Guggenheim & Flavin, 1971), addition of various amounts of cysteine (0.5–5 mM) to a mixture of the *E. coli* enzyme and OSHS changes the reaction from elimination to replacement, as indicated by the transient cessation of α-ketobutyrate production (not shown).

Formation of L-[^{14}C]cystathionine from L-cysteine and *O*-succinyl-L-[^{14}C]homoserine by the replacement reaction

(30-min incubation at 25 °C) was linear with enzyme concentrations up to 3 μg/mL. The optimum pH for the replacement reaction was determined by measuring reaction velocities over the pH range 6.8–9.0. Maximum velocity was observed at pH 7.8 with inflection points at pH 7.4 and 8.7. The value for the optimum pH agrees with that determined for the enzyme from *S. typhimurium* (Kaplan & Flavin, 1966a). No product inhibition was observed with either cystathionine (3 mM) or succinate (60 mM).

The K_m values for both substrates and the maximum velocity (or k_{cat}) were determined by varying both cysteine and OSHS concentrations. The double-reciprocal plots from such a set of experiments have approximately parallel lines, consistent with a ping-pong mechanism commonly seen with PLP enzymes. The data were fit to initial velocity equations by using the program Ping-Pong (Cleland, 1979). The best fit resulted from a ping-pong model where OSHS binds first: the K_m for OSHS was 1.0 mM and for cysteine 0.05 mM, and the maximal velocity was 700 min^{-1}, approximately twice that observed for the elimination reaction using OSHS. The K_m values are similar to those reported by Kaplan and Flavin (1966a) and Johnston et al. (1979) for the *S. typhimurium* enzyme (0.2–0.3 and 0.07 mM), but the maximal velocity at 25 °C (700 min^{-1}) is lower than might be expected since, with the enzyme from *S. typhimurium* at 37 °C, the rate of the replacement reaction has been reported to be 5 times (Kaplan & Flavin, 1966a) or 9 times (Johnston et al., 1979) that of the elimination reaction. The reason for this discrepancy is not known. It is possible that the rate of the replacement reaction is affected by the reduction in temperature to a greater extent than that of the elimination reaction or that the difference in rates represents a real difference in the enzymes from the two organisms. Alternatively, the apparent discrepancy may reflect differences in the assay procedures employed (others assay the disappearance of cysteine rather than the formation of cystathionine) or inhibitory impurities present in the substrates. In any case, the enzyme used during these studies is fully active as evidenced by the virtually complete loss of the 422-nm peak upon reaction with OSHS and full recovery after substrate depletion.

Steady-State Spectra of Reaction Complexes. As shown in Figure 4, spectra of the enzyme taken about 10 s after mixing with OSHS or VG, with or without cysteine, are similar to those described by Guggenheim and Flavin (1971) or by Johnston et al. (1979). As also reported by these workers, after continued incubation, substrate depletion results in restoration of the original enzyme spectrum above 350 nm (not shown). In the steady-state reaction mixture with OSHS (Figure 4A), very little of the 422-nm-absorbing species remains and a small amount of material that absorbs between 460 and 500 nm appears. When the reaction mixture contains both OSHS and cysteine, there is a single peak that is red shifted and slightly less intense than that of the enzyme without substrates. The species that absorb near 500 nm are not detectible. Brzovič et al. (1990) have measured spectral changes over millisecond time periods with these substrates using rapid scanning stopped-flow spectrophotometry. They conclude that with OSHS as substrate the longer wavelength absorbing materials are intermediates in the elimination reaction and show that the presence of cysteine inhibits their accumulation at the shortest observable time period.

The steady-state reaction mixture with VG (Figure 4B) has more residual 422-nm absorbance than that with OSHS, which is probably due in part to enzyme without substrate, since VG has a higher K_m than OSHS. The amount of the longer

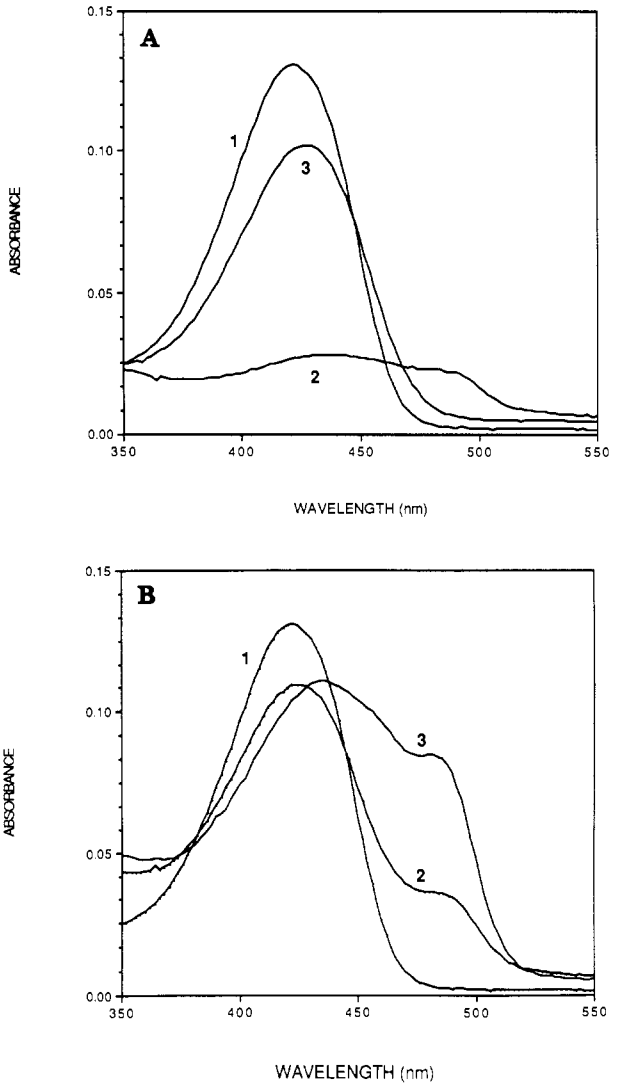

FIGURE 4: Spectra of steady-state reaction mixtures. (A) Curve 1, 3.9 μM cystathionine γ-synthase (CS); curve 2, 10 s after mixing 3.9 μM CS with 10 mM OSHS; curve 3, 10 s after mixing 3.9 μM CS with 10 mM OSHS and 10 mM L-cysteine. (B) Curve 1, 3.9 μM CS; curve 2, 10 s after mixing 3.9 μM CS with 10 mM VG; curve 3, 9 s after mixing 3.9 μM CS with 10 mM VG and 10 mM L-cysteine.

wavelength absorbing intermediates is higher in the VG reaction mixture, which suggests that these compounds are more readily formed from VG than from OSHS. In contrast to the results with OSHS, the addition of cysteine to the VG reaction mixtures causes a large increase in the absorbance near 500 nm and an apparent red shift of the shorter wavelength peak. Difference spectra (Figure 5) show that the enzyme VG reaction mixture has a peak that plateaus between 450 and 500 nm and that addition of cysteine increases the intensity of the peak while about the same shape is kept. The new peak appears to have two components. The long-wavelength peak of the OSHS reaction mixture may be similar to that of the VG reactions, but its intensity is too small for meaningful comparison.

In summary, with OSHS as substrate in the elimination reaction, accumulation of long-wavelength-absorbing intermediates is observed along with substantial reduction of the major visible peak of the unreacted enzyme (Figures 4A and 5). Addition of cysteine causes a complete shift from elimination to replacement (Guggenheim & Flavin, 1971; Holbrook, 1984). Concomitantly, the accumulation of the long-wavelength-absorbing intermediates ceases and a new species, slightly red shifted from the unreacted enzyme, appears

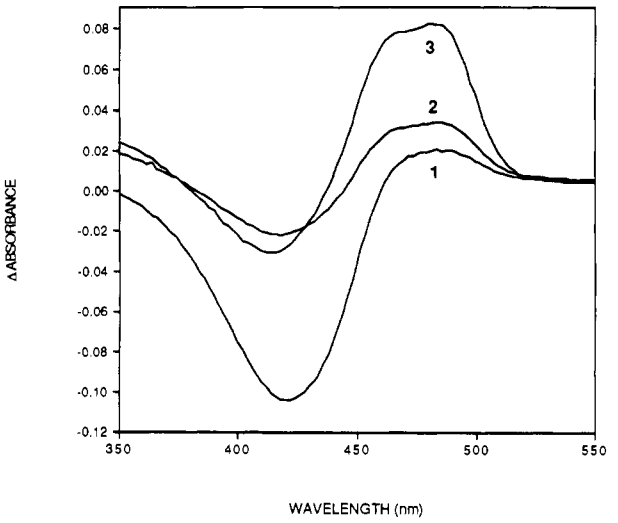

FIGURE 5: Difference spectra of steady-state reaction mixtures (spectrum of reaction mixture − spectrum of cystathionine γ-synthase). Curve 1, 10-s reaction mixture with OSHS; curve 2, 10-s reaction mixture with VG; curve 3, 9-s reaction mixture with VG and cysteine.

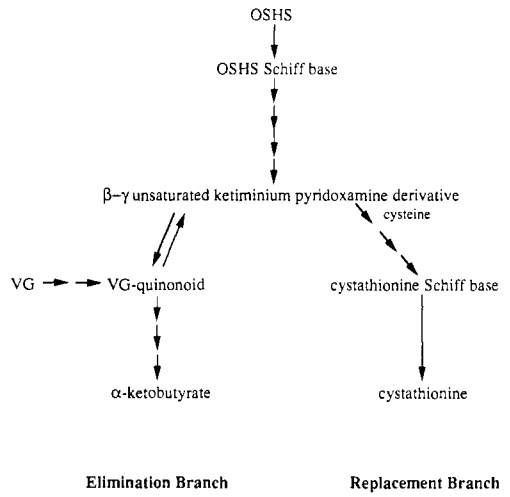

FIGURE 6: Schematic representation of elimination and replacement reactions catalyzed by cystathionine γ-synthase. This is a simplified version of Scheme II in Brzovič et al. (1990) with many intermediates deleted and implied by multiple arrows. Shown are the proposed partitioning intermediate (β–γ unsaturated ketiminium derivative of pyridoxamine) and the VG quinonoid (which is formed by removal of a proton from the VG pyridoxal Schiff base). The reversibility of reactions has not been specified.

(Figure 4A). Figure 6 shows a greatly simplified version of the reaction mechanism proposed by Brzovič et al. (1990). OSHS reacts with the enzyme to form a partitioning intermediate (β-γ unsaturated ketiminium pyridoxamine derivative), which then proceeds along a γ-elimination or a γ-replacement pathway depending on the availability of cysteine. The absorbance in the 460–500-nm region appears to be due to intermediates on the γ-elimination branch, most likely the α-aminocrotonate derivative of pyridoxal (derived by protonation of the VG quinonoid) (Brzovič et al., 1990). With VG instead of OSHS as substrate, larger amounts of the long-wavelength intermediates accumulate (Figures 4B and 5), which we propose results from the point of entry of VG into the reaction path on the elimination branch (see Figure 6). This proposal is consistent with the observation that the maximal rate of α-ketobutyrate formation is faster and that of cystathionine synthesis is slower (Johnston et al., 1979) when VG replaces OSHS as substrate. Instead of suppressing their formation (as is the case for OSHS), addition of cysteine to the enzyme VG reaction mixture greatly increases the steady-state concentration of long-wavelength-absorbing intermediates (Figures 4B and 5). It is likely that these intermediates are the same as those observed during reaction with VG or OSHS alone. If so, it appears that in addition to reacting as a cosubstrate cysteine changes the reactivity of the enzyme, which results in an increase in the accumulation of long-wavelength-absorbing intermediates from VG, even though these intermediates, on the elimination branch, do not directly react with cysteine.

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Registry No. CS, 9030-70-0; OSHS, 1492-23-5; VG, 52773-87-2; L-Cys, 52-90-4.

REFERENCES

Bevington, P. R. (1969) *Data Reduction and Error Analysis for the Physical Sciencies*, McGraw-Hill, New York.

Brzovič, P., Holbrook, E. L., Greene, R. C., & Dunn, M. F. (1990) *Biochemistry* (following paper in this issue).

Cleland, W. W. (1979) *Methods Enzymol. 63*, 103–137.

Cohn, E. J., & Edsall, J. T. (1943) *Proteins, Amino Acids and Peptides*, Reinhold, New York.

Davis, B. D., & Mingioli, E. S. (1950) *J. Bacteriol. 60*, 17–28.

Duchange, N., Zakin, M. M., Ferrera, P., Saint-Girons, I., Park, I., Tran, S. V., Py, M. C., & Cohen, G. N. (1983) *J. Biol. Chem. 258*, 14868–14871.

Gray, H. B., Jr., Ostrander, D. A., Hodnett, J. L., Legerski, R. J., & Robberson, D. L. (1975) *Nucleic Acids Res. 2*, 1459–1492.

Greene, R. C. (1973) *J. Bacteriol. 116*, 230–234.

Greene, R. C. (1977) *Anal. Biochem. 78*, 182–187.

Greene, R. C., Hunter, J. S. V., & Coch, E. H. (1973) *J. Bacteriol. 115*, 57–67.

Guggenheim, S., & Flavin, M. (1969) *J. Biol. Chem. 244*, 6217–6227.

Guggenheim, S., & Flavin, M. (1971) *J. Biol. Chem. 246*, 3562–3568.

Holbrook, E. L. (1984) Dissertation, Duke University.

Johnston, M., Marcotte, P., Donovan, J., & Walsh, C. (1979) *Biochemistry 18*, 1729–1738.

Kaplan, M. M., & Flavin, M. (1966a) *J. Biol. Chem. 241*, 5781–5789.

Kaplan, M. M., & Flavin, M. (1966b) *J. Biol. Chem. 241*, 4463–4471.

Krueger, J. H., Johnson, R. J., & Greene, R. C. (1978) *J. Bacteriol. 133*, 1351–1357.

Laemmli, U. K. (1970) *Nature 227*, 680–685.

Maniatis, T., Fritsch, E. F., & Sambrook, J. (1982) *Molecular Cloning. A Laboratory Manual*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Martel, A., Bouthier de la Tour, C., & Le Goffic, F. (1987) in *Biochemistry of Vitamin B6* (Korpela, T., & Christen, P., Eds.) pp 341–344, Berkhauser Verlag, Boston.

Meister, A. (1950) *J. Biol. Chem. 184*, 117–129.

Miller, J. H. (1972) *Experiments in Molecular Genetics*, Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY.

Roberts, T. M., Bikel, I., Yocum, R. R., Livingston, D. M., & Ptashne, M. (1979) *Proc. Natl. Acad. Sci. U.S.A. 76*, 5596–5600.

Saint-Girons, I., Duchange, N., Cohen, G. N., & Zakin, M. M. (1984) *J. Biol. Chem. 259*, 14282-14285.
Savin, M. A., Flavin, M., & Slaughter, C. (1972) *J. Bacteriol. 111*, 547-556.
Smith, A. A., & Greene, R. C. (1984) *J. Biol. Chem. 259*, 14279-14281.
Sutcliffe, J. G. (1979) *Cold Spring Harbor Symp. Quant. Biol. 43*, 77-90.
Tran, S. V., Schaeffer, E., Bertrand, O., Mariuzza, R., & Ferrara, P. (1983) *J. Biol. Chem. 258*, 14872-14873.
Wada, H., & Snell, E. E. (1961) *J. Biol. Chem. 236*, 2089-2095.
Wood, W. I. (1976) *Anal. Biochem. 73*, 250-257.
Zakin, M. M., Greene, R. C., Dautry-Varsat, A., Duchange, N., Ferrara, P., Py, M.-C., Margarita, D., & Cohen, G. N. (1982) *MGG, Mol. Gen. Genet. 187*, 101-106.

Reaction Mechanism of *Escherichia coli* Cystathionine γ-Synthase: Direct Evidence for a Pyridoxamine Derivative of Vinylglyoxylate as a Key Intermediate in Pyridoxal Phosphate Dependent γ-Elimination and γ-Replacement Reactions†

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ABSTRACT: Cystathionine γ-synthase catalyzes a pyridoxal phosphate dependent synthesis of cystathionine from *O*-succinyl-L-homoserine (OSHS) and L-cysteine via a γ-replacement reaction. In the absence of L-cysteine, OSHS undergoes an enzyme-catalyzed, γ-elimination reaction to form succinate, α-ketobutyrate, and ammonia. Since elimination of the γ-substituent is necessary for both reactions, it is reasonable to assume that the replacement and elimination reaction pathways diverge from a common intermediate. Previously, this partitioning intermediate has been assigned to a highly conjugated α-iminovinylglycine quininoid (Johnston et al., 1979a). The experiments reported herein support an alternative assignment for the partitioning intermediate. We have examined the γ-replacement and γ-elimination reactions of cystathionine γ-synthase via rapid-scanning stopped-flow and single-wavelength stopped-flow UV-visible spectroscopy. The γ-elimination reaction is characterized by a rapid decrease in the amplitude of the enzyme internal aldimine spectral band at 422 nm with a concomitant appearance of a new species which absorbs in the 300-nm region. A 485-nm species subsequently accumulates in a much slower relaxation. The γ-replacement reaction shows a red shift of the 422-nm peak to 425 nm which occurs in the experiment dead time (~3 ms). This relaxation is followed by a decrease in absorbance at 425 nm that is tightly coupled to the appearance of a species which absorbs in the 300-nm region. Reaction of the substrate analogues L-alanine and L-allylglycine with cystathionine γ-synthase results in bleaching of the 422-nm absorbance and the appearance of a 300-nm species. In the absence of L-cysteine, L-allylglycine undergoes facile proton exchange; in the presence of L-cysteine, L-allylglycine undergoes a γ-replacement reaction to form a new amino acid, γ-methylcystathionine. No long-wavelength-absorbing species accumulate during either of these reactions. These results establish that the partitioning intermediate is an α-imino β,γ-unsaturated pyridoxamine derivative with $\lambda_{max} \simeq 300$ nm and that the 485-nm species which accumulates in the elimination reaction is not on the replacement pathway.

Cystathionine γ-synthase is an important pyridoxal phosphate (PLP)[1] dependent enzyme involved in methionine biosynthesis. It is the only known enzyme with a physiological function to catalyze a replacement reaction at the γ-carbon of an amino acid. The enzyme from *Escherichia coli* catalyzes the production of L,L-cystathionine from *O*-succinyl-L-homoserine (OSHS) and L-cysteine (L-Cys) (eq 1). In the absence of L-Cys, the enzyme catalyzes a net γ-elimination reaction from OSHS to yield succinate, α-ketobutyrate, and ammonia (eq 2). The turnover rate for the γ-elimination reaction is only 5-10-fold slower than the γ-replacement reaction (Kaplan & Flavin, 1966; Johnston et al., 1979a; Holbrook et al., 1990). The enzyme also supports certain β-elimination, β-replacement,

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[1] Abbreviations: PLP, pyridoxal phosphate; PMP, pyridoxamine phosphate; OSHS, *O*-succinyl-L-homoserine; LDH, lactate dehydrogenase; NADH, reduced nicotinamide adenine dinucleotide; RSSF, rapid-scanning stopped-flow; SWSF, single-wavelength stopped-flow; NMR, nuclear magnetic resonance; EDTA, ethylenediaminetetraacetate; DSS, 2,2-dimethyl-2-silapentene-5-sulfonate-2,2,3,3-d_4; L-Cys, L-cysteine; DTE, dithioerythritol.

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